(19) World Intellectual Property Organization International Bureau
(43) International Publication Date 30 November 2000 (30.11.2000)


PCT

(10) International Publication Number WO 00/71564 A2
(51) International Patent Classification':
(21) International Application Number: PCT/USO0/11456
(22) International Filing Date: 1 May 2000 ( 01.05 .2000 )
(25) Filing Language:
(26) Publication Language:
(30) Priority Data:

60/131,607
09/302,457
09/499,817 26 November 1999 (26.11.1999) US
(71) Applicant (for all designated States except US): THE UNIVERSITY OF TENNESSEE RESEARCH CORPORATION [US/US]; Suite 403, 1534 White Avenue, Knoxville, TN 37996-1527 (US).
(72) Inventors; and
(75) Inventors/Applicants (for US only): STEINER, Mitchell, S. [US/US]; 8894 Silverbark Drive, Germantown, TN 38138 (US). WANG, Chiang [US/US]; 132 Magnolia Drive, Memphis, TN 38117 (US). RINALDY, Augustinus [US/US]; 5453 North Rolling Oaks Drive, Memphis, TN 38119 (US). MENON, Rema [IN/US]; 1350 Walnut Hall Ct.No.5, Memphis, TN 38119 (US).
(74) Agent: COHEN, Mark, S.; Eitan, Pearl, Latzer \& CohenZedek, Suite 210, One Crystal Park, 2011 Crystal Drive, Arlington, VA 22202-3709 (US).
(81) Designated States (national): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.
(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

## Published:

- Without international search report and to be republished upon receipt of that report.

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: ISOLATED NUCLEIC ACIDS OF THE P-HYDE FAMILY, P-HYDE PROTEINS, AND METHODS OF INDUCING

 SUSCEPTIBILITY TO INDUCTION OF CELL DEATH IN CANCER(57) Abstract: This invention provides isolated nucleic acid of the p-Hyde gene, analogs, fragments, mutants, and variants thereof of the p-Hyde family. The invention provides polypeptides, fusion proteins, chimerics, fusion proteins, antisense molecules, antibodies, and uses thereof. Also, this invention is directed to a method of inducing susceptibility to apoptosis with p-Hyde, a method of suppressing tumor growth with p -Hyde, and a method of treating a subject with cancer with p -Hyde alone or in combination with radiation, chemotherapy, or UV mimetic drugs. The invention also relates to the therapy of human cancers which have a mutation in the p-Hyde gene, including gene therapy, protein replacement therapy and protein mimetics. The invention further relates to the screening of drugs for cancer therapy. Finally, the invention relates to the screening of the p-Hyde gene for mutations, which are useful for diagnosing the predisposition to cancer.

# ISOLATED NUCLEIC ACLDS OF THE P-HYDE FAMILY, P-HYDE PROTEINS, AND METHODS OF INDUCING SUSCEPTBILITY TO INDUCTION OF CELL DEATH IN CANCER 

## FIELD OF INVENTION

This invention provides isolated nucleic acids of p -Hyde genes, proteins, analogs, fragments, mimetics, mutants, synthetics, and variants thereof of the p-Hyde family. This invention is directed to a method of inducing susceptibility to apoptosis with p-Hyde, a method of suppressing tumor growth with p-Fiyde, and a method of treating a subject with cancer with p -Hyde alone or in combination with radiation, chemotherapy, or UV mimetic drugs.

## BACKGROUND OF THE INVENTION

Prostate cancer is the most common malignancy in men with over 317,000 new cases and the second leading cause of male cancer deaths in the United States (Boring et al., 1993; Steiner et. al, 1995). The molecular mechanisms responsible for the development, progression, and metastasis of prostate cancer remain largely unknown. Up to $20 \%$ of prostate cancers occur in men under the age of 65 years of age (Silverberg, 1986) suggesting that prostate carcinogenesis is not only associated with aging, but also to hereditary factors (Silverberg, 1987; McLellan and Norman, 1995; Carter et al., 1992). Genetic linkage studies of 691 affected families have revealed that an earlier age of onset of the disease in the proband and the presence of multiple affected family members are important determinants that increase the risk of prostate cancer. The pattern of inheritance of the putative prostate cancer gene appears to be autosomal dominant with an $88 \%$ penctrance rate (Steinberg, 1990). Thus, hereditary factors play an important role in prostate oncogenesis.

Like many carcinomas, prostate cancer formation is a multistep process involving tumor initiation, promotion, conversion, and progression (Carter et al., 1990; Sandberg, 1992). This process is driven by chromosomal instability, spontaneous mutations, and carcinogen induced genctic and epigenetic changes. Chromosomal instability leads to the total or partial gain or loss of chromosomes, translocations, and other abnormalities. Spontaneous mechanisms are age-related and include activation of oncogenes or inactivation of tumor suppressor genes by genetic mutations. These mutations result in
the misincorporation of nucleotides during DNA replication of the coding region, alteration of the intron-exon junction sequences affecting the splicing mechanism, and aberrations of regulatory sequences changing the control of critical genes. These mutations escape genetic surveillance by a battery of DNA repair mechanisms and its associated gene products, such as p53 (Effert et al., 1992, Isaacs et al, 1991; Mellon et al., 1992) and p21 (El-Deiry et al., 1994) and PCNA (Templeton et al., 1996). Carcinogen-induced genetic and epigenetic changes initiate tumors as a consequence of the direct damaging effects of carcinogenic agents of the DNA altering gene expression. Tumor initiation is subsequently followed by tumor promotion as affected cells have selective reproductive and clonal expansion capabilities through altered signaling transduction and proliferation responses to growth factors, resistance to cytotoxicity, and deregulation of terminal differentiation (Yuspa and Poirier, 1988; Weinstein, 1987). Finally, tumor promotion is succeeded by other genetic mutational events that lead to loss of hormone sensitivity, increased cell motility, invasion, alterations in programmed cell death and metastasis. Accordingly, the initiation and progression of cancer is a multistep process whereby genetic alterations or mutations of critical genes ultimately dictate defined cell phenotypes which differ in regard to many important cellular activities including cell proliferation, differentiation, and programmed cell death. The exact mutational events responsible for the multistep progression of prostate cancer, however, is unknown. A better understanding of the molecular mechanisms responsible for prostate cancer may lead to new therapies to combat, and perhaps, to even prevent prostate cancer.

## SUMMARY OF THE INVENTION

The present invention provides isolated nucleic acids encoding P-Hyde genes of the p -Hyde family. The p-Hyde gene as shown herein is associated with: (1) the regression of tumor growth in vivo (2) the induction to susceptibility to apoptosis caused by UV or chemotherapy induced DNA damage, and (3) prevention of DNA repair with the upregulation of apoptosis as the result of UV damage and the failure to repair DNA.

This invention provides a novel class of genes which act as inhibitor of a DNA repair enzyme and induce susceptibility of cancer cells to cell death. Also, this invention
provides isolated nucleic acids which encodes a mammalian p -Hyde protein which induce susceptibility of a cancer cell to cell death, including allelic, analogs, fragments, mimetics, mutants, synthetics, or variants thereof. This invention provides an isolated nucleic acids which encodes a human p-Hyde protein which induces susceptibility of a cancer cell to cell death, including allelic, analogs, fragments, mimetics, mutants, synthetics, or variants thereof.

Within this invention is provided a nucleic acid which has the nucleotide sequence as shown SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO:9.


#### Abstract

Also, within the invention is a nucleic acid molecule which encodes a fragment of a polypeptide having the amino acid sequence of SEQ ID NO: 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8 or SEQ ID NO: 10, the fragment including at least $15(25,30,50$, 60 , or 63 ) contiguous amino acids of SEQ ID NO: 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8 or SEQ ID NO 10.


Also, within the invention is a nucleic acid molecule having the nucleotide sequence which is at least about $82 \%, 84 \%, 85 \%, 87 \%, 90 \%, 92 \%, 95 \%$, or $98 \%$ identical to the nucleic acid sequence as shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO: 9.

Also, within this invention is a nucleic acid molecule which encodes a naturally occurring allelic variant of a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8, or SEQ ID NO 10 wherein the nucleic acid molecule hybridizes to a nucleic acid molecule comprising SEQ ID NO: 2, SEQ ID NO 4, SEQ ID NO 6, SEQ ID NO 8 or SEQ ID NO 10 or the complement thereof under stringent conditions.

Also within the invention are isolated p-Hyde proteins having an amino acid sequence that is at least about $82 \%, 84 \%, 85 \%, 87 \%, 90 \%, 95 \%$, or $98 \%$ identical to the amino acid sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO 9.

Also within the invention are: an isolated p -Hyde protein protein which is encoded by a nucleic acid molecule having a nucleotide sequence that is at least about $65 \%$, preferably $75 \%, 80 \%, 85 \%$, or $95 \%$ identical to SEQ ID NO: 1 , SEQ ID NO: 3 , SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO 9; and an isolated p-Hyde protein protein which is encoded by a nucleic acid molecule having a nucleotide sequence which hybridizes under stringent hybridization conditions to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO: 1 , SEQ ID NO: 3, SEQ ID NO:5, SEQ ID NO:7 or SEQ ID NO 9, or the complement thereof.

The p-Hyde protein of the present invention, or biologically active portions thereof, can be operably linked to a non- p-Hyde polypeptide (e.g., heterologous amino acid sequences) to form p -Hyde fusion proteins.

This invention provides a vector comprising the isolated mucleic acid encoding P-Hyde gene. This invention provides a replication-defective recombinant E1/E3 deleted adenovirus containing a truncated RSV promoter and the P-Hyde cDNA gene (AdRSVpHyde).

This invention provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within a nucleic acid which encodes the p -Hyde, or a sequence which is complementary to the nucleic acid which encodes the p-Hyde. This invention provides an antisense molecule, triplex oligonucleotide, or ribozyme which is capable of specifically hybridizing with the isolated nucleic acid encoding p -Hyde.

This invention provides a method for producing a polypeptide which comprises growing the host vector system under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced. In one embodiment the method of obtaining a polypeptide in purified form comprises: (a) introducing the vector into a suitable host cell; (b) culturing the resulting cell so as to produce the polypeptide; (c)
recovering the polypeptide produced in step (b); and (d) purifying the polypeptide so recovered.

This invention provides a polypeptide comprising the amino acid sequence of a p-Hyde. This invention provides a fusion protein or chimeric comprising the polypeptide. This invention provides an antibody which specifically binds to the polypeptide. This invention provides a pharmaceutical composition comprising an amount of the polypeptide and a pharmaceutically effective carrier or diluent.

This invention provides a method for determining whether a subject carries a mutation in the p-Hyde gene which comprises: (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant $p$-Hyde so as to thereby deternine whether a subject carries a mutation in the p-Hyde gene. In one embodiment the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant p-Hyde, and whercin the determining of step (b) comprises: (i) contacting the mRNA with the oligonucleotide undet conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant p-Hyde.

This invention provides a method for screening a tumor sample from a human subject for a somatic alteration in a p-Hyde gene in said tumor which comprises gene comparing a first sequence selected form the group consisting of a p-Hyde gene from said tumor sample, p-Hyde RNA from said tunor sample and p-Hyde cDNA made from mRNA from said tumor sample with a second sequence selected from the group consisting of p-Hyde gene from a nontumor sample of said subject, p-Hyde RNA from said nontumor sample and p-Hyde cDNA made from mRNA from said nontumor sample, wherein a difference in the sequence of the p-Hyde gene, p-Hyde RNA or p-Hyde cDNA from said tumor sample from the sequence of the p-Hyde gene, p-Hyde RNA or p-Hyde cDNA
from said nontumpor sample indicates a somatic alteration in the p -Hyde gene in said tumor sample.

This invention provides a method for screening a tumor sample from a human subject for the presence of a somatic alteration in a p-Hyde gene in said tumor which comprises comparing p-Hyde polypeptide from said tumor sample from said subject to p-Hyde polypeptide from a nontumor sample from said subject to analyze for a difference between the polypeptides, wherein said comparing is performed by (i) detecting either a full length polypeptide or a truncated polypeptide in each sample or (ii) contacting an antibody which specifically binds to either an epitope of an altered $p$-Hyde polypeptide or an epitope of a wild-type p-Hyde polypeptide to the p-Hyde polypeptide from each sample and detecting antibody binding, wherein a difference between the p-Hyde polypeptide from said tumor sample from the p-Hyde polypeptide from said nontumor sample indicates the presence of a somatic alteration in the p-Hyde gene in said tumor sample.

This invention provides a method for identifying a chemical compound which is capable inducing susceptibility to cell death which comprises: (a) contacting the p-Hyde with a chemical compound under conditions permitting binding between the p -Hyde and the chemical compound; (b) detecting specific binding of the chemical compound to the p-Hyde; and (c) determining whether the chemical compound inhibits the p -Hyde so as to identify a chemical compound which is capable of capable inducing susceptibility to cell death.

This invention provides a method of inhibiting the growth of cancer cells, comprising the steps of obtaining the cells and contacting the cells of the subject with a replication-deficient adenovirus type 5 expression vector comprising an adenovirus genome having a deletion in the E1 and E3 region of the genome and an insertion within the region of a nucleic acid encoding p-Hyde under the control of a Rous Sarcoma virus promoter, thereby inhibiting the growth of the prostate cancer cells.

This invention provides a method of inhibiting the growth a prostate cancer cells, comprising: 1) obtaining a sample of prostate cells from a subject; 2) contacting the cells with a replication deficient adenovirus type 5 expression vector which comprises an adenovirus genome having a deletion in the E 1 and E 3 regions of the genome and an insertion within the regions of a p-Hyde cDNA under the control of a Rous Sarcoma virus promoter; and 3) introducing the cells into the subject, thereby inhibiting the growth of the cancer cells.

This invention provides a method of suppressing the growth of cancer cells in a subject, comprising introducing into the caucer cell an amount of a nucleic acid encoding a p -Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p -Hyde protein, thereby suppressing the growth of cancer cells in the subject.

This invention provides a method of suppressing growth of cancer cells in a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutically effective anount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p-Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby suppressing the growth of cancer cells in the subject.

This invention provides a method of inducing susceptibility to apoptosis of cancer cells in a subject, comprising introducing into the cancer cell an amount of a nucleic acid encoding a p -Hyde protein, a nucleic acid encoding a fragment of p-Hyde protein, or the uucleic acid encoding a mutant p-Hyde protein, thereby inducing susceptibility to apoptosis.

This invention provides a method of inducing susceptibility to apoptosis of cancer cells in a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of $p$-Hyde protein, or the nucleic acid
encoding a mutant p-Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby inducing susceptibility to apoptosis.

This invention provides a method of treating a subject with cancer which comprises administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p-Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein and a pharmaccutical acceptable carrier or diluent, thereby treating the subject with cancer.

This invention provides a method of treating a subject with cancer, comprising: 1) administering to the subject a phannaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p -Hyde protein in combinatiox with radiation, chemotherapy, or UV mimetic drugs; and 2) a pharmaceutical acceptable carrier or diluent, thereby treating the subject with cancer.

This invention a method of treating a subject with cancer, comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising: 1) an adenovirus type 5 expression vector which comprises an adenovirus genome having a deletion in the E1 and E3 regions of the genome and an insertion within the regions of a full length sense p -Hyde cDNA under the control of a Rous Sarcoma virus promoter, and 2) a suitable carricr or diluent, thereby treating the subject with cancer. In one embodiment the cancer is selected from a group consisting of: melanoma; lymphoma; leukemia; and prostate, colorectal, pancreatic, breast, brain, or gastric carcinoma.

Lastly, the present invention provides the means necessary for production of gene-based therapies directed at cancer cells. These therapeutic agents may take the form of polynucleotides comprising all or a portion of the p-Hyde locus placed in appropriate vectors or delivered to target cells in more direct ways such that the function of the p-Yyde protein is reconstituted. Therapeutic agents may also take the
form of polypeptides based on either a portion of, or the entire protein sequence of p -Hyde. These may functionally replace the activity of p -Hyde in vivo.

## BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. Schematic presentation of AdRSVpHyde structure. The 2664 bp inserted fragment contains a 1467 bp full-length pFlyde cDNA gene (SEQ ID NO: 1) and 1166 bp 3' untranslated downstream region. The complete sequence of AdRSVpHyde is set forth in Figure 10, Specifically, the nucleic acid sequence of region A in Figure 1 is set forth in Figure 10 Region $A$ and the nucleic acid sequence of region $B$ in Figure 1 is set forth in Figure 10 at Region B.

Figure 2. Expression of pHyde by AdRSVpHyde. DU145 cells transduced by AdRSVpHyde at MOI=200 were harvested in 48 h post infection for either mRNA or protein extraction. (A) Expression of pHyde at mRNA level in DU145 cells. Sample wells were each loaded with 10 mg of total RNA, electrophoresed in $12.5 \%$ agarose gel, transferred to nylon membrane, and hybridized with ${ }^{32} \mathrm{p}$-labeled pHyde cDNA. Northem blot was stripped and rehybridized with GAPDH to assess gel loading. (B) Expression of pHyde at protein level in DU145 cells. Protein extracts ( 50 mg ) were loaded on a $12 \%$ SDS-PAGE gel. Rabbit anti-rat pHyde antibody was used as the primary antibody.

Figure 3. Inhibitory effects of pHydc on prostate cancer cell growth. DU145 (A) and LNCaP (B) cells were transduced with or without adenoviral vectors at MOI=100. Cell numbers were counted at day 5 after viral transduction. The data represent the results from two independent experiments with each in duplicates.

Figure 4. AdRSVpHyde inhibits prostate tumor growth in vivo. DU145 cells ( $1.4 \times 10^{7}$ cells) were injected subcutaneously into the flanks of nude mice. When tumors reached an average volume of $80 \mathrm{~mm}^{3}$ (about one month after tumor cell inoculation), tumors were untreated (control), or intratumorally injected (day 0 ) either by $5 \times 10^{9}$ pfu control virus AdRSVlacZ (control virus), or $5 \times 10^{9} \mathrm{pfu}$ AdRSVpHyde (AdRSVpHyde).

The tumor sizes were periodically measured at times shown in the figure up to day 52 days post viral injection.

Figure 5. Morphological changes of DU145 and LNCaP cells transduced by AdRSVpHyde. Cells were transduced by control adenovirus AdRSVlacZ or by AdRSVpHyde at MOI=100. The morphologic features of untreated control cells and viral-transduced cells were recorded at day 5 post viral transduction. All the photos are at the same magnification (66X). (A) and (D): Untreated control cells; (B) and (E): Viral control AdRSVlacZ treated cells; (C) and (F): AdRSVpHyde treated cells.

Figure 6. Morphological changes of PC-3, TSU, and PPC-1 cells transduced by AdRSVpHyde. Cells were transduced by control adenovirus AdRSVlacZ or by AdRSVpHyde at MOI=100. The morphologic features of untreated control cells and viral-transduced cells were recorded at day 5 post viral transduction. All the photos are at the same magnification (66X). (A, D, G): Untreated control cells; (B, E, H): Viral control AdRSVlacZ treated cells; (C, F, I): AdRSVpHyde treated cells.

Figure 7. Expression of $\mathbf{p} 53$ and Rb mRNA in various prostate cancer cell lines. Sample wells were each loaded with 10 mg of total RNA, electrophoresed in $12.5 \%$ agarose gel, transferred to nylon membrane, and hybridized with ${ }^{32} \mathrm{P}$-labeled p 53 or Rb cDNA probe. Northern blot was stripped and rehybridized with GAPDH to normalize the gel loading.

Figure 8. AdRSVpHyde induced p53 expression in DU145 cells. The same Northern blot in Fig. 2A was stripped and rehybridized with ${ }^{32} \mathrm{P}$-labeled p53 cDNA.

Figure 9. AdRSVpHyde induced apoptosis in LNCaP cells. Cells were untreated or transduced by AdRSVpHyde at MOI=100, supernatant were collected 48 h post transduction. Soluble DNA was extracted from floating cells and electrophoresed on a $2 \%$ agarose gel

Figure 10. The sequence of AdRSVpHyde .

Figure 11. Comparison of the nucleotide sequence of the p -Hyde family genes between human p-Hyde (I) and human p-Hyde 40 (II).

Figure 12.. Comparison of the amino acid sequence of the p-Hyde family genes between human p-Fyde (I) and human p-Hyde 40 (II).

Figure 13. A) Comparison of the nucleotide sequence of the p-Hyde family genes, between rat p -Hyde and human p -Hyde. B) Comparison of the amino acid sequence of the p -Hyde family genes between rat p -Hyde and human p -Hyde.

Figure 14. TUNEL, assay of DU145 cells in vitro. DU145 cells were either untreated (A,D), or transduced with control virus (B, E) or AdRSVpFHyde (C, F) at MOI=200, three days after transduction the cells were fixed and proceeded for TUNEL assay. The cells were then visualized by fluorescence microscopy. The arrows indicated some apoptotic cells. Magnification: A, B, C: x2O. D, E, F: x4O.

Figure 15. TUNEL, assay of DU145 cells in vivo. DU145 xenograft tumors (about 80 $\mathrm{mm}^{3}$ ) growing on nude mice were either untreated (A) or injected with $5 \times 10^{9}$ pfu AdRSVpHyde (B). Tumors were harvested in 21 days. Tumor sections were fixed and proceeded for TUNEL assay. The sections were counter stained with propidium iodide (red) to show the nucleus of cells. The bright yellowish stained cells indicated the apoptotic cells. Tumor sections from control virus-treated DU145 tumor showed the similar results as in (A). Maguification: A, B: x2O.

Figure 16. Inhibitory effects of pfyyde on growth of prostate cancer cell lines DU145 and LNCaP. DU145 (A) and LNCaP (B) cells were transduced with or without adenoviral vectors (control virus or AdRSVpHyde) at $\mathrm{MOI}=100$. Celí numbers were counted at day 5 after viral trausduction. The data represent the results from two independent experiments each performed in duplicate.
*Some error bars were too small to see in the figure.

Figure 17. Expression of $\mathbf{R b}$ and $\mathbf{p} 53$ in various prostate cancer cell lines. Various prostate cancer cells were screened for endogenous Rb and p 53 expression at mRNA levels. Each well was loaded with $10 \mu g$ of total RNA. Samples were electrophoresed in $12.5 \%$ agarose gel, transferred to nylon membrane, and hybridized with ${ }^{32} \mathrm{P}$-labeled Rb (which showed an about 4.4 kb transcript) or p53 CDNA (which showed an about 2.5 kb transcript). The Northern blot was then stripped and rehybridized with GAPDH cDNA (which showed a 1.2 kb transcript) to assess RNA integrity and gel loading.

Figure 18. Xnhibitory effects of pHyde on growth of prostate cancer cell lines; PC-3 and TSU. PC-3 (A) and TSU (B) cells were transduced with or without adenoviral vectors (control virus or AdRSVpHyde) at $\mathrm{MOI}=200$. Cell numbers were counted at day 5 after viral transduction. The data represent the results from two independent experiments each performed in duplicate. *Some error bars were too small to see in the figure.

## DETAILED DESCRIPTION OF TERE INVENTION

This invention provides a novel class of genes which act as inhibitor of a DNA repair enzyme and induces susceptibility of cancer cells to cell death. Functionally, P-Hyde is and associated with suppression of tumor growth in vivo and increased susceptibility to apoptosis induced by UV irradiation or FUrD treatment. The upregulation of apoptosis due to UV damage is correlated with the presence of intact photoproduct in prostate cancer cell lines stably transfected with $p$-Hyde. Use of $p$-Hyde in human gene therapy as monotherapy or in combination with radiation or chemotherapy is useful against cancer or hyperproliferative human diseases. In one embodiment, the class of proteins, such as P-Hyde, are DNA repair enzyme inhibitors which downregulate Nucleotide-Excision-Repair (NER) pathway in prostate cancer, MGMT CNA repair pathway in colon cancer cell line, $\mathrm{O}_{6}$ methyl guanine methyl trausferase enzyme $\left(\mathrm{O}_{6} \mathrm{MgMT}\right)$ and 6,4 , photoproducts ( $6,4 \mathrm{PP}$ ). The class of genes is characterized by comprising a leucine zipper binding domain and a death domain which causes the cell to be apoptotic. Human p-Hyde (I) and human p-Hyde 40 (II) are examples set forth
herein, of a family of molecules (the "p-Hyde family") having certain homolgous sequences and conserved structural and functional features.

This invention provides an isolated nucleic acid which encodes a mammalian p-Hyde protein which induces susceptibility of a cancer cell to cell death, including allelic, analogs, fragments, mimetics, mutants, synthetics, or variants thereof. This invention provides an isolated nucleic acid which encodes a human p-Hyde protein which induces susceptibility of a cancer cell to cell death, including allelic, analogs, fragments, mimetics, mutants, synthetics, or variants thereof.

As used interchangeably herein a "p-Hyde activity", "biological activity of p-Hyde" or "functional activity of p -Hyde", refers to an activity exerted by a p -Hyde protein, polypeptide or nucleic acid molecule on a p -Hyde responsive cell as determined in vivo, or in vitro, according to standard techniques which causes apoptosis of the cell. P-Hyde refers to a family of genes having the p-Hyde activity. Examples of such genes are rat p-lhyde, human p-Hyde (I), and human p-Hyde 40 (II). Io one embodiment, these sequences comprise SEQ ID NO: 7 on the nucleotide level and SEQ ID NO: 8 on the amino acid level. Also, examples, of family p-Hyde gene members include isolated nucleic sequences of SEQ ID NOS: $1,3,5$, or 7 on the nucleotide level and amino acid sequences of SEQ ID NOS: $2,4,6$ or 8 on the amino acid level.

In one embodiment the p-Hyde gene has a nucleotide sequence having at least $85 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 1 . In another embodiment the nucleic acid has a nucleotide sequence having at least $87 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 1. Io another embodiment the nucleic acid has a nucleotide sequence having at least $90 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 1. In another embodiment the nucleic acid has a nucleotide sequence having at least $95 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 1. In another embodiment the nucleic acid fragment comprises a fragment which begins at the nucleic acid at position 1 of the SEQ ID NO: 1 and ends at position 557 of SEQ ID NO: 1. In another embodiment the nucleic acid comprises a fragment which begins at the nucleic acid at position 1 of SEQ ID NO: 1 and ends at
position 158 of SEQ ID NO: 1. In another embodiment the nucleic acid comprises a fiagment which begins at the nucleic acid at position 50 of SEQ ID NO: 1 and ends at position 120 of SEQ ID NO: 1. The nucleic acid is DNA, cDNA, genomic DNA, or RNA.

Human p-Hyde nucleic acid coding region:
1 ggggagctge cgeggtcget ccgagcggeg ggcegcagag ccaccaaaat gccagaagag 61 atggacaagc cactgatcag ectccacctg gtggacagcg atagtagcct tgccaaggtc
121 ccogatgagg cccccaaagt gagcatcctg ggtagcgggg acttgcceg ctccetggcc
181 acacgcctgg tgggetctgg cttcaaagtg gtggtgggga gecgcaacce caaacgcaca
241 gccaggetgt ttccctcagc ggcecaagtg acttccaag aggaggcagt gagctccccg
301 gaggtcatct ttgtggctgt gttccgggag cactactctt cactgtgcag tcteagtgac
361 cagctggcgg gcaagatect ggtggatgtg agcaacceta cagagcaaga gcaccttcag
421 catcgtgagt ccaatgctga gtacctggec tccetcttcc ccacttgcac agtggtcaag
481 gcettcaatg tcatctctgc ctggaccetg caggctggcc caagggatgg taacgggcag
541 gtgcccatct geggtgacca gccagaagec aagegtgctg tctcggagat ggegctegcc
601 atgggettca tgcecgtgga catgggatcc ctggegtcag cctgggaggt ggaggccatg
661 cccctgrgce tcctccogge ctggaaggtg cceaccetgc tggecetggg gctettegtc
721 tgcttctarg cctacaactt cgtccgggac gttctgcagc cctatgtgca ggaaagccag
781 aacaagttct tcaagctgce cgtgtccgtg gtcaacacca cactgcogtg cgtggectac
841 gtgctgctgt cactcgtgta cttgccegge gtgctggegg ctgccctgca getgcggcge
901 ggcaccaagt accagcgett ccccgactgg ctggaccact ggctacagca cegcaagcag 961 atcgggctge tcagcttctt ctgcgccgec ctgcacgece tctacagctt ctgcttgecg
1021 ctgcgecgeg cccaccgcta cgacctggtc aacctggcag tcaagcaggt cttggccaac 1081 aagagccacc tctgggtgga ggaggtctgg cggatggaga tctacctctc cctgggagtg 1141 ctggecctcg geacgttgtc cctgctggec gtgacctcac tgccgtccat tgcaaactcg 1201 ctcaactgga gggagttcag cttcgttcag tectcactgg getttgtgge cctcgtgetg 1261 agcacactgc acacgctcac ctacggctgg accogegcct tcgaggagag ccgctacaag 1321 ttctacctgc ctcccacctt cacgetcacg ctgctggtge cctgegtegt catcctggec 1381 aaagccetgt ttctcctgec ctgcatcagc cgcagactey ceaggatcog gagaggetgg 1441 gagagggaga gcaccatcaa gttcacgetg cceacagacc acgecctgge cgagaagacg 1501 agccacgtat gaggtgcctg ccctgggctc tggaccccgg gcacacgagg gacggtgcce 1561 tgagccegtt aggtttctt ttcttggtgg tgcaaagtgg tataactgtg tgcaaatagg
162 laggttgagg tccaaattcc tgggactcaa atgtatgeag tactattcag aatgatatac
1681 acacatatgt gtatatgtat ttacatatat tecacatata taacaggatt tgeaattata
1741 catagctage taaaaagttg ggtctctgag atttcaactt gtagatttaa aaacaagtge
1801 cgtacgttaa gagaagagca gatcatgcta ttgtgacatt tgcagagata tacacacact 1861 ttttgtacag aaaaaaaaaa aaaaaa (SEQ ID NO.: 1)

In another embodiment the nucleic acid encodes an amino acid sequence having the sequence as set forth in SEQ ID NO 2. In one embodiment the the amino acid fragment comprises a fragment which begins at the amino acid at position 1 of SEQ ID NO 2 and ends at position 101. In one embodiment the amino acid fragment comprises a fragment
which begins at the amino acid at position 1 of SEQ ID NO 2 and ends at position 80. In one embodiment the amino acid fragment comprises a fragment which begins at the amino acid at position 1 of SEQ ID NO 2 and ends at position 60 . In one embodiment the amino acid has at least $85 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 2. In another embodiment the amino acid has at least $90 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 2. In another embodiment the amino acid has at least $95 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 2.

Human p-Hyde amino acid sequence:
1 MPEEMDKPLI SLHLVDSDSS LAKVPDEAPK VSILGSGDFA RSLATRLVGS GFKVVVGSRN 61 PKRTARLFPS AAQVTFQEEA VSSPEVIFVA VFREHYSSLC SLSDQLAGKI LVDVSNPTEQ 121 EHLQHRESNA EYLASLFPTC TVVKAFNVIS AWTLQAGPRD GNGQVPICGD QPEAKRAVSE 181 MALAMGFMPV DMGSLASAWE VEAMPLRLLP AWKVPTLLAL GLFVCFYAYN FVRDVLQPYV 241 QESQNKFFKL PVSVVNTTLP CVAYVLLSLV YLPGVLAAAL QLRRGTKYQR FPDWLDHWLQ 301 HRKQIGLLSF FCAALHALYS FCLPLRRAHR YDLVNLAVKQ VLANKSHLWV EEVWRMEIYL 361 SLGVLALGTL SLLAVTSLPS IANSLNWREF SFVQSSLGFV ALVLSTLHTL TYGWTRAFEE 421 SRYKFYLPPT FTLTLLVPCV VILAKALFLL PCISRRLARI RRGWERESTI KFTLPTDHAL 481 AEKTSHV* (SEQ ID NO.: 2)

In one embodiment the p -Hyde gene has a nucleotide sequence having at least $75 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 3. In another embodiment the nucleic acid has a nucleotide sequence having at least $85 \%$ similarity with the uucleic acid coding sequence of SEQ ID NO: 3. In another embodiment the nucleic acid has a nucleotide sequence having at least $90 \%$ similarity with the nucleic acid coding sequence of SEQ D NO: 3 . In another embodiment the nucleic acid has a nucleotide sequence having at least $95 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 3. In another embodiment the nucleic acid fragment comprises a fragment which begins at the nucleic acid at position 1 of the SEQ ID NO: 3 and ends at position 557 of SEQ ID NO: 3. In another embodiment the nucleic acid comprises a fragment which begins at the nucleic acid at position 1 of SEQ ID NO: 3 and ends at position 158 of SEQ ID NO: 3. In another embodiment the nucleic acid comprises a fragment which begins at the nucleic acid at position 50 of SEQ ID NO: 3 and ends at position 120 of SEQ ID NO: 3. The nucleic acid is DNA, cDNA, yenomic DNA, or RNA.

Human P-Hyde 40 nucleic acid sequence:
1 ggggagctgc cgcggtcgct ccgagcggcg ggccgcagag ccaccaaaat gccagaagag
61 atggacaage cactgatcag cotccacctg gtggacageg atagtagcet tgceaaggtc
121 cccgatgagg cccecaaagt gagcatcctg ggtagcgggg actttgccog ctccctggec
181 acacgcctgg tgggctctgg cttcaaagtfg gtggtgggga gecgcaaccc caaacgcaca
241 gccaggctgt ttccctcagc ggcccaagtg actttccaag aggaggcagt gagctccccg
301 gaggtcatct ttgtggctgt gttcegggag cactactctt cactgtgcag tctcagtgac
361 cagctggcgg gcaagatcct ggtggatgtg agcaacccta cagagcaaga gcaccttcag
421 catcgtgagt ccaatgctga gtacctggcc tecctcttcc ccacttgcac agtggtcang
481 gecttcaatg tcatctctge ctggaccetg caggetygec caagggatgg taacgggeag
541 gtgcccatct gcggtgacca gccagaagcc aagegtgctg tctcggayat ggegctcgcc
601 atgggettca tgccogtgga catgggatcc ctggcgtcag cetgggaggt ggaggccatg
661 cccctgcgec tcetccogge ctggaaggtg cccaccetge tggcectggg getcttcgtc
721 tgcttctatg cctacaactt cgtccgggac gttctgcagc cctatgtgca ggaaagccag
781 aacaagttct tcaagctgcc cgtgtcegtg gtcaacacca cactgccgtg cgtggcctac
841 gtgctgctgt cactcgtgta cttgccegge gtyctggcgg ctgcectgca getgcggegc
901 ggcaccaagt accagcgctt ccccgactgg ctggaccact ggctacagca ccgcaagcag
961 atcgggctge tcagcttctt ctgegccgec ctgcacgece tctacagctt ctgcttgecg
1021 ctgcgecgcg cccaccgcta cgacctggtc aacctggcag tcaagcaggt cttggccaac
1081 aagagccacc tctgggtgga ggaggtctgg cggatggaga tctacctctc cetgggagtg
1141 ctggccetcg geacgttgte cetgctggec ytgacctcac tgecgtccat tgcaaacteg
1201 ctcaactgga gggagttcag cttcgttcag tgtgtggcaa cttccagtge aggaaacaca

1261ggcagtggaa cccgaagacc tgaatctcag tcccaagacc cccacttacc tgececgeat 1321 catcagacaa gtttcetagg cectcggage ttctgctgct cacttgtgec tgtgtceacc 1381 ccatatggtc atcaagagga tttgagetgg acacgttaaa tgcaggatge gtgcagccaa 1441 cagtggeatg ctggetttg agtectcact ggectttgtg gecetcgtge tgagcacact

1501 geacacgetc acctacgget ggaccegcge cttcgaggag agccgetaca agttctacct
1561 gectcccace ttcacgetca cgetgctggt gccetgegtc gtcatcctgy ccaaagccet
1621 gtttctcctg ccctgcatca gecgcagact cgccaggatc cggagagget gggagaggga 1681 gagcaccatc aagttcacge tgcceacaga ccacgecctg gecyagaaga cgagccacgt 1741 atgaggtgcc tgecctgggc tctggaccec gggcacacga gggacggtgc cctgagcceg 1801 ttaggtttc ttttcttggt ggtgcaaagt ggtataactg tgtgcaaata ggaggtttga 1861 ggtccaaatt cetgggactc aaatgtatge agtactattc agaatgatat acacacatat
1921 gtgtatatgt atttacatat attccacata tataacagga tttgcaatta tacatagcta
1981gctaaaaagt tgggtctctg agatttcaac ttgtagattt aaaaacaagt gccgtacgtt
2041aagagaagag cagatcatgc tattgtgaca tttgcagaga tatacacaca cttttgtac 2101agaaaaaaaa aaaaaaaa (SEQ ID NO.: 3)

In another embodiment the nucleic acid encodes an amino acid sequence having the sequence as set forth in SEQ ID NO 4. In one embodiment the the amino acid fragment comprises a fragment which begins at the amino acid at position 1 of SEQ ID NO 4 and ends at position 101. In one embodiment the amino acid fragment comprises a fragment which begins at the amino acid at position I of SEQ ID NO 4 and ends at position 80. In one embodiment the amino acid fragment comprises a fragment which begins at the amino acid at position 1 of SEQ ID NO 4 and ends at position 60 . In one embodiment the amino acid has at least $85 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 4. In another embodiment the amino acid has at least $90 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 4. In another embodiment the amino acid has at least $95 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 4.

Human p-Hyde 40 amino acid sequence:
1 MPEEEMDKPLI SLHLVDSDSS LAKVPDEAPK VSILGSGDFA RSLATRLVGS GFKVVVGSRN 61 PKRTARLFPS AAQVTFQEEA VSSPEVIFVA VFREHYSSLC SLSDQLAGKI LVDVSNPTEQ
121 EHLQHRESNA EYLASLFPTC TVVKAFNVIS AWTLQAGPRD GNGQVPICGD QPEAKRAVSE 181 MALAMGFMPV DMGSLASAWE VEAMPLRLLP AWKVPTILLAL GLFVCFYAYN FVRDVLQPYV 241 QESQNKFFKL PVSVVNTTLP CVAYVLLSLV YLPGVLAAAL QLRRGTKY-QR FPDWLDFHLQ 301 HRKQIGLLSF FCAALHALYS FCLPLRRAHR YDLVNLAVKQ VLANKSHLWV EEVWRMEIYL 361 SLGVLALGTL SLLAVTSLPS IANSLNWREF SFVQCVATSS AGNTGSGTRR PESQSQDPHL 421 PAPHHQTSFI GPRSFCCSLV PVSTPYGHQE DLSWTR (SEQ ID No.: 4)

In one embodiment the p-Hyde gene has a nucleotide sequence having at least $75 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 5. In another
embodiment the nucleic acid has a nucleotide sequence having at least $85 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 5. In another embodiment the nucleic acid has a nucleotide sequence having at least $90 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 5. In another embodiment the nucleic acid has a nucleotide sequence having at least $95 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO: 5. In another embodiment the nucleic acid fragment comprises a fragment which begins at the nucleic acid at position 1 of the SEQ ID NO: 5 and ends at position 557 of SEQ ID NO: 5. In another embodiment the nucleic acid comprises a fragment which begins at the nucleic acid at position 1 of SEQ ID NO: 5 and ends at position 1.58 of SEQ ID NO: 5. In another embodiment the nucleic acid comprises a fragment whicl begins at the nucleic acid at position 50 of SEQ ID NO: 5 and ends at position 120 of SEQ ID NO: 5. The nucleic acid is DNA, cDNA, genomic DNA, or RNA.

