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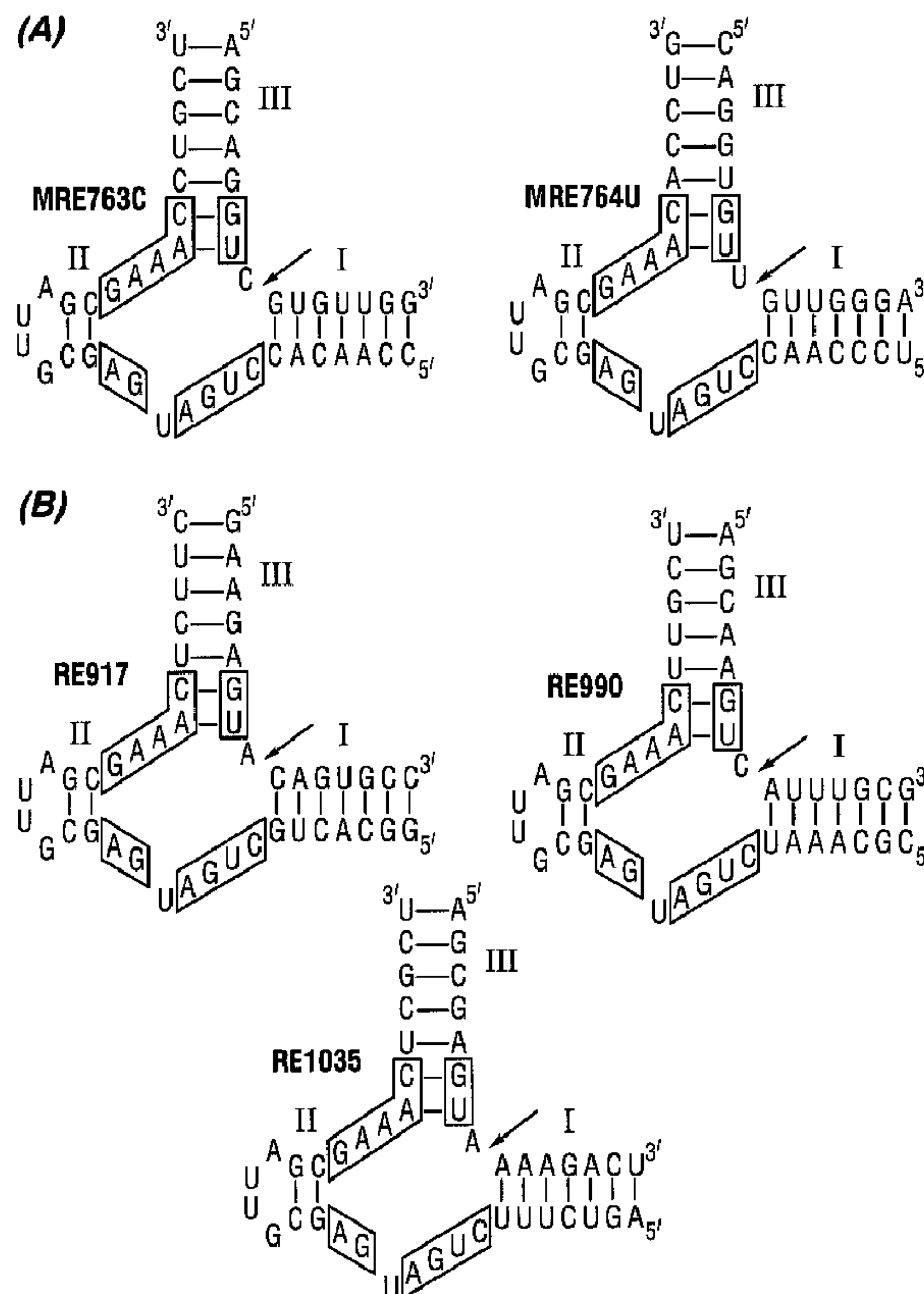
(54) **Titre :** REACTIF ET METHODE D'INHIBITION DE L'EXPRESSION DE N-RAS
(54) **Title:** REAGENT AND METHOD FOR INHIBITION OF N-RAS EXPRESSION



