Ribozyme mediated transformation of malignant cancer cells to normal cancer cells is described.
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RIBOZYME MEDIATED REVERSAL OF TRANSFORMATION
BY CLEAVAGE OF THE HRAS ONCOGENE RNA

Field of the Invention

This application is a continuation-in-part of application PCT/US/90/06226, filed 01 November, 1990, which is a continuation-in-part of application PCT/US/90/03218, filed 07 June, 1990, which is incorporated herein by reference.

This invention relates to a ribozyme effective to cleave oncogene RNA. More particularly the invention relates to a ribozyme effective to cleave Hras RNA at a GUC site on codon 12 where an activating point mutation may appear, to a plasmid into which said ribozyme has been cloned, to mammalian cells into which said plasmid has been transfected and which express said ribozyme and to cancer therapy which entails the administration of such ribozymes per se or of such plasmids to induce in vivo expression of such ribozymes. These ribozymes or ribozyme plasmids can reverse the transformation process to convert malignant cells back to the normal cell phenotype.

Background of the Invention

Ribozyme cleavage of various RNA transcripts is known. See, e.g., published PCT applications no. 89/05852 and no. 88/04300 and pending U.S. Application 9/401,613 filed August 31, 1989.

It is known that a point mutation on codon 12 activates the Hras oncogene to yield a gene product which malignantly transforms cells and may cause a spectrum of neoplasms including human bladder carcinoma. To date no known cancer therapy protocol implicates this phenomenon.
Summary of the Invention

Normal Hras gene RNA does not include a ribozyme cleavage site on codon 12. The point mutation which yields a malignancy inducing Hras transcript does include such a cleavage site. This invention provides a ribozyme effective to cleave the mutated gene both in vitro and in vivo, but not the normal gene. It accordingly provides unique therapy for bladder carcinoma and other malignancies which may be induced by cells transformed by the mutated Hras gene product. The ribozyme of the invention may be administered by a known delivery system such as a liposome or by other means known to the art. It may also be administered in the form of a vector (i.e., RNA tumor virus) into which it has been cloned and which will express the ribozyme.

The invention also includes a ribozyme which inhibits C-fos expression in response to cis-platin or other stimuli.

Detailed Description of the Invention

The DNA sequence flanking codon 12 of the human Hras gene is illustrated by I:

```
I. 5' GTG GTG GGC GCC GGC CGT GTG GGC AAG 3' 
  8  9 10 11 12 13 14 15 16
```

The mutation which activates the Hras gene is shown in brackets above position 2 of codon 12.

The Hras RNA included in mutated gene transcript according includes a GUC site appropriate for ribozyme cleavage as shown in II.
II. 5' GUG GUG GCC GCC GUC GGU GUG GCC AAG 3'  
   +8  9  10  11  12  13  14  15  16

A ribozyme effective to catalytically cleave the mutated Hras DNA at the GUC cleavage site in functional association therewith is shown in III.

III. Hras RNA

5'  G GGC GCC GUC GGU GUG GCC  - 3
   C CCG CGG CA CCA CAC CCG 3'

3'  A    CU
    A    GA
    A    U

Ribozyme  G    AG
          C-G
          A-U
          G-C
          G-C
          G
          A
          GU

Another representation of the ribozyme is shown in IV.
IV. Ribozyme 3' 5' c-Hras RNA

\[
\begin{array}{c}
\text{NORMAL} = G \\
\text{MUTATED} = U
\end{array}
\]

cleavage site

In IV, the nucleotide which is implicated in the mutation is shown by an open circle. As indicated, the nucleotide is "G" in the normal sequence and "U" in the mutation.

The ribozyme per se may be synthesized in known manner by use of a commercially available synthesizer produced, for example, by Applied Biosystems, Inc. or Milligen.

In the preferred practice of the invention a double stranded DNA molecule having one strand, which upon transformation yields the desired ribozyme, is synthesized from single stranded oligodeoxynucleotide molecules. This molecule is then cloned into a plasmid capable of synthesizing the ribozyme in vivo when transfected into a mammalian cell. Appropriate promoters, e.g., T7 RNA polymerase, and terminal sequences may be present and follow the "ribozyme" component of the DNA insert to be cloned into the plasmid. Preferably appropriate nucleotide sequences having sufficient overlapping base pairs are amplified by the polymerase chain reaction to provide
the insert to be cloned. The 3' and 5' termini include restriction sites to insure the correct positioning of the insert in the plasmid. A T7 RNA polymerase promoter may be positioned at the 5' terminus of the "ribozyme" to accommodate in vitro cleavage. One appropriate double stranded construct is shown by V.A and V.B.