Rat p-Hyde mucleic acid sequence:
1 gaattcggca cyaggctgce gaggcactgt gatgtceggg gagatggaca aaccgetcat 61 cagtcgecge ttggtggaca gtgatggcag tctggetgag gtccecaagg aggctcceaa
121 agtggocatc ctgggcageg gggatttgc coggtccetg gccacacgec tggtgggetc
181 tggettcttt gtggtggtgg gaagecgtaa ceccaaacge actgceggec tettccectc 241 cttagcccaa gtgactttcc aggaggagge cgtgagctct ccagaggtca tetttgtgge 301 cgtgttccgg gagcactact cetcactgtg cagtcttgct gaccagttgg ctggcaagat 361 cctagtggat gtaagcaacc ccacggagaa ggaycgtctt cagcaccgec agtcyaacge 421 cgagtacctg gectcectct tcectgcctg cactgtggtc aaggecttca acgtcatctc 481 tgcatgggcc ctacaggctg gcceaaggga tgggaacagg caggtgctca tctgcggtga 541 ccagctggaa gecaageaca ccgtctcaga gatggegcge gccatgggtt tcaccccact 601 ggacatggga tecctggect cagcgaggga ggtagaggcc atacccctgc gecticcttcc 661 atcctggaag gtgcceacce tectggecet ggggctaage acacaaaget atgectacaa 721 cttcatccgg gacgttctac agccgtacat ccggaaagat gagaacaagt tctacaagat 781 gccectgtct gtggtcaaca ccacgatacc etgtgtgget tacgtgctge tglccetggt 841 ttacctgcet ggtgtgetgg cagctgccet tcagctgagg agggggacca agtaccagcg 901 cttcccagac tggctggace attggetgca geaccgcaag cagatcggge tactcagctt 961 tttttcgec atgetgeacg ctctctacag cttctgectg cegctgcgec gctcceaccy 1021 ctatgatctg gtcaacctge ctgtgaagca ggtcctggec aacaagagec gectctgggt 1081tgaggaagaa gtctggcgga tggagatata cctgtccctg ggtgtgetgg ctctgggeat 1141 gctgtcactg ctggcggtta cetcgatccc ttccattgca aactcactca actggaagga 1201 gttcagcttt gtgcagtcca cgctgggctt cgtggccetg atgctgagca caatgcacac 1261 cctcacctac ggctggacce gtgetttga ggaaaaccac tacaagttct acctgccace 1321 cacattcacg ctcacgetge tectgccetg tgtcatcatc ctggccaagg gectcttcct 1381 cctgccetge ctcagccaca gactcaccaa yatccgcagg ggctgggaga gggatggtgc 1441 cgtcaagttc atgctgeccg ctggecacac acaggggyag aaaacaagcc acgtgtgagg 1501 ccctggaaat ggagacaggc acagcttgtg ggggecctgg getgggttcg ggtctettt

1561 ctgggatggt atatgegtgg gtggecgagg tetgaatttc tgggatgcag gtgtatgceg
1621 agatactcag aatggcgtac cacacatgcg ataagtactc acatatattt catatataat
1681aggatttact attattctta gttaaaaaaa aatagtgggt ccttatattt caacttatgc
174 lagggtcccta tatticaact tgagcatttc agagcaaatg ccacacatta aacagcagat
1801 cccaccettg tggtagctgc agagacagac agaaacttct ggttatgaga gagactgtat
1861 tttgttggat tctacettta atccecgitc tctacgttce ectgttagec acatcttaac
1921gttggtgcag agctgggaca agagetggct ctggtgcagc ctcccccatc ccagggctag
1981 gaaacaagce tctgatgaac agagggacca ggtctggace ctcctgctce cycttcectg
2041 ggctcyagtg gggaggetca gegggatcce ccgeaatctg tgcaggagtt ttcacaggtc
2101 tgtccttct tccgggagcg gtctgaageg gecceatctg atcetagetg agcogagatt
2161 gttccecact cectgaaagt ccagagtcac egtggagect geaaattget cettctgega
2221 aggtgtgaag tcaccgtctc accagagcea traacgaacc tgatettcag aagaageata
2281 attgittcec ctccattaag ttggtggtga ccctctttaa accactgtge cttctegect
2341 ttcceatcac taatttgggc atctccatgg agtggactct tgtcggggea gttcaggggg
2401 gagggaagca ttagagattg cggagaataa ccatcgaagc ctcccttgga tgttcccagg
2461 cgtgccttca ttaaattggt ccctaatgag aatgacaggg gaccectgtt gcctgtatgc
252 lagagaaceag cettctgage acccaggaaa cacagtgycc ccacgcectt caggggggtc
2581 ccacgtccce ttteccatge tttgectec ctcectcccg gttacaatca accataaang
2641 tctgcaaaata ttgtttttg aattcttaaa gagaccacat cctttgttat taccaaaaaa
2701aaaaaaaaaa aacc (SEQ ID No.: 5)

In another embodiment the nucleic acid encodes an amino acid sequence having the sequence as set forth in SEQ ID NO 6. In one embodiment the the amino acid fragment comprises a fragment which begins at the amino acid at position 1 of SEQ ID NO 6 and ends at position 101. In one embodiment the amino acid fragment comprises a fragment which begins at the amino acid at position 1 of SEQ ID NO 6 and ends at position 80. In one embodiment the amino acid fragment comprises a fragment which begins at the amino acid at position 1 of SEQ ID NO 2 and ends at position 60. In one embodiment the the amino acid fragment comprises a fragment which begins at the amino acid at position 61 of SEQ ID NO 6 and ends at position 241. In one embodiment the amino acid fragment comprises a fragment which begins at the amino acid at position 81 of SEQ ID NO 6 and ends at position 361. In one embodiment the amino acid fragment comprises a fragment which begins at the amino acid at position 81 of SEQ ID NO 2 and ends at position 421. In another embodiment the amino acid has at least $70 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 6. In another embodiment the amino acid has at least $75 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 6. In another embodiment the amino acid has at least $80 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 6 . In one embodiment the amino acid has at least $85 \%$ similarity with the nucleic acid coding sequence of SEQ

ID NO 6. In another embodiment the amino acid has at least $90 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 6. In another embodiment the amino acid has at least $95 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO 6.

Rat p-Hyde amino acid sequence:
1 MSGEMDKPLI SRRLVDSDGS LAEVPKEAPK VGILGSGDFA RSLATRLVGS GFFVVVGSRN
61 PKRTAGLFPS LAQVTFQEEA VSSPEVIFVA VFREHYSSLC SLADQLAGKI LVDVSNPTEK
121 ERLQHRQSNA EYLASLFPAC TVVKAFNVIS AWALQAGPRD GNRQVLICGD QLEAKHTVSE
181 MARAMGFTPL DMGSLASARE VEAIPLRLLP SWKVPTLLAL GLSTQSYAYN FIRDVLQPYI
241 RKDENKFYKM PLSVVNTTIP CVAYVLLSLV YLPGVLAAAL QLRRGTKYQR FPDWIDHWLQ
301 HRKQIGLLSF FFAMLHALYS FCLPLRRSHR YDLVNLAVKQ VLANKSRLWV EEEVWRMEIY
361 LSLGVLALGM LSLLAVTSIP SIANSLNWKE FSFVQSTLGF VALMLSTMHT LTYGWTRAFE
42I ENHYKFYLPP TFTLTLLLPC VIILAKGLFL LPCLSHRLTK IRRGWERDGA VKFMLPAGHT
481 QGEKTSHV* (SEQ ID NO: 6)

Further this invention provides an isolated nucleic acid which encodes an amino acid sequence tcectggecacacgectggtgggetctggcttc (SEQ ID NO: 7). Further this invention provides an isolated nucleic acid which encodes an amino acid sequence: AAPCVAYVLLSLVYLPGVLAAALQLRRGTKYQRFPDWLDHWLQHRKQIGLLSF F (SEQ ID NO: 8). Further this invention provides an isolated nucleic acid which encodes an anino acid sequence NFIRDVLQPYIRKDENK.

The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence of SEQ ID NO 1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7, due to degeneracy of the genetic code and thus encode the same p-Hyde protein as that encoded by the nucleotide sequence shown in SEQ ID NO: 1, SEQ ID NO:3, SEQ ID NO:5,or SEQ ID NO:7. It will be appreciated by those skilled in the art that DNA sequence polymorphisms that lead to changes in the amino acid sequences of $p$-Hyde may exist within a population (e.g., the human population). Such genetic polymorphism in the p-Hyde gene may exist among individuals within a population due to natural allelic variation. An allele is one of a group of genes which occur alterratively at a given genetic locus. Such natural allelic variations can typically result in $1-5 \%$ variance in the nucleotide sequence of the p-Hyde gene. Altennative alleles can be identified by sequencing the gene of interest in a number of different individuals. This can be readily carried out by using hybridization probes to identify the same genetic locus in a variety of individuals. Any and all such nucleotide variations and resulting amino acid
polymorphisms in p-Hyde that are the result of natural allelic variation and that do not alter the functional activity of p -Hyde are intended to be within the scope of the invention. Moreover, nucleic acid molecules encoding $p$-Hyde proteins from other species ( $p$-Hyde homologues), which have a nucleotide sequence which differs from that of a human p-Hyde, are intended to be within the scope of the invention. Nucleic acid molecules corresponding to natural allelic variants and homologues of the p-Hyde cDNA of the invention can be isolated based on their identity to the human p-Hyde nucleic acids disclosed herein using the human cDNAs, or a portion thereof, as a hybridization probe according to standard hybridization techniques under stringent hybridization conditions. For example, splice variants of human and mouse p-Hyde cDNA can he isolated based on identity to human and mouse p-Hyde.
A.s used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least $60 \%$ $(65 \%, 70 \%$, preferably $75 \%$ ) identical to each orher typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in Current Protocols in Molecular Biology, John Wiley \& Sons, N.Y. (1989), 6.3.1-6.3.6. A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 450 C , followed by one or more washes in $0.2 \mathrm{X} \mathrm{SSC}, 0.1 \%$ SDS at $50-650 \mathrm{C}$. Preferably, an isolated nucleic acid molecule of the invention that hybridizes under stringent conditions to the coding or non-coding (or "sense" or "anti-sense") sequence of SEQ ID NO:1, SEQ ID NO:2, SEQ ID NO:5, or SEQ ID NO:7, corresponds to a naturally- occuring nucleic acid molecule. As used herein, a "naturally-occurring" nucleic acid molecule refers to an RNA or DNA molecule having a nucleotide sequence that occurs in nature (e.g., encodes a natural protein). In addition to naturally-occurring allelic variants of the p -Fyyde sequence that may exist in the population, the skilled artisan will further appreciate that changes can be introduced by mutation into the nuclectide sequence of SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5, or SEQ ID NO:7, thereby leading to changes in the amino acid sequence of the encocled p-Hyde protein, without altering the biological ability of the p-Hyde protein. Mutations cau be introduced by standard techniques, such as site-directed
mutagenesis and PCR-mediated mutagenesis. Preferably, conservative amino acid substitutions are made at one or more predicted non- essential amino acid residues.

A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of annino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta- branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted nonessential amino acid residue in P-Hyde is preferably replaced with another amino acid residue from the same side chain family. Alternatively, mutations can be introduced randomly along all or part of a P-Hyde coding sequence, such as by saturation mutagenesis, and the resultant mutants can be screened for P-Hyde biological activity to identify mutants that retain activity. Following mutagenesis, the encoded protein can be expressed recombinantly and the activity of the protein can be determined.

In order to avoid severely reducing or eliminating biological activity, amino acid residues that are conserved among the p-Hyde proteins of various species are not altered (except by conservative substitution). Both murime and human p-Hyde protein have a conserved pattern of six cysteine residues. Such conserved domains and cysteine residues are less likely to be amenable to mutation. Other amino acid residues, however, (e.g., those that axe not conserved or only semi-conserved among p-Hyde of various species e.g., between murine and human p-Hyde) may not be essential for activity and thus are likely to be amenable to alteration. Accordingly, another aspect of the invention pertains to nucleic acid molecules encoding p-Hyde proteins that contain changes in amino acid residues that are not essential for activity. Such p-Hyde proteins differ in amino acid sequence from SEQ ID NO:2 or SEQ ID NO:4 yet retain biological activity. In one embodiment, the isolated nucleic acid molecule includes a nucleotide sequence encoding a protein that includes an amino acid sequence that is at least about $84 \%$
identical, $85 \%, 90 \%, 95 \%$, or $98 \%$ identical to the amino acid sequence of SEQ ID NO:2, 4, 6, or 8 .

The nucleotide encoding p-Hyde includes RNA, cDNA, genomic DNA, synthetic forms, and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for cxample, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog, internucleotide modifications such as uncharged linkages (e.g., methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), charged linkages (e.g., phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g., polypeptides), intercalators (e.g., acridine, psoralen, etc.), chelators, alkylators, and modified linkages (e.g., alpha anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic nucleotides in their ability to bind to a designated sequence via hydrogen bonding and other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule. substantially homologous to primary structural sequence but which include, e.g., in vivo or in vitro chemical and biochemical modifications or which incorporate unusual amino acids. The nucleic acid may be modified. Such modifications include, for example, acetylation, carboxylation, phosphorylation, glycosylation, ubiquitination, labeling, e.g., with radionuclides, and various enzymatic modifications, as will be readily appreciated by those well skilled in the art. A variety of methods for labeling polypeptides and of substituents or labels useful for such purposes are well known in the art, and include radioactive isotopes such as 32 P , ligands which bind to labeled antiligands (e.g., antibodies), fluorophores, chemiluminescent agents, enzymes, and antiligands which can serve as specific binding pair members for a labeled ligand. The choice of label depends on the sensitivity required, ease of conjuyation with the primer, stability requirements, and available instrumentation. Methods of labeling polypeptides are well known in the art. Sce, e.g., Sambrook et al., 1989 or Ausubel et al., 1992. Besides substantially full-length p-Hyde, the present invention provides for biologically active fragments of the p-hyde which are known to those skilled in the art.

As defined herein an "isolated" or "substantially pure" nucleic acid (e.g., an RNA, DNA or a mixed polymer) is one which is substantially separated from other cellular components which naturally accompany a native human sequence or protein, e.g., ribosomes, polymerases, many other human genome sequences and proteins. The term embraces a nucleic acid sequence or protein which has been removed from its naturally occurring environment, and includes recombinant or cloned DNA isolates and chemically synthesized analogs or analogs biologically synthesized by heterologous systems.
"p-Hyde Allele" refers to normal alleles of the p-Hyde locus as well as alleles carrying variations that predispose individuals to develop cancer of many sites including, for example, breast, ovarian, colorectal and prostate cancer. Such predisposing alleles are also called "p-Hyde susceptibility alleles".
"p-Hyde Locus," "p-Hyde Gene," "p-Hyde Nucleic Acids" or "p-Hyde Polynucleotide" each refer to polynucleotides, all of which are in the p-Hyde region, that are likely to be expressed in normal tissue, certain alleles of which predispose an individual to develop breast, ovarian, colorectal and prostate cancers which have p-Hyde activity.. Mutations at the p-Hyde locus may be involved in the initiation and/or progression of other types of tumors. The locus is indicated in part by mutations that predispose individuals to develop cancer. These mutations fall within the p -Hyde region described infra. The p-Hyde locus is intended to include coding sequences, intervening sequences and regulatory elements controlling transcription and/or translation. The p-Hyde locus is intended to include all allelic variations of the DNA sequence.

A "nucleic acid" refers to the phosphate ester polymeric form of ribonucleosides (adenosine, guanosine, uridine or cytidine; "RNA molecules") or deoxyribonucleosides (deoxyadenosine, deoxyguanosine, deoxythymidine, or deoxycytidine; "DNA molecules") in either single stranded form, or a double-stranded helix. Double stranded DNA-DNA, DNA-RNA and RNA-RNA helices are possible. The term nucleic acid
molecule, and in particular DNA or RNA molecule, refers only to the primary and secondary structure of the molecule, and does not lim it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear or circular DNA molecules (e.g., restriction fragments), plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the nomal convention of giving only the sequence in the $5^{\prime}$ to $3^{\prime}$ direction along the nontranscribed strand of DNA (i.c., the strand having a sequence homologous to the mRNA). A "recombinant DNA" is a DNA that has undergone a molecular biological manipulation.

The pluase "nucleic acid encoding" refers to a nucleic acid molecule which directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA strand sequence that is transcribed into RNA and the RNA sequence that is translated into protein. The nucleic acid molecule iuclude both the full length nucleic acid sequences as well as non-full length sequences derived from the full length protein. It being further understood that the sequence includes the degenerate codons of the native sequence or sequences which may be introduced to provide codon preference in a specific host cell
"Recombinant nucleic acid" is a nucleic acid which is not naturally occurring, or which is made by the artificial combination of two otherwise separated segments of sequence. This artificial combination is often accomplished by either chemical synthesis means, or by the artificial manipulation of isolated segments of nucleic acids, e.g., by genetic engineering techniques. Such is usually done to replace a codon with a redundant codon encoding the same or a conservative amino acid, while typically introducing or removing a sequence recognition site. Altematively, it is performed to join together nucleic acid segments of desired functions to generate a desired combination of functions.

As used herein, the term "cancer cell" means a tissue that grows by cellular proliferation more rapidly than normal, e.g., more rapidly than adjoining cells, or other cells in the tissue. Neoplastic cells continue to grow after growth stimuli cease. Generally, tumors
represent or form a distinct mass of tissue. The present invention relates to both types of tumors, but is particularly valuable in the treatment of cancers.

In one embodiment the cancer cells are selected from a group consisting of: melanoma; lymphoma; leukemia; and prostate, colorectal, pancreatic, breast, brain, or gastric carcinoma. Examples of tumors include but are not limited to: include sarcomas and carcinomas such as, but not limited to: fibrosarcoma, myxosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's sarcoma, leiomyosarcoma, rhabdomyosarcoma, colon carcinoma, pancreatic cancer, breast cancer, ovarian cancer, prostate cancer, squamous cell carcinoma, basal cell carcinoma, adenocarcinoma, sweat gland carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinomas, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilms' tumor, cervical cancer, germ tumor, non-small cell lung carcinoma, small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, and retinoblastoma. In a preferred embodiment the tumor is a melanoma or a prostate cell.

Mutations can be made in a nucleic acid encoding p-Hyde such that a particular codon is changed to a codon which codes for a different amino acid but the induction of susceptibility to cell death is maintained. Such a mutation is generally made by making the fewest nucleotide changes possible. A substitution mutation of this sort can be made to change an amino acid in the resulting protein in a non-conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to another grouping) or in a conservative manner (i.e., by changing the codon from an amino acid belonging to a grouping of amino acids having a particular size or characteristic to an amino acid belonging to the same grouping). Such a conservative change generally leads to less change. in the structure and function of the resulting protein. A non-conservative change
is more likely to alter the structure, activity or function of the resulting protein. The present invention should be considered to include sequences containing conservative changes which do not significantly alter the activity or binding characteristics of the resulting protein. Substitutes for an amino acid within the sequence may be selected from other members of the class to which the amino acid belongs. For example, the nonpolar (hydrophobic) amino acids include alanine, leucine, isoleucine, valine, proline, phenylalanine, tryptophan and methionine. Amino acids containing aromatic ring structures are phenylalanine, tryptophan, and tyrosine. The polar neutral amino acids include glycine, serine, threonine, cysteine, tyrosine, asparagine, and glutamine. The positively charged (basic) amino acids include arginine, lysine and histidine. The negatively charged (acidic) amino acids include aspartic acid and glutamic acid. Such alterations will not be expected to affect apparent molecular weight as determined by polyacrylamide gel electrophoresis, or isoelectric point. This isolated nucleic acid also encodes mutant p-Hyde or the wildtype protein.

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operably linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to p-Hyde mRNA. Regulatory sequences operably linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a vatiety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type sjecific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub et al. (Reviews - Trends in Genetics, Vol. 1(1) 1986).

This invention provides for a replicable vector comprising the isolated nucleic acid molecule of the DNA virus. The vector includes, but is not limited to: a plasmid,
cosmid, phage or yeast artificial chromosome (YAC) which contains at least a portion of the isolated nucleic acid molecule. As an example to obtain these vectors, insert and vector DNA can both be exposed to a restriction enzyme to create complementary ends on both molecules which base pair with each other and are then ligated together with DNA ligase. Alternatively, linkers can be ligated to the insert DNA which correspond to a restriction site in the vector DNA, which is then digested with the restriction enzyme which cuts at that site. Other means are also available and known to an ordinary skilled practitioner. In one embodiment the adenovirus vector is a replication-deficient adenovirus type 5 expression vector. In another exnbodiment the adenovirus vector comprises an adenovirus genome having a deletion in the E1 and E3 region of the genome and an insertion within the region of a nucleic acid encoding p -Hyde under the control of a promoter. The promoter may be a Rous Sarcoma virus promoter.

Knowledge of the genetic organization of adenovirus, a 36 kB , linear and double-stranded DNA virus, allows substitution of a large piece of adenoviral DNA with foreign sequences up to 7 kB (Grunhaus and Horwitz, 1992). In contrast to retrovirus, the infection of adenoviral DNA into host cells does not result in chromosomal integration because adenoviral DNA can replicate in an episomal manner without potential genotoxicity. Also, adenoviruses are structurally stable, and no genome rearrangement has been detected after extensive amplification. Adenovirus can infect virtually all epithelial cells regardless of their cell cycle stage. So far, adenoviral infection appears to be linked only to mild disease such as acute respiratory disease in the buman.

Adenovirus is particulauly suitable for use as a gene transfer vector because of its mid-sized genome, ease of manipulation, high titer, wide target-cell range, and high infectivity. Both ends of the viral genome contain 100-200 base pair Cop) inverted terminal repeats (ITR), which are cis elements necessary for viral DNA replication and packaging. The early ( $E$ ) and late ( $L$ ) regions of the genome contain different transcription units that are divided by the onset of viral DNA replication. The E1 region (E1A and E1B) encodes proteins responsible for the regulation of transcription of the viral genome and a few cellular genes. The expression of the E2 region (E2A and E2B)
results in the synthesis of the proteins for viral DNA replication. These proteins are involved in DNA replication, late gene expression, and host cell shut off (Renan, 1990) The products of the late genes, including the majority of the viral capsid proteins, are expressed only after significant processing of a single primary transcript issued by the major late promoter (MLP). The MLP (located at $16.8 \mathrm{~m} . \mathrm{u}$.) is particularly efficient during the late phase of infection, and all the mRNAs issued from this promotet possess a 5 ' tripartite leader (TL) sequence which makes them preferred mRNAs for translation.

In the current system, recombinant adenovirus is generated from homologous recombination between shuttle vector and provirus vector. Due to the possible recombination between two proviral vectors, wild-type adenovirus may be generated from this process. Therefore, it is critical to isolate a siugle clone of vinus from an individual plaque and examine its genomic structure. Use of the YAC system is an alternative approach for the production of recombinant adenovirus.

Generation and propagation of the current adenovirus vectors, which are replication deficient, depend on a unique helper cell line, designated 293, which was transformed from human embryonic kidney cells by AdS DNA fragments and constitutively expresses El proteins (Graham, et al., 1977). Since the Ej region is dispensable from the adenovirus genome (Jones and Shenk, 1978), the current adenovirus vectots, with the help of 293 cells, carry foreign DNA in either the E1, the E3 or both regions (Graham and Prevec, 1991). In nature, adenovirus can package approximately $105 \%$ of the wild-type genome (Ghosh-Choudhury, et al., 1987), providing capacity for about 2 extra kB of DNA. Combined with the approximately 5.5 kB of DNA that is replaceable in the E1 and E3 regions, the maximun capacity of the current adenovirus vector is under 7.5 kB , or about $15 \%$ of the total length of the vector. More than $80 \%$ of the adenovirus viral genoxne remains in the vector backbone and is the source of vector-borne cytotoxicity. Also, the replication deficiency of the E1 deleted virus is incomplete. For example, leakage of viral gene expression has been observed with the currently available adenovirus vectors at high multiplicities of infection (Mulligan, 1993).

Helper cell lines may be derived from human cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other buman embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are pernissive for human adenovirus. Such cells include, e.g., Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293.

Other than the requirement that the adenovirus vector be replication defective, or at least conditionally defective, the nature of the adenovirus vector is not believed to be crucial to the successful practice of the invention. The adenovirus may be of any of the 42 different known serotypes or subgroups A-F. Adenovirus type 5 of subgroup $C$ is the preferred starting material in order to obtain the conditional replication-defective adenovirus vector for use in the method of the present invention. This is because Adenovirus type 5 is a human adenovirus about which a great deal of biochemical and genetic information is known, and it has historically been used for most constructions employing adenovirus as a vector.

As stated above, the typical vector according to the present invention is replication defective and will not have an adenovirus El region. Thus, it will be most convenient to introduce the nucleic acid encoding p-Hyde at the position from which the El coding sequences have been removed. However, the position of insertion of the $p$-Hyde coding region within the adenovirus sequences is not critical to the present invention. The nucleic acid encoding a p-Hyde transcription unit also may be inserted in lieu of the deleted E3 region in E3 replacement vectors as described previously by Karlsson et. al. (1986) or in the E4 region where a helper cell line or helper virus complements the E4 defect.

Adenovirus is easy to grow and manipulate and exhibits broad host range in vitro and in vivo. This group of viruses can be obtained in high titers, e.g., $10^{9}-10^{11}$ plaque-forming unit (PFU)/ml, and they are highly infective. The life cycle of adenovirus does not require integration into the host cell genome. The foreign genes delivered by adenovirus vectors are episomal, and therefore, have low genotoxicity to host cells. No side effects
have been reported in studies of vaccination with wild-type adenovirus (Couch et al., 1963; Top et al., 1971), demonstrating their safety and therapeutic potential as in vivo gene transfer vectors.

Adenovirus vectors have been used in eulkaryotic gene expression (Levrero et al., 1991; Gomez-Foix et al., 1992) and vaccine development (Grunhaus and Horwitz, 1992; Grahann and Prevec, 1992). Recently, animal studies suggested that recombinant adenovirus could be used for gene therapy (Stratford-Perricaudet and Perricaudet, 1991; Stratford-Penricaudct et al., 1990; Rich et al., 1993). Experiments in administering recombinant adenovirus to different tissues include trachea instillation (Rosenfeld et al., 1991; Rosenfeld et al., 1992), muscle injection (Ragot et al., 1993), peripheral intravenous injection (Herz and Gerard, 1993), and stereotactic inoculation into the brain (Le Gal La Salle et al., 1993).

An appropriate promoter and other necessary vector sequences will be selected so as to be functional in the host, and may include, when appropriate, those naturally associated with p-Hyde genes. Examples of workable combinations of cell lines and expression vectors are described in Sambrook et al., 1989 or Ausubel et al., 1992; see also, e.g., Metzger et al., 1988. Many useful vectors are known in the art and may be obtained from such vendors as Stratagene, New England Biolabs, Promega Biotech, and others. Promoters such as the trp, lac and phage promoters, tRNA promoters and glycolytic enzyme promoters may be used in prokaryotic hosts. Useful yeast promoters include promoter regions for metallothionein, 3-phosphoglycerate kinase or other glycolytic enzymes such as enolase or glyceraldehyde-3-phosphate dehydrogenase, enzymes responsible for maltose and galactose utilization, and others. Vectors and promoters suitable for use in yeast expression are further described in Hitzeman et al., EP 73,675A. Appropriate non-native mammalian promoters might include the early and late promoters from SV40 (Fiers et al., 1978) or promoters derived from murine Moloney leukemia virus, mouse minor virus, avian sarcoma viruses, adenovirus II, bovine papilloma virus or polyoma. In addition, the construct may be joined to aux amplifiable gene (e.g., DHFR) so that multiple copies of the gene may be made. For appropriate enhancer and other expression control sequences, see
also Enhancers and Eukaryotic Gene Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. (1983).

While such expression vectors may replicate autonomously, they may also replicate by being inserted into the genome of the host cell, by methods well known in the art. Expression and cloning vectors will likely contain a selectable marker, a gene encoding a protein necessary for survival or growth of a host cell transformed with the vector. The presence of this gene ensures growth of only those host cells which express the inserts. Typical selection genes encode proteins that a) confer resistance to antibiotics or other toxic substances, e.g. ampicillin, neomycin, methotrexate, etc.; b) complement auxotrophic deficiencies, or c) supply critical nutrients not available from complex media, e.g., the gene encoding D-alanine racemase for Bacilli. The choice of the proper selectable marker will depend on the host cell, and appropriate markers for different hosts are well known in the art.

The vectors containing the nucleic acids of interest can be transcribed in vitro, and the resulting RNA introduced into the host cell by well-known methods, e.g., by injection (see, Kubo et al., 1988), or the vectors can be introduced directly into host cells by methods well known in the art, which vary depending on the type of cellular host, including electroporation; transfection employing calcium chloride, rubidium chloride. calcium phosphate, DEAE-dextran, or other substances; microprojectile bombardment; lipofection; infection (where the vector is an infectious agent, such as a retroviral genome); and other methods. See generally, Sambrook et al., 1989 and Ausubel et al., 1992. The introduction of the polynucleotides into the host cell by any method known in the art, including, inter alia, those described above, will be referred to herein as "transformation." The cells into which have been introduced nucleic acids described above are meant to also include the progeny of such cells.

Regulatory elements required for expression include promoter or enhancer sequences to bind RNA polymerase and transcription initiation sequences for ribosome binding. For example, a bacterial expression vector includes a promoter such as the lac promoter and for transcription initiation the Shine-Dalgarno sequence and the start codon AUG.

Similarly, a eukaryotic expression vector includes a hetcrologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained commercially or assembled from the sequences described by methods well-known in the art, for example the methods described above for constructing vectors in general.

Viral promoters, cellular promoters/enhancers and inducible promoters/enhancers that could be used in combination with the nucleic acid encoding a p-Hyde in an expression construct include but are not limited to the following: Inmunoglobulin Heavy Chain; Tmmunoglobulin Light Chain; T-Cell Receptor; HLA DQ alpha and DQ beta; beta -Interferon; Interleukin-2; Interleukin-2 Receptor; MIIC Class II 5 alpha; MHC Class II HLA-DR alpha; beta -Actin; Muscle Creatine Kinase; Prealbumin (Transthyretin); Elastase I; Metallothionein; Collagenase; Albumin Gene; alpha -Fetoprotein; tau -Globin; beta -Globin; c-fos; c-HA-ras; Neural Cell Adhesion Molecule (NCAM); alpha 1-Antitrypsin; H2B (TH2B) Histonc; Mouse or Type I Collagen; Glucose-Regulated Proteins (GRP94 and GRP78); Rat Growth Hormon; Human Serum Amyloid A (SAA); Troponin I (TN I); Platelet-Derived Growth Factor; Duchenne Muscular; SV40; Polyoma; Retroviruses; Papilloma Virus; Hepatitis B Virus; Human Immunodeficiency Virus; Cytomegalovirus; Gibbon Ape Leukemia Virus; MT II; MMTV (mouse mammary Glucocorticoids; Adenovirus 5 E2.

Additionally any promoter/enhancer combination (as per the Eukaryotic Promoter Data Base EPDB) could also be used to drive expression of a p-Hyde. Use of a T3, T7 or SP6 cytoplasmic expression system is another possible embodiment. Eukaryotic cells can support cytoplasmic transcription from certain bacterial promoters if the appropriate bacterial polymerase is provided, either as part of the delivery complex or as an additional genetic expression construct.

This invention provides a host cell containing the above vector. The host cell may contain the isolated DNA molecule artificially introduced into the host cell. The host cell may be a eukaryotic or bacterial cell (such as E.coli), yeast cells, fungal cells, insect
cells and animal cells. Suitable animal cells include, but are not limited to Vero cells, HeLa cells, Cos cells, CV1 cells and various primary mammalian cells.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein. A host cell can be any prokaryotic or eukaryotic cell. For example, p-Hyde protein can be expressed in bacterial cells such as E. coli, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells ( CHO ) or COS cells) other suitable host cells are known to those skilled in the art. Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co- precipitation, DEAE-dextran-mediated transfection, Iipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (supra), and other laboratory manuals. For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (e.g., for resistance to antibiotics) is generally introduced into the host cells along with the gene of interest.

Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding p-Hyde or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (e.g., cells that have incorporated the selectable marker gene will survive, while the other cells die). A host cell of the invention, such as
a prokaryotic or eukaryotic host cell in culture, can be used to produce (i.e., express) p-Hyde protein.

Accordingly, the invention further provides methods for producing $p$-Hyde protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding p-Hyde has been introduced) in a suitable medium such that $p$-Hyde protein is produced. In another embodiment, the method further comprises isolating p -Hyde from the medium or the host cell. The host cells of the invention can also be used to produce nonhuman transgenic animals.

For example, in one embodiment, a host cell of the invention is a fertilized oocyte or an embryonic stem cell into which p-Hyde-coding sequences have been introduced. Such host cells can then he used to create non-human traxsgenic animals in which exogenous p-Hyde sequences have been introduced iuto their genome or homologous recombinant animals in which endogenous $p$-Hyde sequences have been altered. Such animals are useful for studying the function and/or activity of p-Hyde and for identifying and/or evaluating modulators of p-Hyde activity. As used herein, a "transgenic animal', is a non-human animal, preferably a mammal, more preferably a rodent such as a rat or mouse, in which one or more of the cells of the animal includes a transgene. Other examples of transgenic animals include non-human prinnates, sheep, dogs, cows, goats, chickens, amphibians, etc.

A transgenic anjimal of the invention can be created by introducing p -Hyde-encoding nucleic acid into the male pronuclei of a fertilized oocyte, e.g., by microinjection, retroviral infection, and allowing the oocyte to develop in a pseudopregnant female foster animal. The p-Hyde cDNA sequence e.g., that of (SEQ ID NO:1,3, 5, or 7) can be introduced as a transgene into the genome of a mon-human animal. Alternatively, a nonhuman homologue of the human p-Hyde gene, can be isolated based on hybridization to the human p -Hyde cDNA and used as a transgene. Intronic sequences and polyadenylation signals can also be included in the transgene to increase the efficiency of expression of the transgene. A tissue-specific regulatory sequence(s) can be
operably linked to the p-Hyde transgene to direct expression of p -Hyde protein to particular cells. Methods for generating transgenic animals via embryo manipulation and microinjection, particularly animals such as mice, have become conventional in the art and are described, for example, in U.S. Patent Nos. 4,736,866 and 4,870,009, U.S. Patent No. 4,873,191 and in Hogan, Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Similar methods are used for production of other transgenic animals. A transgenic founder animal can be identified based upon the presence of the p-Hyde transgene in its genome and/or expression of p-Fyde mRNA in tissues or cells of the animals. A transgenic founder animal can then be used to breed additional animals carrying the transgene. Moreover, transgenic animals carrying a trausgene encoding p -Hyde can further be bred to other transgenic animals carrying other transgenes.

To create an homologous recombinant animal, a vector is prepared which contains at least a portion of a p-Hyde gene (e.g., a human or a non-human homolog of the p-Hyde gene, e.g., a murine p-Hyde gene) into which a deletion, addition or substitution has been introduced to thereby alter, e.g., functionally disrupt, the p-Hyde gene. In a preferred embodiment, the vector is designed such that, upon homologous recombination, the endogenous p-Hyde gene is functionally disrupted (i.e., no longer encodes a functional protein; also referred to as a "lurock out" vector). Alternatively, the vector can be designed such that, upon homologous recombination, the endogenous p -Hyde gene is mutated or otherwise altered but still encodes functional protein (e.g., the upstream regulatory region can be altered to thereby alter the expression of the endogenous p -Fyde protein).

The term "vector", refers to viral expression systems, autonomous self-replicating circular DNA (plasmids), and includes both expression and nonexpression plasmids. Where a recombinant microorganism or cell culture is described as hosting an "expression vector," this includes both extrachromosomal circular DNA and DNA that has been incorporated into the host chromosome(s). Where a vector is being maintained by a host cell, the vector may either be stably replicated by the cells during mitosis as an autonomous structure, or is incorporated within the host's genome.

The term "plasmid" refers to an autonomous circular DNA molecule capable of replication in a cell, and includes both the expression and nonexpression types. Where a recombinant microorganism or cell culture is described as hosting an "expression plasmid", this includes latent viral DNA integrated into the host chromosome(s). Where a plasmid is being maintained by a host cell, the plasmid is either being stably replicated by the cells during mitosis as an autonomous structure or is incorporated within the host's genome.

The following tems are used to describe the sequence relationships between two or more nucleic acid molecules or polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparison; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing or may comprise a complete cDNA or gene sequence.

Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) Adv. Appl. Math. 2:482, by the homology alignment algorithm of Needleman and Wunsch (1970) J. Mol. Biol. 48:443, by the search for similarity method of Pearson and Lipman (1988) Proc: Natl. Acad. Sci. (USAA) 85:2444, or by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Dr., Madison, WI).
"Substantial identity" or "substantial sequence identity" mean that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap which share at least 90 percent sequence identity, preferably at least 95 percent sequence identity, more preferably at least 99 percent sequence identity or more. "Percentage amino acid identity" or "percentage amino acid sequence identity" refers to a comparison of the amino acids of two polypeptides which, when optimally aligned, have approximately the designated percentage of the same amino acids. For example,
" $95 \%$ amino acid identity" refers to a comparison of the amino acids of two polypeptides which when optimally aligned have $95 \%$ amino acid identity. Preferably, residue positions which are not identical differ by conservative amino acid substitutions. For example, the substitution of amino acids having similar chemical properties such as charge or polarity are not likely to effect the properties of a protein. Examples include glutamine for asparagine or glutamic acid for aspartic acid.

The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (i.e., \% identity of identical positions/total \# of positions (e.g., overlapping) $\times 100$ ). Preferably, the two sequences are the same length. The determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) Proc. Natl. Acad. Sci. USA. 87:2264-2268, modified as in Karlin and Altschul (1993) Proc. Natl. Acad. Sci. USA 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul, et al. (1990) J. Mol. Biol. 215:403-410. BLAST nucleotide searches can be performed with the NBLAST program, score $=100$, wordlength $=12$ to obtain nucleotide sequences homologous to p-Hyde nucleic acid molecules of the invention. BLAST protein searches can be performed with the XI3LAST program, score $=50$, wordlength $=3$ to obtain amino acid sequences homologous to p-Hyde protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul et al., (1997) Nucleic Acids Res. :3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., X13LAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algoritum utilized for the comparison of sequences is the algorithm of Myers and Miller, CABIOS 4:11-17 (1988). Such an algorithm is incorporated into the ALIGN program (version 2.0 ) which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12 , and a gap penalty of 4 can be used. The percent identity between two sequences
can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, only exact matches are counted.

This invention provides a nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid of the human p-Hyde gene. Specifically, this invention provides an oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of nucleotides present within a nucleic acid which encodes the human p-Hyde. In one embodiment the nucleic acid is DNA or RNA. In another embodiment the oligonucleotide is labeled with a detectable marker. In another embodiment the oligonucleotide is a radioactive isotope, a fluorophor or an enzyme.

Oligonucleotides which are complementary may be obtained as follows: The polymerase chain reaction is then carried out using the two primers. See $P C R$ Protocols: A Guide to Methods and Applications [74]. Following PCR amplification, the PCR-amplified regions of a viral DNA can be tested for their ability to hybridize to the three specific nucleic acid probes listed above. Alternatively, hybridization of a viral DNA to the above nucleic acid probes can be performed by a Southern blot procedure without viral DNA amplification and under stringent hybridization conditions as described herein.

Oligonucleotides for use as probes or PCR primers are chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage and Carruthers [19] using an automated synthesizer, as described in Needham-VanDevanter [69]. Purification of oligonucleotides is by either native acrylamide gel electrophoresis or by anion-exchange YPPLC as described in Pearson, J.D. and Regnier, F.E. [75A]. The sequence of the synthetic oligonucleotide can be verified using the chemical degradation method of Maxam, A.M. and Gilbert, W. [63].

High stringent hybridization conditions are selected at about 5? C lower than the thermal melting point ( Tm ) for the specific sequence at a defined ionic strength and pH . The Tm is the temperature (under defined ionic strength and $\mathrm{p} H$ ) at which $50 \%$ of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions
will be those in which the salt concentration is at least about 0.02 molar at pH 7 and the temperature is at least about 60C. As other factors may significantly affect the stringency of hybridization, including, among others, base composition and size of the complementary strands, the presence of organic solvents, ie. salt or formamide concentration, and the extent of base mismatching, the combination of parameters is more important than the absolute measure of any one. For Example high stringency may be attained for example by ovemight hybridization at about 68C in a 6 x SSC solution, washing at room temperature with 6 x SSC solution, followed by washing at about 68 C in a $6 x$ SSC in a $0.6 x$ SSX solution.

Hybridization with moderate stringency may be attained for example by: 1) filter pre-hybridizing and hybridizing with a solution of $3 x$ sodium chloride, sodium citrate (SSC), $50 \%$ formamide, 0.1 M Tris buffer at Ph 7.5, 5 x Denhardt's solution; 2.) pre-hybridization at 37 ? C for 4 hours; 3) hybridization at 37 ? C with amount of labelled probe equal to $3,000,000 \mathrm{cpm}$ total for 16 hours; 4) wash in $2 \times$ SSC and $0.1 \%$ SDS solution; 5) wash $4 x$ for 1 minute each at room temperature at $4 x$ at 60 ? $C$ for 30 minutes each; and 6) dry and expose to film.

The phrase "selectively hybridizing to" refers to a nucleic acid probe that hybridizes, duplexes or binds only to a particular target DNA or RNA sequence when the target sequences are present in a preparation of total cellular DNA or RNA. By selectively hybridizing it is meant that a probe binds to a given target in a manner that is detectable in a different manner from non-target sequence under high stringency conditions of hybridization. in a different "Complementary" or "target" nucleic acid sequences refer to those nucleic acid sequences which selectively hybridize to a nucleic acid probe. Proper annealing conditions depend, for example, upon a probe's length, base composition. and the number of mismatches and their position on the probe, and must often be detemined empirically. For discussions of nucleic acid probe design and annealing conditions, see, for example, Sambrook et al., [81] or Ausubel, F., et al., [8].

It will be readily understood by those skilled in the art and it is intended here, that when reference is made to particular sequence listings, such reference includes sequences
which substantially correspond to its complementary sequence and those described including allowances for minor sequencing errors, single base changes, deletions, substitutions and the like, such that any such sequence variation corresponds to the nucleic acid sequence of the pathogenic organism or disease marker to which the relevant sequence listing relates.

The primer pairs of the present invention are useful for determination of the nucleotide sequence of a particular p-Hyde allele using PCR. The pairs of single-stranded DNA primers can be amealed to sequences within or surrounding the p -Hyde gene in order to prime amplifying DNA synthesis of the p-Hyde gene itself. A complete set of these primers allows synthesis of all of the nucleotides of the p -Hyde gene coding sequences, i.e., the exons. The set of primers preferably allows synthesis of both intron and exon sequences. Allele-specific primers can also be used. Such primers anneal only to particular p-Hyde mutant alleles, and thus will only amplify a product in the presence of the mutant allele as a template.

Nucleic acid probe technology is well known to those skilled in the art who readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule having the full-length or a fragment of the isolated nucleic acid molecule of the DNA virus into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transforned bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the full length or a fragment of the isolated nucleic acid molecule of the DNA virus downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with a linearized isolated nucleic acid molecule of the DNA virus or its fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

As defixed herein nucleic acid probes may be DNA or RNA fragments. DNA fragments can be prepared, for example, by digesting plasmid DNA, or by use of PCR, or synthesized by either the phosphoramidite method described by Beaucage and Carruthers, [19], or by the triester method according to Matteucci, et al., [62], both incorporated herein by refereuce. A double stranded fragment may then be obtained, if desired, by annealing the chemically synthesized single strands together under appropriate conditions or by synthesizing the complementary strand using DNA polymerase with an appropriate primer secuence. Where a specific sequence for a nucleic acid probe is given, it is understood that the complementary strand is also identified and included. The complementary strand will work equally well in situations where the target is a double-stranded nucleic acid. It is also understood that when a specific sequence is identified for use a nucleic probe, a subsequence of the listed sequence which is 25 basepairs or more in length is also encompassed for use as a probe.

The nucleic acid of the subject invention also include DNA molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a rerminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intemnediate DNA sequences that facilitate construction of readily expressed vectors.

Also, this invention provides an antisense molecule capable of specilically hybridizing with the isolated nucleic acid of the human p-Hyde gene. This invention provides an antagonist capable of blocking the expression of the peptide or polypeptide encoded by the isolated DNA molecule. In one embodiment the antagonist is capable of hybridizing
with a double stranded DNA molecule. In another embodiment the antagonist is a triplex oligonucleotide capable of hybridizing to the DNA molecule. In another embodiment the triplex oligonucleotide is capable of binding to at least a portion of the isolated DNA molecule with a nucleotide sequence.

The antisense molecule may be DNA or RNA or variants thereof (i.e. DNA or RNA with a protein backbone). The present invention extends to the preparation of antisense nucleotides and ribozymes that may be used to interfere with the expression of the receptor recognition proteins at the translation of a specific mRNA, either by masking that MRNA with an antisense nucleic acid or cleaving it with a ribozyme.

Antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific MRNA molecule. In the cell, they hybridize to that MRNA, forming a double stranded molecule. The cell does not translate an MRNA in this double-stranded forme. Therefore, antisense nucleic acids interfere with the expression of MRNA into protein.

Antisense nucleotides or polynucleotide sequences are useful in preventing or diminishing the expression of the p-Hyde gene, as will be appreciated by those skilled in the art. For example, polynucleotide vectors containing all or a portion of the p -Hyde gene or other sequences from the p -Hyde region (particularly those flanking the p-Hyde gene) may be placed under the control of a promoter in an antisense orientation and introduced into a cell. Expression of such an antisense construct within a cell will interfere with p -Hyde transcription and/or translation and/or replication. Oligomers of about fifteen nucleotides and molecules that hybridize to the AUG initiation codon are particularly efficient, since they are easy to synthesize and are likely to pose fewer problems than larger molecules upon introduction to cells.

This invention provides a transgexic nonhuman mammal which comprises at least a portion of the isolated DNA molecule introduced into the mammal at an embryonic stage. Methods of producing a transgenic nonhuman mammal are known to those skilled in the art.

This invention provides a polypeptide comprising the amino acid sequence of a human p-Hyde. In one embodiment the amino acid sequence is set forth in SEQ ID NOs. 2, 4, 6 or 8 . This invention provides a fusion protein or chimeric comprising the polypeptide. This invention provides an antibody which specifically binds to the polypeptide. In one embodiment the antibody is a monoclonal or polyclonal antibody.

The invention also provides p -Hyde chimeric or fusion proteins. As used herein, a p-Hyde "chimeric protein" or "fusion protein" comprises a p-Hyde polypeptide operably linked to a non-p-Hyde polypeptide. A "p-Hyde polypeptide" refers to a polypeptide having an amino acid sequence corresponding to p-Hyde, whereas a "non-p-Hyde polypeptidell refers to a polypeptide having an amino acid sequence corresponding to a protein which is not substantially identical to the p-Hyde protein, e.g., a protein wbich is different from the p-Hyde protein and which is derived from the same or a different organism. Within a p-Hyde fusion protein the p-Hyde polypeptide can correspond to all or a portion of a p-Hyde protein, preferably at least one biologically active portion of a p-Hyde protein.

Within the fusion protein, the term "operably linked" is intended to indicate that the p-Hyde polypeptide and the non-p-Hyde polypeptide are fused in-frame to each other. The non-p-Hyde polypeptide can, be fused to the N-terminus or C-terminus of the p-Hyde polypeptide. In yet another embodiment, the fusion protein is an p -Hyde-immunoglobulin fusion protein in which all or part of p-Hyde is fused to sequences derived from a member of the immunoglobulin protein family. The p-Hyde immunoglobulin fusion proteins of the invention can be incorporated into pharmaceutical compositions and administered to a subject.

The present invention further provides methods of preparing a polynucleotide comprising polymerizing nucleotides to yield a sequence comprised of at least eight consecutive nucleotides of the p -Hyde locus; and methods of preparing a polypeptide comprising polymerizing amino acids to yield a sequence comprising at least five amino acids encoded within the $p$-Hyde locus.

The present invention provides an isolated polynucleotide comprising all, or a portion of the p-Hyde locus or of a mutated p-Hyde locus. Such polynucleotides may be antisense polyuucleotides. The present invention also provides a recombinant construct comprising such an isolated polynucleotide, for example, a recombinant construct suitable for expression in a transformed host cell.

Also provided by the present invention are methods of detecting a polynucleotide comprising a portion of the p-Hyde locus or its expression product in an analyte. Such methods may further comprise the step of amplifying the portion of the p -Hyde locus, and may further include a step of providing a set of polynucleotides which are primers for amplification of said portion of the p-Flyde locus. The method is useful for cither diagnosis of the predisposition to cancer or the diagnosis or prognosis of cancer.

This invention also provides a method of producing a polypeptide encoded by isolated DNA molecule, which comprises growing the above host vector system under suitable
conditions permitting production of the polypeptide and recovering the polypeptide so produced.

Further, the isolated polypeptide encoded by the isolated DNA molecule may be linked to a second polypeptide encoded by a nucleic acid molecule to form a fusion protein by expression in a suitable host cell. In one embodiment the second nucleic acid molecule encodes beta-galactosidase. Other nucleic acid molecules which are used to form a fusion protein are known to those skilled in the art.

This invention provides an antibody which specifically binds to the polypeptide encoded by the isolated DNA molecule. In one embodiment the antibody is a monoclonal antibody. In another embodiment the antibody is a polyclonal antibody. The antibody or DNA molecule may be labelled with a detectable marker including, but not limited to: a radioactive label, or a colorimetric, a luminescent, or a fluorescent marker, or gold. Radioactive labels include, but are not limited to: ${ }^{3} \mathrm{H},{ }^{14} \mathrm{C},{ }^{32} \mathrm{P},{ }^{33} \mathrm{P} ;{ }^{35} \mathrm{~S},{ }^{36} \mathrm{Cl},{ }^{51} \mathrm{Cr},{ }^{57} \mathrm{Co}$, ${ }^{59} \mathrm{Co},{ }^{59} \mathrm{Fe},{ }^{90} \mathrm{Y},{ }^{125} \mathrm{I},{ }^{131} \mathrm{I}$, and ${ }^{186} \mathrm{Re}$. Fluorescent markers include but are not limited to: fluorescein, rhodamine and auramine. Colorimetric markers include, but are not limited to: biotin, and digoxigenin. Methods of producing the polyclonal or monoclonal antibody are known to those of ordinary skill in the art.

Further, the antibody or nucleic acid molecule complex may be detected by a second antibody which may be linked to an enzyme, such as alkaline phosphatase or horseradish peroxidase. Other enzymes which may be employed are well known to one of ordinary skill in the art.
"Specifically binds to an antibody" or "specifically immunoreactive with", when refexring to a protein or peptide, refers to a binding reaction which is determinative of the presence of the p-Hyde of the invention in the presence of a heterogeneous population of proteins and other biologics including viruses other than the p-Hyde. Thus, under designated immunoassay conditions, the specified antibodies bind to the p -Hydc antigens and do not bind in a significant amount to other antigens present in the sample. Specific binding to an antibody under such conditions may require an antibody
that is selected for its specificity for a particular protein. For example, antibodies raised to the human p-Hyde immunogen described herein can be selected to obtain antibodies specifically immunoreactive with the p -Hyde proteins and not with other proteins. These antibodies recognize proteins homologous to the human $p$-Hyde protein. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select monoclonal antibodies specifically immunoreactive with a protein. See Haylow and Lane [32] for a description of inmmonoassay formats and conditions that can be used to determine specific immunoreactivity.

This invention provides a method to select specific regions on the polypeptide encoded by the isolated DNA molecule of the DNA vinus to generate antibodies. The protein sequence may be determined from the cDNA sequence. Amino acid sequences may be analyzed by methods well known to those skilled in the art to determine whether they produce hydrophobic or hydrophilic regions in the proteins which they build. In the case of cell membrane proteins, hydrophobic regions are well known to form the part of the protein that is inserted into the lipid bilayer of the cell membrane, while hydrophilic regions are located on the cell surface, in an aqueous environment. Usually, the hydrophilic regions will be more immunogenic than the hydrophobic regions. Therefore the hydrophilic amino acid sequences may be selected and used to generate antibodies specific to polypeptide encoded by the isolated nucleic acid molecule encoding the DNA virus. The selected peptides may be prepared using commercially available machines. As an alternative, DNA, such as a cDNA or a fragment thereof, may be cloned and expressed and the resulting polypeptide recovered and used as an immunogen.

Polyclonal antibodies against these peptides may be produced by immunizing animals using the selected peptides. Monoclonal antibodies are prepared using hybridoma technology by fusing antibody producing $B$ cells from immunized animals with myeloma cells and selecting the resulting hybridoma cell line producing the desired antibody. Alternatively, monoclonal antibodies may be produced by in vitro techniques
known to a person of ordinary skill in the art. These antibodies are useful to detect the expression of polypeptide encoded by the isolated DNA molecule of the DNA virus in living animals, in humans, or in biological tissues or fluids isolated from animals or humans.

The antibodies may be detectably labelled, utilizing conventional labelling techniques well-known to the art. Thus, the antibodies may be radiolabelled using, for example, radioactive isotopes such as ${ }^{3} \mathrm{H},{ }^{125} \mathrm{I},{ }^{131} \mathrm{I}$, and ${ }^{35} \mathrm{~S}$. The antibodies may also be labelled using fluorescent labels, enzyme labels, free radical labels, or bacteriophage labels, using tecluniques known in the art. Typical fluorescent labels include fluorescein isothiocyanate, rhodamine, phycoerythrin, phycocyanin, alophycocyanin, and Texas Red.

Since specific enzymes may be coupled to other molecules by covalent links, the possibility also exists that they might be used as labels for the production of tracer materials. Suitable enzymes include alkaline phosphatase, beta-galactosidase, glucose-6-phosphate dehydrogenase, maleate dehydrogenase, and peroxidase. Two principal types of enzyme immunoassay are the enzyme-linked immunosorbent assay (ELISA), and the homogeneous enzyme immunoassay, also known as enzyme-multiplied immunoassay (EMIT, Syva Corporation, Palo Alto, CA). In the ELISA system, separation may be achieved, for example, by the use of antibodies coupled to a solid phase. The EMIT system depends on deactivation of the enzyme in the tracer-antibody complex; the activity can thus be measured without the need for a separation step.

Additionally, chemiluminescent compounds may be used as labels. Typical chemiluminescent compounds include luminol, isoluminol, aromatic acridinium esters, imidazoles, acridinium salts, and oxalate esters. Similarly, bioluminescent compounds may be utilized for labelling, the bioluminescent compounds including luciferin, luciferase, and aequorin. Once labeled, the antibody may be employed to identify and quantify immunologic counterparts (autibody or antigenic polypeptide) utilizing techniques well-known to the art.

A description of a radioimmunoassay (RIA) may be found in Laboratory Techniques in Biochemistry and Molecular Biology [52], with particular reference to the chapter entitled "An Introduction to Radioimmune Assay and Related Techniques" by Chard, T., incorporated by reference herein. A description of general immunometric assays of various types can be found in the following U.S. Pat. Nos. 4,376,110 (David et al.) or 4,098,876 (Piasio).

One can use immunoassays to detect for the p-Hyde gene, specific peptides, or for autibodies to the virus or peptides. A general overview of the applicable technology is in Harlow and Lane [32], incorporated by reference herein.

In one embodiment, antibodies to the human p-Hyde can be used to detect the agent in the sample. In brief, to produce antibodies to the agent or peptides, the sequence being targeted is expressed in transfected cells, preferably bacterial cells, and purified. The product is injected into a mammal capable of producing antibodies. Either monoclonal or polyclonal antibodies (as well as any recombinant antibodies) specific for the gene product can be used in various immunoassays. Such assays include competitive irmmoassays, radioimmunoassays, Western blots, ELISA, indirect immunofluorescent assays and the like. For competitive immunoassays, see Harlow and Lane [32] at pages 567-573̂ and 584-589.

In a further embodiment of this invention, commercial test kits suitable for use by a medical specialist may be prepared to determine the presence or absence of predetermined binding activity or predetermined binding activity capability to suspected target cells. In accordance with the testing techniques discussed above, one class of such kits will contain at least the labeled polypeptide or its binding partner, for instance an antibody specific thereto, and directions, of course, depending upon the method selected, e.g., "competitive," "sandwich," "DASP" and the like. The kits may also contain peripheral reagents such as buffers, stabilizers, etc.

Monoclonal antibodies or recombinant antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells or other lymphocytes from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (see, Kohler and Milstein [50], incorporated herein by reference). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. New techniques using recombinant phage antibody expression systems can also be used to generate monoclonal antibodies. See for example: McCafferty, I et al. [64]; Hoogenboom, H.R. et al. [39]; and Marks, J.D. et al. [60].

Such peptides may be produced by expressing the specific sequence in a recombinantly engineered cell such as bacteria, yeast, filamentous fungal, insect (especially employing baculoviral vectors), and mammalian cells. Those of skill in the art are knowledgeable in the numerous expression systems available for expression of herpes virus protein.

Briefly, the expression of natural or synthetic nucleic acids encoding viral protein will typically be achieved by operably linking the desired sequence or portion thereof to a promoter (which is either constitutive or inducible), and incorporated into an expression vector. The vectors are suitable for replication or integration in either prokaryotes or eukaryotes. Typical cloning vectors contain antibiotic resistance markers, genes for selection of transformants, inducible or regulatable promoter regions, and translation terminators that are useful for the expression of viral genes.

Methods for the expression of cloned genes in bacteria are also well known. In general, to obtain high level expression of a cloned gene in a prokaryotic system, it is advisable to construct expression vectors containing a strong promoter to direct mRNA transcription. The inclusion of selection markers in DNA vectors transformed in E. coli is also useful. Examples of such markers include genes specifying resistance to
antibiotics. See [81] supra, for details concerning selection markers and promoters for use in $E$. coli. Suitable eukaryote hosts may include plant cells, insect cells, mammalian cells, yeast, and filamentous fungi.

The peptides derived form the nucleic acids, peptide fragments are produced by recombinant technology may be purified by standard techniques well known to those of skill in the art. Recombinantly produced sequences can be directly expressed or expressed as a fusion protein. The protein is then purified by a combination of cell lysis (e.g., sonication) and affinity chromatography. For fusion products, subsequent digestion of the fusion protein with an appropriate proteolytic enzyme releases the desired peptide.

The proteins may be purified to substantial purity by standard techniques well known in the art, including selective precipitation with such substances as ammonjum sulfate, column chromatography, immunopurification methods, and others. See, for instance, Scopes, R. [84], incorporated herein by reference.

This invention is directed to analogs of the isolated nucleic acid and polypeptide which comprise the amino acid sequence as set forth above. The analog may have an N -terminal methionine or an N -terminal polyhistidine optionally attached to the N or COOH terminus of the polypeptide which comprise the amino acid sequence.

In another embodiment, this invention contemplates peptide fragments of the polypeptide which result from proteolytic digestion products of the polypeptide. In another embodiment, the derivative of the polypeptide has one or more chemical moieties attached thereto. In another embodiment the chemical moiety is a water soluble polymer. In another embodiment the chemical moiety is polyethylene glycol. In another embodiment the chemical moiety is mon-, di-, tri- or tetrapegylated. In another embodiment the chemical moiety is N -terminal monopegylated.

A polypeptide "fragment," "portion" or "segment" is a stretch of amino acid residues of at least about five to seven contiguous amino acids, often at least about seven to
nine contiguous amino acids, typically at least about nine to 13 contiguous amino acids and, most preferably, at least about 20 to 30 or more contiguous amino acids.

The polypeptides of the present invention, if soluble, may be coupled to a solid-phase support, e.g., nitrocellulose, nylon, column packing materials (e.g., Sepharose beads), magnetic beads, glass wool, plastic, metal, polymer gels, cells, or other substrates. Such supports may take the form, for example, of beads, wells, dipsticks, or membranes.
"Target region" refers to a region of the nucleic acid which is amplified and/or detected. The term "target sequence" refers to a sequence with which a probe or primer will form a stable hybrid under desired conditions.

Attachment of polyethylene glycol (PEG) to compounds is particularly useful because PEG has very low toxicity in mammals (Carpenter et al., 1971). For example, a PEG adduct of adenosine deaminase was approved in the United States for use in humans for the treaument of severe combined immunodeficiency syndrome. A second advantage afforded by the conjugation of PEG is that of effectively reducing the immunogenicty and antigenicity of heterologous compounds. For example, a PEG adduct of a human protein might be useful for the treatment of disease in other mammalian species without the risk of triggering a severe immune response. The compound of the present invention may be delivered in a microencapsulation device so as to reduce or prevent an host immune response against the compound or against cells which may produce the compound. The compound of the present invention may also be delivered microencapsulated in a membrane, such as a liposome.

Numerous activated forms of PEG suitable for direct reaction with proteins have been described. Useful PEG reagents for reaction with protein amino groups include active esters of carboxylic acid or carbonate derivatives, particularly those in which the leaving groups are N -hydroxysuccinimide, p-nitrophenol, imidazole or 1-hydroxy-2-nitrobenzene-4-sulfonatc. PEG derivatives containing maleimido or haloacetyl groups are useful reagents for the modification of protein free sulfhydryl
groups. Likewise, PEG reagents containing amino hydrazine or hydrazide groups are useful for reaction with aldehydes generated by periodate oxidation of carbohydrate groups in proteins.

In one embodiment, the amino acid residues of the polypeptide described herein are preferred to be in the "L" isomeric form. In another embodiment, the residues in the "D" isomeric form can be substituted for any L-amino acid residue, as long as the desired functional property of lectin activity is retained by the polypeptide. $\mathrm{NH}_{2}$ refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. Abbreviations used herein are in keeping with standard polypeptide nomenclature, J. Biol. Chem., 243:3552-59 (1969).

It should be noted that all amino-acid residue sequences are represented herein by formulae whose left and right orientation is in the conventional direction of amino-terminus to carboxy-terminus. Furthermore, it should be noted that a dash at the beginning or end of an amino acid residue sequence indicates a peptide bond to a further sequence of one or more amino-acid residues.

Synthetic polypeptide, prepared using the well known techniques of solid phase, liquid phase, or peptide condensation techniques, or any combination thereof, can include natural and unnatural amino acids. Amino acids used for peptide synthesis may be standard Boc (N-amino protected N-t-butyloxycarbonyl) amino acid resin with the standard deprotecting, neutralization, coupling and wash protocols of the original solid phase procedure of Merrifield (1963, J. Am. Chem. Soc. 85:2149-2154), or the base-labile N -amino protected 9 -fluorenylmethoxycarbonyl (Fmoc) amino acids first described by Carpino and Han (1972, J. Org. Chem. 37:3403-3409). Thus, polypeptide of the inveution may comprise D -amino acids, a combination of D - and L -amino acids, and various "designer" amino acids (e.g., methyl amino acids, C-methyl amino acids, and N -methyl amino acids, etc.) to convey special properties. Synthetic amino acids include ornithine for lysine, fluorophenylalanine for phenylalanine, and norleucine for leucine or isoleucine. Additionally, by assigning specific amino acids at specific
coupling steps, alpha-helices, alpha turns, beta sheets, beta-tums, and cyclic peptides can be generated.

In one aspect of the invention, the peptides may comprise a special amino acid at the C-terminus which incorporates either a $\mathrm{CO}_{2} \mathrm{H}$ or $\mathrm{CONH}_{2}$ side chain to simulate a free glycine or a glycine-amide group. Another way to consider this special residue would be as a D or L amino acid analog with a side chain consisting of the linker or bond to the bead. In one embodiment, the pseudo-free $C$-terminal residue may be of the $D$ or the L optical configuration; in another embodiment, a racemic mixture of $D$ and L-isomers may be used.

In an additional embodiment, pyroglutamate may be included as the N-terminal residue of the peptide. Although pyroglutamate is not amenable to sequence by Edman degradation, by limiting substitution to only $50 \%$ of the peptides on a given bead with N-terminal pyroglutamate, there will remain enough non-pyroglutamate peptide on the bead for sequencing. One of ordinary skill would readily recognize that this technique could be used for sequencing of any peptide that incorporates a residue resistant to Edman degradation at the $N$-terminus. Other methods to characterize individual peptides that demonstrate desired activity are described in detail infra. Specific activity of a peptide that comprises a blocked N-terminal group, e.g., pyroglutamate, when the particular N-terminal group is present in $50 \%$ of the peptides, would readily be demonstrated by comparing activity of a completely ( $100 \%$ ) blocked peptide with a non-blocked (0\%) peptide.

In addition, the present invention envisions preparing peptides that have more well defined structural properties, and the use of peptidomimetics, and peptidomimetic bonds, such as ester bonds, to prepare peptides with novel properties. In another embodiment, a peptide may be generated that incorporates a reduced peptide bond, i.e., $\mathrm{R}_{1}-\mathrm{CH}_{2}-\mathrm{NH}-\mathrm{R}_{2}$, where $R_{1}$ and $R_{2}$ are amino acid residues or sequences. A reduced peptide bond may be introduced as a dipeptide subunit. Such a molecule would be resistant to peptide bond hydrolysis, e.g., protease activity. Such peptides would provide ligands with unique function and activity, such as extended half-lives in vivo due to resistance to metabolic
breakdow, or protease activity. Furthermore, it is well known that in certain systems constrained peptides show enhanced functional activity (Hruby, 1982, Life Sciences 31:189-199; Hruby et al., 1990, Biochem J. 268:249-262); the present invention provides a method to produce a constrained peptide that incorporates random sequences at all other positions.

A constrained, cyclic or rigidized peptide may be prepared synthetically, provided that in at least two positions in the sequence of the peptide an amino acid or amino acid analog is inserted that provides a chemical functional group capable of cross-linking to constrain, cyclise or rigidize the peptide after treatment to form the cross-link. Cyclization will be favored when a turn-inducing amino acid is incorporated. Examples of amino acids capable of cross-linking a peptide are cysteine to form disulfide, aspartic acid to form a lactone or a lactase, and a chelator such as carboxyl-glutamic acid (Gla) (Bachem) to chelate a transition metal and form a cross-link. Protected carboxyl glutamic acid may be prepared by modifying the synthesis described by Zee-Cheng and Olson (1980, Biophys. Biochem. Res. Commun. 94:1128-1132). A peptide in which the peptide sequence comprises at least two amino acids capable of cross-linking may be treated, e.g., by oxidation of cysteine residues to form a disulfide or addition of a metal ion to form a chelate, so as to cross-link the peptide and form a constrained, cyclic or rigidized peptide.

The present invention provides strategies to systematically prepare cross-links. For example, if four cysteine residues arc incorporated in the peptide sequence, different protecting groups may be used (Hiskey, 1981, in The Peptides: Analysis, Synthesis, Biology, Vol. 3, Gross and Meienhofer, eds., Academic Press: New York, pp. 137-167; Ponsanti et al., 1990, Tetrahedron 46:8255-8266). The first pair of cysteine may be deprotected and oxidized, then the second set may be deprotected and oxidized. In this way a defined set of disulfide cross-links may be formed. Alternatively, a pair of cysteine and a pair of collating amino acid analogs may be incorporated so that the cross-links are of a different chemical nature.

The following mon-classical amino acids may be incorporated in the peptide in order to introduce particular conformational motifs: 1,2,3,4-tetrahydroisoquinoline-3-carboxylate (Karmierski et al., 1991, J. Am. Chemp. Soc. 113:2275-2283); (2S,3S)-methyl-phenylalanine, (2S,3R)-methyl-phenylalanine, (2R,3S)-methyl-phenylalanine and (2R,3R)-methyl-phenylalanine (Kazmierski and Hruby, 1991, Tetrahedron Lett.); 2-aminotetrahydronaphthalene-2-carboxylic acid (Landis. 1989, Ph.D. Thesis, University of Arizona); hydroxy-1,2,3,4-tetrahydroisoquixoline-3-carboxylate (Miyake et al., 1989, J. Takeda Res. Labs. 43:53-76); ? -carboline (D and L) (Kazmierski, 1988, Ph.D. Thesis, University of Arizona); HIC (histidine isoquinoline carboxylic acid) (Zechel et al., 1991, Int. J. Pep. Protein Res. 43); and HIC (histidine cyclic urea) (Dharanipragada).

The following amino acid analogs and peptidomimetics may be incorporated into a peptide to induce or favor specific secondary structures: LL-Acp (LL-3-amino-2-propenidone-6-carboxylic acid). a dipeptide analog (Kemp et al., 1985, J. Org. Chem. 50:5834-5838); and analogs provided by the following references: Nagai and Sato, 1985, Tetrahedron Lett. 26:647-650; DiMaio et al., 1989, J. Chem. Soc. Perkin Trans. p. 1687; also a Gly-Ala turn analog (Kahn et al., 1989, Tetrahedron Lett. 30:2317); amide bond isostere (Jones et al., 1988, Tetrahedron Lett. 29:3853-3856); tretrazol (Zabrocki ct al., 1988, J. Aun. Chem. Soc. 110:5875-5880); DTC (Samanen et al., 1990, Int. J. Protein Pep. Res. 35:501:509); and analogs taught in Olson et al., 1990, J. Am. Chem. Sci. 112:323-333 and Garvey et al., 1990. J. Ory. Chem. 56:436. Conformationally restricted mimetics of beta turns and beta bulges, and peptides containing them, are described in U.S. Patent No. 5,440,013, issued August 8, 1995 to Kahn.

The present iuvention further provides for modification or derivatization of the polypeptide or peptide of the invention. Modifications of peptides are well known to one of ordinary skill, and include phosphorylation, carboxymethylation, and acylation. Modifications may be effected by chemical or enzymatic means. In another aspect, glycosylated or fatty acylated peptide derivatives may be prepared. Preparation of
glycosylated or fatty acylated peptides is well known in the art. Fatty acyl peptide derivatives may also be prepared. For example, and not by way of limitation, a free amino group ( $N$-terminal or lysyl) may be acylated, e.g., myristoylated. In another embodiment an amino acid comprising an aliphatic side chain of the structure - $\left(\mathrm{CH}_{2}\right)_{n} \mathrm{CH}_{3}$ may be incorporated in the peptide. This and other peptide-fatty acid conjugates suitable for use in the present invention are disclosed in U.K. Patent GB-8809162.4, International Patent Application PCT/AU89/00166, and reference 5, supra.

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the ant. Such techniques are explained fully in the literature. See, c.g., Sambrook et al, "Molecular Cloning: A Laboratory Manual" (1989); "Current Protocols in Molecular Biology" Volumes I-III [Ausubel, R. M., ed. (1994)]; "Cell Biology: A Laboratory Handbook" Volumes I-III [J. E. Celis, ed. (1994))]; "Current Protocols in Immunology" Volumes I-III [Coligan, J. E., ed. (1994)]; "Oligonucleotide Synthesis" (M.I. Gait ed. 1984); "Nucleic Acid Hybridization" [B.D. Hames \& S.J. Higgins eds. (1985)]; "Transcription And Translation" [B.D. Hames \& S.J. Higgins, eds. (1984)]; "Animal Cell Culture" [R.I. Fresiney, ed. (1986)]; "Immobilized Cells And Enzymes" [IRL Press, (1986)]; B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

Chemical Moieties For Derivatization. Chemical moieties suitable for derivatization may be selected from among water soluble polymers. The polymer selected should be water soluble so that the component to which it is attached does not precipitate in an aqueous environment, such as a physiological environment. Preferably, for therapeutic use of the end-product preparation, the polymer will be pharmaceutically acceptable. One skilled in the att will be able to sclect the desired polymer based on such considerations as whether the polymer/component conjugate will be used therapeutically, and if so, the desired dosage, circulation time, resistance to proteolysis, and other considerations. For the present component or components, these may be ascertained using the assays provided herein.

The water soluble polymer may be selected from the group consisting of, for example, polyethylene glycol, copolymers of ethylene glycol/propylene glycol, carboxymethylcellulose, dextran, polyvinyl alcohol, polyvinyl pyrrolidone, poly-1, 3-dioxolane, poly-1,3,6-trioxane, ethylene/maleic anhydride copolymer, polyaminoacids (either homopolymers or random copolymers), and dextran or poly(n-vinyl pyrrolidone)polyethylene glycol, propropylene glycol homopolymers, prolypropylene oxide/ethylene oxide co- polymers, polyoxyethylated polyols and polyvinyl alcohol. Polyethylene glycol propionaldenhyde may have advantages in manufacturing due to its stability in water.

The number of polymer molecules so attached may vary, and one skilled in the art will be able to ascertain the effect on function. One may mono-derivatize, or may provide for a di-, tri-, tetra- or some combination of derivatization, with the same or different chemical moieties (e.g., polymers, such as different weights of polyethylene glycols). The proportion of polymer molecules to component or components molecules will vary, as will their concentrations in the reaction mixture. In general, the optimum ratio (in terms of efficiency of reaction in that there is no excess unreacted component or components and polymer) will be determined by factors such as the desired degree of derivatization (e.g., mono, di-, tri-, etc.), the molecular weight of the polymer selected, whether the polymer is branched or unbranched, and the reaction conditions.

This invention provides a method for determining whether a subject carries a mutation in the p-Hyde gene which comprises: a) obtaining an appropriate nucleic acid sample from the subject; and(b) detemining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant $p$-Hyde so as to thereby determine whether a subject carries a mutation in the p-Hyde gene. In one embodiment, the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant p-Hyde, and wherein the determining of step (b) comprises: (i) contacting the mRNA with the oligonucleotide under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes
mutant p-Hyde. In another embodiment, the determining of step (b) comprises: i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid; (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant p-Hyde.

The present invention further provides methods of preparing a polynucleotide comprising polymerizing nucleotides to yield a sequence comprised of at least eight consecutive nucleotides of the p-Hyde gene; and methods of preparing a polypeptide comprising polymerizing amino acids to yield a sequence comprising at least five amino acids encoded within the p -Hyde gene.

This invention provides a pharmaceutical composition comprising an amount of the polypeptide and a pharmaceutically effective carrier or diluent.

This invention provides a method for determining whether a subject carries a mutation in the p -Hyde gene which comprises: (a) obtaining an appropriate nucleic acid sample from the subject; and (b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant p-Hyde so as to thereby determine whether a subject carxies a mutation in the p -Hyde gene. In one embodiment the uucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant p -Hyde, and wherein the determining of step (b) comprises: (i) contacting the mRNA with the oligonucleotide under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex; (ii) isolating the complex so formed; and (iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant p-Hyde. In another embodiment the determining of step (b) comprises: (i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid
sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid; (ii) isolating the pieces of nucleic acid; and (iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucleic acid derived from the isolated nucleic acid so as to thereby determine whether the mucleic acid sample is, or is derived from, a nucleic acid which encodes mutant p-Hyde.

Detection of point mutations or variations may be accomplished by molecular cloning of the p-Hyde allele(s) and sequencing the allele(s) using techniques well known in the art. Alternatively, the gene sequences can be amplified directly from a genomic DNA preparation from the tumor tissue, using known techniques. The DNA sequence of the amplified sequences can then be determined. There are six well known methods for a more complete, yet still indirect, test for confirming the presence of a susceptibility allele: 1) single stranded conformation analysis (SSCA) (Orita et al., 1989); 2) denaturing gradient gel electrophoresis (DGGE) (Wartell et al., 1990; Sheffield et al., 1989); 3) RNase protection assays (Finkelstein et al., 1990; Kinszler et al., 1991); 4) allele-specific oligonucleotides (ASOs) (Conner et al., 1983); 5) the use of proteins which recognize nucleotide mismatches, such as the E. coli mutS protein (Modrich, 1991); and 6) allele-specific PCR (Rano \& Kidd, 198.9). For allele-specific PCR, primers are used which hybridize at their $3^{\prime}$ ends to a particular p-Hyde mutation. If the particular p-Hyde mutation is not present, an amplification product is not observed. Amplification Refractory Mutation System (ARMS) can also be used, is disclosed in European Patent Application Publication No. 0332435 and in Newton et al., 1989. Insertions and deletions of genes cax also be detected by cloning, sequencing and amplification. In addition, restriction fragment length polymorphism (RFLP) probes for the gene or surrounding marker genes can be used to score alteration of an allele or an insertion in a polymorphic fragment. Such a method is particularly useful for screening relatives of an affected individual for the presence of the p -Hyde mutation found in that individual. Other techniques for detecting insertions and deletions as known in the art can be used.

In similar fashion, DNA probes can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., Cotton et al., 1988; Shenk et al., 1975; Novack et al.,
1986. Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes. See, e.g., Cariello, 1988. With either riboprobes or DNA probes, the cellular mRNA or DNA which might contain a mutation can be amplified using PCR (see below) before hybridization. Changes in DNA of the p-Hyde gene can also be detected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

As noted above, non-PCR based screening assays are also contemplated in this invention This procedure hybridizes a nucleic acid probe (or an analog such as a methyl phosphonate backbone replacing the normal phosphodiester), to the low level DNA target. This probe may have an enzyme covalently linked to the probe, such that the covalent linkage does not interfere with the specificity of the hybridization. This enzyme-probe-conjugate-target nucleic acid complex can then be isolated away from the free probe enzyme conjugate and a substrate is added for enzyme detection. Enzymatic activity is observed as a change in color development or luminescent output resulting in a 10 sup $3-10$ sup 6 increase in sensitivity. For an example relating to the preparation of oligodeoxynucleotide-alkaline phosphatase conjugates and their use as hybridization probes see Jablonski et al., 1986.

Two-step label amplification methodologies are known in the art. These assays work on the principle that a small ligand (such as digoxigenin, biotin, or the like) is attached to a nucleic acid probe capable of specifically binding p-Hyde. Allele specitic probes are also contemplated within the scope of this example. In one example, the small ligand attached to the nucleic acid probe is specifically recognized by an antibody-enzyme conjugate. In one embodiment of this example, digoxigenin is attached to the nucleic acid probe. Hybridization is detected by an antibody-alkaline phosphatase conjugate which turns over a chemiluminescent substrate, For methods for labeling nucleic acid probes according to this embodiment see Martin et al. , 1990.

In a second example, the small ligand is recognized by a second ligand-enzyyne conjugate that is capable of specifically complexing to the first ligand. A well known embodiment of this example is the biotin-avidin type of interactions. For methods for
labeling nucleic acid probes and their use in biotin-avidin based assays see Rigby et al., 1977 and Nguyen et al., 1992.

It is also contemplated within the scope of this invention that the nuclejc acid probe assays of this invention will employ a cocktail of nucleic acid probes capable of detecting p-Hyde. Thus, in one example to detect the presence of p-Hyde in a cell sample, more than one probe complementary to p -Hyde is employed and in particular the number of different probes is alternatively 2 , 3 , or 5 different nucleic acid probe sequences. In another example, to detect the presence of mutations in the p -Hyde gene sequence in a patient, more than one probe complementary to p -Hyde is employed where the cocktail includes probes capable of patients with alternate-specific mutations identified in populations of patients with alterations in p-Hyde. In this embodiment, any number of probes can be used, and will preferably ixclude probes corresponding to the major gene mutations identified as predisposing an individual to breast cancer.

This invention provides a method for screening a tumor sample from a human subject for a somatic alteration in a p-Hyde gene in said tumor which comprises gene comparing a first sequence selected form the group cousisting of a p-Hyde gene from said tumor sample, p-Hyde RNA from said tumor sample and p-Hyde cDNA made from mRNA from said tumor sample with a second sequence selected from the group consisting of p -Hyde gene from a nontumor sample of said subject, p-Hyde RNA from said nontumor sample and p-Hyde cDNA made from mRNA from said nontumor sample, wherein a difference in the sequence of the p-Hyde gene,p-Hyde RNA or p-Hyde cDNA from said tumor sample from the sequence of the p-Hyde gene, p-Hyde RNA or p -Hyde cDNA from said nontumor sample indicates a somatic alteration in the p -Hyde gene in said tumor sample.

This invention provides a method for screening a tumor sample from a human subject for the presence of a somatic alteration in a p-Hyde gene in said tumor which comprises comparing p-Hyde polypeptide from said tumor sample from said subject to p -Hyde polypeptide from a nontumor sample from said subject to analyze for a difference
between the polypeptides, wherein said comparing is performed by (i) detecting either a full length polypeptide or a truncated polypeptide in each sample or (ii) contacting an antibody which specifically binds to either an epitope of an altered p -Hyde polypeptide or an epitope of a wild-type $p$-Hyde polypeptide to the p -Hyde polypeptide from each sample and detecting antibody binding, wherein a difference between the p-Hyde polypeptide from said tumor sample from the p -Hyde polypeptide from said nontumor sample indicates the presence of a somatic alteration in the $p$-Hyde gene in said tumor sample.

This invention is particularly useful for screening compounds by using the p-Hyde polypeptide or binding fragment thereof in any of a variety of drug screening techniques. The p-Hyde polypeptide or fragment employed in such a test may either be free in solution, affixed to a solid support, or borne on a cell surface. One method of drug screening utilizes eucaryotic or procaryotic host cells which are stably transformed with recombinant polynucleotides expressing the polypeptide or fragment, preferably in competitive binding assays. Such cells, either in viable or fixed form, can be used for standard binding assays. One may measure, for example, for the formation of complexes between a p-Hyde polypeptide or fragment and the agent being tested, or examine the degree to which the formation of a complex between a p-Hyde polypeptide or fragment and a known ligand is interfered with by the agent being tested.

Thus, the present invention provides methods of screening for drugs comprising contacting such an agent with a p-Hyde polypeptide or fragment thereof and assaying (i) for the presence of a complex between the agent and the p-Hyde polypeptide or fragment, or (ii) for the presence of a complex between the p -Hyde polypeptide or fragment and a ligand, by methods well known in the art. In such competitive binding assays the p -Hyde polypeptide or fragment is typically labeled. Free p-Hyde polypeptide or fragment is separated from that present in a protein:protein complex, and the amount of free (i.e., uncomplexed) label is a measure of the binding of the agent being tested to p -Hyde or its interference with p -Hyde:ligand binding, respectively.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to the p -Hyde polypeptides and is described in detail in Geysen, PCT published application WO 84/03564, published on Sep. 13, 1984. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with p -Hyde polypeptide and washed. Bound p-Hyde polypeptide is then detected by methods well known in the art.

Purified p-Hyde can be coated directly onto plates for use in the aforementioned drug screening techniques. However, non-ncutralizing antibodies to the polypeptide can be used to capture antibodies to immobilize the p-Hyde polypeptide on the solid phase. This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of specifically binding the p-Hyde polypeptide compete with a test compound for binding to the p-Hyde polypeptide or fragments thereof. In this manner, the antibodies can be used to detect the presence of any peptide which shares one or more antigenic determinants of the p-Hyde polypeptide.

A further technique for drug screening involves the use of host eukaryotic cell lines or cells (such as described above) which have a nonfunctional p-Hyde gene. These host cell lines or cells are defective at the p-Hyde polypeptide level. The host cell lines or cells are grown in the presence of drug compound. The rate of growth of the host cells is measured to determine if the compound is capable of regulating the growth of p-Hyde defective cells.

The goal of rational drug design is to produce structural analogs of biologically active polypeptides of interest or of small molecules with which they interact (e.g., agonists, antagonists, inhibitors) in order to fashion drags which are, for example, more active or stable foums of the polypeptide, or which, e.g., enhance or interfere with the function of a polypeptide in vivo. See, e.g., Hodgson, 1991. In one approach, one first determines the three-dimensional structure of a protein of interest (e.g., p-Hyde polypeptide) or, for example, of the p -Hyde-receptor or ligand complex, by x -ray
crystallography, by computer modeling or most typically, by a. combination of approaches. Less often, useful information regarding the structure of a polypeptide may be gained by modeling based on the structure of homologous proteins. An example of rational drug design is the development of HIV protease unhibitors (Erickson et al., 1990). In addition, peptides (e.g., p-Hyde polypeptide) are analyzed by an alaninc scan (Wells, 1991). In this technique, an amino acid residue is replaced by Ala , and its effect on the peptide's activity is determined. Each of the amino acid residues of the peptide is analyzed in this mannet to determine the important regions of the peptide.