V.A Sequence 1

T7 RNA polymerase promoter

5' GGT CGA CTA ATA CGA CTC ACT ATA GGC CCA CAC CCT GAT
Sal I

3'----C GTG GGA CTA

V.B Sequence 2

GA - 3'

CTC AGG CAC TCC TGC TTT GCC GCG GGT TCG AAC - 5'
Hind III

The single stranded synthetic oligodeoxynucleotides with flanking Sal I and Hind III restriction sites are annealed to provide an insert with sticky ends including the ribozyme to be cloned into the plasmid.

The T7 RNA polymerase promoter sequence shown in V.A may be omitted if in vitro cleavage of Hras RNA is not contemplated.

The sal I and Hind III sequences are included to insure that the double stranded product which results from PCR amplification of the V.A-V.B construct appears in the proper orientation when cloned into a plasmid to produce a vector.
The selection of an appropriate plasmid is within the skill of the art. A preferred plasmid is pH-β-Apr.1, more specifically pH-β Apr.1-neo which yields the corresponding vector pH-β Apr.1-Hras-R in which R is the double stranded PCR product, one strand of which, V.A includes the Hras ribozyme. Other plasmids useful in the invention include pH α pKoneo, the pSV2 cat plasmid and pMAMneo.

The DNA fragments provided by the PCR amplification product are cloned by sticky end ligation into a selected site of the plasmid.

Cells transfected, e.g., by lipofection or electroporation, with the vector pH-β-Apr.1 Hras R express the Hras ribozyme shown in III and IV.

DESCRIPTION OF THE FIGURES

Figure 1 is a block diagram schematic illustration of certain steps included in the invention.

Figure 2 illustrates a plasmid including a ribozyme of the type shown in III and IV. The sequence below the plasmid illustrates the "ras-ribozyme" insert.

Figure 3 illustrates the position of the ribozyme IV and a synthetic RNA (1685-1761) substrate used to test the ribozyme with respect to exon 1 of H-ras mRNA. Cleavage of the synthetic H-ras RNA with increasing proportions (0.1-2.0 fold) of the ras ribozyme (3A). All sizes shown are in bases. The oligodeoxyribonucleotide containing the sequence of a bacteriophage T7 RNA polymerase promoter (H-rasR-1:5'TAA TAC GAC TCA CTA TAG CTG GTG GTG GTG GCC GCC GTC GTG GTG GCC AAG A-3' or the ribozyme H-rasRb-3:5' GGT CGA CTA ATA CGA CTC ACT ATA GGC CCA CAC CCT GAT GA-3') and a sequence complementary to the desired RNA product, was mixed with an oligodeoxyribonucleotide (H-rasR-2:5' TCG TCC ACA AAA TGG TTC TGG ATC AGC TGG ATG GTC AGC GCA CTC TTG CCC A-3' or the ribozyme H-rasRb-4:5' CCA GCT TGG GCG CCG TTT CGT
CCT CAC GGA CTC ATC AGG GTG C-3') complementary to the
T7 promoter segment to form a hemi-duplex (H-rasR-1,
H-rasR-2, and H-rasRb-3, H-rasRb-4). DNA PCR was
performed to generate a 95-base pair sequence for
synthetic H-ras RNA or a 72-base pair sequence for the
H-ras ribozyme (Figure 3B). T-7 polymerase-generated
synthetic H-ras RNA and ribozyme RNA were purified
by 12% PAGE and the ribozyme assay performed as
previously described.