It is also possible to isolate a target-specific antibody, selected by a functional assay, and then to solve its crystal structure. In principle, this approach yields a pharmacore upon which subsequent drug design can be based. It is possible to bypass protein crystallography altogether by generating anti-idiotypic antibodies (anti-ids) to a functional, pharmacologically active antibody. As a mirror image of a mirror image, the binding site of the anti-ids would be expected to be an analog of the original receptor. The anti-id could then be used to identify and isolate peptides from banks of chemically of biologically produced banks of peptides. Sclected peptides would then act as the pharmacore.

Thus, one may design drugs which have, e.g., improved p-Hyde polypeptide activity or stability or which act as inhibitors, agonists, antagonists, etc. of p -Hyde polypeptide activity. By virtue of the availability of cloned p-Hyde sequences, sufficient amounts of the p-Hyde polypeptide may be made available to perform such analytical studies as $x$-ray crystallography. In addition, the knowledge of the p-Hyde protein sequence provided herein will guide those employing computer modeling techniques in place of, or in addition to $x$-ray crystallography.

This invention provides a method for identifying a chemical compound which is capable inducing susceptibility to cell death which comprises: (a) contacting the p-Hyde with a chemical compound under conditions permitting binding between the p -Hyde and the chemical compound;(b) detecting specific binding of the chemical compound to the p -Hyde; and (c) determining whether the chemical compound inhibits the p -Hyde so as
to identify a chemical compound which is capable of capable inducing susceptibility to cell death. Useful diagnostic techniques include, but are not limited to fluorescent in situ hybridization (FISH), direct DNA sequencing, PFGE analysis, Southern blot analysis, single stranded conformation analysis (SSCA), Rnase protection assay, allele-specific oligonuclcotide (ASO), dot blot analysis and PCR-SSCP, as discussed in detail further below.

A rapid preliminary analysis to detect polymorphisms in DNA sequences can be performed by looking at a series of Southern blots of DNA cut with one or more restriction enzymes, preferably with a large number of restriction enzymes. Each blot contains a series of normal individuals and a series of tumors. Southern blots displaying hybridizing fragments (differing in length from control DNA whex probed with sequences near or including the p -Hyde gene) indicate a possible mutation. If restriction enzymes which produce very large restriction fragments are used, then pulsed field gel electrophoresis (PFGE) is employed.

Detection of point mutations may be accomplished by molecular cloning of the p-Hyde allele(s) and sequencing the allele(s) using techniques well known in the art. Alternatively, the gene sequences can be amplified directly from a genomaic DNA preparation from the tumor tissue, using known techuiques. The DNA sequence of the amplified sequences can then be determined. There are six well known methods for a more complete, yet still indirect, test for confirming the presence of a susceptibility allele: 1) single stranded conformation analysis (SSCA) (Orita et al., 1989); 2) denaturing gradient gel electrophoresis (DGGE) (Wartell et al., 1990; Sheffield et al., 1989); 3) RNase protection assays (Finkelstein et al., 1990; Kinszleret al., 1991); 4) allele-specific oligonucleotides (ASOs) (Conner et al., 1983); 5) the use of proteins which recognize nucleotide mismatches, such as the E. coli mutS protein (Modrich, 1991); and 6) allele-specific PCR (Rano \& Kidd, 1989). For allele-specific PCR, primers are used which hybridize at their 3' ends to a particular p-Hyde mutation. If the particular p-Hyde mutation is not present, an amplification product is not observed. Amplification Refractory Mutation System (ARMS) can also be used, as disclosed in European Patent Application Publication No. 0332435 and in Newton et al.,
1989. Insertions and deletions of genes can also be detected by cloning, sequencing and amplification. In addition, restriction fragment length polymorphism (RFLP) probes for the gene or surrounding marker genes can be used to score alteration of an allele or an insertion in a polymorphic fragment. Such a method is particularly useful for screening relatives of an affected individual for the presence of the p-Hyde mutation found in that individual. Other techniques for detecting insertions and deletions as known in the art can be used.

In similar fashion, DNA probes can be used to detect mismatches, through enzymatic or chemical cleavage. See, e.g., Cotton et al., 1988; Shenk et al., 1975; Novack et al., 1986. Alternatively, mismatches can be detected by shifts in the electrophoretic mobility of mismatched duplexes relative to matched duplexes. See, e.g., Cariello, 1988. With either riboprobes or DNA probes, the cellular mRNA or DNA which might contain a mutation can be amplified using PCR (see below) before hybridization. Changes in DNA of the p-Hyde gene can also be delected using Southern hybridization, especially if the changes are gross rearrangements, such as deletions and insertions.

DNA sequences of the p -Hyde gene which have been amplified by use of $P C R$ may also be screened using allele-specific probes. These probes are mucleic acid oligomers, each of which contains a region of the p-Hyde gene sequence harboring a known mutation. For example, one oligomer may be about 30 nucleotides in length, corresponding to a portion of the p-Hyde gene sequence. By use of a battery of such allele-specific probes, PCR amplification products can be screened to identify the presence of a previously identified mutation in the p-Hyde gene. Hybridization of allele-specific probes with amplified p-Hyde sequences can be performed, for example, on a nylon filter. Hybridization to a particular probe under stringent hybridization conditions indicates the presence of the same mutation in the tumor tissue as in the allele-specific probe.

Alteration of p -Hyde mRNA expression can be detected by any techniques known in the art. These include Northern blot analysis, PCR amplification and RNase protection. Diminished mRNA expression indicates an alteration of the wild-type p-Hyde gene.

Alteration of wild-type p-Hyde genes can also be detected by screening for alteration of wild-type $p$-Hyde protein. For example, monoclonal antibodies immunoreactive with $p$-Hyde can be used to screen a tissue. Lack of cognate antigen would indicate a p-Hyde mutation. Antibodies specific for products of mutant alleles could also be used to detect mutant p-Hyde gene product. Such immunological assays can be done in any convenient formats known in the art. These include Western blots, immunohistochemical assays and ELISA assays. Any means for detecting an altered p-Hyde protein can be used to detect alteration of wild-type p-Hyde genes. Functional assays, such as protein binding determinations, can be used. In addition, assays can be used which detect p-Hyde biochemical function. Finding a mutant p-Hyde gene product indicates alteration of a wild-type p-Hyde gene. Mutant p-Hyde genes or gene products can also be detected in other human body samples, such as senun, stool, urine and sputum.

The present invention also provides for fusion polypeptides, comprising p-Hyde polypeptides and fragments. Homologous polypeptides may be fusions between two or more $p$-Hyde polypeptide sequences or between the sequences of p -Hyde and a related protcin. Likewise, heterologous fusions may be constructed which would exhibit a combination of properties or activities of the derivative proteins. For example, ligand-binding or other domains may be "swapped" between different new fusion polypeptides or fragments. Such homologous or heterologous fusion polypeptides may display, for example, altered strength or specificity of binding. Fusion partners include immunoglobulins, bacterial beta -galactosidase, $\operatorname{trp} \mathrm{E}$, protein A , beta -lactamase, alpha amylase, alcohol dehydrogenase and yeast alpha mating factor. See, e.g., Godowski et al. , 1988. Fusion proteins will typically be made by either recombinant mucleic acid methods, as described below, or may be chemically synthesized. Techniques for the synthesis of polypeptides are described, for example, in Merrifield, 1963.

Probes for p -Hyde alleles may be derived from the sequences of the p -Hyde region or its cDNAs. The probes may be of any suitable length, which span all or a portion of the p -Hyde region, and which allow specific hybridization to the p -Hyde region. If
the target sequence contains a sequence identical to that of the probe, the probes may be short, e.g., in the range of about $8-30$ base pairs, since the hybrid will be relatively stable under even stringent conditions. If some degree of mismatch is expected with the probe, i.e., if it is suspected that the probe will hybridize to a variant region, a longer probe may be employed which hybridizes to the target sequence with the requisite specificity.

The probes will include an isolated polynucleotide attached to a label or reporter molecule and may be used to isolate other polynucleotide sequences, having sequence similarity by standard methods. For techniques for preparing and labeling probes see, e.g., Sambrook et al., 1989 or Ausubel et al., 1992.

Probes comprising synthetic oligonucleotides or other polynucleotides of the present invention may be derived from naturally occurring or recombinant single- or double-stranded polynucleotides, or be chemically synthesized. Probes may also be labeled by nick translation, Klenow fill-in reaction, or other methods known in the art.
Portions of the polynucleotide sequence having at least about eight nucleotides, usually at least about 15 nucleotides, and fewer than about 6 kb , usually fewer than about 1.0 kb , from a polynucleotide sequence encoding p -Hyde are preferred as probes. The probes may also be used to determine whether mRNA encoding p-Hyde is present in a cell or tissue.

This invention provides a method of inhibiting the prowth of cancer cells, comprising the steps of obtaining the cells and contacting the cells of the subject with a replication-deficient adenovirus type 5 expression vector comprising an adenovirus genome having a deletion in the E1 and E3 region of the genome and an insertion within the region of a nucleic acid encoding p-Hyde under the control of a Rous Sarcoma virus promoter, thereby inhibiting the growth of the prostate cancer cells.

This invention provides a method of inhibiting the growth a prostate cancer cells, comprising: 1) obtaining a sample of prostate cells from a subject; 2) contacting the cells with a replication deficient adenovirus type 5 expression vector which comprises an
adenovirus genome having a deletion in the El and E 3 regions of the genome and an insertion within the regions of a p-Hyde cDNA under the control of a Rous Sarcoma virus promoter; and 3) introducing the cells into the subject, thereby inhibiting the growth of the cancer cells.

This invention provides a method of suppressing the growth of cancer cells in a subject, comprising introducing into the cancer cell an amount of a nucleic acid encoding a p -Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant $p$-Hyde protein, thereby suppressing the growth of cancer cells in the subject.

This invention provides a method of suppressing growth of cancer cells in a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p-Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby suppressing the growth of cancer cells in the subject.

As demonstrated herein apoptotic response was assessed using DNA laddering assay. DNAs were extracted after the respective treatment ( 24 hours with 1 mM Hydroxyurea followed by 24 hours with 0.1 mM 5 '-dFUrd) and analyzed on $1.6 \%$ agarose gel electrophoresis. In agreement with cell cycle analyses, apoptotic response of the stable transfectants AT1-H1 and AT3-H1 (pcHYDE transfected AT-1 and AT-3) are consistently and significantly higher relative to both parental (AT-1 and AT-3) and pcDNA-transfected parental cell lines (AT1-pcl and AT3-pcl). In particular, the highest apoptotic response occurred in synchronized culture under the induction with 0.1 mM 5 '-dFUrd as shown in Fig.15. The enhanced apoptotic response in AT1-H1 and AT3-H1 transfectant after hydroxyurea treatment is the result of "thymineless death" (Kyprianou, 1994, Kyprianou et al., 1994) leading to depletion of intracellular thymidine-5-triphosphate (TTP) pools through indirect inhibition of thymidylate synthetase by fluorodeoxyuridine. However, the exact mechanism of the apoptosis itself in association with TTP depletion is not known. Taken together, these data suggest that
the apoptotic response in the pcHYDE stable transfectants is likely due to the downstream effect of pcHYDE gene product.

This invention provides a method of inducing susceptibility to apoptosis of cancer cells, comprising introducing into the cancer cell an amount of a nucleic acid encoding a p -Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein, thereby inducing susceptibility to apoptosis.

This invention provides a method of inducing susceptibility to apoptosis of cancer cells in a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p-Hyde protein, or the nucleic acid encoding a mutant $p$-Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby inducing susceptibility to apoptosis.

As demonstrated hercin, p-Hyde suppresses cancer growth in vivo. The lowest level of p-Hyde expression was observed in AT3 cell line as shown in Fig. 9. For this reason, AT3 cell line was transfected with pcHYDE, a construct of $p$-Hyde in mammalian expression vector of pcDNA3.1(-) under G418 selection. As negative control, AT-3 cell line was also transfected with the vector only and the stable transfectants obtained was designated as AT3-pc. The tumor growth of the parental cell line of AT-3 and in stable transfectant AT3-HI and AT3-H2 have been evaluated in vivo. One million cells of each cell lines in 0.3 ml of Hanks solution were inoculated subcutaneously in each flank of inbred male Copenhagen rat. In this initial experiment, three groups of each five rats were injected with each cell line. The size of tumors were scored after a tine schedule shown in Fig.18. These preliminary results indicated that both AT3-H1 and AT3-H2 stable transfectant grew significantly slower than the AT-3 parental cell line demonstrating that the tumor growth regression in both stable transfectants and regulated by the pcHYDE gene product.

Interestingly, p-Hyde has the dual ability to act like a tumor suppressor gene and induce susceptibility to apoptosis by what may be p53 independent pathways. The growth of
prostate twnors in rats was greatly inhibited by p-Hyde. Moreover, prostate cancer cells expressing $p-H y d e$ were more sensitive to UV DNA damage driving these cells into cell programmed death. Analysis of DNA repair enzyme activity suggests a defect resulting in the presence of intact (6-4) PP and decreased cell survival by colony forming assay. However, the capacity of p-Hyde to induce susceptibility to apoptosis is not limited to UV DNA damage. Chemotherapy agent, Fluorodeoxyuridine, a pyrimidine antimetabolite which is related to fluorouracil ( $5-\mathrm{FU}$ ) and has been used for treatment of a wide variety of human epithelial malignancies, also more readily induces apoptosis in prostate cancer cell expressixg $p$-Hyde. Moreover, caxcer cells expressing $p$-Hyde are also more susceptible to gamma radiation. Thus, the mechanisms of cellular DNA injury are different for UV, gamma radiation, and Fluorodeoxyuridine suggesting that the ability to make cells more susceptible to apoptosis is more global in action. This unique function of p-Hyde may represent a new class genes that induce susceptibility to apoptosis. This is different than the function ascribed to tumor suppressor genes like p53 which directly induces apoptosis, not sensitivity to apoptosis (Yonish-Rouach er al., 1991). Moreover, p-Hyde activity is in contrast to bcl-2 where the absence of bcl-2, not the presence of, makes the cancer cell more susceptible to cell programmed death (McDonnellet al., 1992).

This invention provides a method of suppressing cancer cells, comprising introducing into the cancer cell an amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein, thereby inducing susceptibility to apoptosis.

This invention provides a method of suppressing cancer cells a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p -Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby suppressing cancer cells.

This invention provides a method of treating a subject with cancer which comprises administering to the subject a pharmaceutical composition comprising a therapeutically
effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p -Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby treating the subject with cancer.

This invention provides a method of treating a subject with cancer, comprising: 1) administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a mucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p -Hyde protein in combination with radiation, chemotherapy, or UV mimetic drugs; and 2) a pharmaceutical acceptable carrier or diluent, thereby treating the subject with cancer.

The unique functional features of the $p$-Hyde gene may be exploited for the treatment of hyperproliferative disorders and cancer. One effective therapeutic strategy, for example, may be the treatment of carcinoma cells expressing $p$-Hyde with chemotherapy agents or UV mimetic drugs (such as acetylaminofluorine). However, cancer cells are not likely to produce significant levels of the growth inhibition $p$-Hyde. Consequently, the p-Hyde gene my be introduced into cancer cells by gene therapy. Tumnors transduced with vectors containing $p$-Hyde may not only be directly suppressed by p-Hyde as demonstrated in this study, but also when treated in combination with DNA damaging therapy such as chemotherapy, UV mimetic drugs, or radiation, have even a greater anti-cancer effect. Since gene therapy will target cancer cells, then enhancement of apoptosis will occur more selectively in cancer cells following DNA damage (UV, radiation, or chemotherapy).

Three DNA enzyme repair systems were evaluated in parental compared to p-Hfyde transfected cells: uridine phosphorylase, uridine kinase, and UV damage repair. UV damage repair was impaired in the $p$-Hyde transfected cells. Figure 16 shows that decreased DNA repair activity results in higher levels of intact photoproducts (64PP). Consistent with these data, pHyde transfected cells also had a significant reduction in survival following UV exposure compared to parental AT3 cells as determined by
colony formation assay. Thus, DNA repair enzyme inpaimment correlated with shorter survival and induction of apoptosis in prostate cancer cells transfected with p-Hyde.

This invention a method of treating a subject with cancer, comprising administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising: 1) an adenovirus type 5 expression vector which comprises an adenovirus genome having a deletion in the E1 and E3 regions of the genome and an insertion within the regions of a full length sense p-Hyde cDNA under the control of a Rous Sarcoma virus promoter, and 2) a suitable carrier or diluent, thereby treating the subject with cancer. In one embodiment the cancer is selected from a group consisting of: melanoma; lymphoma; leukemia; and prostate, colorectal, pancreatic, breast, brain, or gastric carcinoma.

The present invention provides the means necessary for production of gene-based therapies directed at cancer cells. These therapeutic agents may take the form of polynucleotides comprising all or a portion of the p-Hyde locus placed in appropriate vectors or delivered to target cells in more direct ways such that the function of the p-Hyde protein is reconstituted. Therapeutic agents may also take the forn of polypeptides based on either a portion of, or the entire protein sequence of p -Hyde. These may functionally replace the activity of $p$-Hyde in vivo.

A suitable bodily fluid includes, but is not limited to: serum, plasma, cerebrospinal fluid, lymphocytes, urine, transudates, or exudates. In the preferred embodiment, the suitable bodily fluid sample is serun or plasma. In addition, the bodily fluid sample may be cells from bone marrow, or a supernatant from a cell culture. Methods of obtaining a suitable bodily fluid sample from a subject are known to those skilled in the art. Methods of determining the level of antibody or antigen include, but are not limited to: ELISA, IFA, and Western blotting.

The diagnostic assays of the invention can be nucleic acid assays such as nucleic acid hybridization assays and assays which detect amplification of specific nucleic acid to detect for a nucleic acid sequence of the human $p$-Hyde described herein.

Target specific probes may be used in the nucleic acid hybridization diagnostic. The probes are specific for or complementary to the target of interest. For precise allelic differentiations, the probes should be about 14 nucleotides long and preferably about 20-30 nucleotides. For more general detection of the human p-Hyde of the invention, nucleic acid probes are about 50 to about 1000 nucleotides, most preferably about 200 to about 400 nucleotides.

The specific nucleic acid probe can be RNA or DNA polynucleotide or oligonucleotide, or their analogs. The probes may be single or double stranded nucleotides. The probes of the invention may be synthesized enzymatically, using methods well known in the art (e.g., nick translation, primer extension, reverse transcription, the polymerase chain reaction, and others) or chemically (e.g., by methods such as the phosphoramidite method described by Beaucage and Carruthers [19], or by the triester method according to Matteucci, et al. [62], both incorporated herein by reference).

An alternative means for determining the presence of the human p -Hyde is in situ hybridization, or more recently, in situ polymerase chain reaction. In situ PCR is described in Neuvo et al. [71], Intracellular localization of polymerase chain reaction (PCR)-amplified Hepatitis C cDNA; Bagasra et al. [10], Detection of Human Immunodeficiency virus type 1 provirus in mononuclear cells by in situ polymerase chain reaction; and Heniford et al. [35], Variation in cellular EGF receptor mRNA expression demonstrated by in situ reverse transcriptase polymerase chain reaction. In situ hybridization assays are well known and are generally described in Methods Enzymol. [67] incorporated by reference herein. In an in situ hybridization, cells are fixed to a solid support, typically a glass slide. The cells are then contacted with a hybridization solution at a moderate temperature to permit annealing of target-specific probes that are labelled. The probes are preferably labelled with radioisotopes or fluorescent reporters.

The above described probes are also useful for in-situ hybridization or in order to locate tissues which express this gene, or for other hybridization assays for the presence of this gene or its MRNA in various biological tissues. In-situ hybridization is a sensitive localization method which is not dependent on expression of antigens or native vs. denatured conditions.

In brief, inhibitory mucleic acid therapy approaches can be classified into those that target DNA sequences, those that target RNA sequences (including pre-mRNA and mRNA), those that target proteins (sense strand approaches), and those that cause cleavage or chemical modification of the target mucleic acids.

Approaches targeting DNA fall into several categories. Nucleic acids can be designed to bind to the major groove of the duplex DNA to forn a triple helical or "triplex" structure. Alternatively, inhibitory nucleic acids are designed to bind to regions of single stranded DNA resulting from the opening of the duplex DNA during replication or transcription.

More commonly, inhibitory nucleic acids are designed to bind to mRNA or mRNA precursors. Inhibitory nucleic acids are used to prevent maturation of pre-mRNA. Inhibitory nucleic acids may be designed to interfere with RNA processing, splicing or translation.

The inhibitory nucleic acids can be targeted to mRNA. In this approach, the inhibitory nucleic acids are designed to specifically block translation of the encoded protein. Using this approach, the inhibitory nucleic acid can be used to selectively suppress certain cellular functions by inhibition of translation of mRNA encoding critical proteins. For example, an inhibitory nucleic acid complementary to regions of c-myc mRNA inhibits c-myc protein expression in a human promyelocytic leukemia cell line, HL60, which overexpresses the c-myc proto-oncogene. See Wickstrom E.L., et al. [93] and Harel-Bellan, A., et al. [31A]. As described in Helene and Toulme, inhibitory nucleic acids targeting mRNA have been shown to work by several different mechanisms to inhibit translation of the encoded protein(s).

Lastly, the inhibitory nucleic acids can be used to induce chemical inactivation or cleavage of the target genes or mRNA. Chemical inactivation can occur by the induction of crosslinks between the inhibitory nucleic acid and the target nucleic acid within the cell. Other chemical modifications of the target nucleic acids induced by appropriately derivatized inhibitory nucleic acids may also be used.

Cleavage, and therefore inactivation, of the target nucleic acids may be effected by attaching a substituent to the inhibitory nucleic acid which can be activated to induce cleavage reactions. The substituent can be one that affects either chemical, or enzymatic cleavage. Alternatively, cleavage can be induced by the use of ribozymes or catalytic RNA. In this approach, the inhibitory nucleic acids would comprise either naturally occurring RNA (ribozymes) or synthetic nucleic acids with catalytic activity.

As used herein, "pharmaceutical composition" could mean therapeutically effective amounts of polypeptide products of the invention together with suitable diluents, preservatives, solubilizers, emulsifiers, adjuvant and/or carriers useful in SCF (stem cell factor) therapy. A "therapeutically effective amount" as used herein refers to that amount which provides a therapeutic effect for a given condition and administration regimen. Such compositions are liquids or lyophilized or otherwise dried fomulations and include diluents of various buffer content (e.g., Tris-HCl., acetate, phosphate), pH and ionic strength, additives such as albumin or gelatin to prevent absorption to surfaces, detergents (c.g., Tween 20, Tween 80, Pluronic F68, bile acid salts). solubilizing agents (e.g., glycerol, polyethylene glycerol), anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), preservatives (e.g., Thimerosal, benzyl alcohol, parabens), bulking substances or tonicity modifiers (e.g., lactose, mannitol), covalent attachment of polymers such as polyethylene glycol to the protein, complexation with metal ions, or incorporation of the material into or onto particulate preparations of polymeric compounds such as polylactic acid, polglycolic acid, hydrogels, etc, or onto liposomes, microemulsions, micelles, unilamellar or multilamellar vesicles, erytbrocyte ghosts, or spheroplasts. Such compositions will influence the physical state, solubility, stability, rate of in vivo release, and rate of in vivo clearance of SCF. The choice of compositions
will depend on the physical and chemical properties of the protein having SCF activity. For example, a product derived from a membrane-bound form of SCF may require a formulation containing detergent. Controlled or sustained release compositions include formulation in lipophilic depots (e.g., fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated wirh polymers (e.g., poloxamers or poloxamines) and SCF coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral. In one embodiment the pharmaceutical composition is administered parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intradermaly, subcutaneously, intraperitonealy, intraventricularly, intracranialy.

Further, as used hercin "pharmaceutically acceptable carricr" are well kriown to those skilled in the art and include, but are not limited to, $0.01-0.1 \mathrm{M}$ and preferably 0.05 M phosphate buffer or $0.8 \%$ saline. Additionally, such pharmaceutically acceptable carriers may be aqueous or non-aqueous solutions, suspensions, and emulsions. Examples of non-aqueous solvents are propylene glycol, polyethylene glycol, vegetable oils such as olive oil, and injectable organic esters such as ethyl oleate. Aqueous carriers include water, alcoholic/aqueous solutions, emulsions or suspensions, including saline and buffered media. Parenteral vehicles include sodium chloride solution, Ringer's dextrose, dextrose and sodium chloride, lactated Ringer's or fixed oils. Intravenous vehicles include fluid and nutrient replenishers, electrolyte replenishers such as those based on Ringer's dextrose, and the like. Preservatives and other additives may also be present, such as, for example, antimicrobials, antioxidants, collating agents, inert gases and the like.

The term "adjuvant" refers to a compound or mixture that enhances the immune response to an antigen. An adjuvant can serve as a tissue depot that slowly releases the antigen and also as a lymphoid system activator that non-specifically enhances the immune response (Hood et al., Immunology, Second Ed., 1984, Benjamin/Cummings:

Menlo Park, California, p. 384). Often, a primary challenge with an antigen alone, in the absence of an adjuvant, will fail to elicit a humoral or cellular immune response. Adjuvant include, but are not limited to, complete Freund's adjuvant, incomplete Freund's adjuvant, saponin, mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil or hydrocarbon emulsions, keyhole limpet hemocyanins, dinitrophenol, and potentially useful human adjuvant such as BCG (bacille Calmette-Guerin) and Corynebacterium parvum. Preferably, the adjuvant is pharmaceutically acceptable.

Controlled or sustained release compositions include formulation in lipophilic depots (e.g. fatty acids, waxes, oils). Also comprehended by the invention are particulate compositions coated with polymers (e.g. poloxamers or poloxamines) and the compound coupled to antibodies directed against tissue-specific receptors, ligands or antigens or coupled to ligands of tissue-specific receptors. Other embodiments of the compositions of the invention incorporate particulate forms protective coatings, protease inhibitors or permeation enhancers for various routes of administration, including parenteral, pulmonary, nasal and oral.

When administered, compounds are often cleared rapidly from mucosal surfaces or the circulation and may therefore elicit relatively short-lived pharmacological activity. Consequently, frequent administrations of relatively large doses of bioactive compounds may by required to sustain therapeutic efficacy. Compounds modified by the covalent attachment of water-soluble polymers such as polyethylene glycol, copolymers of polyethylene glycol and polypropylene glycol, carboxymethyl cellulose, dextran, polyvinyl alcohol, polyvinylpyrrolidone or polyproline are known to exhibit substantially longer half-lives in blood following intravenous injection than do the corresponding unmodified compounds (Abuchowski et al., 1981; Newmark et al., 1982; and Katre et al., 1987). Such modifications may also increase the compound's solubility in aqueous solution, eliminate aggregation, enhance the physical and chemical stability of the compound, and greatly reduce the immunogenicity and reactivity of the compound. As a result, the desired in vivo biological activity may be achieved by the administration of such polymer-compound abducts less frequently or in lower doses than with the unnodified compound.

Dosages. The sufficient amount may include but is not limited to from about $1 \mu \mathrm{~g} / \mathrm{kg}$ to about $1000 \mathrm{mg} / \mathrm{kg}$. The amount may be $10 \mathrm{mg} / \mathrm{kg}$. The pharmaceutically acceptable form of the composition includes a pharmaceutically acceptable carrier.

The preparation of therapeutic compositions which contain an active component is well understood in the art. Typically, such compositions are prepared as an aerosol of the polypeptide delivered to the nasopharynx or as injectables, either as liquid solutions or suspensions, however, solid forms suitable for solution in, or suspension in, liquid prior to injection can also be prepared. The preparation can also be emulsified. The active therapeutic ingredient is often mixed with excipients which are pharmaceutically acceptable and compatible with the active ingredient. Suitable excipients are, for example, water, saline, dextrose, glycerol, ethanol, or the like and combinations thereof. In addition, if desired, the composition can contain minor amounts of auxiliary substances such as wetting or emulsifying agents, pHy buffering agents which enhauce the effectiveness of the active ingredient.

An active component can be formulated into the therapeutic composition as neutralized pharmaceutically acceptable salt forms. Pharmaceutically acceptable salts include the acid addition salts (formed with the free amino groups of the polypeptide or antibody molecule) and which are formed with inorganic acids such as, for example, hydrochloric or phosphoric acids, or such organic acids as acetic, oxalic, tartaric, mandelic, and the like. Salts formed from the free carboxyl groups can also be derived from inorganic bases such as, for example, sodium, potassium, ammonium, calcium, or ferric hydroxides, and such organic bases as isopropylamine, trimethylamine, 2-ethylamino ethanol, histidine, procaine, and the like.

A composition comprising " A " (where " A " is a single protein, DNA molecule, vector, etc.) is substantially free of " B " (where " B " comprises one or more contaminating proteins, DNA molecules, vectors, etc.) when at least about $75 \%$ by weight of the proteins, DNA, vectors (depending on the category of species to which A and B belong)
in the composition is "A". Preferably, "A" comprises at least about $90 \%$ by weight of the $A+B$ species in the composition, most preferably at least about $99 \%$ by weight.

The phrase "therapeutically effective amount" is used herein to mean an amount sufficient to reduce by at least about 15 percent, preferably by at least 50 percent, more preferably by at least 90 percent, and most preferably prevent, a clinically significant deficit in the activity, function and response of the host.

According to the invention, the component or components of a therapeutic composition of the invention may be introduced parenterally, transmucosally, e.g., orally, nasally, pulmonarailly, or rectally, or transdempnally. Preferably, administration is parenteral, e.g., via intravenous injection, and also including, but is not limited to, intra-arteriole, intramuscular, intradermal, subcutaneous, intraperitoneal, intraventricular, and intracranial administration. Oral or pulmonary delivery may be preferred to activate mucosal immunity; since pneumococci generally colonize the nasopharyngeal and pulmonary mucosa, mucosal immunity may be a particularly effective preventive treatment. The term "unit dose" when used in reference to a therapeutic composition of the present invention refers to physically discrete units suitable as unitary dosage for humans, each unit containing a predetermined quantity of activc material calculated to produce the desired therapeutic effect in association with the required diluent; i.e., carrier, or vehicle.

According to the present invention, a method is also provided of supplying wild-type p-Hyde function to a cell which carries mutant p-Hyde alleles. Supplying such a function should suppress neoplastic growth of the recipient cells. The wild-type p-Flyde gene or a part of the gene may be introduced into the cell in a vector such that the gene remains extrachromosomal. In such a situation, the gene will be expressed by the cell from the extrachromosomal location. If a gene fragment is introduced and expressed in a cell carrying a mutant p-Hyde allele, the gene fragment should encode a part of the p-Hyde protein which is required for non-neoplastic growth of the cell. More preferred is the situation where the wild-type p-Hyde gene or a part thereof is introduced into the mutant cell in such a way that it recombines with the
endogenous mutant p-Hyde gene present in the cell. Such recombination requires a double recombination event which results in the correction of the p-Hyde gene mutation. Vectors for introduction of genes both for recombination and for extrachromosomal maintenance are known in the art, and any suitable vector may be used. Methods for introducing DNA into cells such as electroporation, calciumphosphate co-precipitation and viral transduction are known in the art, and the choice of method is within the competence of the routineer. Cells transformed with the wild-type p-Hyde gene can be used as model systems to study cancer remission and drug treatments which promote such remission.

As generally discussed above, the p-Hyde gene or fragment, where applicable, may be employed in gene therapy methods in order to increase the amount of the expression products of such genes in cancer cells. Such gene therapy is particularly appropriate for use in both cancerous and pre-cancerous cells, in which the level of p-Hyde polypeptide is absent or diminished compared to normal cells. It may also be useful to increase the level of expression of a given p-Hyde gene even in those rumor cells in which the mutant gene is expressed at a "normad" level, but the gene product is not fully functional.

Gene therapy would be carried out according to generally accepted methods, for example, as described by Friedman, 1991. Cells from a patient's tumor would be first analyzed by the diagnostic methods described above, to ascertain the production of p-Hyde polypeptide in the rumor cells. A virus or plasmid vector (see further details below), containing a copy of the p-Hyde gene linked to expression control elements and capable of replicating inside the rumor cells, is prepared. Suitable vectors are known, such as disclosed in U.S. Pat. No. 5,252,479 and PCT published application WO $93 / 07282$. The vector is then injected into the patient, either locally at the site of the rumor or systemically (in order to reach any rumor cells that may have metastasized to other sites). If the transfected gene is not permanently incorporated into the genome of each of the targeted minor cells, the treatinent may have to be repeated periodically.

Gene transfer systems known in the art may be useful in the practice of the gene therapy methods of the present invention. These include viral and nonviral transfer methods. A number of viruses have been used as genc transfer vectors, including papovaviruses, e.g., SV40 (Madzak et al., 1992), adenovirus (Berkner, 1992; Berkner et al., 1988; Gorziglia and Kapikian, 1992; Quantin et al., 1992; Rosenfeld et al., 1992; Wilkinson et al., 1992; Stratford-Perricaudet et al., 1990), vaccinia virus (Moss, 1992), adeno-associated virus (Muzyczka, 1992; Ohi et al., 1990), herpesviruses including HSV and EBV (Margolskee, 1992; Johnson et al., 1992; Fink et al., 1992; Breakfield and Geller, 1987; Freese et al., 1990), and retroviruses of avian (Brandyopadhyay and Temin, 1984; Petropoulos et al., 1992), murine (Miller, 1992; Miller et al., 1985; Sorge et al., 1984; Mann and Baltimore, 1985; Miller et al., 1988), and human origin (Shimada et al., 1991; Helseth et al., 1990; Page et al., 1990; Buchschacher and Panganiban, 1992). Most human gene therapy protocols have been based on disabled murine retroviruses.

Peptides which have p-Hyde activity can be supplied to cells which carry mutant or missing p-Hyde alleles. The sequence of the p-Hyde protein is disclosed (SEQ ID NO:2). Protein can be produced by expression of the cDNA sequence in bacteria, for example, using known expression vectors. Alternatively, p-Hyde polypeptide can be extracted from $\dot{p}$-Hyde-producing mammalian cells. In addition, the techniques of synthetic chemistry can be employed to synthesize p-Hyde protein. Any of such tecloniques can provide the preparation of the present invention which comprises the p-Hyde protein. The preparation is substantially free of other human proteins. This is most readily accomplished by synthesis in a microorganism or in vitro.

P-Hyde molecules can be introduced into cells by microinjection or by use of liposomes, for example. Alternatively, some active molecules may be taken up by cells, actively or by diffusion. Extracellular application of the p-Hyde geñe product may be sufficient to affect rumor growth. Supply of molecules with p-Hyde activity should lead to partial reversal of the neoplastic state. Other molecules with p-Fyde activity (for example, peptides, drugs or organic compounds) may also be used to effect such
a reversal. Modified polypeptides having substantially similar function are also used for peptide therapy.

In amother embodiment, the active compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid).

In yet another embodiment, the therapeutic compound can be delivered in a controlled release system. For example, the polypeptide may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)). Preferably, a controlled release device is introduced into a subject in proximity of the site of inappropriate immune activation or a tumor. Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

A subject in whom administration of an active component as set forth above is an effective therapeutic regimen for a bacterial infection is preferably a human, but can be any animal. Thus, as can be readily appreciated by one of ordinary skill in the art, the methods and pharmaceutical compositions of the present invention are particularly suited to administration to any animal, particularly a manmal, and including, but by no
means limited to, domestic animals, such as feline or canine subjects, farm animals, such as but not limited to bovine, equine, caprine, ovine, and porcine subjects, wild animals (whether in the wild or in a zoological garden), research animals, such as mice, rats, rabbits, goats, sheep, pigs, dogs, cats, etc., i.e., for veterinary medical use.

In the therapeutic methods and compositions of the invention, a therapeutically effective dosage of the active component is provided. A therapeutically effective dosage can be determined by the ordinary skilled medical worker based on patient characteristics (age, weight, sex, condition, complications, other diseases, etc.), as is well known in the art. Furthermore, as further routine studies are conducted, more specific information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, is able to ascertain proper closing. Generally, for intravenous injection or infusion, dosage may be lower than for intraperitoneal, intramuscular, or other route of administration. The dosing schedule may vary, depending on the circulation half-life, and the formulation used. The compositions are administered in a manner compatible with the dosage formulation in the therapeutically effective, amount. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20 , preferably about 0.5 to about 10 , and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

It is contemplated by this invention that p -Hyde replacement therapy could be used similarly in conjunction with chemo- or radiotherapeutic intervention. To induce susceptibility to cell dcath or to inhibit cell growth or to kill cells, such as malignant or metastatic cells, using the methods and compositions of the present invention, one would
contact a "target" cell with the expression vector and at least one DNA damaging agent In oen embodiment the cell is contacted with a single composition or pharmacological formulation that includes both agents, or by contacting the cell with two distinct compositions or formulations, at the same time, wherein one composition includes the vector and the other includes the DNA damaging agent. In another embodiment, treatment with the vector may precede or follow the DNA damaging agent treatment by intervals ranging from minutes to weeks. Protocols and methods are known to those skilled in the art.

DNA damaging agents or factors are known to those skilled in the art and means any chemical compound or treatment method that induces DNA damage when applied to a cell. Such agents and factors include radiation and waves that induce DNA damage such as, gamma -irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, and the like. A variety of chemical compounds, also described as "chemotherapeutic agents", function to induce DNA damage, all of which are intended to be of use in the combined treatment metbods disclosed herein. Chemotherapeutic agents contemplated to be of use, include, e.g., adriamycin, 5-fluorouracil (5FU), etoposide (VP-16), camptothecin, actinomycin-D, mitomycin $C$, cisplatin (CDDP) and even hydrogen peroxide. The invention also encompasses the use of a combination of one or more DNA damaging agents, whether radiation-based or actual compounds, such as the use of X-rays with cisplatin or the use of cisplatin with etoposide.

In another embodiment one may irradiate the localized tumor site with DNA damaging radiation such as X-rays, UV-light, gamma -rays or even microwaves. Alternatively, the tumor cells may be contacted with the DNA damaging agent by administering to the subject a therapeutically effective amount of a pharmaceutical composition comprising a DNA damaging compound such as, adriamycin, 5-fluorouracil, etoposide, camptothecin, actinomycin-D, mitomycin C , or more preferably, cisplatin. The DNA damaging agent may be prepared and used as a combined therapeutic composition, or kit, by combining it with a p-Hyde expression construct, as described above. Agents that directly cross-link nucleic acids, specifically DNA, are envisaged and are shown herein, to eventuate DNA damage leading to a synergistic antineoplastic combination. Agents such
as cisplatin, and other DNA alkylating may be used. Cisplatin has been widely used to treat cancer, with efficacious doses used in clinical applications of $20 \mathrm{mg} / \mathrm{m}<2>$ for 5 days every three weeks for a total of three courses. Cisplatin is not absorbed orally and must therefore be delivered via injection intravenously, subcutaneously, intratumorally or intraperitoneally. Agents that damage DNA also include compounds that interfere with DNA replication, mitosis and chromosomal segregation. Such chemotherapeutic compounds include adriamycin, also known as doxorubicin, etoposide, verapamil, podophyllotoxin, and the like. Widely used in a clinical setting for the treatment of neoplasms, these compounds are administered through bolus injections intravenously at doses ranging from $25-75 \mathrm{mg} / \mathrm{m}<2>$ at 21 day intervals for adriamycin, to $35-50$ $\mathrm{mg} / \mathrm{m}<2>$ for etoposide intravenously or double the intravenous dose orally.

Agents that disrupt the synthesis and fidelity of nucleic acid precursors and subunits also lead to DNA damage. As such a number of nucleic acid precursors have been developed. Particularly useful are agents that have undergone extensive testing and are readily available. As such, agents such as 5 -fluorouracil ( $5-\mathrm{FU}$ ), axe preferentially used by neoplastic tissue, making this agent particularly useful for targeting to neoplastic cells. Althougl quite toxic, $5-\mathrm{FU}$, is applicable in a wide range of carriers, including topical, however intravenous administration with doses ranging from 3 to $15 \mathrm{mg} / \mathrm{kg} /$ day being commonly used.

Other factors that cause DNA damage and have been used extensively include what are commonly known as ganma -rays, X-rays, and/or the directed delivery of radioisotopes to tumor cells. Other forms of DNA damaging factors are also contemplated such as microwaves and UV-irradiation. It is most likely that all of these factors effect a broad range of damage DNA, on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of 50 to 200 roentgens for prolonged periods of time ( 3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

Several non-viral methods for the transfer of expression constructs into cultured mammalian cells also are contemplated by the present invention. These include calcium phosphate precipitation (Graham and Van Der Eb, 1973; Chen and Okayama, 1987; Rippe et al., 1990) DEAE-dextran (Gopal, 1985), electtoporation (Tur-Kaspa et al., 1986; Potter et al., 1984), direct microinjection (Harland and Weintraub, 1985), DNA-loaded liposomes (Nicolau and Sene, 1982; Fraley et al., 1979) and lipofectamine-DNA complexes, cell sonication (Fechlheimer et al., 1987), gene bombardment using high velocity microprojectiles (Yang et al., 1990), and receptor-mediated transfection (Wu and Wu, 1987; Wu and Wu, 1988). Some of these techniques may be successfully adapted for in vivo or ex vivo use. Also, helper cell lines may be derived from humaux cells such as human embryonic kidney cells, muscle cells, hematopoietic cells or other human embryonic mesenchymal or epithelial cells. Alternatively, the helper cells may be derived from the cells of other mammalian species that are permissive for human adenovirus. Such cells include, e.g. , Vero cells or other monkey embryonic mesenchymal or epithelial cells. As stated above, the preferred helper cell line is 293 .

In another embodiment, the active compound can be delivered in a vesicle, in particular a liposome (see Langer, Science 249:1527-1533 (1990); Treat et al., in Liposomes in the Therapy of Infectious Disease and Cancer, Lopez-Berestein and Fidler (eds.), Liss, New York, pp. 353-365 (1989); Lopez-Berestein, ibid., pp. 317-327; see generally ibid).

In yet another embodiment, the therapeutic compound can be delivered in a controlled release system. For example, the polypeptide may be administered using intravenous infusion, an implantable osmotic pump, a transdermal patch, liposomes, or other modes of administration.. In one embodiment, a pump may be used (see Langer, supra; Sefton, CRC Crit. Ref. Biomed. Eng. 14:201 (1987); Buchwald et al., Surgery 88:507 (1980); Saudek et al., N. Engl. J. Med. 321:574 (1989)). In another embodiment, polymeric materials can be used (see Medical Applications of Controlled Release, Langer and Wise (eds.), CRC Pres., Boca Raton, Florida (1974); Controlled Drug Bioavailability, Drug Product Design and Performance, Smolen and Ball (eds.), Wiley, New York (1984); Ranger and Peppas, J. Macromol. Sci. Rev. Macromol. Chem. 23:61 (1983); see also

Levy et al., Science 228:190 (1985); During et al., Ann. Neurol. 25:351 (1989); Howard et al., J. Neurosurg. 71:105 (1989)). In yet another embodiment, a controlled release system can be placed in proximity of the therapeutic target, i.e., the brain, thus requiring only a fraction of the systemic dose (see, e.g., Goodson, in Medical Applications of Controlled Release, supra, vol. 2, pp. 115-138 (1984)). Preferably, a controlled release device is introduced into a subject in proxinnity of the site of inappropriate immune activation or a tumor. Other controlled release systems are discussed in the review by Langer (Science 249:1527-1533 (1990)).

As can be readily appreciated by one of ordinary skill in the art, the methods and pharmaceutical compositions of the present invention are particularly suited to administration to a mammal, preferable a human subject.

In the therapeutic methods and compositions of the invention, a therapeutically effective dosage of the active component is provided. A therapeutically effective dosage can be determined by the ordinary skilled medical worker based on patient characteristics (age, weight, sex, condition, complications, other diseases, etc.), as is well known in the art. Furthermore, as further routine studies are conducted, more specific information will emerge regarding appropriate dosage levels for treatment of various conditions in various patients, and the ordinary skilled worker, considering the therapeutic context, age and general health of the recipient, is able to ascertain proper dosing. Generally, for intravenous injection or infusion, dosage may be lower than for intraperitoneal, intrannuscular, or other route of administration. The dosing schedule may vary, depending on the circulation half-life, and the formulation used. The compositions are administered in a manner compatible with the dosage formulation in the therapeutically effective amount. Precise amounts of active ingredient required to be administered depend on the judgment of the practitioner and are peculiar to each individual. However, suitable dosages may range from about 0.1 to 20 , preferably about 0.5 to about 10 , and more preferably one to several, milligrams of active ingredient per kilogram body weight of individual per day and depend on the route of administration. Suitable regimes for initial administration and booster shots are also variable, but are typified by an initial administration followed by repeated doses at one or more hour intervals by a
subsequent injection or other administration. Alternatively, continuous intravenous infusion sufficient to maintain concentrations of ten nanomolar to ten micromolar in the blood are contemplated.

The present invention provides a kit comprising the all the essential materials and reagents required for inhibiting prostate tumor cell proliferation, transforming prostate cells or detecting prostate cancer cells, may be assembled together in a kit. This generally will comprise selected expression constructs. Also included may be various media for replication of the expression constructs and host cells for such replication. Such kits will comprise distinct containers for each individual reagent. When the components of the kit are provided in one or more liquid solutions, the liquid solution preferably is an aqueous solution, with a sterile aqueous solution being particularly preferred. For in vivo use, the expression construct may be formulated into a pharmaceutically acceptable syringeable composition. In this case, the container means may itself be an inhalent, syringe, pipette, eye dropper, or other such like apparatus, from which the formulation may be applied to an infected area of the body, such as the Jungs, injected into an animal, or even applied to and mixed with the other components of the kit. The components of the kit may also be provided in dried or lyophilized forms. When reagents or components are provided as a dried form, reconstitution generally is by the addition of a suitable solvent. It is envisioned that the solvent also may be provided in another container means.

The kits of the present invention also will typically include a means for containing the vials in close confinement for commercial sale such as, e.g., injection or blow-molded plastic containers into which the desired vials are retained. Irrespective of the number or type of containers, the kits of the invention also may comprise, or be packaged with, an instrument for assisting with the injection/administration or placement of the ultimate complex composition within the body of an animal. Such an instrument may be an inhalent, syringe, pipette, forceps, measured spoon, eye dropper or any such medically approved delivery vehicle.

The following examples are presented in order to more fully illustrate the preferred embodiments of the invention. They should in no way be construed, however, as limiting the broad scope of the invention.

## EXPERIMIENTAL DETAILS SECTION

## EXAMPLE: p-Ffyde induces susceptibility to induction of cell programmed cell death in prostate cancer

## MATERIALS AND METHODS

Cell lines: Two rat prostatic cancer cell lines (amaplastic tumor, low metastatic AT-1 and highly metastatic MAT-LyLu) were used for the cDNA competition hybridization strategy (Rinaldy and Steiner, 1997). Other Dunning rat prostate cancer cell lines used for Northern analyses were AT-3, MAT-LyLu, MAT-Lu and G. These cell lines were derived from established in-vivo Dunning R3327 rat prostatic tumor sublines and further developed as in-vitro cell lines by Isaacs et al. at Johns Hopkins Oncology Center, Baltimore, Maryland (Isaacs et al., 1986). Human prostate cancer cell lines PPC-1, LNCaP, TSU and DU145 used for Northem analyses were obtained from American Tissue Culture Collection, Rockville, Maryland. All cells were grown in RPMI 1640 medium (Mediatech, Herndon, Virginia) in the presence of $10 \%$ fetal calf serum, 50 units of penicillin G and $50 \mu \mathrm{~g}$ streptomycin sulfate per ml and $250 \mu \mathrm{M}$ dexamethasone as previously described (Isaacs et al, 1986).

Cloning strategy: Radiolabeled MAT-LyLu cDNA population in the presence of vast excess amount of competitor non-radiolabeled AT-1 cDNA population was used to identify cDNAs clones in the MAT-LyLu cDNA library (Rinaldy and Steiner, 1997). One of these cDNAs was novel and desiguated as $p$-Hyde. The prostate cancer associated p-Hyde cDNA was further characterized.

Characterization of cDNA: Sequencing-p-Hyde cDNA was originally obtained as a ? ZAP Uni XR clone, and was further subcloned into pBluescript SK vector through in vivo excision protocol as described (Stratagene. La Jolla, California). This
double-stranded cDNA was further subjected for Dye Terminator Cycle Sequencing (Perkin Elmer, Foster City, California) using ABI 377 automatic DNA sequencer Version 3.0. The open reading frame of p-Hyde cDNA was determined using the DNA Strider program (Pasteur Institute, Paris).

Northern blot analyses: Total RNAs were isolated from cell lines during exponential growth ( $70 \%$ confluence) by using RNAZol B as suggested by the supplier (Tel-Test, Inc., Friendswood, Texas) and subjected to Northern blot analyses. Reverse and forward PCR primer were designed and used to amplify 1.35 kb of the p-Hyde sequence representing the 1467 bases of its open reading frame. The PCR product was radiolabeled by random oligo labeling technique (Multiprime DNA Labeling System, Amersham) and used as a probe for Northern analyses. Relative expression of $p$-Hyde gene was compared to an internal control (cyclophylin) by scanning of the autoradiogram with a PDI Discovery Series Scanner equipped with Quantity One software.

In vitro transcription and translation: The open reading frame of the cDNA was confirmed using in-vitro transcription (capped mRNA) as described followed by in-vitro translation using rabbit reticulocyte lysate (Stratagene, La Jolla, California) in the presence of 75 Ci of $\mathrm{L}-\left[{ }^{35} \mathrm{~S}\right]$ Methionine (Amersham, Arlington Heights, Illinois). The in vitro translation product was further identified on $10 \%$ SDS polyacrylamide gel electrophoresis by autoradiography.

Subcloning of p-Ytyide into pcDNA3.1 (-): cDNA insert was released from the pBluescript SK* vector through double digests by KpnI and SacI (SK fragment). This fragment represents the intact $p$-Hyde sequence and was then ligated into dephosphorylated KpnI-SacI double digests of mammalian shuttle vector pcDNA $3.1(-)$ (Invitrogen). Ligation mix was used to transfect competent DH5?, selected for ampicillin resistance followed by plasmid preparation using standard cesium chloride density gradient centrifugation. The new construct of $p$-Hyde was then used to transfect AT3 rat prostatic cancer subline by using lipofectamine (Gibco/BRL) followed by G418 selection (Rinaldy et al., 1988). Eight clones were obtained and two of them, AT3-H1
and AT3-H2, were used to assess the function of p-Hyde in its association with apoptosis. In addition, AT3 cell line was also transfected with pcDNA3.1(-) vector only and its stable transfected cel 1 line, designated as AT3-pc was used as negative control relative to stable transfectant of $\mathrm{AT} 3-\mathrm{Hl}$ and $\mathrm{AT} 3-\mathrm{H} 2$ for the functional assessment of the p-Hyde.

Apoptosis assay: Apoptosis intensity in AT3 parental cell line was assessed in comparison with AT3-H1, AT3-H2 and AT3-pe as negative control. Two apoptotic agents were employed for this assessment: (1) UV damage of the DNA using UV dosage of $200 \mathrm{~J} / \mathrm{m} 2$; apoptosis intensity was assayed at 36 h post-UV irradiation and (2) $100 \mu \mathrm{M}$ Fluorodeoxyuridine (FUrD) treatment for 36 hours followed by the apoptosis assay. After these apoptotic induction, cells were collected in two fractions: floating and attached cells. Both cell fractions were counted using Neubauer claamber and trypan blue exclusion. DNA were extracted separately from both fractions and analyzed on 1.6 \% agarose gel electrophoresis to visualize the DNA laddering.

UV-damage repair assay: Ultraviolet induced damage in the DNA was assayed using mouse monoclonal antibody specifically cross reacts with cyclobutane pyrimidine dimer (TDM-2) and photoproduct ( $64 \mathrm{M}-2$ ). The presence of cyclobutane dimer and 64 photoproducts in DNAs were assessed in microtiter plates (100 and $200 \mathrm{ng} /$ well ) using both antibodies separately in a standard ELISA technique. In addition, the UV resistance was also assessed by using UV gradient assay as published (Rinaldy et al, 1988).

Uridine Phosphorylase Assay: Cell extract will be prepared from the cell pellet Before and after the induction with $1 \mathrm{mM} 5-\mathrm{dFUrd}$ for 24 hours, the corresponding cell extract will be prepared in 50 mM Potassium Phosphate buffer pH 7.4 through sonication followed by dialysis against the reaction buffer ( 50 mM potassium phosphate, pH 7.4). The amount of protein will be determined by using standard Lowry or Biorad assay. The same amount of protein from all cell lines will be assayed for UP activity in 50 mM potassium phosphate buffer ( pH 7.4 ) containing 10 mM uridine or thymidine as substrate. After 30 min incubation at $37^{\circ} \mathrm{C}$, the reaction will be terminated by adding
methanol followed by centrifugation. An aliquot of the supernatant will be run on HPLC column ( $6 \times 200 \mathrm{~mm}$ ) of ERC-ODS-1171 (ERMA CR, Inc). The amount of reaction product Uracil or Thymidine can be measured with UV detector at 265 nm compared to the standard. As negative control, the similar reaction mixture will be boiled before the incubation.

Coastruction of cDNA libraries: In the first stage, cDNA. libraries derived from AT-1 and MAT-LyLu cell lines were generated using Uni ZAP XR vector based on the protocol of Stratagene. The independent clones obtained were 1.9 and 3.4 million clones for MAT-LyLu and AT-1, respectively. These unamplified libraries were subjected to PCR amplification of the cDNA insert population. Reverse primers (RP) and forward primers (FP), downstream and upstrean of Xhol and Eco RI cloning site, were used to amplify the cDNA insert population. The distance between both primers in? Uni ZAP or pBluescript was 228 bases.

Design of competition probes. Two PCR probes were amplified: radiolabeled MAT-LyLu cDNA population probe and the non-radiolabeled AT-1 cDNA population probe (the cold competitor). The radiolabeled MAT-LyLu PCR product was enriched using S400 Sephacryl spin columa. The majority of the unincorporated ${ }^{32}$ P-dCTP, primer dimer, and 228 bp of PCR product resulting from the amplification of ? DNA without insert, was separated from the cDNAs. The purified radiolabeled cDNAs were mixed with 30 fold excess of non-radiolabeled cold competitor AT-1 PCR products and used as a competition-probe to screen the MAT-LyLu cDNA library.

Two kinds of unexpected radiolabeled PCR products that may potentially interfere with the hybridization between the radiolabeled cDNA of the competition probe and the screened cDNA of the library were: 1) the non-exponential amplification of the cDNAs, and 2) 228 bp PCR product derived from the ? DNA without cDNA insert. In order to reduce the possible cross-hybridization between these two unexpected PCR products with the vector of the screened library, excess amounts of HindIII-digested_DNA, PvuII-digested-pBluescript DNA, and the 228 bp PCR product of the pBluescript based on both primers were mixed with the competition probe. Preliminary assessment of this
complete mixed competition probe indicated that the hybridization of the MAT-LyLu cDNA library with this probe was extremely weak; whereas the duplicate filter hybridized with the same probe, but without non-radiolabeled AT-1 PCR product, was extremely positive. This clearly indicate that the positive hybridization of the MAT-LyLu products was due to the radiolabeled MAT-LyLu cDNAs of the PCR products which was not competed by the AT-1 cDNA.s.

Screening of MAT-LyLu cDNA Libraries. Independent cDNA clones of the unamplified MAT-LyLu phage cDNA library ( 250,000 clones) were screened with the competition probe as described above. Nineteen enhanced signals were observed; these putative prostate cancer- or metastasis-associated plaques were then rescreened. As the result of this rescreening, 12 individual plaques were purified. The cDNAs were excised and subcloned into plasmid based vectors (pBluescript). This is possible due to the ability of helper phage ExAssist (Stratagene) to excise the cDNA with the pBluescript sequence in circular forma as a filamentous phage and secreted from the cell (Fig. 2). Recombinant pBluescript plasmids were recovered by infecting an $F^{\prime}$ strain of E. coli (SOLR strain, Stratagene), ampicillin resistant colonies were selected, and the plasmid DNA was extracted. The obtained plasmids were digested with Eco RI and XhoI to identify the cDNA inserts by agarose gel electrophoresis. The results indicated that only four plasmids carried cDNA inserts.

Sequencing of four candidate genes: All four cDNAs were partially sequenced at both the 3 ' and 5'-ends. These initial sequences were used to search the Genbauk nucleic acid database of NCBI for homology or similarities. Three of the cDNAs matched known sequences: 1) rat mitochondrial genes coding for $16 s$ and 12 s rRNAs and tRNAs specific for valine and phenylalanine (Accession \# emb/V00680/MIRNR2). 2) rat nucleolar proteins B23.1 mRNA (Accession \# gb/T03969/RATB23NP) and 3) rat nucleolar proteins B23.1 and B23.2 (Accession \# gb/M37041/RATNUCBA7). The results of this competitive hybridization were consistent with the recognized phenotypic difference between both cell lines. MAT-LyLu exhibits twice the number of both nucleoli and mitochondria (Isaacs et al, 1986). This also suggests that the MAT-LyLu cell line is
metabolically more active due to higher gene-dosage or gene-amplification of nucleolar and mitochondrial rRNA and its associated genes.

The complete sequence of the putative gene, p-Hyde: The fourth cDNA (lane 1), designated as $p$-Hyde, had an initial sequence which did not match any known full length sequences in the BLASTN mucleic acid database of the NCBI. Accordingly, p-Hyde cDNA was completely sequenced by using a walk-through sequencing strategy in both directions for three redundancies. Nine contigs from each sequencing direction were obtained and compounded as a full length composite of 2694 bases. The poly(A) tail on the $3^{\prime}$-end and polyadenylation signal sequence of GAGAAA (a slight modification of the conserved AATAAA sequence) located at the position 27 upstream from the poly(A) was also identified. Fig. 6 shows the sequencing strategy and the restriction map illustrating the open reading frame of $p$-Hyde. SEQ ID No. 3 sets forth the resulting nucleic acid sequence of the rat $p$-Hyde and the resulting amino acid sequence of the rat $p$-Hyde.

Generation of PCR probe representing open reading frame p-Hyde Gene: Figure 8 shows the PCR amplification of the CDNA insert using three sets of sequencing primers. PCR product based on primer 11 and 5 represents the open reading frame of the $p$-Hyde cDNA. It was further radiolabeled by Random-Oligo-Labeling technique and used as a representative probe for the cDNA insert in Northern analysis.

Northern blot analysis of p-Hyde. Some of the rat prostate-cancer sublines (AT-1, MAT-LyLu, MAT-Lu, AT-3 and G) and human prostate cancer cell line (PPC, LNCaP, DU145 and TSU) were assessed by Northern blot analysis using PCR radiolabeled probe. The result indicated that the transcript of the human counterpart was slightly smaller relative to the rat $p$-fyyde mRNA. The same blot was further hybridized with cyclophylin cDNA as internal control. In this autoradiogram, 28 S and 18 S ribosomal RNA were used as marker. The length of the transcript was calculated based on the Spirin's formula $M=1550 \times \mathrm{S}^{2.1}$ where $M=$ molecular weight and $S=$ Svedberg's constant (McConkey, 1967). Signals of the p-Hyde and cyclophylin transcripts were quantitated and normalized using a computerized densitometer (Quantity-One software,

PDI. The levels of $p$-Hyde mRNA expression from both rat and human prostate cancer cell lines were compared (Table I). The data indicated that there was differential expression of p-Hyde gene in both Dumning rat and human prostate cancer cell lines which suggests that there may be a functional correlation between $p$-Hyde expression and prostate cancer progression. MAT-Lu showed the highest level of transcription, whereas AT-3 had the lowest. These data also indicated that the level of transcription in MAT-LyLu was relatively higher than that of AT-1, suggesting that the novel cDNA was the result of cDNA competition between MAT-LyLu and AT-1 cDNAs. More importantly, the human homologue of $p$-Hyde does exist as demonstrated in the human prostate cancer cell lines. The highest level of $p$-Hyde transcription occurred in PPC-1, whereas the lowest was in LNCaP cell line (Table 1).

Analysis of the deduced amino acid sequence of p-Hyde derived from ORF: Open reading of the cDNA consists of 1467 bases coding for 489 amino acid residues; the calculated molecular weight of this protein is 54.8 kD . Further molecular analyses of this deduced amino acid sequence is based on the Kyte-Doolittle hydrophilicity plot, James-Wolf antigenic index and Emini surface probability plot using Laser Gene DNAstar software. In addition, hydrophilicity profile of the deduced amino acid sequence was deduced based on Hopp and Woods using Antigen program for the prediction of its antigenic determinants (Table II). The results of the first three analyses were in agreement with the fourth one. Two peptide regions (residues 113 through 131 and residues 223 through 249) exhibited the highest points of lyydrophilicity and the antigenic index. Both peptide sequences can be used as immunogenic peptide to generate antibody. Its application to detect the translation product of $p$-Hyde in Western analysis was in agreement with the highest score of its surface probability.

Table II. Average FIydrophilicity Amino-Acid Sequence

| 2.53 | 241 to 246 |
| :--- | :--- |
| 1.93 | 117 to 122 |
| 1.73 | 119 to 124 |

Confirmation of the ORF by in vitro transcription and translation: To confirm the reading frame based on the sequencing data, the p-Hyde cDNA which is constructed in pBluescript was digested with KpnI rendering the linearized construct to be accessible for T3-RNA polymerase. This enzyme directs the synthesis of the riboprobe with 5'cap structure ( 5 'me7Gppps'G analog) similar to those present in cukaryotic mRNA (Stratagene). The $5^{\prime}$ cap is important to increase the yield and the stability of the synthesized mRNA as well as to enhance the in-vitro translation efficiency of the $m$ mNA. This capped mRNA was then used as a template for in-vitro translation using rabbit reticulocyte lysates (Stratagene) in the presence of ${ }^{35} \mathrm{~S}$-Methionine. The translation products were identified under denaturing conditions using polyacrylamide gel electrophoresis containing 8 M urea. The autoradiograph shown in Fig. 13 revealed that two translation products ( 55 and 55.5 kDa protein) were observed.

Molecular weights of these two proteins are in agreement with the calculated molecular weight of the deduced amino acid sequence of the reading frame. In addition, two start codons were also identified in this reading frame correlating with the two translation products obtained through in vitro translation of the riboprobe. The difference in molecular weight between both translation products is in agreement with the difference in 4 amino acid residues (MSGE) on the C terminal between both start codons. The molecular weight difference between both translation products is in agreement with the 0.5 kDa molecular weight of MSGE.

Subcloning of $p$-Hyde insert into pcDNA 3.1 (-) and transfection into rat and human prostate cancer cell lines: Referring to the data of differential expression of $p$-Hyde in rat and human prostate cancer cell lines, it is tempting to integrate this gene into cell line
expressing the lowest level of $p$-Hyde followed by the assessment of the function of this gene in its stable transfectants. For this purpose, p-Hyde cDNA was subcloned into pcDNA3.1 (-) mammalian expression vector (Invitrogen). The following features of pcDNA3.1 facilitate both subcloning and gene expression: (1) An extensive multiple cloning site to facilitate cloning in one direction. (2) A CMV promoter to drive the constitutive transcription and translation of the inserted $p$-Hyde cDNA, and (3) An SV40 splice acceptor linked to a SV40 polyadenylation signal to facilitate expression. The KpnI-Xbal fragment of the $p$-Hyde cDNA containing an intact open reading frame was successfully subcloned into pcDNA. $3.1(-)$ vector. The CsCl -banding-purified-pcDNA containing HYDE insert (designated as pcFYDE) was then transfected into rat (AT-1 and AT-3) and human (DU145 and PPC-1) prostate cancer cell line using Lipofectamine (Gibco/BRL) under G418 selection. Iu addition to this pcHYDE, all cell lines were also successfully transfected with the pcDNA3.1(-) vector as negative control. The results of obtaining the stable transfectants indicated that pcHYDE gene product is not toxic to the host cell.

In vitro assessment of stable p-Hyde transfectants: The function of pcHYDE in stable transfectants (AT1 and AT3 group) listed were assessed in vitro in association with the cell cycle and apoptosis. The objective for the apoptosis assessment is based on the hypothesis that $p$-Hyde is a rat homologue of the murine putative TSAP-6 gene which is associated with the upregulation of apoptosis response under the induction of tumor suppressor p53 (Amson et al., 1996). Whereas the cell cycle analysis is referred to the fact that MAT-LyLu cell line is highly metastatic and the level of $p$-Hyde expression in this cell line is relatively higher than AT-1 cell line.

Cell cycle analysis: The strategy of the cell cycle analysis is based on the arrest of cell population in Gl and $S$ boundary after 24 hours treatment with 1 mM Hydroxyurea (Iwasaki et al., 1995). As a result, the G2 phase should be zero. Cells were harvested after hydroxyurea release at 0,10 and 24 hours and subjected to flowcytometer analysis using standard propidium iodide staining. The result is shown in TABLE III and showed that both AT1-H1 and AT3- HI , at 10 hours after the release of Hy droxyurea, were relatively slow in entering the S-phase, whereas the parental cell line (AT1 and

AT3) were faster. At 24 hours after the release, the G2 cells were all elevated indicating that cell cycle was in progress. Overall, the slow entrance into $S$ phase in both pcHYDE stable transfectants does not corroborate directly that it is correlated with the slower tumor growth in vivo. The progress of re-entering the cell cycle at 0,10 and 24 hours after the release of Hydroxyurea were followed by flowcytometer. Consistently, no G2 phase was detected at to - after 24 hours treatment with Hydroxyurea - demonstrating the cell cycle arrest in the G1 and S boundary.

Overall, the arrest of all cell cultures in Gl and S boundary by l mM Hydroxyurea treatment for 24 hours was confirmed referring to the $0 \%$ population of G 2 . These results demonstarte that the slower response in entering the S phase in AT1-H1 and AT3-H1 is likely due to the effect of the pcHYDE gene product that may reflect the growth characteristic of these stable transfectants in vivo.

Induction of susceptibility to apoptosis: Apoptotic response was assessed using DNA laddering assay. DNAs were extracted after the respective treatment ( 24 hours with 1 mM Hydroxyurea followed by 24 hours with $0.1 \mathrm{mM} 5^{\prime}$-dFUrd) and analyzed on $1.6 \%$ agarose gel electrophoresis. In agreement with cell cycle analyscs, apoptotic response of the stable transfectants AT1-Hl and AT3-H1 (pcHYDE transfected AT-1 and AT-3) are consistently and significantly higher relative to both parental (AT-1 and AT-3) and pcDNA-transfected parental cell lines (AT1-pcl and AT3-pc1). In particular, the highest apoptotic response occurred in syncluronized culture under the induction with 0.1 mM 5'-dFUrd.

The enhanced apoptotic response in AT1-H1 and AT3-H1 transfectant after hydroxyurea treatment is the result of "thymineless death" (Kyprianou, 1994, Kyprianou et al., 1994) leading to depletion of intracellular thymidine-5-triphosphate (TTP) pools through indirect inhibition of thymidylate synthetase by fluorodcoxyuridine. Taken together, these data demonstrate that the apoptotic response in the pcl-CYDE stable transfectants is due to the downstream effect of pcHYDE gene product.

UV-damaged DNA cannot be repaired in prostate cells transfected with p-Hyde:Three DNA enzyme repair systems were evaluated in parental compared to p-Hyde transfected cells: uridine phosphorylase, uridine kinase, and UV damage repair. UV damage repair was impaired in the $p$-Hyde transfected cells. Decreased DNA repair activity results in higher levels of intact photoproducts (64PP). Consistent with these data, pHyde transfected cells also had a significant reduction in survival following UV exposure compared to parental AT3 cells as deternined by colony fomnation assay. Thus, DNA repair enzyme impairment correlated with shorter survival and induction of apoptosis in prostate cancer cells transfected with p-Hyde.

Northern RNA analysis of $p$-HYyde confirms overcxpression of p-Hyde: To confirm the correlation between apoptotic response and p-Hyde on the transcription level, Northern analysis of the total RNA derived from AT3-H1, AT3-H2 relative to AT3 parental cell line was performed. The result clearly demonstrated that the transcription level of pcHYDE in stable transfectant of AT3-H1 and AT3-H2 were significantly higher relative to AT3 parental cell line. This Northern analysis was carried out only in AT3, AT3-H1 and AT3-H2 using Hyde PCR product and internal control cyclophilin as a probe.
p-Hyde suppresses prostate cancer growth in vivo: The lowest level of p-Hyde expression was observed in AT3 cell line. For this reason, AT3 cell line was transfected with pcHYDE, a construct of $p$-Hyde in mammalian expression vector of pcDNA3.1(-) under G418 selection. As negative control, AT-3 cell line was also transfected with the vector only and the stable trausfectants obtained was desiguated as AT3-pc.

The tumor growth of the parental cell line of AT-3 and in stable transfectant AT3-H1 and AT3-H2 have been evaluated in vivo. One million colls of each cell lines in 0.3 ml of Hanks solution were inoculated subcutaneously in each flauk of inbred male Copenhagen rat. In this initial experiment, three groups of each five rats were injected with each cell line. The size of tumors were scored after a time schedule. These results indicated that both AT3-H1 and AT3-H2 stable transfectant grew significantly slower
than the AT-3 parental cell line suggesting that the tumor growth regression in both stable transfectants are regulated by the pcHYDE gene product.

Tumor progression represents an accumulation of genetic changes that affect oncogene and tumor suppressor gene expression, thereby altering the responsiveness of the cell to autocrine and paracrine positive and negative growth regulators. The Dunning tumor rat model of prostate cancer tumor progression consists of a spectrum of prostate cancer phenotypes ranging from well differentiated to poorly differentiated with differing responses to androgens. Moreover, the sublines originated and evolved from the same original spontaneous rat prostate tumor, thus making this present study of tumor progression unique. The developinent of these cell lines as the results of progression of tumor within a single and multiple serial passage .

The screening strategy of competition hybridizatiou of cDNA library generated from highly metastatic rat MAT-LyLu cell line resulted in a novel cDNA clone designated as p-Hyde (Rinaldy and Steiner, 1997). The full length of this cDNA, indicated as its restriction map, consists of 2713 nucleic acid residues; it contains two reading frames consisting of 1467 and 1452 residues, respectively. Forty six percent of the sequence is the untranslated region and majority of this is in the $3^{\prime}$ end of the gene. The nucleic acid sequence of the rat p-Hyde is set forth in SEQ ID NO 3 and the nucleic acid sequence of the human $p$-Hyde is set forth in SEQ ID NO 1.

The level of expression of $p$-Hyde was determined by Northern blot analyses in both rat and human prostate cancer cell lines and compared to the cyclophylin expression as internal control. The Dunning rat prostatic cancer cell line AT-1, MAT-LyLu, MAT-Lu, AT-3 and G and human prostate cancer cell lines PPC1, LNCaP, TSU and DU145 are all positive with different level of expression (Table I). The transcript size of the human counterpart is slightly smaller relative to the rat $p$-Hyde mRNA. The data also indicated that there is a differential expression of p-Hyde gene in both rat and human prostatic cancer cell lines suggesting that there may be a functional correlation between $p$-Hyde expression and prostate cancer progression. MA.T-Lu showed the highest level of rranscription, whereas AT-3 had the lowest. The striking difference between these two
cell lines is important in its association with the growth characteristic of MAT-Lu cell line which is higldy metastatic. These data also indicated that the level of transcription in MAT-LyLu was relatively ( $10 \%$ ) higher than that of AT-1, demonstrating that the novel cDNA was the result of cDNA competition between MAT-LyLu and AT-1 cDNAs (Rinaldy and Steiner, 1997). Of significance, the human homologue of p-Hyde does exist in the human prostate cancer cell lines. The highest level of $p$-Hyde transcription occured in PPC1, a primary androgen sensitive human prostate cancer cell line, whereas it was lowest in LNCaP cell line, an androgen-insensitive prostate cancer cell line. Thus, p-Hyde does not appear to be specific to mammalian prostate tissue, but has been found in other tissues including placenta and breast and in other species such as mouse and human suggesting that its role is important in fundamental cellular biology.

Tumor suppression and induction of susceptibility to apoptosis: Interestingly, $p$-Hyde has the dual ability to act like a tumor suppressor gene and induce susceptibility to apoptosis by what may be p53 independent pathways. The growth of prostate tumors in rats was greatly inhibited by $p$-Hyde. Moreover, prostate cancer cells expressing $p-H y d e$ were more sensitive to UV DNA damage driving these cells into cell programmed death. Analysis of DNA repair enzyme activity suggests a defect resulting in the presence of intact ( $6-4$ ) PP and decreased cell survival by colony forming assay. However, the capacity of $p$-Hyde to induce susceptibility to apoptosis is not limited to UV DNA damage. Chemotherapy agent, Fluorodeoxyuridine, a pyrimidine antimetabolite which is related to fluorouracil ( $5-\mathrm{FU}$ ) and has been used for treatment of a wide variety of human epithelial malignancies, also more readily induces apoptosis in prostate cancer cell expressing p-Hyde. Moreover, cancer cells expressing p-Hyde are also more susceptible to gamma radiation. Thus, the mechanisms of cellular DNA injury are different for UV, gamma radiation, and Fluorodeoxyuridine suggesting that the ability to make cells more susceptible to apoptosis is more global in action. This unique function of $p$-Hyde represents a new class genes that induce susceptibility to apoptosis. This is different than the function ascribed to tumor suppressor genes like p53 which directly induces apoptosis, not sensitivity to apoptosis (Yonish-Rouach et al., 1991). Moreover, p-Hyde activity is in contrast to bcl-2 where the absence of bcl-2, not the
presence of, makes the cancer cell more susceptible to cell programmed death (McDonnellet al., 1992).

Use in therapy of human disease: These unique functional features of the p-Hyde gene may be exploited for the treatment of hyperproliferative disorders and cancer. One effective therapeutic strategy, for example, may be the treatment of carcinoma cells expressing p-Flyde with chemotherapy agents or UV mimetic drugs (such as acetylaminofluorine). However, cancer cells are not likely to produce significant levels of the growth inhibition $p$-Hyde. Consequently, the $p$-Hyde gene my be introduced into cancer cells by gene therapy. Tumors transduced with vectors containing $p$-Hyde may not only be directly suppressed by $p$-Hyde as demonstrated in this study, but also when treated in combination with DNA damaging therapy such as chemotherapy, UV mimetic drugs, or radiation, have even a greater anti-cancer effect. Since gene therapy will target cancer cells, then enlancement of apoptosis will occur more selectively in cancer cells following DNA damage (UV, radiation, or chemotherapy).

## EXAMPLE 2: Prostate Cancer Gene Therapy Using Adenovirus Expressing A Novel Tumor Suppressor Gene pHyde

## MATERIALS AND METHODS

Cell lines and tissue culture condition: Human prostate cancer cell lines (obtained from ATCC, Rockville, Maryland) PPC-1, DU145, PC-3, LNCaP, and TSU-Prl were grown in RPMI-1640 medium (Cellgro, Herndon, VA) containing $10 \%$ fetal bovine serum (Hyclone Laboratories, Logan, UT) at $37^{\circ} \mathrm{C}$ and $5 \% \mathrm{CO}_{2}$. Human embryonic kidney cell line 293 (ATCC) was grown in D-MEM medium (Cellgro) containing 10\% heat inactivated fetal bovine serum at $37^{\circ} \mathrm{C}$ and $5 \% \mathrm{CO}_{2}$.

Construction of $\operatorname{AdRSVpHyde:~A~rat~pHyde~cDNA~gene~was~isolated~as~described~in~}$ U.S Serial No: 09/302,457. After digestion with EcoR I, a 2.6 kb fragment which contains the 1467 bp full-length coding sequence of pHyde cDNA was subcloned under the control of a truncated RSV promoter ( 395 bp ) into an E1/E3 deleted adenoviral
shuttle vector. The resultant adenoviral shuttle vector was cotransfected into 293 cells with pJM17, an adenoviral type 5 genome plasmid, by calcium phosphate method. Individual plaques were screened for recombinant AdRSVpHyde by PCR using specific primers for both the RSV promoter and pHyde cDNA sequences. Single viral clones were propagated in 293 cells. The culture medium of the 293 cells showing the completed cytopathic effect (CPE) was collected, and the adenovirus was purified and concentrated by twice CsCl 2 gradient ultracentrifugation. The viral titration and transduction were performed as previously described. The schematic diagram of AdRSVpHyde was illustrated in Fig. 1. The sequence of AdRSVpHyde is set forth in Figure 10.

Northern blot: Cells were extracted and total RNA was isolated by RNeasy Kit (Qiagen, Santa Clarita, CA). Total RNA was loaded on a $1.2 \%$ polyacrylamide gel and processed to electrophoresis. The standard Northern blot transfer to a Nylon membrane (Hybond- $\mathrm{N}^{+}$, Amersham Life Science, Buckinghankshire, England) was performed as previously described. The cDNA probes ( pHyde or p 53 ) were labeled by a- ${ }^{32} \mathrm{P}$-dCTP using random primer method (Prime-It II Kit, Stratagene, La Jolla, CA). The membrane was hybridized with the probe in Rapid-hyb buffer (Amershaun Life Science) according to the Manufacturer's protocol. The membrave was exposed to a Kodak X-ray film under one intensifying screens at $-80^{\circ} \mathrm{C}$ for autoradiography. GAPDH CDNA probe was labeled as described above and used as an internal control for normalization.

Western blot: Cells were extracted and processed for gel electrophoresis as previously described. ( Lu Y, Whitaker $\mathrm{L}, \mathrm{Li} \mathrm{X}$, et al. Coexpression of galectin-1 and its complementary glycoconjugates laminin and lysosome-associated membrane proteins in murine PCC4.azalR embryonal carcinoma cells induced to differentiation by butyrate. Mol Cell Differ. 1995;3:175-191). Cell extract lysates ( 100 mg ) were loaded on $12 \%$ polyacrylamide gels and subjected to sodium dodecylsulfate (SDS) gel electrophoresis, then transferred to a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was treated with blocking solution ( $15 \%$ nonfat milk, $0.02 \%$ sodium
azide in phosphate-buffered saline) overnight at $4^{\circ} \mathrm{C}$. The membrane was incubated for 1 hr at room temperature with rabbit anti-rat pHyde polyclonal antibody (specifically generated by Research Genetics, Inc. based on the computer-created antigenic peptide derived from pHyde coding sequences). The membrane was then incubated for 1 hr at room temperature with ${ }^{125}$ I-labeled second antibody (Amersham Life Science, Arlington Heights, IL). The membrane was exposed to a Kodak X-ray film between two intensifying screens at $-80^{\circ} \mathrm{C}$ for autoradiography.

AdRSVpHyde in vitro studies: Human prostate cancer cells were infected with AdRSVpHyde in vitro with a multiplicity of infection (MOI) of 100 or 200. After viral infection, cells were incubated at $37^{\circ} \mathrm{C}$ and cell numbers were determined at day 5 after viral infection.

In vivo studies using DU145 xenograft tumors: DU145 cells ( $1.4 \times 10^{7}$ cells in 0.2 ml of PBS) were injected subcutaneously into the flank of male nude mice (Harlan Sprague Dawley). When the tumors reached an average volume of $80 \mathrm{~mm}^{3}, 5 \times 10^{9}$ pfu adenoviral vectors (AdRSVpHyde or control adenovirus AdRSVlacZ) or PBS alone for untreated controls were injected directly into the tumor. Tumor volume was measured every three to four days until the animals were euthanized. All the animals were sacrificed at day 52 after viral injection when several of them showed distress or had a tumor burden greater than $15 \%$ of total body weight. Tumor samples were collected and processed for $H \& E$ staining.

DNA extraction and gel electrophoretic analysis of DNA fragmentation: Soluable
DNA was extracted as described previously (in Oridate N, Lotan D, Xu X-C, Hong WK, and Lotan R. Differentiation induction of apoptosis by all-trans-retinoic acid and N -(4-hydroxyphenyl)retinamide in human bead and neck squamous cell carcinoma cell lines. Clin. Cancer Res. 1996;2:855-863) Briefly, the cells floating in medium were collected 48 h post transduction by centrifugation. The pellet was resuspended in Tris-EDTA buffer ( pH 8.0 ). The cells were lysed in $10 \mathrm{mM} \operatorname{Tr}-\mathrm{s}-\mathrm{HCl}$ ( pH 8.0 ), 10 mM EDTA, and $0.5 \%$ Triton X-100 on ice for 15 min . The lysate was centrifuged at $12,000 \mathrm{xg}$ for 15 min to separate soluble (fragmented) DNA from pellet (intact genomic) DNA. Soluble DNA was treated with Rnase A ( $50 \mathrm{ug} / \mathrm{ml}$ ) at 37 C for 1 h , followed by treatment with proteinase $\mathrm{K}(100 \mathrm{ug} / \mathrm{ml})$ in $0.5 \%$ SDS, at 50 C for 2 h . The residual material was extracted with phenol/chloroform, precipitated in ethanol, electrophoresed on a $2 \%$ agarose gel.

## RESULTS

Exogenous plYyde expression in DU145 cells: To determine whether AdRSVpHyde is able to successfully transfer and express rat pflyde at mRNA and protein levels, DU145 cells were tranduced by AdRSVpHyde at MOI=200. The cell extract were harvested 48 h after viral transduction to determine the pHyde expression. While there was a minor endogenous expression of pHyde at mRNA level (Fig. 2A) but not at protein level (Fig. 2B) in DU145 cells, there was an apparent high exogenous pHyde expression induced by AdRSVpHyde at both mRNA (Fig. 2A) and protein (Fig. 2B) levels.

Prostate cancer cell growth inhibited by AdRSVHyde: To determine the effects of pHyde on cell growth of human prostate cancer cell lines, DU145 and LNCaP were treated with AdRSVpHyde, AdRSVlacZ (control vector), and no virus in vitro. AdRSVpHyde significantly inhibited the growth of DU145 and LNCaP cells, with $76.9 \%$ (Fig. 3A) and $83.1 \%$ (Fig. 3B) inhibition respectively, compared to untreated control cells.