Figure 4 shows detection of H-ras ribozyme by PCR
(Figure 4A). A radiolabelled H-ras ribozyme probe
(H-rasRb-5:5' GAC CAG TGT TTG CCT TTT A-3') was
hybridized to the PCR product from 100 ng of EJ RNA
(lane 1), EJ pHβ (vector alone) RNA (lane 2), and
EJpHβHras ribozyme clones 4.1, 4.7, 9B, 4.10 and 4.5
(lanes 3 to 7 respectively). H-ras expression in EJ
cells (Figure 4B). Total RNA (10μg/lane) was obtained
for EJ cells (lane 1), EJpHβ (vector alone) and EJpHβ
H-ras ribozyme clones 4-5, 4-1, 4-7, 4-10 and 9B
(lanes 3 to 7 respectively). RNA was isolated by using
the guanidium isothiocyanate method, electrophoreses on
horizontal agarose gels, hybridization and a densitome-
tric analysis (Ambis, San Diego, CA) were performed as
previously described. The probe (100Ng) was labelled
with about 5x10^7 dpm using a nick translation kit
(BRL). Figure 4C exhibits cleavage of synthetic H-ras
RNA by extracts of 1000 EJ cells. Lane 1, EJ cells;
lane 2, EJpHβ (vector only), and EJpHβ H-ras ribozyme
clones 4-1, 9B, 4-10 and 4-5 (lanes 3 to 7,
respectively).

Figure 5 is a morphology of (A) EJ cells and (B) EJ
cells transfected with the ras ribozyme, (C) EJ cells in
nude mice and (D) EJ cells with the ras ribozyme in nude
mice. The plasmid used for cloning the H-ras ribozyme
was prepared from two synthetic single-stranded
oligodeoxyribonucleotides (H-rasRb-1:5' TCG ACG GCC CAC
ACC CTG ATG AGT CCG TGA GGA CGA AAC GGC GCC CA-3' and
H-rasRB-2:5' AGC TTG GGC GCC GTT TCG TCA CGG ACT CAT
CAG GGT GTG GGC CG-3') and contained a 72 base pair
sequence with flanking restriction sites. The sequence
and orientation of the inserts were confirmed by using
the Sequenase kit (U.S. Biochemical Corp.).

The Hras ribozymes or the plasmid vectors which
express such ribozymes of the invention may be
administered by injection of appropriate delivery
systems such as liposomes. Hence, one aspect of the
invention includes liposomes in which the Hras ribozymes
are encapsulated or are included in the liposomal
bilayers. In vivo ribozymes expressed by cells
transfected with a plasmid, as described, express the
ribozyme shown in III or IV which cleaves the RNA
expressed by malignant Hras genes as evidenced by
Example 1.

EXAMPLE 1

A control group and an experimental group of nude
mice were treated with one of the following treatments:

1. EJ human bladder carcinoma cells exposed
   (treated) to the plasmid pH-β Apr.1-neo (no
   ribozyme); and

2. EJ human bladder carcinoma cells exposed
   (treated) to the Figure 2 plasmid pH-β
   Apr.1-neo containing the H-ras ribozyme as
   shown by Figure 2.

Cells (2 x 10^6) from each group were injected into
the bladder of the nude mice.

The control group received the EJ cells without the
H-ras ribozyme. Seven of eight control mice died with
an average survival of 4.5 weeks. Pathology studies
revealed metastatic invasion of lung, kidney and liver
of seven out of eight control mice within four to six
weeks of treatment.
The experimental group received the EJ cells with the H-ras ribozyme. This group had only three deaths after 11.0 weeks. The pathology of these dead animals indicated no invasive tumor. This data is summarized in Table 1.

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<th>Control</th>
<th>EJ pH 6HrasR</th>
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<tr>
<td>Dead/Mice</td>
<td>7/8</td>
<td>3/53</td>
</tr>
<tr>
<td>% Survival</td>
<td>12%</td>
<td>94.4%</td>
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</table>

In summary, the control animals had a 12% survival rate at six weeks while 94.4% of the experimental animals (with H-ras ribozyme) were alive after 11.0 weeks.

Further describing the invention, a hammerhead ribozyme IV was designed to target the activated codon 12 region of the H-ras gene in EJ bladder carcinoma cells. The mutation converts GGC (gly) to GUC (val), simultaneously creating a target site for a hammerhead ribozyme. Synthetic oligonucleotides encoding the catalytic core, flanking sequences complementary to the target sequence in H-ras mRNA, and a T7 promoter were synthesized (Figure 3A). The oligonucleotides were annealed and extended using Taq polymerase to generate a double-stranded DNA template. Transcription of the template using T7 polymerase and α-(32P)-UTP generated a radioactive 47 base ribozyme. Likewise, oligonucleotides encoding a portion of H-ras mRNA were used to create a template such that a small (77 base) H-ras substrate could be synthesized using T7 polymerase (Figure 3A). Following purification on polyacrylamide gels, these molecules were used to determine the specificity of ribozyme cleavage and
optimize cleavage conditions in vitro. Figure 3A shows that incubation of the H-ras RNA substrate with increasing amounts of ras ribozyme results in specific cleavage of the RNA into two products, 57 and 20 bases long. In contrast, a substrate containing the normal H-ras sequence at codon 12 was not cleaved by the ribozyme.