AdRSVpHyde inhibited prostate tumor growth in vivo: To evaluate the effects of AdRSVpHyde treatment of prostate cancer cell growth in vivo, DU145 human prostate tumors were established in nude mice by injecting $1.4 \times 10^{7} \mathrm{PPC}-1$ cells subcutaneously into the flanks of nude mice. When mice developed tumors with an averaging $80 \mathrm{~mm}^{3}$ volume, the mice were divided into three groups: AdRSVpHyde treated ( $n=7$ ), AdRSVlacZ control virus treated ( $n=7$ ), and untreated groups ( $n=7$ ). Treated tumors were injected with a single dose of $5 \times 10^{9}$ pfu of either the control virus or AdRSVpHyde. As shown in Fig. 4, untreated and control virus treated DU145 tumors grew rapidly relative to the AdRSVpHyde treated tumors. By day 53 following viral injection, the tumor burden in mude mice bearing untreated and control virus treated DU145 tumors reached $5953 \mathrm{~mm}^{3}$ and $4777 \mathrm{~mm}^{3}$ respectively. In contrast, DU145 tumors transduced by AdRSVpHyde had a significant reduction in tumor volume (1515 $\mathrm{mm}^{3}$ ) compared to untreated and control virus treated-DU145 tumors, that is, $25.4 \%$ of untreated and $31.7 \%$ of control virus treated DU145 tumor volunne (Fig. 4).

Growth inhibition by AdRSVpHyde correlated to p53 expression in prostate cancer cells: One observation in the in vitro study was that the phenotype of DU145 and LNCaP cells transduced by AdRSVpHyde were also altered. Unlike the control and control virus treated cells, AdRSVpF-Hyde-transduced cells had a marked morphology of round, detaching and floated dying cells, a characteristic of cells undergoing apoptosis, and cell number decreased quickly over time (Fig. 5). Consistently, AdRSVpHyde showed a strong inhibition on the growth of these two cell lines (Fig. 3). However, AdRSVpHyde did not inhibit the cellular growth of PC-3 and TSU-Pr cells, and had only a minor growth inhibition on PPC-1 cells (data not shown). Consistently, there was no evident morphological differences between untreated control, control virus-treated and AdRSVpHyde-treated cells in these lines (Fig. 6). To determine whether the differential expression of various genes, especially those involved in apoptosis pathway, accounted for the differential inhibitory effect by AdRSVpHyde on different prostate cancer cell lines, several genes including p 53 and Rb were screened at mRNA level by Northern hybridization. Interestingly but not too surprisingly, p53 was found to only express in DU145 and LNCaP cells but not in PC-3. TSU-Pr, and PPC-1 cells; in contrast, Rb gene were all expressed at the mRNA level in these cells (Fig. 7).

Therefore, it appeared that there was a correlation between p53 expression and AdRSVpHyde-mediated inhibition. To determine whether pHyde regulated p53 expression, the same Northern blot in Fig. 2A was stripped and rehybridized with p53 probe. Indeed an induction of p53 mRNA was observed in AdRSVpHyde transduced DU145 cells (Fig. 8). Furthermore, transduction of LNCaP cells by AdRSVpHyde showed DNA laddering pattern, a marker for cells undergoing apoptosis (Fig. 9), indicate that pHyde expression induced apoptosis in LNCaP cells. Taken together, these results demonstrate that pHyde may function via p 53 pathway to induce apoptosis and its growth inhibition may depend on p 53 expression in the target cells.

The previous study showed that pHyde has the dual ability to act like a tumor suppressor gene and induce susceptibility to apoptosis. The growth of prostate tumors in rats was greatly inhibited by p-Hyde. Moreover, prostate cancer cells expressing pHyde were more sensitive to UV DNA damage driving these cells into cell programmed death. In this study AdRSVpHyde was shown to have an effective inhibition on cell growth both in vitro and in vivo for the prostate cancer cells expressing p53, but not for the prostate cancer cells missing p53 expression. One possibility is that pHyde can induces apoplosis by p53 dependent pathway (such as in DU145 and LNCaP cells) and p53 independent pathway which requires an outside cell death trigger such as UV or chemical (such as methylnitrosourea) to act as a co-inducer for apoptosis. Consistent with our result that only DU145 and LNCaP, but not PPC-1, PC-3 and TSU-Pr, expressed p53 at mRNA level (Fig. 10), other groups found that only DU145 and LNCaP cells expressed p53 at protein level but not PC-3 and TSU-Pr. Interestingly, p53 protein in DU145 was claimed to be a mutant p53. ${ }^{21}$ Therefore, it seems that existence of p53 protein, regardless of its wild-type or mutant status, is required for pHyde to act as a tumor suppressor gene alone. The possibility, that the mutation in p53 protein in DU145 cells may not affect its ability for pHyde-mediated growth inhibition, need to be further studied. Moreover, the potential inhibitory effects by combination of AdRSVpHyde and UV (or methylnitrosourea) on cells missing p53 protein expression (such as PC-3 and TSU-Pr) will be further characterized.

One interesting and important finding of this study was that AdRSVpHyde induced p53 expression in DU145 cells. Not only this may explain that pHyde acts in a more global manner to induce susceptibility to apoptosis, but also partially explains why pHyde has a partial sequence homologue with TSAP-6, a human protein claimed to be involved in p53-associated pathway: because p.Hyde could be a rat homologue of TSAP-6 gene, or a member of the TSAP-6 like family which is involved in p53-associated pathway. Furthermore, there is very few, if any, identified cellular proteius which act as regulators for p53 gene, which is a transcription factor to act many downstream genes. The significance of the finding that pHyde up-regulates p53 expression and the consequent exploration for a new cellular regulation mechanism are exciting. Nevertheless, whether pHyde directly regulates p53 gene at the transcriptional level will be determined in our current study by employing p53 promoter/CAT reporter chimeric gene in the presence and absence of pHyde protein in DU145 cells.

In summary, pHyde is a novel a tumor suppressor gene and AdRSVpHyde effectively inhibites prostate cancer both in vitro and in vivo. The monotherapy of AdRSVpHyde alone or combined therapy of AdRSVpHyde with radio- or chemo-therapy should have an effective therapeutic potential for treatment of locally advanced prostate cancer.

## EXAMPLE: 3

The other classical method for detecting apoptosis, TUNEL assay, was also used to demonstrate that AdRSVpHyde indeed caused DU145 cells to apoptosis. Seventy two hrs after viral transduction, DU145 cells growing on culture dish were subjected to TUNEL assay. There were more fluorescence-stained cells in AdRSVpHyde transduced cells (Fig. 15C and 15F) compared to untreated control (Fig. 15A and 15D) or control virus transduced cells, (Fig. 15B and 15E), indicating that there were more apoptotic cells in AdRSVpHyde treated cells than the latter two kinds. In addition, tumor sections from DU145 xenograft tumors growing in nude mice showed that there was a significant apoptosis occurring in AdRSVpHyde treated tumors (Fig. 17B), compared to that of untreated tumors (Fig. 17A) and control virus treated tumors.

The TUNEL assay of AdRSVpHyde transduced DU145 cells did not show comparable amount of apoptotic cells (about $10 \%$ stained cells, Fig. 15) to the extent that matches the growth inhibition ( $76.9 \%$ inhibition, Fig. 17A). One explanation is that the growth inhibition of cell was recorded at day 5 after AdRSVplIyde transduction (Fig. 17A), whereas the in vitro TUNEL staining was performed on cells 3 days after AdRSVpHyde transduction (Fig. 15). P-Hyde-mediated apoptosis may take time to show the peak of the apoptosis that might not be available yet in the cells at day 3 after viral transduction, because in vivo) TUNEL staining of the AdRSVpHyde treated DU145 xenograft tumox sections, that were derived from 21 days after AdRSVpHyde transduction, showed a significantly higher amount of apoptotic cells (Fig. 16) compared to that of in vitro TUNEL staining (Fig. 15). Another explanation is that apoptosis may accounts for only part of the pHyde-mediated growth inhibition, in other words, pHyde-mediated growth inhibition may be composed of apoptosis and some other unknown mechanisms. As described below, AdRSVpHyde was able to inhibit cell growth of another human prostate cancer cell line TSU, however, no apoptosis was observed in AdRSVpHyde transduced TSU cells.

To determine whether there is an association between p53 status and susceptibility to apoptosis by pHyde, four different human prostate cancer cell lines PC3, TSU, LNCaP and DU145 were screened for the endogenous expression of p53 and their sensitivity to the pHyde-mediated growth inhibition and apoptosis were compared. The Northern blot analysis showed that PC-3 and TSU cells did not express p53 at the mRNA levels, whereas both LNCaP and DU145 cells expressed p53 mRNA. In contrast, all of the four cell lines expressed comparable Rb at the mRNA levels (Fig. 18). Consistently, other group has showed that DU145 and LNCaP cells, but not PC-3 cells, expressed p53 protein by Western blot analysis.

To determine the sensitivity of these four cell lines to the pHyde-mediated growth inhibition and apoptosis, cells were transduced with AdRSVpHyde and the growth was monitored. AdRSVpHyde had a strong growth inhibition on DU145 and LNCaP cells ( $76.9 \%$ and $83.1 \%$ inhibition compared to the untreated control, respectively, Fig. 17), the two cell lines that expressed p53. Interestingly, for the two P53 not-expressing cell
lines PC-3 and TSU, AdRSVpHyde had no inhibitory effect on the growth of PC-3 cells, but had a minor inhibition ( $24.5 \%$ inhibition compared to untreated control) on the growth of TSU cells (Fig. 19). However, both AdRSVpHyde transduced PC-5 and TSU cells did nor show any apoptosis as detected by DNA fragmentatin and TUNEL assays. 5 These results suggest that there may be an association between the presence of p53 and the susceptibility of cell to the pHyde-mediated apoptosis. p53 may be required for the pHyde-mediated apoptotic induction. In addition, preliminary results showed that caspase-3, a key apoptotic protease was elevated after AdRSVpHyde transduction in DU145 cells.

## REFERENCES

Amson RB, Nemani M, Roperch JP, Israeli D, Bougueleret L, Le Gall I, Medhioub M, Linares-Gruz G, Lethrosne F, Pasturaud P, Piouffre L, Prieur S, Susini L, Alvaro V, Millasseau P, Guidicelli C, Bui FI, Massart C, Cazed. L, Dufour F, Bruzzoni-Giovanelli $H$, Owadi $H$, Hennion C, Charpak G, Dausset J, Calvo F, Oren M, Cohen D and A Telerman (1996). Isolation of 10 differentially expressed cDNAs in p53-induced apoptosis: Activation of the vertebrate homologue of the Drosophila seven in absentia gene, Proc.Natl.Acad.Sci.USA, 93, 3953-3957.

Anand S, Verma H, Kumar L and N Singh (1995) Induction of apoptosis in chronic myelogenous leukemia lymphocytes by hydroxyurea and adriamycin, Cancer Letters, 88, 101-105.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman IG, Smith JA and K Strulal (1987-1997) Current protocols in molecular biology, John Wiley \& Sons, Inc.

Boring CC, Squires T'S and T Tong (1993) Cancer Statistics, Cancer 43, 7-26.

Carter BH, Piantadosi S and JT Isaacs (1990) Clinical evidence for and implications of the multistep development of prostate cancer, The Journal of Urol., 143, 742 746.

Carter BS, Beaty TH, Steinberg GD, Childs B and PC Walsh (1992) Mendelian inheritance of prostate cancer, Proc. Natl. Acad. Sci. USA, 89, 3367-3371.

Effert PJ, Neubauer A, Walter PJ, et al. (1992) Alteration of the p53 gene are associated with the progression of a human prostate carcinoma, J Urol, 147, 789 793.

El Deiry WS, Harper JW, O'Connor PM, Velculescu VE, Canman CE, Jackinan J, Pietenpol JA, Burrell M, Hill DE, Wang Y, Wiman KG, Mercer WE, Kastan MB, Kohn KW, Elledge SJ, Kinzler KW, and B Vogelstcin (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis Cancer Research, 54, 1169-1174.

Findenig G,Mader RM, Fritzer-Szekerez M, Steger GG and T Szekerez (1996), Modulation of 5 -fluorouracil resistance in human colon tumor cell lines by azidothymidine,Ocol. Res. (US), 8, 189-196.

Fournier REK, and FH Ruddle (1977) Microcell mediated transfer of murine chromosomes into mouse, chinese hamster and human somatic cells, Proc Natl.Acad.Sci USA, 74, 319-323.

Gerschenfeldt HK and Weissman IL (1986), Cloning of a cDNA for a T cell-specific serine protease from a cytotoxic T lymphocyte, Science, 232, 854-858.

Grem JL (1990) Fluoronated pyrimidines, in Cancer Chemotherapy:Principle and Practice, ed. by BA Chapner and JM Collins. Philadelphia: JB Lippincott, pp. 89190.

Inaba M, Mitsuhashi J, Sawada H, Miike N, Naoe Y, Daimon A, Koizumi K, Tsujimoto $H$ and M Fukushima (1996), Reduced activity of anabolizing enzymes in 5-fluorouracil-resistant human stomach cancer cells, Jpn J Cancer Res (Japan), 87, 212-220.

Isaacs WB, Carter BS, Ewing CM (1991) Wild-type pS3 suppresses growth of humian prostate cancer cells containing mutant p53 alleles, Cancer Res 51, 4716 4720.

Isaacs JT, Isaacs WB, Feitz WFJ, and Scheres J (1986) Establishment and characterization of seven Dunning rat prostatic cancer cell lines and their use in developing methods for predicting metastatic abilities of prostate cancer, The Prostate, 9, 261-281.

Isaacs JT, Wake N, Coffey DS, and Sandberg AA (1982), Cancer Res, 42, 2353-2361.

Iwasaki T, Shinkai K, Mukai M, Yoshioka K, Fuji Y, Nakahara K, Matsuda H and H Akedo (1995) Cell cycle dependent invasion in vitro by rat ascites hepatoma cells, Int. J. Cancer, 63, 282 -287.

Kaur GP, Rinaldy A, Lloyd RS and RS Athwal (1992) A gene that partially complements xeroderma pigmentosum group A cells maps to human chromosome 8 , Somatic Cell and Molecular Genetics, 18, 371-379.

Kyprianou, N, Bains AK, and Jacobs SC. (1994) Induction of apoptosis in androgen-independent human prostate cancer cells undergoing thymineless death. Prostate 25, 66-75.

Kyprianou, N. (1994) Apoptosis: Therapeutic significance in the treatment of androgen-dependent and androgen-independent prostate cancer. World J Urol 12, 299-303.

Lennon G, Auffray C, Polymeropoulos A and MB Soares (1996) The I.M.A.G.E. Consortium: An Integrated Molecular Analysis of Genomes and Their Expression, Genomics, 33, 151152.

Lowe SW, Schmitt EM, Smith SW, Osborne BA and T Jacks (1993) Nature (Loudon) 362, 847-849.

Mangiarotti G, Chung S, Zucker C, and Lodish HF (1981) Selection and analysis of cloned develomentally regulated Dictiostelium discoidum genes by hybridization-competition, Nucleic Acids Res. 9, 947-963.

McConkey EH (1967) The fractionation of RNA's by sucrose gradient centrifugation, Methods in Enzymology, 12A, 620-634.

McDonnell TJ, Troncoso P, Brisbay SM et al. (1992) Expression of the protooncogene bcl-2 in the prostate and its association with the emergence of androgen independent prostate cancer. Can Res 52, 6940-6944.

McLellan DL and RW Norman (1995) Hereditary aspects of prostate cancer, Can. Med. Assoc. J., 153, 895 -900.

Mellon K, Thomson S, Charlton RG et al. (1992) p53, c-erB-2 and the epidernal growth factor receptor in the benign and malignant prostate, J Urol, 147, 496-499.

Michalovitz D, Halevy O and M Oren (1990) Conditional inhibition of transcription and of cell proliferation by temperature sensitive mutant of p53, Cell 62, 671-680.

Rinaldy A, Dodson ML, Darling TL, and Lloyd RS (1988) Gene cloning using cDNA librraies in a differential competition hybridization strategy: application to cloning XP-A related genes, DNA 7, 563-570.

Rinaldy A, Bellew T, Egli E, and Lloyd RS (1990) Increased UV resistance in xeroderma pigmentosunn group A cells after transformation with a human genomic DNA clone, Proc Natl Acad Sci USA, 87, 6818-6822.

Sachs L (1996) Proc.Natl.Acad.Sci USA 93, 4742-4749.

Sachs L and J Lotem (1995) in Apoptosis and the Immune Response, ed. Gregory CD (Wiley, New York), pp. 371-403.

Sandberg AA (1992) Chromosome abnormalities and related events in prostate cancer, Human Pathology, 23, 368-380.

Sherr C (1993) Mammalian G1 cyclins. Cell 73, 1059-1065.

Shields PG and CC Harris (1991) Molecular epidemiology and the genetics of environmental cancer, J. of Am. Med. Assoc., 266, 681-687.

Silverberg E (1987) Statistical and epidemiologic data on urologic cancer, Cancer, 6, 692-717.

Steinberg GD, Carter BS, Beaty TH, Childs B and PC Walsh (1990) Family history and the risk of prostate cancer, Prostate, 17, 337-341.

Steiner MS and Barrack ER (1992) Transforming growth factor-? 1 overproduction in prostate cancer: Effects on growth in vivo and in vitro, Molec. Endocrin., 6, 15-25.

Steiner MS, Satterwhite D.J and HL Moses (1995) Molecular insights into altered cell cycle regulation and genitourinary malignancy, Urol. Oncol., 1, 3-17.

Templeton L, K Alderink and A Rinaldy (1996) Down-regulation of wild-type p53 and up-regulation of PCNA by pXPA2 gene are associated with the partial correction of NER in XP-A transfectant, Proc. of the Am. Assoc. for Cancer Res., 37, 499.

Vogelstein B, Fearon ER, Hamilton $S R$, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AMM and JL Bos (1988) Genetic alterations during 'colorectal-tumor development, New Engl. J. Med., 319, 525-532.

Wadhwa R, Pereira-Smith OM, Reddel RR, Sugimoto Y, Mitsui Y and C Kaul (1995) Correlation between complementation group for immortality and the cellular distribution of mortalin, Exp. Cell Res., 216, 101-106

Weinstein IB (1987) Growth factors, oncogenes, and multistage carcinogenesis, J. of Cell Biochem., 33, 213-224.

Weil M, MD Jacobson, HSR Coles, TJ Davies, RL Gardner, KD Raff and MC Raff (1996) Constitutive expression of the machinery for programmed cell death, The Journal of Cell Biology, 133, 1052-1059.

Yonish-Rouach E, Renitzky D, Lotem J, Sachs L, Kimchi A and M Oren (1991) Wild-type p53 induces apoptosis of nyyeloid leukemic cells that is inhibited by interlekin-6, Nature (London), 352, 345-347.

Yuspa SH and MC Poirier (1988) Chemical carcinogenesis; from animal models to molecular models in one decade, Adv. Cancer Res., 50, 25-70.

## WHAT IS CLAIMED IS:

1. An isolated nucleic acid which encodes a mammalian p-Hyde protein of the p-Hyde family which induces susceptibility of a cancer cell to cell death, and comprises a nucelic acid sequence as set forth in SEQ ID NO. 1, 3, 5, or 7 including variants, and mutants, thercof.
2. The isolated nucleic acid of claim 1, wherein the nucleic acid has a nucleic acid sequence having at least $82 \%$ similarity with the nucleic acid coding sequence of SEQ $\operatorname{DD}$ NOs: $1,3,5$, or 7 .
3. The isolated nucleic acid of clain 1 , wherein the nucleic acid has a nucleic acid sequence having at least $85 \%$ similarity with the nucleic acid coding sequence of SEQ ID NOs: $1,3,5$, or 7 .
4. The isolated nucleic acid of claim 1, wherein the nucleic acid has a nucleic acid having at least $95 \%$ similarity with the nucleic acid coding sequence of SEQ ID NO:s 1, 3, 5, or 7 .
5. The isolated nucleic acid of claim 1 , wherein the nucleic acid fragment is set forth in SEQ ID NOs: 7.
6. The isolated nucleic acid of claim 1, wherein the nucleic acid fragment code for an amio acid fragment as set forth in SEQ ID NOs: 8 .
7. The isolated nucleic acid of claim 1, wherein the nucleic acid is DNA or RNA
8. Thic isolated nucleic acid of claim 2 , wherein the nucleic acid is cDNA or genomic DNA.
9. The isolated nucleic acid of claim 1, wherein the nucleic acid encodes an amino acid sequence having the sequence as set forth in SEQ ID NOs: 2,4 , or 6 .
10. The isolated nucleic acid of claim 1, wherein the nucleic acid is labeled with a detectable marker.
11. The isolated nucleic acid of claim 10, wherein the detectable marker is a radioactive, colorimetric, luminescent, fluorescent marker, or gold label.
12. An oligonucleotide of at least 15 nucleotides capable of specifically hybridizing with a sequence of the nucleic acid which encodes the human p-Hyde of claim 1 .

The oligonucleotide of claim 12, wherein the nucleic acid is DNA or RNA.
14. The oligonucleotide of claim 12, wherein the oligonucleotide is labeled with a detectable marker.
15. The oligonucleotide of clain 13, wherein the oligonucleotide is a radioactive, colorimetric, luminescent, fluorescent marker, or gold label
16. A nucleic acid having a sequence complementary to the sequence of the isolated nucleic acid of claim 1 .
17. An antisense molecule capable of specifically hybridizing with the isolated nucleic acid of claim 3 .
18. A vector comprising the isolated nucleic acid of claim 1.
19. The vector of clain 18, further comprising a promoter of RNA transcription operatively, or an expression element linked to the nucleic acid.
20. The vector of claim 18, wherein the promoter comprises a bacterial, yeast, insect or mammalian promoter.
21. The vector of claim 20, wherein the vector is a plasmid, cosmid, yeast artificial chromosome (YAC), BAC, adenovirus, adeno-associated virus, retovirus, Pl , bacteriophage or eukaryotic viral DNA.
22. The adenovirus vector of claim 21, wherein the adenovirus vector is a replication-deficient adenovirus type 5 expression vector.
23. The adenovirus vector of claim 22, wherein the adenovirus vector comprises an adenovirus genome having a deletion in the E1 and E3 region of the genome and an insertion within the region of a nucleic acid encoding p-Hyde, allele, fragment or variant thereof under the control of a promoter.
24. The vector of claim 23, wherein the promoter is a Rous Sarcoma virus promoter.
25. A host vector system for the production of a polypeptide which comprises the vector of claim 18 in a suitable host.
26. The host vector system of claim 25, wherein the suitable host is a prokaryotic or eukaryotic cell.
27. The host vector system of clain 26, wherein the eukaryotic cell is a yeast, insect, plant or mammalian cell.
28. A method for producing a polypeptide which comprises growing the host vector system of clain 18 under suitable conditions permitting production of the polypeptide and recovering the polypeptide so produced.
29. A method of obtaining a polypeptide in purified form which comprises:
(a) introducing the vector of claim 18 into a suitable host cell;
(b) culturing the resulting cell so as to produce the polypeptide;
(c) recovering the polypeptide produced in step (b); and
(d) purifying the polypeptide so recovered.
30. A p-Hyde family polypeptide comprising the amino acid sequence of a mammalian p-Hyde.
31. The polypeptide of claim 30 , wherein the amino acid sequence is set forth in SEQ D NOs: $2,4,6$, or 8 .
32. A fusion protein or chimeric comprising the polypeptide of claim 30.
33. An antibody which specifically binds to the polypeptide of claim 30.
34. The antibody of claim 33, wherein the antibody is a monoclonal or polyclonal antibody.
35. A pharmaceutical composition comprising an amount of the polypeptide of claim 30 and a pharmaceutically effective carrier or diluent.
36. A transgenic, nonhuman mammal comprising the isolated nucleic acid of claim 1.
37. A method for determining whether a subject carries a mutation in the p-Hyde gene which comprises:
(a) obtaining an appropriate nucleic acid sample froin the subject; and
(b) determining whether the nucleic acid sample from step (a) is, or is derived from, a nucleic acid which encodes mutant p-Hyde so as to thereby determine whether a subject carries a mutation in the p-Hyde gene.
38. The method of claim 37, wherein the nucleic acid sample in step (a) comprises mRNA corresponding to the transcript of DNA encoding a mutant p-Hyde, and wherein the determining of step (b) comprises:
(i) contacting the mRNA with the oligonucleotide of claim 12 under conditions permitting binding of the mRNA to the oligonucleotide so as to form a complex;
(ii) isolating the complex so formed; and
(iii) identifying the mRNA in the isolated complex so as to thereby determine whether the mRNA is, or is derived from, a nucleic acid which encodes mutant p-Hyde.
39. The method of claim 38, wherein the determining of step (b) comprises:
(i) contacting the nucleic acid sample of step (a), and the isolated nucleic acid of claim 1 with restriction enzymes under conditions permitting the digestion of the nucleic acid sample, and the isolated nucleic acid into distinct, distinguishable pieces of nucleic acid;
(ii) isolating the pieces of nucleic acid; and
(iii) comparing the pieces of nucleic acid derived from the nucleic acid sample with the pieces of nucjeic acid derived from the isolated nucleic acid so as to thereby determine whether the nucleic acid sample is, or is derived from, a nucleic acid which encodes mutant p-Hyde.
40. A method for screening a tumor sample from a human subject for a somatic alteration in a p-Hyde gene in said tumor which comprises gene comparing a first sequence selected form the group consisting of a p -Hyde gene from said tumor sample, p-Hyde RNA from said tumor sample and p -Hyde cDNA made from mRNA from said tumor sample with a second sequence selected from the group consisting of p-Hyde gene from a nontumor sample of said subject, p-Hyde RNA from said
nontumor sample and p-Hyde cDNA made from mRNA from said nontumor sample, wherein a difference in the sequence of the p-Hyde gene, p-Hyde RNA or p-Hyde cDNA from said tumor sample from the sequence of the p-Hyde gene, p-Hyde RNA or p-Hyde cDNA from said nontumor sample indicates a somatic alteration in the p -Hyde gene in said tumor sample.
41. A method for screening a tumor sample from a human subject for the presence of a somatic alteration in a p-Hyde gene in said tumor which comprises comparing p -Hyde polypeptide from said tumor sample from said subject to p-Hyde polypeptide from a nontumor sample from said subject to analyze for a difference between the polypeptides, wherein said comparing is performed by (i) detecting either a full length polypeptide or a truncated polypeptide in each sample or (ii) contacting an antibody which specifically binds to either an epitope of an altered p -Hyde polypeptide or an epitope of a wild-type p-Hyde polypeptide to the p-Hyde polypeptide from each sample and detecting antibody binding, wherein a difference between the p-Hyde polypeptide from said tumor sample from the p-Hyde polypeptide from said nontumor sample indicates the presence of a somatic alteration in the p -Hyde gene in said tumor sample.
42. A method for identifying a chemical compound which is capable inducing susceptibility to cell death which comprises:
(a) contacting the p-Hyde with a chemical compound under conditions permitting binding between the p-Hyde and the chemical compound;
(b) detecting specific binding of the chemical compound to the p-Hyde; and
(c) determining whether the chemical compound inhibits the $\mathrm{p}-\mathrm{Hyde}$ so as to identify a chemical compound which is capable of capable inducing susceptibility 10 cell death.
43. A method of inhibiting the growth of cancer cells, comprising the steps of obtaining the cells and contacting the cells of the subject with a replication-deficient adenovirus type 5 expression vector comprising an adenovirus genome having a deletion in the E1 and E3 region of the genome and an insertion within the region of a nucleic acid encoding p-Hyde under the control of a Rous Sarcoma virus promoter, thereby inhibiting the growth of the cancer cells.
44. A method of inhibiting the growth a cancer cells, comprising: 1) obtaining a sample of prostate cells from a subject; 2) contacting the cells with a replication deficient adenovirus type 5 expression vector which comprises an adenovirus genome having a deletion in the E1 and E3 regions of the genome and an insertion within the regions of a p-Hyde cDNA under the control of a Rous Sarcoma virus promoter; and 3) introducing the cells into the subject, thereby inhibiting the growth of the cancer cells.
45. A method of suppressing the growth of cancer cells in a subject, comprising introducing into the cancer cell an amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of $p$-Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein, thereby suppressing the growth of cancer cells in the subject.
46. A method of suppressing growth of cancer cells in a subject, comprising administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby suppressing the growth of cancer cells in the subject.
47. A method of inducing susceptibility to apoptosis of cancer cells in a subject, comprising introducing into the cancer cell an amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein, thereby inducing susceptibility to apoptosis.
48. A method of inducing susceptibility to apoptosis of cancer cells in a subject, comprising adminjstering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a mucleic acid encoding a fragment of $p$-Hyde protein, or the nucleic acid encoding a mutant $p$-Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby inducing susceptibility to apoprosis.
49. A method of treating a subject with cancer which comprises administering to the subject a phamaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p -Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein and a pharmaceutical acceptable carrier or diluent, thereby treating the subject with cancer.
50. A method of treating a subject with cancer, comprising: 1) administering to the subject a pharmaceutical composition comprising a therapeutically effective amount of a nucleic acid encoding a p-Hyde protein, a nucleic acid encoding a fragment of p-Hyde protein, or the nucleic acid encoding a mutant p-Hyde protein in combination with radiation, chemotherapy, or UV mimetic drugs; and 2) a pharmaceutical acceptable carrier or diluent, thereby treating the subject with cancer.
51. A method of treating a subject with cancer, comprising administering to the subject a therapeutically effective annount of a phammaceurical composition comprising: 1) an adenovirus type 5 expression vector which
comprises an adenovirus genome having a deletion in the E1 and E3 regions of the genome and an insertion within the regions of a full length sense p-Hyde cDNA under the control of a Rous Sarcoma virus promoter, and 2) a suitable carrier or diluent, thereby treating the subject with cancer.
52. The method of claims 43-51, wherein the cancer is selected from a group consisting of: melanoma; lymphoma; leukemia; and prostate, colorectal, pancreatic, breast, brain, or gastric carcinoma.
53. The method of claim 50, wherein the pharmaceutical composition is administered parenterally, paracancerally, transmucosally, transdermally, intramuscularly, intravenously, intradermaly, subcutaneously, intraperitonealy, intraventricularly, or intracranialy.


FIG. 1


FIG. 2A


FIG. 2B
$4 / 43$

A


B


FIG. 3

## 5 / 43



FIG. 4


DU145/AdRSVpHyde


DU145/Control Virus


FIG. 5



## PC-3/Control



## TSU/Control



PPC-1/Control

FIG. 6

## $8 / 43$




FIG. 7


FIG. 8


FIG. 9

Sequence of Region A of AdRSVpHyde:
GCGGCCGCCATCATCAATAATATACCITATITTGGATTGAAG CCAATATGATAATGAGGGGGTGGAGTTTGTGACGIGGC GCGGGGCGTGGGAACGGGGCGGGTGACGTAGTAGTGTGGC GGAAGTGTGATGTIGCAAGTGTGGCGGAACACATGTAAGC GACGGATGTGGCAAAAGTGACGIIIITGGTGTGCGCCGGTG TACACAGGAAGTGACAATITTCGCGCGGTITTAGGCGGA TGTTGTAGTAAATITGGGCGTAACCGAGTAAGATITGGCCAT TTTCGCGGGAAAACTGAATAAGAGGAAGTGAAATCTGA ATAATTTTGTGTTACTCATAGCGCGTAATATTTGTCTAGGGCC GCGGGGACTTTGACCGTITACGTGGAGACTCGCCCAG GGCGCGCCCCGATGTACGGGCCAGATATACGCGTATCTGAG GGGACTAGGGTGTGTTTAGGCGAAAAGCGGGGCTTCGGT TGTACGCGGTTAGGAGTCCCCTCAGGATATAGTAGTTICGCT TTTGCATAGGGAGGGGGAAATGTAGTCTTATGCAATAC
TCTTGTAGTCTTGCAACATGGTAACGATGAGTIAGCAACATG CCTTACAAGGAGAGAAAAAGCACCGTGCATGCCGATITG GTGGAAGTAAGGTGGTACGATCGTGCCTTATTAGGAAGGCA ACAGACGGGTCTGACATGGATTGGACGAACCACTGAATT CCGCATTGCAGAGATATTGTATTTAAGTGCCTAGCTCGATAC AATAAACGCCATITGACCATTCACCACATTGGTGTGCA
CCTCCGGCCCTGGCCACTCTCITCCGCATCGCTGTCTGCGGG GGCCAGCTGTTGGGCTCGCGGITGAGGACAAACTCTTC GCGGTCTITCCAGTACTCTTGGATCGGAAACCCGTCGGCCTC CGAACGGTACTCCGCCGCCGAGGGACCTGAGCGAGTCC GCATCGACCGGATCGGAAAACCTCTCGAGAAAGGCGTGTAA CCAGTCACAGTCGCTCTAGAACTAGTGGATCCCCCGGGC TGCAGGAATTCGATAATICGGCACGAGGCTGCCGAGGCACT GTGATGTCCGGGGAGATGGACAAACCGCTCATCAGTCGC CGCTTGGTGGACAGTGATGGCAGTCTGGCTGAGGTCCCCAA GGAGGCTCCCAAAGTGGGCATCCTGGGCAGCGGGGATTT TGCCCGGTCCCTGGCCACACGCCTGGTGGGCTCTGGCTTCT TIGTGGTGGTGGGAAGCCGTAACCCCAAACGCACTGCCG GCCTCTTCCCCTCCITAGCCCAAGTGACTITCCAGGAGGAGG CCGTGAGCTCTCCAGAGGTCATCIITGTGGCCGTGTTC
CGGGAGCACTACTCCTCACTGTGCAGTCTIGCTGACCAGTTG GCTGGCAAGATCCTAGTGGATGTAAGCAACCCCACGGA GAAGGAGCGTCTTCAGCACCGCCAGTCGAACGCCGAGTACC TGGCCTCCCTCTICCCTGCCTGCACTGTGGTCAAGGCCT TCAACGTCATCICTGCATGGGCCCTACAGGCTGGCCCAAGG GATGGGAACAGGCAGGTGCTCATCTGCGGTGACCAGCTG GAAGCCAAGCACACCGTCTCAGAGATGGCGCGCGCCATGG GTITCACCCCACTGGACATGGGATCCCTGGCCTCAGCGAG GGAGGTAGAGGCCATACCCCTGCGCCTCCITCCATCCTGGA AGGTGCCCACCCTCCTGGCCCTGGGGCTAAGCACACAAA GCTATGCCTACAACITCATCCGGGACGTTCTACAGCCGTACA TCCGGAAAGATGAGAACAAGTICTACAAGATGCCCCTG TCTGTGGTCAACACCACGaTACCCTGTGTGGCTTACGTGCTG CTGTCCCTGGTIIACCTGCCTGGTGTGCTGGCAGCTGC CCITCAGCTGAGGAGGGGGACCAAGTACCAGCGCTTCCCAG ACTGGCTGGACCATIGGCTGCAGCACCGCAAGCAGATCG GGCTACTCAGCTIIIIIITCGCCATGCTGCACGCTCTCTACAG CTTCTGCCTGCCGCTGCGCCGCTCCCACCGCTATGAT CTGGTCAACCTGGCTGTGAAGCAGGTCCTGGCCAACAAGAG CCGCCTCTGGGTTGAGGAAGAAGTCTGGCGGATGGAGAT

FIG. 10
ATACCTGTCCCTGGGTGTGCTGGCTCTGGGCATGCTGTCACT GCTGGCGGTTACCTCGATCCCTTCCAITGCAAACTCAC TCAACTGGAAGGAGTTCAGCITTGTGCAGTCCACGCTGGGC TTCGTGGCCCTGATGCTGAGCACAATGCACACCCTCACC TACGGCTGGACCCGTGCIIITGAGGAAAACCACTACAAGITC TACCTGCCACCCACATTCACGCTCACGCTGCTCCTGCC CTGTGTCATCATCCTGGCCAAGGGCCTCITCCTCCTGCCCTG CCTCAGCCACAGACICACCAAGATCCGCAGGGGCTGGG AGAGGGATGGTGCCGTCAAGTTCATGCTGCCCGCTGGCCAC ACACAGGGGGAGAAAACAAGCCACGTGTGAGGCCCTGGA AATGGAGACAGGCACAGCITGTGGGGGCCCTGGGCTGGGT TCGGGTCTCTTTTCTGGGATGGTATATGCGTGGGTGGCCG AGGTCTGAATITCTGGGATGCAGGTGTATGCCGAGATACTCA GAATGGCGTACCACACATGCGATAAGTACTCACATATA TITCATATATAATAGGATITACTATTATTCITAGITAAAAAAAA ATAGTGGGTCCITATATITCAACTTATGCAGGGTCC CTATATITCAACTTGAGCATITCAGAGCAAATGCCACACATTA AACAGCAGATCCCACCCITGTGGTAGCTGCAGAGACA GACAGAAACITCTGGTIATGAGAGAGACTGTATIITGTTGGAT TCTACCTITAATCCCCGTTCTCTACGTTcCCCTGTTA GCCACATCITAACGTTGGTGCAGAGCTGGGACAAGAGCTGG CTCTGGTGCAGCCTCCCCCATCCCAGGGCTAGGAAACAA GCCTCTGATGAACAGAGGGACCAGGTCTGGACCCTCCTGCT CCCGCTTCCCTGGGCTCGAGTGGGGAGGCTCAGCGGGAT CCCCCGCAATCTGTGCAGGAGIIITCACAGGTCTGTCCTITC TTCCGGGAGCGGTCTGAAGCGGCCCCATCTGATCCTAG CTGAGCCGAGATTGTTCCCCACTCCCTGAAAGTCCAGAGTCA CCGTGGAGCCTGCAAATTGCTCCTTCTGCGAAGGTGTG AAGTCACCGTCTCACCAGAGCCATTAACGAACCTGATCITCA GAAGAAGCATAATTGTTTCCCCTCCATTAAGTTGGTGG TGACCCTCTITAAACCACTGTGCCTTCTCGCCITTCCCATCAC TAATITGGGCATCTCCATGGAGTGGACICIIGTCGGG GCAGTTCAGGGGGGAGGGAAGCATTAGAGATTGCGGAGAA TAACCATCGAAGCCTCCCITGGATGITCCCAGGCGTGCCT TCATTAAATTGGTCCCTAATGAGAATGACAGGGGACCCCTGT TGCCTGTaTGCAGAGAACCAGCCITCTGAGCACCCAGG AAACACAGTGGCCCCACGCCCTTCAGGGGGGTCCCACGTCC CCTITCCCATGCTITTGCCTCCCTCCCTCCCGGTTACAA TCAACCATAAAAGTCTGCAAATATTGTITTITGAATTATCAAG CITATCGATACCGTCGAAACTIGTITATTGCAGCITA TAATGGTTACAAATAAAGCAATAGCATCACAAATITCACAAAT AAAGCATTTTTITCACTGCATTCTAGTTGTGGTTTGT CCAAACTCATCAATGTATCTTATCATGTCTGGATCCGACCTCG G

## Sequence of Region $B$ of AdRSYpHydc:

ATCTGGAAGGTGCTGAGGTACGATGAGACCCGCACCAGGTG CAGACCCTGCGAGTGTGGCGGTAAACATATTAGGAACCA GCCTGTGATGCTGGATGTGACCGAGGAGCTGAGGCCCGATC ACITGGTGCTGGCCTGCACCCGCGCTGAGIITGGCTCTA GCGATGAAGATACAGATTGAGGTACTGAAATGTGTGGGCGT GGCTTAAGGGTGGGAAAGAATATATAAGGTGGGGGTCTT ATGTAGTITTGTATCTGTTITGCAGCAGCCGCCGCCGCCATG AGCACCAACTCGTITGATGGAAGCATTGTGAGCTCATA

## 13 / 43

TTTGACAACGCGCATGCCCCCATGGGCCGGGGTGCGTCAGA ATGTGATGGGCTCCAGCATTGATGGTCGCCCCGTCCTGC CCGCAAACTCTACTACCTTGACCTACGAGACCGTGTCTGGAA CGCCGTTGGAGACTGCAGCCTCCGCCGCCGCTTCAGCC GCTGCAGCCACCGCCCGCGGGATTGTGACTGACITTGCTITC CTGAGCCCGCTIGCAAGCAGTGCAGCIICCCGTICATC CGCCCGCGATGACAAGTTGACGGCTCTITTGGCACAATTGG ATTCTTTGACCCGGGAACITAATGTCGTTTCTCAGCAGC TGTTGGATCTGCGCCAGCAGGTITCTGCCCTGAAGGCTTCCT CСССTCCCAATGCGGTITAAAACATAAATAAAAAACCA GACTCTGTITGGATITGGATCAAGCAAGTGTCTIGCTGTCTIT ATTTAGGGGTITTGCGCGCGCGGTAGGCCCGGGACCA GCGGTCTCGGTCGTTGAGGGTCCTGTGTATTITTTCCAGGAC GTGGTAAAGGTGACTCTGGATGTICAGATACATGGGCA TAAGCCCGTCTCTGGGGTGGAGGTAGCACCACTGCAGAGCT TCATGCTGCGGGGTGGTGTTGTAGATGATCCAGTCGTAG CAGGAGCGCTGGGCGTGGTGCCTAAAAATGTCTITCAGTAG CAAGCTGATTGCCAGGGGCAGGCCCTTGGTGTAAGTGTT TACAAAGCGGTTAAGCTGGGATGGGTGCATACGTGGGGATA TGAGATGCATCITGGACTGTATITTTAGGTTGGCTATGT TCCCAGCCATATCCCTCCGGGGATTCATGTTGTGCAGAACCA CCAGCACAGTGTATCCGGTGCACTTGGGAAATITGTCA TGTAGCTTAGAAGGAAATGCGTGGAAGAACTIGGAGACGCC CITGTGACCTCCAAGATITTCCATGCATTCGTCCATAAT GATGGCAATGGGCCCACGGGCGGCGGCCTGGGCGAAGATA IITCTGGGATCACTAACGTCATAGTTGTGITCCAGGATGA GATCGTCATAGGCCAIIITTACAAAGCGCGGGCGGAGGGTG CCAGACTGCGGTATAATGGTTCCATCCGGCCCAGGGGCG TAGTTACCCTCACAGATITGCATTTCCCACGCTITGAGTTCAG ATGGGGGGATCATGTCTACCTGCGGGGCGATGAAGAA AACGGTITCCGGGGTAGGGGAGATCAGCTGGGAAGAAAGC AGGTTCCTGAGCAGCTGCGACTTACCGCAGCCGGTGGGCC CGTAAATCACACCTATTACCGGGTGCAACTGGTAGTTAAGAG AGCTGCAGCTGCCGTCATCCCTGAGCAGGGGGGCCACT TCGTTAAGCATGTCCCTGACTCGCATGTITTCCCTGACCAAAT CCGCCAGAAGGCGCTCGCCGCCCAGCGATAGCAGTTC TTGCAAGGAAGCAAAGTIIITCAACGGITIGAGACCGTCCGC CGTAGGCATGCTTTTGAGCGITTGACCAAGCAGTTCCA GGCGGTCCCACAGCTCGGTCACCTGCTCTACGGCATCTCGA TCCAGCATATCTCCTCGTITCGCGGGTTGGGGCGGCIIT CGCTGTACGGCAGTAGTCGGTGCTCGTCCAGACGGGCCAGG GTCATGTCTITCCACGGGCGCAGGGTCCTCGTCAGCGTA GTCTGGGTCACGGTGAAGGGGTGCGCTCCGGGCTGCGCGC TGGCCAGGGTGCGCTTGAGGCTGGTCCTGCTGGTGCTGAA GCGCTGCCGGTCTTCGCCCTGCGCGTCGGCCAGGTAGCATT TGACCATGGTGTCATAGTCCAGCCCCTCCGCGGCGTGGC CCTTGGCGCGCAGCITGCCCITGGAGGAGGCGCCGCACGA GGGGCAGTGCAGACIIITGAGGGCGTAGAGCITGGGCGCG AGAAATACCGATTCCGGGGAGTAGGCATCCGCGCCGCAGGC CCCGCAGACGGTCTCGCATTCCACGAGCCAGGTGAGCTC TGGCCGTTCGGGGTCAAAAACCAGGTTTCCCCCATGCTITIT GATGCGIITCTTACCTCTGGIITCCATGAGCCGGTGTC CACGCTCGGTGACGAAAAGGCTGTCCGTGTCCCCGTATACA GACTTGAGAGGCCTGTCCTCGAGCGGTGTTCCGCGGTCC TCCTCGTATAGAAACTCGGACCACTCTGAGACAAAGGCTCGC GTCCAGGCCAGCACGAAGGAGGCTAAGTGGGAGGGGTA

[^0]CATCCTGCACGAGGTTGACCTGACGACCGCGCACAAGGAAG CAGAGTGGGAATITGAGCCCCTCGCCTGGCGGGTITGGC TGGTGGTCITCTACTTCGGCTGCTTGTCCTTGACCGTCTGGC TGCTCGAGGGGAGTTACGGTGGATCGGACCACCACGCC GCGCGAGCCCAAAGTCCAGATGTCCGCGCGCGGCGGTCGG ^GCTTGATGACAACATCGCGCAGATGGGAGCTGTCCATGG TCTGGAGCTCCCGCGGCGTCAGGTCAGGCGGGAGCTCCTGC AGGTITACCTCGCATAGACGGGTCAGGGCGCGGGCTAGA TCCAGGTGATACCTAAIITCCAGGGGCTGGTIGGTGGCGGC GTCGATGGCTTGCAAGAGGCCGCATCCCCGCGGCGCGAC TACGGTACCGCGCGGCGGGCGGTGGGCCGCGGGGGTGTCC TTGGATGATGCATCTAAAAGCGGTGACGCGGGCGAGCCCC CGGAGGTAGGGGGGGCTCCGGACCCGCCGGGAGAGGGGG CAGGGGCACGTCGGCGCCGCGCGCGGGCAGGAGCTGGTGC T
GCGCGCGTAGGTIGCTGGCGAACGCGACGACGCGGCGGTT GATCTCCTGAATCTGGCGCCTCTGCGTGAAGACGACGGGC CCGGTGAGCITGAGCCTGAAAGAGAGTTCGACAGAATCAAT TTCGGTGTCGTTGACGGCGGCCTGGCGCAAAATCTCCTG CACGTCTCCTGAGTTGTCITGATAGGCGATCTCGGCCATGAA CTGCTCGATCTCTICCTCCTGGAGATCTCCGCGICCGG CTCGCTCCACGGTGGCGGCGAGGTCGITGGAAATGCGGGC CATGAGCTGCGAGAAGGCGTTGAGGCCTCCCTCGTTCCAG ACGCGGCTGTAGACCACGCCCCCTTCGGCATCGCGGGCGCG CATGACCACCTGCGCGAGATTGAGCTCCACGTGCCGGGC GAAGACGGCGTAGIITCGCAGGCGCTGAAAGAGGTAGTTGA GGGTGGTGGCGGTGTGTTCTGCCACGAAGAAGTACATAA CCCAGCGTCGCAACGTGGATTCGTTGATATCCCCCAAGGCCT CAAGGCGCTCCATGGCCTCGTAGAAGTCCACGGCGAAG TTGAAAAACTGGGAGITGCGCGCCGACACGGITAACTCCTC CTCCAGAAGACGGATGAGCTCGGCGACAGTGTCGCGCAC CTCGCGCTCAAAGGCTACAGGGGCCTCITCTTCTTCTICAAT СТССТСТТССАТААGGGCCTCCCCTTCTTCTTCTTCTG GCGGCGGTGGGGGAGGGGGGACACGGCGGCGACGACGGC GCACCGGGAGGCGGTCGACAAAGCGCICGATCATCTCCCCG CGGCGACGGCGCATGGTCTCGGTGACGGCGCGGCCGTTCT CGCGGGGGCGCAGTTGGAAGACGCCGCCCGTCATGTCCCG GTTATGGGTTGGCGGGGGGCTGCCATGCGGCAGGGATACG GCGCTAACGATGCATCTCAACAATIGITGTGTAGGTACTC CGCCGCCGAGGGACCTGAGCGAGTCCGCATCGACCGGATC GGAAAACCTCTCGAGAAAGGCGTCTAACCAGTCACAGTCG CAAGGTAGGCTGAGCACCGTGGCGGGCGGCAGCGGGCGGC GGTCGGGGTTGTITCTGGCGGAGGTGCTGCTGATGATGTA ATTAAAGTAGGCGGTCTTGAGACGGCGGATGGTCGACAGAA GCACCATGTCCTTGGGTCCGGCCTGCTGAATGCGCAGGC GGTCGGCCATGCCCCAGGCTTCGIITTGACATCGGCGCAGG TCITTGTAGTAGTCITGCATGAGCCITTCTACCGGCACT TCTTCITCTCCITCCTCTTGTCCTGCATCTCITGCATCTATCGC TGCGGCGGCGGCGGAGTTTGGCCGTAGGTGGCGCCC TCTTCCTCCCATGCGTGTGACCCCGAAGCCCCTCATCGGCTG AAGCAGGGCTAGGTCGGCGACAACGCGCTCGGCTAATA TGGCCTGCTGCACCTGCGTGAGGGTAGACTGGAAGTCATCC ATGTCCACAAAGCGGTGGTATGCGCCCGTGTTGATGGTG TAAGTGCAGTTGGCCATAACGGACCAGTTAACGGTCTGGTG ACCCGGCTGCGAGAGCTCGGTGTACCTGAGACGCGAGTA AGCCCTCGAGTCAAATACGTAGTCGTTGCAAGTCCGCACCA GGTACTGGTATCCCACCAAAAAGTGCGGCGGCGGCTGGC
gGTAGAGGGGCCAGCGTAGGGTGGCCGGGGCTCCGGGGG CGAGATCTTCCAACATAAGGCGATGATATCCGTAGATGTAC CTGGACATCCAGGTGATGCCGGCGGCGGTGGTGGAGGCGC GCGGAAAGTCGCGGACGCGGTTCCAGATGTTGCGCAGCGG CAAAAAGTGCTCCATGGTCGGGACGCTCTGGCCGGTCAGGC GCGCGCAATCGTTGACGCTCTACCGTGCAAAAGGAGAGC CTGTAAGCGGGCACTCTTCCGTGGTCTGGTGGATAAATTCGC AAGGGTATCATGGCGGACGACCGGGGTTCGAGCCCCGT ATCCGGCCGTCCGCCGTGATCCATGCGGTTACCGCCCGCGT GTCGAACCCAGGTGTGCGACGTCAGACAACGGGGGAGTG CTCCTTMTGGCTTCCTTCCAGGCGCGGCGGCTGCTGCGCTAG CTIMITTGGCCACTGGCCGCGCGCAGCGTAAGCGGTTA GGCTGGAAAGCGAAAGCATTAAGTGGCTCGCTCCCTGTAGC CGGAGGGTTATTITCCAAGGGTTGAGTCGCGGGACCCCC GGTTCGAGTCTCGGACCGGCCGGACTGCGGCGAACGGGGG TITGCCTCCCCGTCATGCAAGACCCCGCTTGCAAATTCCT CCGGAAACAGGGACGAGCCCCTITITTGCTITICCCAGATGC ATCCGGTGCTGCGGCAGATGCGCCCCCCTCCTCAGCAG CGGCAAGAGCAAGAGCAGCGGCAGACATGCAGGGCACCCT CCCCTCCTCCTACCGCGTCAGGAGGGGCGACATCCGCGGT TGACGCGGCAGCAGATGGTGATTACGAACCCCCGCGGCGCC GGGCCCGGCACTACCTGGACTTGGAGGAGGGCGAGGGCC TGGCGCGGCTAGGAGCGCCCTCTCCTGAGCGGTACCCAAGG GTGCAGCTGAAGCGTGATACGCGTGAGGCGTACGTGCCG CGGCAGAACCTGITTCGCGACCGCGAGGGAGAGGAGCCCG AGGAGATGCGGGATCGAAAGTTCCACGCAGGGCGCGAGCT GCGGCATGGCCTGAATCGCGAGCGGTTGCTGCGCGAGGAG GACTTTGAGCCCGACGCGCGAACCGGGATTAGTCCCGCGC GCGCACACGTGGCGGCCGCCGACCTGGTAACCGCATACGA GCAGACGGTGAACCAGGAGATTAACITTCAAAAAAGCTIT AACAACCACGTGCGTACGCTTGTGGCGCGCGAGGAGGTGG CTATAGGACTGATGCATCTGTGGGACTITGTAAGCGCGCT GGAGCAAAACCCAAATAGCAAGCCGCTCATGGCGCAGCTGT TCCTTATAGTGCAGCACAGCAGGGACAACGAGGCATTCA GGGATGCGCTGCTAAACATAGTAGAGCCCGAGGGCCGCTG GCTGCTCGATITGATAAACATCCTGCAGAGCATAGTGGTG CAGGAGCGCAGCTTGAGCCTGGCTGACAAGGTGGCCGCCAT CAACTATTCCATGCTTAGCCTGGGCAAGTIITACGCCCG CAAGATATACCATACCCCTTACGTTCCCATAGACAAGGAGGT AAAGATCGAGGGGTTCTACATGCGCATGGCGCTGAAGG TGCTTACCTTGAGCGACGACCTGGGCGTITATCGCAACGAG CGCATCCACAAGGCCGTGAGCGTGAGCCGGCGGCGCGAG CTCAGCGACCGCGAGCTGATGCACAGCCTGCAAAGGGCCCT GGCTGGCACGGGCAGCGGCGATAGAGAGGCCGAGTCCTA CTTTGACGCGGGCGCTGACCTGCGCTGGGCCCCAAGCCGAC GCGCCCTGGAGGCAGCTGGGGCCGGACCTGGGCTGGCGG TGGCACCCGCGCGCGCTGGCAACGTCGGCGGCGTGGAGGA ATATGACGAGGACGATGAGTACGAGCCAGAGGACGGCGAG TACTAAGCGGTGATGTTTCTGATCAGATGATGCAAGACGCAA CGGACCCGGCGGTGCGGGCGGCGCTGCAGAGCCAGCCG TCCGGCCTTAACTCCACGGACGACTGGCGCCAGGTCATGGA CCGCATCATGTCGCTGACTGCGCGCAATCCTGACGCGTT CCGGCAGCAGCCGCAGGCCAACCGGCTCTCCGCAATTCTGG AAGCGGTGGTCCCGGCGCGCGCAAACCCCACGCACGAGA AGGTGCTGGCGATCGTAAACGCGCTGGCCGAAAACAGGGC CATCCGGCCCGACGAGGCCGGCCTGGTCTACGACGCGCTG

[^1]AACGAAGCCTTCCATCCAGACATCATITTGCTGCCAGGATGC GGGGTGGACTTCACCCACAGCCGCCTGAGCAACTTGTT GGGCATCCGCAAGCGGCAACCCTTCCAGGAGGGCTITAGGA TCACCTACGATGATCTGGAGGGTGGTAACATTCCCGCAC TGITGGATGTGGACGCCTACCAGGCGAGCITGAAAGATGAC ACCGAACAGGGCGGGGGTGGCGCAGGCGGCAGCAACAGC AGTGGCAGCGGCGCGGAAGAGAACTCCAACGCGGCAGCCG CGGCAATGCAGCCGGTGGAGGACATGAACGATCATGCCAT TCGCGGCGACACCTTTGCCACACGGGCTGAGGAGAAGCGC GCTGAGGCCGAAGCAGCGGCCGAAGCTGCCGCCCCCGCTG CGCAACCCGAGGTCGAGAAGCCTCAGAAGAAACCGGTGATC AAACCCCTGACAGAGGACAGCAAGAAACGCAGTTACAAC CTAATAAGCAATGACAGCACCTTCACCCAGTACCGCAGCTGG TACCTTGCATACAACTACGGCGACCCTCAGACCGGAAT CCGCTCATGGACCCTGCTTTGCACTCCTGACGTAACCTGCGG CTCGGAGCAGGTCTACTGGTCGTTGCCAGACATGATGC AAGACCCCGTGACCITCCGCTCCACGCGCCAGATCAGCAAC TTTCCGGTGGTGGGCGCCGAGCTGTTGCCCGTGCACTCC AAGAGCTTCTACAACGACCAGGCCGTCTACTCCCAACTCATC CGCCAGTITACCTCTCTGACCCACGTGTTCAATCGCTT TCCCGAGAACCAGATTTTGGCGCGCCCGCCAGCCCCCACCA TCACCACCGTCAGTGAAAACGTTCCTGCTCTCACAGATC ACGGGACGCTACCGCTGCGCAACAGCATCGGAGGAGTCCA GCGAGTGACCATTACTGACGCCAGACGCCGCACCTGCCCC TACGTITACAAGGCCCTGGGCATAGTCTCGCCGCGCGTCCTA TCGAGCCGCACII ITTGAGCAAGCATGTCCATCCTTAT ATCGCCCAGCAATAACACAGGCTGGGGCCTGCGCTTCCCAA GCAAGATGTTTGGCGGGGCCAAGAAGCGCTCCGACCAAC ACCCAGTGCGCGTGCGCGGGCACTACCGCGCGCCCTGGGG CGCGCACAAACGCGGCCGCACTGGGCGCACCACCGTCGAT GACGCCATCGACGCGGTGGTGGAGGAGGCGCGCAACTACA CGCCCACGCCGCCACCAGTGTCCACAGTGGACGCGGCCAT TCAGACCGTGGTGCGCGGAGCCCGGCGCTATGCTAAAATGA AGAGACGGCGGAGGCGCGTAGCACGTCGCCACCGCCGCC GACCCGGCACTGCCGCCCAACGCGCGGCGGCGGCCCTGCT TAACCGCGCACGTCGCACCGGCCGACGGGCGGCCATGCGG GCCGCTCGAAGGCTGGCCGCGGGTATIGTCACTGTGCCCCC CAGGTCCAGGCGACGAGCGGCCGCCGCAGCAGCCGCGGC CATTAGTGCTATGACTCAGGGTCGCAGGGGCAACGTGTATT GGGTGCGCGACTCGGTTAGCGGCCTGCGCGTGCCCGTGC GCACCCGCCCCCCGCGCAACTAGATTGCAAGAAAAAACTAC TTAGACTCGTACTGTTGTATGTATCCAGCGGCGGCGGCG CGCAACGAAGCTATGTCCAAGCGCAAAATCAAAGAAGAGAT GCTCCAGGTCATCGCGCCGGAGATCTATGGCCCCCCGAA GAAGGAAGAGCAGGATTACAAGCCCCGAAAGCTAAAGCGG GTCAAAAAGAAAAAGAAAGATGATGATGATGAACTTGACG ACGAGGTGGAACTGCTGCACGCTACCGCGCCCAGGCGACG GGTACAGTGGAAAGGTCGACGCGTAAAACGTGTITTGCGA CCCGGCACCACCGTAGTCITTACGCCCGGTGAGCGCTCCAC CCGCACCTACAAGCGCGTGTATGATGAGGTGTACGGCGA CGAGGACCTGCITGAGCAGGCCAACGAGCGCCTCGGGGAG TTTGCCTACGGAAAGCGGCATAAGGACATGCTGGCGTTGC CGCTGGACGAGGGCAACCCAACACCTAGCCTAAAGCCCGTA ACACTGCAGCAGGTGCTGCCCGCGCTTGCACCGTCCGAA GAAAAGCGCGGCCTAAAGCGCGAGTCTGGTGACITGGCACC CACCGTGCAGCTGATGGTACCCAAGCGCCAGCGACTGGA

[^2]AAAAAACTCACGTATTTGGGCAGGCGCCTTATTCTGGTATAA ATATIACAAAGGAGGGTATICAAATAGGTGTCGAAGGT CAAACACCTAAATATGCCGATAAAACATITCAACCTGAACCT CAAATAGGAGAATCTCAGTGGTACGAAACTGAAATTAA TCATGCAGCTGGGAGAGTCCTTAAAAAGACTACCCCAATGAA ACCATGTTACGGTTCATATGCAAAACCCACAAATGAAA ATGGAGGGCAAGGCATTCTTGTAAAGCAACAAAATGGAAAG CTAGAAAGTCAAGTGGAAATGCAAMIITCTCAACTACT GAGGCGACCGCAGGCAATGGTGATAACTTGACTCCTAAAGT GGTATTGTACAGTGAAGATGTAGATATAGAAACCCCAGA САСТСАТАТТТСТTACATGCCCACTATTAAGGAAGGTAACTCA CGAGAACTAATGGGCCAACAATCTATGCCCAACAGGC CTAATTACATTGCTTTTAGGGACAATITTATIGGTCTAATGTA TTACAACAGCACGGGTAATATGGGTGTTCTGGCGGGC CAAGCATCGCAGTTGAATGCTGITGTAGATITGCAAGACAGA AACACAGAGCTITCATACCAGCTITTGCTTGATTCCAT TGGTGATAGAACCAGGTACTITTCTATGTGGAATCAGGCTGT TGACAGCTATGATCCAGATGTTAGAATTATTGAAAATC ATGGAACTGAAGATGAACTTCCAAATTACTGCTTTCCACTGG GAGGTGTGATTAATACAGAGACTCTTACCAAGGTAAAA CCTAAAACAGGTCAGGAAAATGGATGGGAAAAAGATGCTAC AGAAIITTCAGATAAAAATGAAATAAGAGTTGGAAATAA TITTGCCATGGAAATCAATCTAAATGCCAACCTGTGGAGAAA IITCCTGTACTCCAACATAGCGCTGTATITGCCCGACA AGCTAAAGTACAGTCCTTCCAACGTAAAAATTTCTGATAACC CAAACACCTACGACTACATGAACAAGCGAGTGGTGGCT CCCGGGTTAGTGGACTGCTACATTAACCTTGGAGCACGCTG GTCCCTTGACTATATGGACAACGTCAACCCATTTAACCA CCACCGCAATGCTGGCCTGCGCTACCGCTCAATGTTGCTGG GCAATGGTCGCTATGTGCCCTTCCACATCCAGGTGCCTC AGAAGTTCTITGCCATTAAAAACCTCCTTCTCCTGCCGGGCT CATACACCTACGAGTGGAACITCAGGAAGGATGTTAAC ATGGTTCTGCAGAGCTCCCTAGGAAATGACCTAAGGGITGA CGGAGCCAGCATTAAGTTTGATAGCATITGCCTTTACGC CACCTTCTTCCCCATGGCCCACAACACCGCCTCCACGCTTGA GGCCATGCTTAGAAACGACACCAACGACCAGTCCTITA
ACGACTATCTCTCCGCCGCCAACATGCTCTACCCTATACCCG CCAACGCTACCAACGTGCCCATATCCATCCCCTCCCGC AACTGGGCGGCTTTCCGCGGCTGGGCCTTCACGCGCCTIAA GACTAAGGAAACCCCATCACTGGGCTCGGGCTACGACCC
TLATTACACCTACTCTGGCTCTATACCCTACCTAGATGGAACC TTITACCTCAACCACACCTITAAGAAGGTGGCCATTA CCTITGACTCTTCTGTCAGCTGGCCTGGCAATGACCGCCTGC ITACCCCCAACGAGTTTGAAATTAAGCGCTCAGITGAC GGGGAGGGTTACAACGTIGCCCAGTGTAACATGACCAAAGA CTGGTTCCTGGTACAAATGCTAGCTAACTACAACATTGG CTACCAGGGCTTCTATATCCCAGAGAGCTACAAGGACCGCAT GTACTCCTTCTITAGAAACTICCAGCCCATGAGCCGTC AGGTGGTGGATGATACTAAATACAAGGACTACCAACAGGTG GGCATCCTACACCAACACAACAACTCTGGATITGTTGGC
TACCTTGCCCCCACCATGCGCGAAGGACAGGCCTACCCTGC TAACITCCCCTATCCGCTTATAGGCAAGACCGCAGTTGA CAGCATTACCCAGAAAAAGITTCTTTGCGATCGCACCCTTTG GCGCATCCCATTCTCCAGTAACTTTATGTCCATGGGCG CACTCACAGACCTGGGCCAAAACCTTCTCTACGCCAACTCCG CCCACGCGCTAGACATGACTITTGAGGTGGATCCCATG

GACGAGCCCACCCTTCTITATGTITTGIITGAAGTCITTGACG TGGTCCGTGTGCACCGGCCGCACCGCGGCGTCATCGA AACCGTGTACCTGCGCACGCCCITCTCGGCCGGCAACGCCA CAACATAAAGAAGCAAGCAACATCAACAACAGCTGCCGC CATGGGCTCCAGTGAGCAGGAACTGAAAGCCATTGTCAAAG ATCTTGGTTGTGGGCCATATITIITGGGCACCTATGACA AGCGCTITCCAGGCITTGTTTCTCCACACAAGCTCGCCTGCG CCATAGTCAATACGGCCGGTCGCGAGACTGGGGGCGTA CACTGGATGGCCTITGCCTGGAACCCGCACTCAAAAACATGC TACCTCTITGAGCCCTITGGCTIITCTGACCAGCGACT CAAGCAGGTTTACCAGIITGAGTACGAGTCACTCCTGCGCCG TAGCGCCATTGCTTCTTCCCCCGACCGCTGTATAACGC TGGAAAAGTCCACCCAAAGCGTACAGGGGCCCAACTCGGCC GCCTGTGGACTATTCTGCTGCATGITTCTCCACGCCTII GCCAACTGGCCCCAAACTCCCATGGATCACAACCCCACCATG AACCTTATTACCGGGGTACCCAACTCCATGCTCAACAG TCCCCAGGTACAGCCCACCCTGCGTCGCAACCAGGAACAGC TCTACAGCTTCCTGGAGCGCCACTCGCCCTACTICCGCA GCCACAGTGCGCAGATTAGGAGCGCCACTTCTTTTTGTCACT TGAAAAACATGTAAAAATAATGTACTAGAGACACTITC AATAAAGGCAAATGCIITTATITGTACACTCTCGGGTGATTAT TTACCCCCACCCTTGCCGTCTGCGCCGTITAAAAATC AAAGGGGTTCTGCCGCGCATCGCTATGCGCCACTGGCAGGG ACACGTTGCGATACTGGTGTTTAGTGCTCCACTTAAACT CAGGCACAACCATCCGCGGCAGCTCGGTGAAGITITCACTC CACAGGCTGCGCACCATCACCAACGCGTTTAGCAGGTCG GGCGCCGATATCTTGAAGTCGCAGTTGGGGCCTCCGCCCTG CGCGCGCGAGITGCGATACACAGGGTTGCAGCACTGGAA CACTATCAGCGCCGGGTGGTGCACGCTGGCCAGCACGCTCT TGTCGGAGATCAGATCCGCGTCCAGGTCCTCCGCGTTGC TCAGGGCGAACGGAGTCAACTITGGTAGCTGCCTTCCCAAA AAGGGCGCGTGCCCAGGCTITGAGTTGCACTCGCACCGT AGTGGCATCAAAAGGTGACCGTGCCCGGTCTGGGCGTTAGG ATACAGCGCCTGCATAAAAGCCITGATCTGCITAAAAGC CACCTGAGCCTTTGCGCCTTCAGAGAAGAACATGCCGCAAG ACTTGCCGGAAAACTGATTGGCCGGACAGGCCGCGTCGT GCACGCAGCACCTIGCGTCGGTGITGGAGATCTGCACCACA. TITCGGCCCCACCGGTTCTTCACGATCTTGGCCITGCTA GACTGCTCCTTCAGCGCGCGCTGCCCGTTTTCGCTCGTCACA TCCATITCAATCACGTGCTCCITATTTATCATAATGCT TCCGTGTAGACACTTAAGCTCGCCTTCGATCTCAGCGCAGCG GTGCAGCCACAACGCGCAGCCCGTGGGCTCGTGATGCT TGTAGGTCACCTCTGCAAACGACTGCAGGTACGCCTGCAGG AATCGCCCCATCATCGTCACAAAGGTCTTGITGCTGGTG AAGGTCAGCTGCAACCCGCGGTGCTCCTCGITCAGCCAGGT CTTGCATACGGCCGCCAGAGCTTCCACITGGTCAGGCAG TAGTTTGAAGTTCGCCTITAGATCGTTATCCACGTGGTACTTG TCCATCAGCGCGCGCGCAGCCTCCATGCCCTTCTCCC ACGCAGACACGATCGGCACACTCAGCGGGITCATCACCGTA ATTTCACTTTCCGCTTCGCTGGGCTCTTCCTCTTCCTCT TGCGTCCGCATACCACGCGCCACTGGGTCGTCITCAITCAGC CGCCGCACTGTGCGCITACCTCCTITGCCATGCTTGAT TAGCACCGGTGGGTTGCTGAAACCCACCATTTGTAGCGCCA CATCTTCTCTTTCTTCCTCGCTGTCCACGATTACCTCTG GTGATGGCGGGCGCTCGGGCTTGGGAGAAGGGCGCTTCTTT TTCTTCTTGGGCGCAATGGCCAAATCCGCCGCCGAGGTC

GATGGCCGCGGGCTGGGTGTGCGCGGCACCAGCGCGTCTT GTGATGAGTCTTCCTCGTCCTCGGACTCGATACGCCGCCT CATCCGCTTTITTGGGGGCGCCCGGGGAGGCGGCGGCGAC GGGGACGGGGACGACACGTCCTCCATGGTTGGGGGACGTC GCGCCGCACCGCGTCCGCGCTCGGGGGTGGTITCGCGCTG СTCCTCTTCCCGACTGGCCATTTCCTTCTCCTATAGGCAG AAAAAGATCATGGAGTCAGTCGAGAAGAAGGACAGCCTAAC CGCCCCCTCTGAGTTCGCCACCACCGCCTCCACCGATGC CGCCAACGCGCCTACCACCTTCCCCGTCGAGGCACCCCCGC TTGAGGAGGAGGAAGTGATTATCGAGCAGGACCCAGGTI TTGTAAGCGAAGACGACGAGGACCGCTCAGTACCAACAGAG GATAAAAAGCAAGACCAGGACAACGCAGAGGCAAACGAG GAACAAGTCGGGCGGGGGGACGAAAGGCATGGCGACTACC TAGATGTGGGAGACGACGTGCTGTTGAAGCATCTGCAGCG CCAGTGCGCCATTATCTGCGACGCGTTGCAAGAGCGCAGCG ATGTGCCCCTCGCCATAGCGGATGTCAGCCITGCCTACG AACGCCACCTATICTCACCGCGCGTACCCCCCAAACGCCAAG AAAACGGCACATGCGAGCCCAACCCGCGCCTCAACITC TACCCCGTATITGCCGTGCCAGAGGTGCTTGCCACCTATCAC ATCIIITICCAAAACTGCAAGATACCCCTATCCTGCCG TGCCAACCGCAGCCGAGCGGACAAGCAGCTGGCCTTGCGG CAGGGCGCTGTCATACCTGATATCGCCTCGCTCAACGAAG TGCCAAAAATCTITGAGGGICTTGGACGCGACGAGAAGCGC GCGGCAAACGCTCTGCAACAGGAAAACAGCGAAAATGAA AGTCACTCTGGAGTGTTGGTGGAACTCGAGGGTGACAACGC GCGCCTAGCCGTACTAAAACGCAGCATCGAGGTCACCCA CTITGCCTACCCGGCACTTAACCTACCCCCCAAGGTCATGAG CACAGTCATGAGTGAGCTGATCGTGCGCCGTGCGCAGC CCCTGGAGAGGGATGCAAATTTGCAAGAACAAACAGAGGAG GGCCTACCCGCAGTTGGCGACGAGCAGCTAGCGCGCTGG CTTCAAACGCGCGAGCCTGCCGACTTGGAGGAGCGACGCAA ACTAATGATGGCCGCAGTGCTCGTTACCGTGGAGCITGA GTGCATGCAGCGGTTCIITGCTGACCCGGAGATGCAGCGCA AGCTAGAGGAAACATTGCACTACACCTITCGACAGGGCT ACGTACGCCAGGCCTGCAAGATCTCCAACGTGGAGCTCTGC AACCTGGTCTCCTACCTTGGAATTTTGCACGAAAACCGC CTTGGGCAAAACGTGCTTCATTCCACGCTCAAGGGCGAGGC GCGCCGCGACTACGTCCGCGACTGCGIITACTIATITCT ATGCTACACCTGGCAGACGGCCATGGGCGTTTGGCAGCAGT GCTTGGAGGAGTGCAACCTCAAGGAGCTGCAGAAACTGC TAAAGCAAAACTTGAAGGACCTATGGACGGCCTTCAACGAG CGCTCCGTGGCCGCGCACCTGGCGGACATCATITTCCCC GAACGCCTGCTTAAAACCCTGCAACAGGGTCTGCCAGACTTC ACCAGTCAAAGCATGTTGCAGAACTTTAGGAACTTTAT CCTAGAGCGCTCAGGAATCTTGCCCGCCACCTGCTGTGCACT TCCTAGCGACTITGTGCCCATTAAGTACCGCGAATGCC CTCCGCCGCTITGGGGCCACTGCTACCITCTGCAGCTAGCCA ACTACCTTGCCTACCACTCTGACATAATGGAAGACGTG AGCGGTGACGGTCTACTGGAGTGTCACTGTCGCTGCAACCT ATGCACCCCGCACCGCTCCCTGGTITGCAATTCGCAGCT GCITAACGAAAGTCAAATTATCGGIACCTITGAGCTGCAGGG TCCCTCGCCTGACGAAAAGTCCGCGGCTCCGGGGTTGA AACTCACTCCGGGGCTGTGGACGTCGGCITACCITCGCAAAT TTGTACCTGAGGACTACCACGCCCACGAGATTAGGTTC TACGAAGACCAATCCCGCCCGCCAAATGCGGAGCTTACCGC CTGCGTCATTACCCAGGGCCACATTCTTGGCCAATTGCA

[^3]ACAACAGTTTCAACCCAGACGGAGTGAGTCTACGAGAGAAC CTCTCCGAGCTCAGCTACTCCATCAGAAAAAACACCACC CTCCTTACCTGCCGGGAACGTACGAGTGCGTCACCGGCCGC TGCACCACACCTACCGCCTGACCGTAAACCAGACTTTTT CCGGACAGACCTCAATAACTCTGTTTACCAGAACAGGAGGT GAGCTTAGAAAACCCITAGGGTATTAGGCCAAAGGCGCA GCTACTGTGGGGTITATGAACAATTCAAGCAACTCTACGGGC TATTCTAATTCAGGTTTCTCTAATCGGGGTTGGGGTTA TTCTCTGTCTTGTGATTCTCTITATTCTTATACTAACGCTTCTC TGCCTAAGGCTCGCCGCCTGCTGTGTGCACATTTGC
aTTIATTGTCAGCTITTTAAACGCTGGGGTCGCCACCCAAGA TGATTAGGTACATAATCCTAGGTTTACTCACCCTTGCG TCAGCCCACGGTACCACCCAAAAGGTGGATTTTAAGGAGCC aGCCTGTAATGTTACATTCGCAGCTGAAGCTAATGAGTG CACCACTCTTATAAAATGCACCACAGAACATGAAAAGCTGCT TATTCGCCACAAAAACAAAATTGGCAAGTATGCTGTTT ATGCTATTTGGCAGCCAGGTGACACTACAGAGTATAATGTTA CAGTITTCCAGGGTAAAAGTCATAAAACTITTATGTAT ACITTTCCATITTATGAAATGTGCGACATTACCATGTACATGA GCAAACAGTATAAGTTGTGGCCCCCACAAAATTGTGT GGAAAACACTGGCACTITCTGCTGCACTGCTATGCTAATTAC AGTGCTCGCTTTGGTCTGTACCCTACTCTATATTAAAT
ACAAAAGCAGACGCAGCTTTATTGAGGAAAAGAAAATGCCIT AATTTACTAAGTTACAAAGCTAATGTCACCACTAACTG CTTTACTCGCTGCTTGCAAAACAAATTCAAAAAGTTAGCATTA TAATTAGAATAGGATTTAAACCCCCCGGTCATTTCCT GCTCAATACCATTCCCCTGAACAATTGACTCTATGTGGGATA TGCTCCAGCGCTACAACCTTGAAGTCAGGCTTCCTGGA TGTCAGCATCTGACTTTGGCCAGCACCTGTCCCGCGGATTTG TTCCAGTCCAACTACAGCGACCCACCCTAACAGAGATG ACCAACACAACCAACGCGGCCGCCGCTACCGGACTTACATC TACCACAAATACACCCCAAGTITCTGCCTTTGTCAATAA CTGGGATAACTTGGGCATGTGGTGGTTCTCCATAGCGCTTAT GTTTGTATGCCTTATTAITATGTGGCTCATCTGCTGCC TAAAGCGCAAACGCGCCCGACCACCCATCTATAGTCCCATCA TTGTGCTACACCCAAACAATGATGGAATCCATAGATTG GACGGACTGAAACACATGTTCTITTCTCTTACAGTATGATTAA atGaGACATGATTCCTCGAGTTITTATATTACTGACC CTIGTTGCGCTITITTGTGCGTGCTCCACATTGGCTGCGGTTT CTCACATCGAAGTAGACTGCATTCCAGCCITCACAGT CTATTTGCTTTACGGATTTGTCACCCTCACGCTCATCTGCAGC CTCATCACTGTGGTCATCGCCTTTATCCAGTGCATTG ACTGGGTCTGTGTGCGCTITGCATATCTCAGACACCATCCCC AGTACAGGGACAGGACTATAGCTGAGCTTCTTAGAAAT GGACGGAATTATTACAGAGCAGCGCCTGCTAGAAAGACGCA GGGCAGCGGCCGAGCAACAGCGCATGAATCAAGAGCTCC AAGACATGGTTAACTTGCACCAGTGCAAAAGGGGTATCTITT GTCTGGTAAAGCAGGCCAAAGTCACCTACGACAGTAAT ACCACCGGACACCGCCTTAGCTACAAGTTGCCAACCAAGCG TCAGAAATTGGTGGTCATGGTGGGAGAAAAGCCCATTAC CATAACTCAGCACTCGGTAGAAACCGAAGGCTGCATTCACTC ACCTTGTCAAGGACCTGAGGATCTCTGCACCCTTATTA AGACCCTGTGCGGTCTCAAAGATCTIATTCCCTITAACTAATA AAAAAAAAATAATAAAGCATCACTTACTTAAAATCAGT TAGCAAATTTCTGTCCAGTTTATTCAGCAGCACCTCCTTGCCC TCCTCCCAGCTCTGGTATTGCAGCTTCCTCCTGGCTG