An additional set of oligonucleotides, containing the ras ribozyme and flanking restriction sites, was used to clone the construct into the mammalian expression vector, pHEAPr-1, which utilizes the human β-actin promoter to drive expression of clone sequences, and carries the neo gene for selection of transformants. DNA sequence analysis confirmed insertion of the correct sequence orientation. The ras ribozyme expression plasmids were introduced into EJ cells by electroporation. G418-resistant stable clones were screened for integration of the ras ribozyme plasmid by polymerase chain reaction (PCR) analysis of their DNA. Expression of the ribozyme was demonstrated in total RNA isolated from five clones by another PCR assay, using a β-actin 5'UT region primer and the plasmid polylinker primer to amplify a 118 base, mature (spliced) ribozyme RNA product (Figure 4A). The five clones were subdivided into three groups, according to their level of ribozyme expression: group I, including clones 4-7 and 4-10, with low level expression; group II, consisting of clone 4-5, representing intermediate ribozyme expression; and group III, with clones 4-1 and 9-b, exhibiting the highest expression of the H-ras ribozyme (Figure 4A and Table 2). These clones were selected for further analysis, as well as control clones transfected with the expression vector but missing the ribozyme (EJpHβ, Table 2).
The level of H-ras gene expression was then examined in the aforementioned clones. Northern and PCR analysis both indicated that the level of endogenous H-ras mRNA was dramatically reduced as compared to parental EJ cells (Figure 4B). In fact, in clones 4-1 and 9B, analysis failed to reveal any H-ras mRNA. Of even greater interest was the finding that no p21 expression could be detected by Western blot analysis (data not shown), suggesting that much of the detected H-ras RNA represented cleavage products after ribozyme action (Figure 4B). To demonstrate that the ribozyme transcribed in vitro retained its cleavage ability, cellular extracts of various EJ clones were incubated with radiolabelled synthetic H-ras RNA. The results reveal, in addition to RNA degradation, cleavage of the 77-base substrate into 57- and 20-base moieties in ribozyme-containing clones but not in EJpHb cells (Figure 4C).

EJ transfectants expressing the ras ribozyme revealed markedly different growth characteristics and morphology in culture (Figure 5, and Table 2). Whereas the parental EJ tumor line exhibits long, spindly cells that rapidly spread to cover the growth surface, the transfected clones in general were more rounded or cuboidal, and tended to grow in patches rather than spread (Figure 5A). The generation time was substantially longer from 30.5 to 62.3 hours in EJ ras ribozyme clones versus 24 hours in EJ cells (Table 2); the plating efficiency in agar was lower in five EJ ribozyme clones at 1% or 20% serum (Table 2); and the rate of DNA synthesis in the five EJ ribozyme clones was lower as evidenced by the (3H)-thymidine incorporation assay. Interestingly, there was a correlation between level of ribozyme and H-ras gene expression and these growth characteristics. Thus, group I clones with low levels of ribozyme and a correspondingly higher H-ras
gene expression exhibited a lower generation time as well as enhanced plating efficiency in soft agar and 
\((^{3}H)\)-thymidine uptake. In contrast, group III clones, 
with high ribozyme expression, grew more slowly, with 
poor plating efficiency in soft agar and decreased DNA 
synthesis (Table 2).