CAAACTITCTCCACAATCTAAATGGAATGTCAGTTTCCTCCTG TTCCTGTCCATCCGCACCCACTATCTTCATGTTGTTG CAGATGAAGCGCGCAAGACCGTCTGAAGATACCTTCAACCC CGTGTATCCATATGACACGGAAACCGGTCCTCCAACTGT GCCTTTTCTTACTCCTCCCTITGTATCCCCCAATGGGTITCAA GAGAGTCCCCCTGGGGTACTCTCTTTGCGCCTATCCG AACCTCTAGTTACCTCCAATGGCATGCTTGCGCTCAAAATGG GCAACGGCCTCTCTCTGGACGAGGCCGGCAACCTTACC
TCCCAAAATGTAACCACTGTGAGCCCACCTCTCAAAAAAACC AAGTCAAACATAAACCTGGAAATATCTGCACCCCTCAC
AGTTACCTCAGAAGCCCTAACTGTGGCTGCCGCCGCACCTCT AATGGTCGCGGGCAACACACTCACCATGCAATCACAGG CCCCGCTAACCGTGCACGACTCCAAACTTAGCATTGCCACCC AAGGACCCCTCACAGTGTCAGAAGGAAAGCTAGCCCTG CAAACATCAGGCCCCCTCACCACCACCGATAGCAGTACCCTT ACTATCACTGCCTCACCCCCTCTAACTACTGCCACTGG TAGCTTGGGCATTGACTTGAAAGAGCCCATITATACACAAAA TGGAAAACTAGGACTAAAGTACGGGGCTCCTTTGCATG TAACAGACGACCTAAACACTTTGACCGTAGCAACTGGTCCAG GTGTGACTATTAATAATACTTCCTTGCAAACTAAAGTT
ACTGGAGCCTTGGGTTTTGATTCACAAGGCAATATGCAACTT AATGTAGCAGGAGGACTAAGGATTGATTCTCAAAACAG ACGCCITATACTTGATGTTAGTTATCCGTTTGATGCTCAAAAC CAACTAAATCTAAGACTAGGACAGGGCCCTCTITITA TAAACTCAGCCCACAACTTGGATATTAACTACAACAAAGGCC TTTACTTGTTTACAGCTTCAAACAATTCCAAAAAGCTT GAGGTIAACCTAAGCACTGCCAAGGGGTTGATGTITGACGC TACAGCCATAGCCATTAATGCAGGAGATGGGCTTGAATT TGGTTCACCTAATGCACCAAACACAAATCCCCTCAAAACAAA AATTGGCCATGGCCTAGAATTTGATTCAAACAAGGCTA TGGTTCCTAAACTAGGAACTGGCCITAGTTITGACAGCACAG GTGCCATTACAGTAGGAAACAAAAATAATGATAAGCTA ACTITGTGGACCACACCAGCTCCATCTCCTAACTGTAGACTA. AATGCAGAGAAAGATGCTAAACTCACTITGGTCTTAAC AAAATGTGGCAGTCAAATACTTGCTACAGTTTCAGTITTGGC TGTTAAAGGCAGTTTGGCTCCAATATCTGGAACAGTTC AAAGTGCTCATCTTATTATAAGATTTGACGAAAATGGAGTGC TACTAAACAATTCCTTCCTGGACCCAGAATATTGGAAC TITAGAAATGGAGATCTTACTGAAGGCACAGCCTATACAAAC GCTGTTGGATMTATGCCTAACCTATCAGCTTATCCAAA ATCTCACGGTAAAACTGCCAAAAGTAACATTGTCAGTCAAGT TTACTTAAACGGAGACAAAACTAAACCTGTAACACTAA CCATTACACTAAACGGTACACAGGAAACAGGAGACACAACT CCAAGTGCATACTCTATGTCATITTCATGGGACTGGTCT GGCCACAACTACATTAATGAAATATITGCCACATCCTCTTACA CTITITCATACATTGCCCAAGAATAAAGAATCGTITG TGTTATGTTTCAACGTGTTTATTTTTCAATTGCAGAAAATITCA aGTCATTTTTCATTCAGTAGTATAGCCCCACCACCA CATAGCTTATACAGATCACCGTACCTTAATCAAACTCACAGA ACCCTAGTATTCAACCTGCCACCTCCCTCCCAACACAC AGAGTACACAGTCCTTTCTCCCCGGCTGGCCTTAAAAAGCAT CATATCATGGGTAACAGACATATTCTTAGGTGTTATAT TCCACACGGTITCCTGTCGAGCCAAACGCTCATCAGTGATAT TAATAAACTCCCCGGGCAGCTCACTTAAGTTCATGTCG CTGTCCAGCTGCTGAGCCACAGGCTGCTGTCCAACTTGCGG TTGCTTAACGGGCGGCGAAGGAGAAGTCCACGCCTACAT
gGgGgTAgagTCATAATCGTGCATCAGGATAGGGCGGTGGT GCTGCAGCAGCGCGCGAATAAACTGCTGCCGCCGCCGCT CCGTCCTGCAGGAATACAACATGGCAGTGGTCTCCTCAGCG ATGATTCGCACCGCCCGCAGCATAAGGCGCCTTGTCCTC CGGGCACAGCAGCGCACCCTGATCTCACTTAAATCAGCACA GTAACTGCAGCACAGCACCACAATATTGTTCAAAATCCC ACAGTGCAAGGCGCTGTATCCAAAGCTCATGGCGGGGACCA CAGAACCCACGTGGCCATCATACCACAAGCGCAGGTAGA TTAAGTGGCGACCCCTCATAAACACGCTGGACATAAACATTA CCTCTTTTGGCATGTTGTAATTCACCACCTCCCGGTAC
CATATAAACCTCTGATTAAACATGGCGCCATCCACCACCATC CTAAACCAGCTGGCCAAAACCTGCCCGCCGGCTATACA CTGCAGGGAACCGGGACTGGAACAATGACAGTGGAGAGCC CAGGACTCGTAACCATGGATCATCATGCTCGTCATGATAT CAATGTTGGCACAACACAGGCACACGTGCATACACTTCCTCA GGATTACAAGCTCCTCCCGCGTTAGAACCATATCCCAG GGAACAACCCATTCCTGAATCAGCGTAAATCCCACACTGCAG GGAAGACCTCGCACGTAACTCACGTTGTGCATTGTCAA AGTGTTACATTCGGGCAGCAGCGGATGATCCTCCAGTATGG TAGCGCGGGTTTCTGTCTCAAAAGGAGGTAGACGATCCC TACTGTACGGAGTGCGCCGAGACAACCGAGATCGTGTTGGT CGTAGTGTCATGCCAAATGGAACGCCGGACGTAGTCATA TTTCCTGAAGCAAAACCAGGTGCGGGCGTGACAAACAGATC TGCGTCTCCGGTCTCGCCGCTTAGATCGCTCTGTGTAGT aGTTGTAGTATATCCACTCTCTCAAAGCATCCAGGCGCCCCC TGGCTTCGGGTTCTATGTAAACTCCTTCATGCGCCGCT GCCCTGATAACATCCACCACCGCAGAATAAGCCACACCCAG CCAACCTACACATTCGTTCTGCGAGTCACACACGGGAGG AGCGGGAAGAGCTGGAAGAACCATGTITTTTITITTATTCCA AAAGATTATCCAAAACCTCAAAATGAAGATCTATTAAG TGAACGCGCTCCCCTCCGGTGGCGTGGTCAAACTCTACAGC CAAAGAACAGATAATGGCATTTGTAAGATGTTGCACAAT GGCTTCCAAAAGGCAAACGGCCCTCACGTCCAAGTGGACGT AAAGGCTAAACCCTTCAGGGTGAATCTCCTCTATAAACA TTCCAGCACCITCAACCATGCCCAAATAATTCTCATCTCGCCA CCTTCTCAATATATCTCTAAGCAAATCCCGAATATTA
AGTCCGGCCATTGTAAAAATCTGCTCCAGAGCGCCCTCCACC TTCAGCCTCAAGCAGCGAATCATGATTGCAAAAATTCA GGTTCCTCACAGACCTGTATAAGATTCAAAAGCGGAACATTA acalanatiaccgcgatcccgTagGTCCCTTCGCAGGGC CAGCTGAACATAATCGTGCAGGTCTGCACGGACCAGCGCGG CCACTTCCCCGCCAGGAACCTTGACAAAAGAACCCACAC TGATTATGACACGCATACTCGGAGCTATGCTAACCAGCGTAG CCCCGATGTAAGCTITGTTGCATGGGCGGCGATATAAA ATGCAAGGTGCTGCTCAAAAAATCAGGCAAAGCCTCGCGCA AAAAAGAAAGCACATCGTAGTCATGCTCATGCAGATAAAA GGCAGGTAAGCTCCGGAACCACCACAGAAAAAGACACCATT ITTCTCTCAAACATGTCTGCGGGTTTCTGCATAAACACA AAATAAAATAACAAAAAAACATTTAAACATTAGAAGCCTGTCT TACAACAGGAAAAACAACCCTTATAAGCATAAGACGG ACTACGGCCATGCCGGCGTGACCGTAAAAAAACTGGTCACC GTGATTAAAAAGCACCACCGACAGCTCCTCGGTCATGTC CGGAGTCATAATGTAAGACTCGGTAAACACATCAGGTTGATT CATCGGTCAGTGCTAAAAAGGGACCGAAATAGCCCGGG GGAATACATACCCGCAGGCGTAGAGACAACATTACAGCCCC CATAGGAGGTATAACAAAATTAATAGGAGAGAAAAACAC


#### Abstract

ATAAACACCTGAAAAACCCTCCTGCCTAGGCAAAATAGCACC CTCCCGCTCCAGAACAACATACAGCGCTTCACAGCGGC AGCCTAACAGTCAGCCTTACCAGTAAAAAAGAAAACCTATTA AAAAAACACCACTCGACACGGCACCAGCTCAATCAGTC ACAGTGTAAAAAAAGGGCCAAGTGCAGAGCGAGTATATATAG GACTAAAAAATGACGTAACGGTTAAAGTCCACAAAAAAC ACCCAGAAAACCGCACGCGAACCTACGCCCAGAAACGAAAG CCAAAAAAACCCACAACTTCCTCAAATCGTCACTTCCGTT TTCCCACGTTACGTAACTTCCCATTTTAAGAAAACTACAATTC CCAACACATACAAGTTACTCCGCCCTAAAACCTACGT CACCCGCCCCGTTCCCACGCCCCGCGCCACGTCACAAACTC CACCCCCTCATTATCATATTGGCTTCAATCCAAAATAAG gTATAT


Query $=$ Human Hyde, Sbjct $=$ Human Hyde-40 (II)


8 Sbjct: 1

Query: 61

| Query: 61 | atggacaagccactgatcagectrcacctggtggacagcgatagtagcettgccaaggtc 120 |
| :--- | :--- |
| Sbjct: 61 | 111111111111111111111111111111111111111111111111111 |
| atggacaagccactgatcagcctccacctggtggacagcgatagtagecttgccaaggtc 120 |  |

Sbjct: 61
Query: 121 cecgatgaggececcaaagtgagcatcctqggtagegggactttgecegetecetggec 100


Query: 181
Sbjct: 101
Query: 241
Sbjct: 241
Query: 301
Shict 301 llllllll11111111111111111111111111111111111111111111 360
gaggtcatctttgtggctgtgttcegggagcactactcttcactgtgcagtctcagtgac 360
cagctggcgggcaagatcctggtggatgtgagcaaccetacagagcaagagcacettcag 420
cagctggcgggcaagatect ggtggatgtgagcaaccctacagagcaagagcaccttcag 420
Query: 361
Sbjct: 361
Query: 421 catcgtgagtccaatgctgagtacctggcctccctcttccccacttgcacagtggtcaag 400



Query: 601 atgggcttcatgcccgtggacatgggatccctggcotcagcctgggaggtggaggccatg 660


Query: 721 tgcttctatgcctacaacttcgtccgggacgttctgcagccetatgtgcaggaaagccag 780 Hillilililililililililililililifilillilililililililill 780
Query: 781 aacpagttcttcaagctgccegtgtccgtggtcaacaccacactgccgtgcgtggcctac 840
sbjct: 781 aacaagttcttcaagetgccegtgtccgtggtcaacaccacactgecgtgegtggcetac 840
Query: 841 gtgctgctgtcactcgtgtacttgeccggcgtgetggeggetgecetgcagctgeggcge 900

gtgctgctgtcactcgtgtacttgcceggegtgctggcggctgccetgcagctgcggcgc 900


## atcgggctgctcagcttcttctgcgccgccctgcacgecctctacagcttctgcttgccg 1020

sbjet: 961 atcgggetgctcagcttcttctgcgccgccetgcacgccetctacagcttctgcttgecg 1020
Query: 1021 ctgcgccgegcccaccgctacgacctggtcaacctggcagtcaagcaggtcttggccaac 1000
sbjct: 1021 ctgcgccgcgcccaccgctacgacctggtcaacctggcagtcaagcaggtcttggccaac 1000
aagagccacctctgggtggaggaggtctggcggatggagatctacctctccctgggagtg 1140
Sbjct: 2082 aagagccacctctgggtggaggaggtctggcggatggagatctacctctccetgggagtg 1140
Query: 1141 ctggccctcggcacgttgtccetgctggccgtgacctcactgccgtccattgcaaactcg 1200
Sbjct: 1141 ctggccetcggcacgttgtccetgctggecgtgacctcactgccgtccattgcaaactcg 1200
Query: 1201 ctcaactggagggagttcagcttcgttcagt 1231
\|llllllllllll|lllllll|l
Sbjct: 1201 ctcaactggagggagttcagcttcgttcagt 1231

Quexy: 1229 agtcctcactgggctttgtggccctcgtgctgagcacactgcacacgctcacctacggct 1208
Sbjct: 1461 agtcctcactgggetttgtggccetcgtgctgagcacactgcacacgctcacctacgget 1520

Query: 1349 cgetgetggtgecctgegtcgtcatcetggccaaagccctgtttctcctgccetgcatca 1408
Sbjct: 1581 cgetgetggtgecctgcgtcgtcatcctggecaaagccetgtttctcctgccetgcatca 1640
Query: 1409 gccgcagactcgccaggatccggagaggetgggagagggagagcaccatcaigttcacge 1460


Sbjet: 1641 gcegcagactcgccaggatccggagaggetgggagagggagagcaccatcaagttcacge 1700
Query: 1469 tgcccacagaccacgccctggccgagaagacgagccacgtatgaggtgcctgccctgggc $152 \theta$
Sbjct: 1701 tgcccacagaccacgecetggcegagaagacgagceacgtatgaggtgcctgccetgggc 1760
Query: 1529 tctggaccccgggcacacgagggacggtgccetgagcccgttaggttttcttttcttggt 1580
Sbjct: 1761 tctggaccccgggcacacgagggacggtgccetgagccegttaggtttetttictiggt 1820
Query: 1589 ggtgcaaagtggtataactgtgtgcaaztaggaggtttgaggtccaaattcctgggactc 1648

Ouery: 1649 aaatgtatgcagtactattcagaatg-2674
Sbjct: 1881 aatgtatgcagtactattcagaatg
Query: 1723 acaggatttgcaattatacatagctagctaaaaagttgggtctctgagatttcaacttgt 1782
Sbjct: 1955 acaggatttgcaattatacatagctagctaaazagttgggtctctgagatttcaacttgt 2014
Query: 1783 agatttaaaaacaagtgccgtacgttaagagaagagcagatcatgctartgtgacatttg 1842 sbjct: 2015 agatttaaaaacaagtgccotacgttaagagaagagcagatcatgctattgtgacatttg 2074.
Query: 1843 cagagatatacacacacttttgtacag 1870
sbjct: 2075 cagagatatacacacactttttgtacag 2102
Query＝Human Hyde，sbjet＝Human Hyde－40（Hyde II）
QU日IY： 1 MPEEMDKPLISLHLVDSDSSIARVPDEAPKVSILGSGDFARSIATRIVGSGERVVVGSRNMPEEMDREIISLHLVDSDSSLARUPDEAPRVSILGSGDEARSLATRLVGSGEKVVVGSRNSbjet： 1 MPEEMDKPLISLELVDSDSSLAKVPDEAPKVSIEG5GDFARSILATRLVGSGEKVVTGSRNQUEIY： 61 PKRTARLFPSAAQVTEQEEAVSSPEVIFVAVEREHYSSICSLSDQLAGKILVDVSNPTEQPKRTARJEPSAAOVTEOEEAVSSPEVI FVAVEREHYSSLCELSDOLAGKILVDVSNETEQ
Sbjet： 61 PKRTARLFPSAAQYTFQEEAUSSPEUITVAVEREHYSSLCSLEDOLAGKILVDVBNPTEQ
QuEIY： 121 EHLOFRESNAEYIASLFPTCTVIKAFNVISAWTLQAGPRDGNGQVPICGDOPEARRAVSEEHLQHRESNAEYLASLEPTCTVYKAFMVISANTLQAGPRDGNGQVPICGDQPEARRAVSE
Sbjct： 121 EHLQFRESNAEYIASLERTCTVTKAFNVISAMTLQAGPRDGNGOVPICGDOPEARRAVSE
Query： 181 RALAMGFMPVDMGSLASAMEVEAMPLRLLPAMKVPTLIALGLFVCFYAYNEVRDVLQPYVMR工AMGEMFVDMGSLASAWEVEAMRLRIIPAMRVPTLLALGLFVCFYAINEVRDVLQPYV
Sbjet： 181 MA工AMGEMPVDMGSLASANEVEAMPLRLLPAWKUPTLLALGLFVCFYAYNFVRDVLQPYV
Quezy： 241 QESQNKFFKLPVSUNTTLPCVAYTLLSLVYIPGULAAALOLRRGTXYQRFFDWIDFTLQQESQNKFEKLPVSWNTTIPCVAYVLLSLVYLPGVLARA工QLRRGTKYQRFPDWIDFKLQ
Sbjct： 241 QESQNKFFKLPVSVVNTTIPCVAYULLSLVYIPGVLAAALQLRRGTKYQRFPDNLDHKLQ
Query： 301 HRKQIGLISFFCAA工HALYSFCLPLRRAHRYDLVNLAVKOVIANKSHLNVEEVWRMEIYX HRKOIGLISEECAALHAIYSECLELRRAHRYDIVNLAVROVLANRSHLWVEEVNRMEIY
Sbjct： 301 HRKRQTGLISFFCAXIERIYSFCLPLRRASRYDINNLAVKOVLANKSHLNEEEVDRMEIYI
Quezy： 361 XXXXXXXXXXXXXXXXOXPSIANSINWREFSEVQ ..... 394
PSIANSINWREFSFVQ
Sbjot： 361 SLGVLALGTLSLIAVISIPSIANSLNWREFSFYQ 394
Figure 12

## Query $=$ Human, Sbjct $=$ rat


Query: 178 gccacacgectggtoggctctggcttcaeag 237

Sbjct: 161 gccacacgcetggtgggctctggcttctttgtggtggtgggaagccgtaaccccaaacgc 220
Query: 23 B acagccaggctgtttecetcageggcccaagtgactttccaagaggaggcagtgagctcc 297
Sbjct: 221 actgccggectcttcccctcettageccaagtgactttccaggaggaggecgtgagctct 280
Query: 298 coggaggtcatctttgtggctgtgttccgggagcactactcttcactgtgcagtctcagt 357
Sbjct: 201 l| $\begin{aligned} & \text { Ccagaggtcatctttgtggecgtgttccgggagcactactcctcactgtgcagtcttgct } 340\end{aligned}$
Onery: 358 gaccagctggcgggcaagatcctggtggatgtgagcaaccctacagagcaagagcacctt 417


Query: 410 cagcatcgtgagtccaatgctgagtacctggcctccetcttccccacttgcacagtggtc 477
sbjct: 401 cagcaccgccagtcoaacgccgagtacctggcctccctcttccctgcctgcactgtggtc 460
Figure 13 A

Query: 538 caggtgeccatctgeggtgaccagceagaagecaagegtgctgtetcggagatggegetc 597
Sbjet: 521 caggtgetcatctgcggtgaccagctggaagccaagcacaccgtctcagagatggegcge 580
Query: 598 gecatgggcttcatgccegtggacatgggatccctggcgtcagcctgggaggtggaggec 657
Sbjct: 501 gecatgggtttcaccecactggacatgggatccetggectcagcgagggaggtagaggec 640
Query: 658 atgeccetgegectectcceggectggeaggtgcccaccctgetggeectggqgetcttc 717

Query: 71.0 gtctgcttctatgectacaacttcgtccgggacgttctgcagccctatgtgcaggaaage 777
Sbjct: 701 acacaaagctatgectacaacttcatccgggacgttctacagccgtacatccggaaagat 760
Query: 778 cagaacaagttcttcaagctgccegtgtccgtggtcaacaccacactgcegtgcgtggec 837
Sbjct: $761 \begin{gathered}\text { l1111111111 } \\ \text { gagaacaagttctacaagatgccectgtctgtggtcaacaccacgataccetgtgtgget }\end{gathered} \mathbf{2 0}$
Query: 83日 tacgtgetgetgtcactegtgtacttgeceggegtgctggeggetgcectgeagetgegg 897

Query: 898 cgcggcaccaagtaccagcgcttccecgactggetggaccactggctacagcaccgcaag 957
Sbjct: 881 agggggaccaagtaccagcgetteccagactggetggaccattggetgeagcaccgcaag 940
Q 950 cagatcgggetgctcagcttcttctgcgecgccetgcacgecctctacagettctgettg 1017


Query: 1195 aactcgctcaactggagggagttcagcttcgttcagtcctcactgggetttgtggccetc 1254
Sbjct: 1181 aactcactcaactggaaggagttcagetttgtgcagtccacgetgggettcgtggecctg 1240
Query: 1255 gtgctgagcacactgcacacgetcacctacggctggaccegeqcettegaggagagecge 1314
sbjct: 1241 atgctgagcacaatgcacaccetcacctacggctggaccegtgcttttgaggaaaaccac 1300
Query: 1315 tacaagttctacctgecteccaccttcacgetcacgetgetggtgecetgegtcgtcatc 1374
Sbjct: 1301 tacaagttetacctgecacccacattcacgetcacgetgetectgcectgtgtcatcatc 1360

## Query=Human, Sbjct=rat

Quezy: 1 MPEEMDKPIISLHLYDSDSSIAKVRDEAPKVSILGSGDFARSIATRLVGSGFKNVVGSRN $M$ EMDKPIIS LVDSD SIATVP EAPRV ILGSGDFARSLATRLVGSGF VVVGSRN SbJCE: 1 MSGEMDKPLISRRHVDSDGSLAEVPKEAPRVGILGSGDEARSLATRLVGSGFPVVVGSRN

QUETY: 61 PKRTARLFPSAAQVTFQEEAVSSPEVIFVAVRREHYSSLCSLSDQLARKIIVDVSNRTEQ PKRTA LFPS AOVTFQEEAVSSPEVIFVAVFREHYSSICSI+DQIAGKIIUDVSNPTE+
Sbjct: 61 PKRTAGLFRSLAQVTEQEEAVSSPEVIEVAVRREHYSSICSIADOLAGKIIVDVSNPTEK
Query: 121 EHLQHRESNAEYIASLFPTCTVVKAFNVISAWTIQAGPRDGNGQVPICGDQPEAKRAVSE E LQHRTSNAEYLASLEF CTVIKCAFNUISAN LQAGPRDGN OV ICGDQ EAK VSE
Sbjct: 121 ERLQHRQSNAEYLASLEPACTVVKAFAYISANALQAGPRDGNRQVLICGDQLEAKHTVSE
QLEIY: 181 MRIAMGMPVDMGSLASANEVEAMPLRLLPANKVPTLILALGLEVCFYAYNFVRDVLQFYV MA AMGF P+DMGSLASA EVEA+PLRUIP+WKVPTIIALGL YAYNE+RDVLQEY+
SbJCt: $1 B 1$ MARAMGETPLDMGSILASAREVEAIPLRIIFSNKVETLIALGLSTQSYAYNFIRDVLQPYI
QueIy: 241 OESONKFFKLEVSVNNTTIPCVAYVLLSLVYIPEVLAAALOURRGTKYQRFPDWLDFWLQ ++ +NKI+K+P+SVVNTT+PCVAYVLISLVYIPGVLAAALQLRRGTKYYRFEDWUDFILQ
Sbjct: 241 RKDENKFYKMPLSVVNTTIPCVAYVLLSLVYLPGVAAAALOLRRGTKYQRFPDRIDHKLQ
QUEIY: 301 FRKQIGLLSFFCAALFALYSFCLPLRRAFRYDLUNLAVKOVLANKSHLRV-EEVWRMEIY HRKQIGLLSFE A LHALYSFCLFLRR +HRYDIVNLAVKOVLANKS LRN EEVNRMEIY
Sbjct: 301 HRKQIGLLSFFFAMLHALYSFCLPIRRSFRYDIVNLAVKOVLANKSRLWVEEEVWRMEIY
Query: 360 XXXXXXXXXXXXXXXXXXXPSIANSINWREFSEVQSSLGFVALVLSTLETLTYGWTRAEE PSIANSLNW+ERSEVQS+LGEVAI+IST+HTITYGWTRAFE
Sbjct: 361 LSLGVLALGMLSLLAVTSIPSIANSLNWEEFSTVQSTLGFVAIMUSTMHTITYGWTRAFE
QLeIy: 420 ESRYRFYXXXXXXXXXXXXXXKXIIARKAIFLI PCISRRLARIRRGKERESTIKFTLPTDHA Et YKFY ILAK LELIPC+S RL +IRRGWER+ +KE LP i Sbjct: 421 ENHYKFYLPPTFTITLIIPCVIILARGLILLPCLSHRLTKIRRGWERDGAVRIMLPAGHT

Query: 480 LAEKTSHV 487
EKTSHO
sbjct: 481 QGEKTSHV 488

$$
\text { Figure } 13 \mathrm{~B}
$$

## 39/43



FIG. 14


Fig. 15

(roryi) yaquou lio

4

(solxi) saquina lipo

## $\pm$

FIG. 16


FIG. 17

43 / 43

A


B


FIG. 18


[^0]:    gCGGTCGTTGTCCACTAGGGGGTCCACTCGCTCCAGGGTGT GAAGACACATGTCGCCCTCTTCGGCATCAAGGAAGGTGA TTGGTTTGTAGGTGTAGGCCACGTGACCGGGTGTTCCTGAA gGGgGgctatanafggGggtggaggcgcgitccicctca CTCTCTTCCGCATCGCTGTCTGCGAGGGCCAGCTGTTGGGG TGAGTACTCCCTCTGAAAAGCGGGCATGACTTCTGCGCT AAGATTGTCAGTTTCCAAAAACGAGGAGGATITGATAITCAC CTGGCCCGCGGTGATGCCTMTGAGGGTGGCCGCATCCA TCTGGTCAGAAAAGACAATCIITITGTTGTCAAGCTTGGTGG CAAACGACCCGTAGAGGGCGTTGGACAGCAACTTGGCG ATGGAGCGCAGGGTTTGGTTTTTGTCGCGATCGGCGCGCTC CTTGGCCGCGATGTTTAGCTGCACGTATTCGCGCGCAAC GCACCGCCATTCGGGAAAGACGGTGGTGCGCTCGTCGGGC ACCAGGTGCACGCGCCAACCGCGGTTGTGCAGGGTGACAA GGTCAACGCTGGTGGCTACCTCTCCGCGTAGGCGCTCGTTG GTCCAGCAGAGGCGGCCGCCCTTGCGCGAGCAGAATGGC GGTAGGGGGTCTAGCTGCGTCTCGTCCGGGGGGTCTGCGTC CACGGTAAAGACCCCGGGCAGCAGGCGCGCGTCGAAGTA GTCTATCTIGCATCCTTGCAAGTCTAGCGCCTGCTGCCATGC GCGGGCGGCAAGCGCGCGCTCGTATGGGTTGAGTGGGG gaccccatggcatggGgTgGgTgagcgcggaggcgtacat GCCGCAAATGTCGTAAACGTAGAGGGGCTCTCTGAGTATT CCAAGATATGTAGGGTAGCATCTTCCACCGCGGATGCTGGC GCGCACGTAATCGTATAGTTCGTGCGAGGGAGCGAGGAG GTCGGGACCGAGGTTGCTACGGGCGGGCTGCTCTGCTCGG AAGACTATCTGCCTGAAGATGGCATGTGAGTTGGATGATA TGGTTGGACGCTGGAAGACGTTGAAGCTGGCGTCTGTGAGA CCTACCGCGTCACGCACGAAGGAGGCGTAGGAGTCGCGC AGCTTGTTGACCAGCTCGGCGGTGACCTGCACGTCTAGGGC GCAGTAGTCCAGGGTTTCCTTGATGATGTCATACTTATC CTGTCCCTIITITITCCACAGCTCGCGGTTGAGGACAAACTCT TCGCGGTCTITCCAGTACTCTTGGATCGGAAACCCGT CGGCCTCCGAACGGTAAGAGCCTAGCATGTAGAACTGGTTG ACGGCCTGGTAGGCGCAGCATCCCTTTTCTACGGGTAGC GCGTATGCCTGCGCGGCCTICCGGAGCGAGGTGTGGGTGA GCGCAAAGGTGTCCCTGACCATGACTITGAGGTACTGGTA TTTGAAGTCAGTGTCGTCGCATCCGCCCTGCTCCCAGAGCAA AAAGTCCGTGCGCTITITGGAACGCGGATTTGGCAGGG CGAAGGTGACATCGTTGAAGAGTATCITTCCCGCGCGAGGC ATAAAGTTGCGTGTGATGCGGAAGGGTCCCGGCACCTCG GAACGGTTGTTAATTACCTGGGCGGCGAGCACGATCTCGTC AAAGCCGTTGATGTTGTGGCCCACAATGTAAAGTTCCAA GAAGCGCGGGATGCCCTTGATGGAAGGCAATITITTAAGTTC CTCGTAGGTGAGCTCTTCAGGGGAGCTGAGCCCGTGCT CTGAAAGGGCCCAGTCTGCAAGATGAGGGTTGGAAGCGAC GAATGAGCTCCACAGGTCACGGGCCATTAGCATTIGCAGG TGGTCGCGAAAGGTCCTAAACIGGCGACCTATGGCCATIITT TCTGGGGTGATGCAGTAGAAGGTAAGCGGGTCTTGTTC CCAGCGGTCCCATCCAAGGTTCGCGGCTAGGTCTCGCGCGG CAGTCACTAGAGGCTCATCTCCGCCGAACTTCATGACCA GCATGAAGGGCACGAGCTGCTTCCCAAAGGCCCCCATCCAA GTATAGGTCTCTACATCGTAGGTGACAAAGAGACGCTCG GTGCGAGGATGCGAGCCGATCGGGAAGAACTGGATCTCCC gCCACCAATTGGAGGAGTGGCTATTGATGTGGTGAAAGTA GAAGTCCCTGCGACGGGCCGAACACTCGTGCTGGCTTTTGT aAAAACGTGCGCAGTACTGGCAGCGGTGCACGGGCTGTA

[^1]:    CTTCAGCGCGTGGCTCGTTACAACAGCGGCAACGTGCAGAC CAACCTGGACCGGCTGGTGGGGGATGTGCGCGAGGCCGT GGCGCAGCGTGAGCGCGCGCAGCAGCAGGGCAACCTGGGC TCCATGGTTGCACTAAACGCCTTCCTGAGTACACAGCCCG CCAACGTGCCGCGGGGACAGGAGGACTACACCAACTTTGTG AGCGCACTGCGGCTAATGGTGACTGAGACACCGCAAAGT GAGGTGTACCAGTCTGGGCCAGACTATIIITICCAGACCAGT AGACAAGGCCTGCAGACCGTAAACCTGAGCCAGGCTIT CAAAAACTTGCAGGGGCTGTGGGGGGTGCGGGCTCCCACA GGCGACCGCGCGACCGTGTCTAGCTTGCTGACGCCCAACT CGCGCCTGTTGCTGCTGCTAATAGCGCCCTTCACGGACAGT GGCAGCGTGTCCCGGGACACATACCTAGGTCACTTGCTG ACACTGTACCGCGAGGCCATAGGTCAGGCGCATGTGGACGA GCATACTITCCAGGAGATTACAAGTGTCAGCCGCGCGCT GGGGCAGGAGGACACGGGCAGCCTGGAGGCAACCCTAAAC TACCTGCTGACCAACCGGCGGCAGAAGATCCCCTCGTTGC ACAGTTTAAACAGCGAGGAGGAGCGCATTTTGCGCTACGTG CAGCAGAGCGTGAGCCTTAACCTGATGCGCGACGGGGTA ACGCCCAGCGTGGCGCTGGACATGACCGCGCGCAACATGG AACCGGGCATGTATGCCTCAAACCGGCCGTITATCAACCG CCTAATGGACTACTTGCATCGCGCGGCCGCCGTGAACCCCG AGTATTTCACCAATGCCATCTTGAACCCGCACTGGCTAC CGCCCCCTGGTTTCTACACCGGGGGATTCGAGGTGCCCGAG GGTAACGATGGATTCCTCTGGGACGACATAGACGACAGC GTGTITTCCCCGCAACCGCAGACCCTGCTAGAGTTGCAACAG CGCGAGCAGGCAGAGGCGGCGCTGCGAAAGGAAAGCTT CCGCAGGCCAAGCAGCTTGTCCGATCTAGGCGCTGCGGCCC CGCGGTCAGATGCTAGTAGCCCATTTCCAAGCTIGATAG GGTCTCTTACCAGCACTCGCACCACCCGCCCGCGCCTGCTG GGCGAGGAGGAGTACCTAAACAACTCGCTGCTGCAGCCG CAGCGCGAAAAAAAACCTGCCTCCGGCATITCCCAACAACGG GATAGAGAGCCTAGTGGACAAGATGAGTAGATGGAAGAC GTACGCGCAGGAGCACAGGGACGTGCCAGGCCCGCGCCCG CCCACCCGTCGTCAAAGGCACGACCGTCAGCGGGGTCTGG TGTGGGAGGACGATGACTCGGCAGACGACAGCAGCGTCCT GGATITGGGAGGGAGTGGCAACCCGTTTGCGCACCTTCGC CCCAGGCTGGGGAGAATGITIIIAAAAAAAAAAAAGCATGAT GCAAAATAAAAAACTCACCAAGGCCATGGCACCGAGCGT TGGTITTCTTGTATTCCCCTTAGTATGCGGCGCGCGGCGATG TATGAGGAAGGTCCTCCTCCCTCCTACGAGAGTGTGGT GAGCGCGGCGCCAGTGGCGGCGGCGCTGGGTTCTCCCTTC GATGCTCCCCTGGACCCGCCGTITGTGCCTCCGCGGTACC TGCGGCCTACCGGGGGGAGAAACAGCATCCGTTACTCTGAG TTGGCACCCCTATTCGACACCACCCGTGTGTACCTGGTG GACAACAAGTCAACGGATGTGGCATCCCTGAACTACCAGAA CGACCACAGCAACTTTCTGACCACGGTCATTCAAAACAA TGACTACAGCCCGGGGGAGGCAAGCACACAGACCATCAATC TTGACGACCGGTCGCACTGGGGCGGCGACCTGAAAACCA TCCTGCATACCAACATGCCAAATGTGAACGAGTTCATGTTTA CCAATAAGTTTAAGGCGCGGGTGATGGTGTCGCGCTTG CCTACTAAGGACAATCAGGTGGAGCTGAAATACGAGTGGGT GGAGTTCACGCTGCCCGAGGGCAACTACTCCGAGACCAT GACCATAGACCTTATGAACAACGCGATCGTGGAGCACTACTT GAAAGTGGGCAGACAGAACGGGGTTCTGGAAAGCGACA. TCGGGGTAAAGTITGACACCCGCAACTTCAGA.CTGGGGTTT GACCCCGTCACTGGTCTTGTCATGCCTGGGGTATATACA

[^2]:    AGATGTCTTGGAAAAAATGACCGTGGAACCTGGGCTGGAGC CCGAGGTCCGCGTGCGGCCAATCAAGCAGGTGGCGCCGG GACTGGGCGTGCAGACCGTGGACGTTCAGATACCCACTACC agTagCaccagtattgccaccaccacagaggacatggag ACACAAACGTCCCCGGTTGCCTCAGCGGTGGCGGATGCCGC GGTGCAGGCGGTCGCTGCGGCCGCGTCCAAGACCTCTAC GGAGGTGCAAACGGACCCGTGGATGTTTCGCGTTTCAGCCC CCCGGCGCCCGCGCGGTTCGAGGAAGTACGGCGCCGCCA GCGCGCTACTGCCCGAATATGCCCTACATCCTTCCATTGCGC CTACCCCCGGCTATCGTGGCTACACCTACCGCCCCAGA AGACGAGCAACTACCCGACGCCGAACCACCACTGGAACCCG CCGCCGCCGTCGCCGTCGCCAGCCCGTGCTGGCCCCGAT TTCCGTGCGCAGGGTGGCTCGCGAAGGAGGCAGGACCCTG GTGCTGCCAACAGCGCGCTACCACCCCAGCATCGTTTAAA AGCCGGTCTTTGTGGTTCTTGCAGATATGGCCCTCACCTGCC GCCTCCGTTTCCCGGTGCCGGGATTCCGAGGAAGAATG CACCGTAGGAGGGGCATGGCCGGCCACGGCCTGACGGGCG GCATGCGTCGTGCGCACCACCGGCGGCGGCGCGCGTCGCA CCGTCGCATGCGCGGCGGTATCCTGCCCCTCCTTATTCCACT GATCGCCGCGGCGATTGGCGCCGTGCCCGGAATTGCAT CCGTGGCCTTGCAGGCGCAGAGACACTGATTAAAAAACAAGT TGCATGTGGAAAAATCAAAATAAAAAGTCTGGACTCTCA CGCTCGCTTGGTCCTGTAACTATITTGTAGAATGGAAGACAT CAACTTTGCGTCTCTGGCCCCGCGACACGGCTCGCGCC CGTTCATGGGAAACTGGCAAGATATCGGCACCAGCAATATG AGCGGTGGCGCCTTCAGCTGGGGCTCGCTGTGGAGCGGC ATTAAAAATTTCGGTTCCACCGTTAAGAACTATGGCAGCAAG GCCTGGAACAGCAGCACAGGCCAGATGCTGAGGGATAA GTTGAAAGAGCAAAATTTCCAACAAAAGGTGGTAGATGGCC TGGCCTCTGGCATTAGCGGGGTGGTGGACCTGGCCAACC AGGCAGTGCAAAATAAGATTAACAGTAAGCTTGATCCCCGCC CTCCCGTAGAGGAGCCTCCACCGGCCGTGGAGACAGTG TCTCCAGAGGGGCGTGGCGAAAAGCGTCCGCGCCCCGACA GGGAAGAAACTCTGGTGACGCAAATAGACGAGCCTCCCTC GTACGAGGAGGCACTAAAGCAAGGCCTGCCCACCACCCGTC CCATCGCGCCCATGGCTACCGGAGTGCTGGGCCAGCACA CACCCGTAACGCTGGACCTGCCTCCCCCCGCCGACACCCAG CAGAAACCTGTGCTGCCAGGCCCGACCGCCGTTGTTGTA ACCCGTCCTAGCCGCGCGTCCCTGCGCCGCGCCGCCAGCGG TCCGCGATCGTTGCGGCCCGTAGCCAGTGGCAACTGGCA AAGCACACTGAACAGCATCGTGGGTCTGGGGGTGCAATCCC TGAAGCGCCGACGATGCTICTGAATAGCTAACGTGTCGT ATGTGTGTCATGTATGCGTCCATGTCGCCGCCAGAGGAGCT GCTGAGCCGCCGCGCGCCCGCTITCCAAGATGGCTACCC CTTCGATGATGCCGCAGTGGTCTTACATGCACATCTCGGGCC AGGACGCCTCGGAGTACCTGAGCCCCGGGCTGGTGCAG TITGCCCGCGCCACCGAGACGTACTTCAGCCTGAATAACAAG ITTAGAAACCCCACGGTGGCGCCTACGCACGACGTGAC CACAGACCGGTCCCAGCOTTTGACGCTGCGGTTCATCCCTGT GGACCGTGAGGATACTGCGTACTCGTACAAGGCGCGGT TCACCCTAGCTGTGGGTGATAACCGTGTGCTGGACATGGCTT CCACGTACTTTGACATCCGCGGCGTGCTGGACAGGGGC CCTACTTTTAAGCCCTACTCTGGCACTGCCTACAACGCCCTG GCTCCCAAGGGTGCCCCAAATCCTTGCGAATGGGATGA aGCTGCTACTGCTCTTGAAATAAACCTAGAAGAAGAGGACG ATGACAACGAAGACGAAGTAGACGAGCAAGCTGAGCAGC

[^3]:    aGCCATCAACAAAGCCCGCCAAGAGTTTCTGCTACGAAAGG GACGGGGGGTTTACTTGGACCCCCAGTCCGGCGAGGAGC TCAACCCAATCCCCCCGCCGCCGCAGCCCTATCAGCAGCAG CCGCGGGCCCTTGCTTCCCAGGATGGCACCCAAAAAGAA GCTGCAGCTGCCGCCGCCACCCACGGACGAGGAGGAATACT GGGACAGTCAGGCAGAGGAGGTTTTGGACGAGGAGGAGG AGGACATGATGGAAGACTGGGAGAGCCTAGACGAGGAAGC TTCCGAGGTCGAAGAGGTGTCAGACGAAACACCGTCACCC TCGGTCGCATTCCCCTCGCCGGCGCCCCAGAAATCGGCAAC CGGTTCCAGCATGGCTACAACCTCCGCTCCTCAGGCGCC GCCGGCACTGCCCGTTCGCCGACCCAACCGTAGATGGGACA CCACTGGAACCAGGGCCGGTAAGTCCAAGCAGCCGCCGC CGTTAGCCCAAGAGCAACAACAGCGCCAAGGCTACCGCTCA TGGCGCGGGCACAAGAACGCCATAGTTGCTTGCTTGCAA GACTGTGGGGGCAACATCTCCTTCGCCCGCCGCTITCTTCTC TACCATCACGGCGTGGCCTTCCCCCGTAACATCCTGCA TTACTACCGTCATCTCTACAGCCCATACTGCACCGGCGGCAG CGGCAGCGGCAGCAACAGCAGCGGCCACACAGAAGCAA AGGCGACCGGATAGCAAGACTCTGACAAAGCCCAAGAAATC CACAGCGGCGGCAGCAGCAGGAGGAGGAGCGCTGCGTCT GGCGCCCAACGAACCCGTATCGACCCGCGAGCTTAGAAACA GGATITITCCCACTCTGTATGCTATATTTCAACAGAGCA GGGGCCAAGAACAAGAGCTGAAAATAAAAAACAGGTCTCTG CGATCCCTCACCCGCAGCTGCCTGTATCACAAAAGCGAA GATCAGCTTCGGCGCACGCTGGAAGACGCGGAGGCTCTCTT CAGTAAATACTGCGCGCTGACTCTIAAGGACTAGTITCG CGCCCTTICTCAAATTTAAGCGCGAAAACTACGTCATCTCCA GCGGCCACACCCGGCGCCAGCACCTGTCGTCAGCGCCA TTATGAGCAAGGAAATTCCCACGCCCTACATGTGGAGTTACC agCCACAAATGGGACTTGCGGCTGGAGCTGCCCAAGAC TACTCAACCCGAATAAACTACATGAGCGCGGGACCCCACAT GATATCCCGGGTCAACGGAATCCGCGCCCACCGAAACCG AATTCTCTTGGAACAGGCGGCTATTACCACCACACCTCGTAA TAACCTTAATCCCCGTAGTTGGCCCGCTGCCCTGGTGT ACCAGGAAAGTCCCGCTCCCACCACTGTGGTACTTCCCAGA GACGCCCAGGCCGAAGTTCAGATGACTAACTCAGGGGCG CAGCTTGCGGGCGGCTTTCGTCACAGGGTGCGGTCGCCCGG GCAGGGTATAACTCACCTGACAATCAGAGGGCGAGGTAT TCAGCTCAACGACGAGTCGGTGAGCTCCTCGCTTGGTCTCC GTCCGGACGGGACATITCAGATCGGCGGCGCCGGCCGTC CTTCATTCACGCCTCGTCAGGCAATCCTAACTCTGCAGACCT CGTCCTCTGAGCCGCGCTCTGGAGGCATTGGAACTCTG CAATTTATTGAGGAGTTTGTGCCATCGGTCTACTITAACCCCT TCTCGGGACCTCCCGGCCACTATCCGGATCAATITAT TCCTAACITTGACGCGGTAAAGGACTCGGCGGACGGCTACG ACTGAATGTTAAGTGGAGAGGCAGAGCAACTGCGCCTGA AACACCTGGTCCACTGTCGCCGCCACAAGTGCTITGCCCGC GACTCCGGTGAGTITTGCTACTITGAATIGCCCGAGGAT CATATCGAGGGCCCGGCGCACGGCGTCCGGCTTACCGCCCA GGGAGAGCTTGCCCGTAGCCTGATTCGGGAGTITACCCA GCGCCCCCTGCTAGTTGAGCGGGACAGGGGACCCTGTGTTC TCACTGTGATTTGCAACTGTCCTAACCTTGGATTACATC AAGATCTTTGTTGCCATCTCTGTGCTGAGTATAATAAATACAG AAATTAAAATATACTGGGGCTCCTATCGCCATCCTGT
    AAACGCCACCGTCTTCACCCGCCCAAGCAAACCAAGGCGAA CCTTACCTGGTACTITTAACATCTCTCCCTCTGTGATTT