Finally, the effect of downregulating activated 
\(H\text{-ras}\) expression was tested in a nude mouse model, which 
preserves the invasiveness and metastatic capacity of 
human bladder carcinoma cells (Table 3). Inoculation of 
EJ cells transfected with the expression plasmid 
\(p\text{H\#Apr-1}\) also resulted in death of the mice in an 
average of 42 days (Table 3). Remarkably, mice 
implanted with EJ clones expressing the \(ras\) ribozyme 
survived significantly longer (80 days) (Table 3). 
Furthermore, the pattern of invasion and spread was 
strikingly different from that observed for the parental 
EJ line (Figure 3B). Control and experimental mice 
frequently had kidney as well as bladder tumors due to 
the tendency of these animals to reflux their urine. 
However, in contrast with the infiltrating invasive 
pattern of EJ tumor cells, the ribozyme-containing cells 
grew as more circumscribed masses and demonstrated much 
more limited local invasion. Distant metastases 
(primarily in the lung) were detected at a primary in EJ 
cells in contrast to only one out of 16 EJ ribozyme mice.

The present invention thus uses a novel approach to 
reduce tumorigenicity of the normally malignant EJ 
bladder carcinoma cell line. The insertion of a 47 bp 
synthetic DNA sequence encoding the \(H\text{-ras}\) ribozyme was 
sufficient to decrease \(H\text{-ras}\) gene expression and induce 
a dramatic change in the fundamental characteristics of 
the EJ bladder carcinoma cell line. Thus a cell line 
which normally produces tumors in nude mice at a high 
frequency was shown to have markedly decreased 
tumorigenicity. These results underscore the role that
the H-ras gene product plays in the various steps in the transformation from the normal to the neoplastic phenotype. Recent studies have implicated ras gene activation preceded the onset of neoplasia in a mouse mammary model. The results suggest that, despite the possible requirement for a "second hit" event for achievement of full tumorigenic potential, the targeting of H-ras genes still constitutes a valid approach as the H-ras ribozyme decreased incidence of tumor take in nude mice. The control cases consisted of 11 mice infused with EJpH6 (plasmid vector only), while the ribozyme cases consisted of 44 infused with EJpH6 H-ras ribozyme cells and the results were summarized in Table 3. Overall, the control group mean survival was 34 days as compared to 78.8 days in the ribozyme group. Autopsy of control mice demonstrated an extensive aggressive pattern of invasion with a 30% metastases rate. Ribozyme tumor mice exhibited a focal benign pattern of invasion with a 7.1% metastasis rate (Figure 5). The data indicates that the ras ribozyme suppressed the invasive and metastatic phenotype of a human bladder cancer cell line in an orthotopic nude mouse model and this alteration was associated with a prolonged survival.

as 4-1 and 9B) displayed a correspondingly less invasive phenotype. Ras has been previously implicated in metastasis in studies using transfection of the EJ/T24 H-ras genes into a variety of cell lines to demonstrate enhanced metastatic and malignant potential. In this study, insertion of the H-ras ribozyme into EJ cells leads to a 50% decrease in incidence of metastasis upon orthotopic transplantation into nude mice. Thus, decreasing expression of activated ras genes not only significantly inhibits metastatic spread, but also results in generation of a tumor cell type devoid of its aggressive, invasive characteristics in vivo.
### TABLE 2
Growth Characteristics of EJ Cells

<table>
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<tr>
<th>Cell Lines</th>
<th>Ribozyme Expression</th>
<th>Generation Time (h)</th>
<th>Colonies²/ Serum:1%/20%</th>
<th>(³H)dThd Incorporation²/</th>
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<tr>
<td>EJ</td>
<td>-</td>
<td>24.1</td>
<td>17/84</td>
<td>100</td>
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<tr>
<td>EJpHb (Vector only)</td>
<td>-</td>
<td>24.5</td>
<td>19/68</td>
<td>100</td>
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<td>EJpHbH-ras Ribozyme clones</td>
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<td></td>
</tr>
<tr>
<td>I 4-7</td>
<td>+</td>
<td>30.5</td>
<td>4/34</td>
<td>19.1</td>
</tr>
<tr>
<td>4-10</td>
<td>+</td>
<td>31.6</td>
<td>3/44</td>
<td>18.3</td>
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<tr>
<td>II 4-5</td>
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<td>III 4-1</td>
<td>+++</td>
<td>62.3</td>
<td>0/18</td>
<td>9.2</td>
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<tr>
<td>9B</td>
<td>+++</td>
<td>62.1</td>
<td>0/20</td>
<td>8.6</td>
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a/ EJ cell lines (250 cells/35mm²) were grown in 0.3% agar with 1% or 20% fetal bovine serum (Sigma Co.). Colonies were counted 13 days later with Giemsa dye. The colonies were counted and the control equaled 84 colonies in 20% serum and 17 colonies in 1% serum.

b/ To determine the rate of (³H)dThd incorporation in acid insoluble material EJ cells (2.5 x 10³ cells/35mm² dish) were grown for 48 hours, then incubated for two hours with (³H)dThd (10^6 dpm/dish), washed, acid precipitated and counted as previously described. The EJ cell control (100%) represented 2.12 ±0.3 fmol/mg DNA/hr.
<table>
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<tr>
<th>Cells Injected</th>
<th># of Mice</th>
<th># With Tumor</th>
<th>Invasive</th>
<th>Metastasis</th>
<th>Survival Days</th>
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<tr>
<td>EJpHb (vector only)</td>
<td>11</td>
<td>10</td>
<td>Extensive</td>
<td>3/10</td>
<td>34</td>
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<td></td>
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<tr>
<td>I 4-7</td>
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<td>8</td>
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<td>2/8</td>
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<td>9B</td>
<td>17</td>
<td>10</td>
<td>Focal</td>
<td>0/10</td>
<td>83</td>
</tr>
<tr>
<td>Total</td>
<td>44</td>
<td>28</td>
<td>Focal</td>
<td>2/28</td>
<td>78.8 Average</td>
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Pathogen-free mice were given intravesical infusions of 2x10^6 cells (PV is plasmid vector; RP is ribozyme plasmid). Mice were autopsied if they showed signs of distress, unexpectedly died, or if they survived 90 days. Athymic mice were anesthetized with i.p. pentobarbital. EJ cells (2x10^6) transfected with vector only or a ribozyme-expressing construct were instilled into the bladder via a 22 gauge angiocatheter. Mice were observed for signs of tumor burden (loss of weight, diminished activity level, impaired mobility) and euthanized when they exhibited signs of distress.
CLAIMS

1. A ribozyme which cleaves Hras RNA at a GUC cleavage site.

2. A ribozyme having the sequence of the ribozyme shown in III.

3. A plasmid having as a cloned insert, a double stranded DNA fragment one strand of said fragment having the sequence of the ribozyme shown in III.

4. A cell including a plasmid, as defined by Claim 3, said cell expressing a ribozyme as shown in III.

5. The plasmid depicted by Figure 2.

6. A method of converting a malignant cancer cell to a nonmalignant cell which comprises incorporating a plasmid, as defined by Claim 5, into said cell.

7. A method as defined by claim 6 in which said malignant cancer cell is a malignant EJ human bladder cancer cell.

8. A method for treating human bladder carcinoma which comprises administering to a patient in need of such treatment an effective amount of a plasmid, as defined by Claim 5.
PRO AMPLIFICATION

CLONE INSERT INTO PLASMID

TRANSFECT PLASMID INTO MALIGANT BLADDER CANCER CELL

EXPRESSED RIBOZYME CLEAVAGE H RAS-CONVERTS MALIGNANT CELL TO NORMAL CELL

FIG. 1
5' - TCG ACG GCC CAC ACC CTG ATG AGT CCG TGA GGA CQA AAC GGC GCC CA - - - - - - 3'
3' - - - - - GC CGG GTG TGG GAC TAC TCA GGC ACT CCT GCT TTG CCG CGG GTT CCA - 5'

FIG. 2
**FIG. 3A**

**FIG. 3B**

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<td>FIG. 4A</td>
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<td>FIG. 4B</td>
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<td>FIG. 4C</td>
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FIG. 5
## INTERNATIONAL SEARCH REPORT

### I. CLASSIFICATION OF SUBJECT MATTER

According to International Patent Classification (IPC) or to both National Classification and IPC.

IPC(5):A61K 37/54; 31/70; C07H 15/12; C12N 15/00

U.S. CL.: 424/94.6; 514/44; 536/27; 435/172.1, 172.3, 320.1

### II. FIELDS SEARCHED

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<td>U.S.</td>
<td>424/94.6; 514/44; 536/27; 435/172.1, 172.3, 320.1</td>
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</table>

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched.

### III. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
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<th>Category</th>
<th>Citation of Document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to Claim No.</th>
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### IV. CERTIFICATION

Date of the Actual Completion of the International Search: 26 February 1991

Date of Mailing of the International Search Report: 2 May 1991

International Search Authority: ISA/US

ISA/US
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