(57) **Abrégé/Abstract:**
The invention relates to enzymatic RNA molecules which cleave mutant N-ras mRNA, preferably at a NUX cleavage site (N = any base, X = A, C or U), having preferably the sequences 5'-CCAACACCUGAUGAGCGUUAGCGAAACCUGCU-3' or 5'-

(57) **Abrégé(suite)/Abstract(continued):**
UCCCAACCUGAUGAGCGUUAGCGAAACACCUG-3', modified derivatives thereof, pharmaceuticals containing such molecules and use of such molecules for the preparation of pharmaceuticals for the treatment of diseases involving abnormal cell growth and/or differentiation.

Abstract

Reagent and method for inhibition of N-ras expression

The invention relates to enzymatic RNA molecules which cleave mutant N-ras mRNA, preferably at a NUX cleavage site (N = any base, X = A, C or U), having preferably the sequences
5'-CCAACACCUGAUGAGCGUUAGCGAAACCUGCU-3' or
5'-UCCCAACCUGAUGAGCGUUAGCGAAACACCUG-3',
modified derivatives thereof, pharmaceuticals containing such molecules and use of such molecules for the preparation of pharmaceuticals for the treatment of diseases involving abnormal cell growth and/or differentiation.

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Description

5 Reagent and method for inhibition of N-ras expression

The invention relates to enzymatic RNA molecules which cleave mutant N-ras mRNA, preferably at a NUX cleavage site (N = any base, X = A, C or U), pharmaceuticals containing such molecules and use of such molecules for the
10 preparation of pharmaceuticals for the treatment of diseases involving abnormal cell growth and/or differentiation.

The growth and differentiation of cells depends on a variety of parameters and signal pathways. The inhibition of expression of certain signal transduction
15 proteins may lead to an efficient therapy. Three ras- genes (Ha-ras, N-ras, Ki-ras) coding for ras-proteins are essentially involved in cell signal transduction and are members of the super gene family of small GTP/GDP binding proteins. Ras mutations were detected in a wide variety of tumors such as pancreatic carcinomas, tumors in stomach and breast (Bos, J.L. (1989) Cancer Research
20 49, 4682-4689). N-ras mutations have been found in neuroblastoma, melanoma, acute myeoloblastic leukaemia (AML), chronic myelogenous leukaemia (CML) and multiple myeloma (Portier, M., Moles, J.-P., Mazars, G.-R., Jeanteur, P., Bataille, R., Klein, C. (1992) Oncogene 7, 2539-2543). Studies of ras-oncogenes in tumors revealed point mutations leading to amino acid
25 substitutions. Point mutations in the codons 12, 13, 59 and 61 cause structural changes of the GTP binding site and a reduced GTPase activity.

Enzymatic RNA molecules include Group I- and Group II-introns: Hammerhead ribozymes, hairpin ribozymes, hepatitis delta virus ribozymes ("axehead"), self-
30 splicing introns and the subunit of RNAse P. Ribozymes of the hammerhead type are the smallest catalytic RNA ever found. They contain a consensus sequence which is part of the hammerhead structure. They recognize

substrates containing NUX base triplett (N can be any base; X can be A, C, or U) and cleave the phosphodiester bond on the 3' side of X in trans position specifically. The GUC base triplett is cleaved most efficiently (Dahm, S.C. and Uhlenbeck, O.C. (1991) Biochemistry 30, 9464-9469). Hammerhead ribozymes
5 consist of three stems and 11 of 13 conserved nucleotides.

Enzymatic RNA molecules which specifically cleave mRNA have been described previously (WO 93 / 23057). Although enzymatic cleavage of ras mRNA has been mentioned in this application, it has not been disclosed which type of ras
10 gene (Ha-ras, Ki-ras or N-ras) can be cleaved, and more importantly, it has not been shown which region within the ras mRNA sequence could be cleaved. Furthermore, only wildtype ras gene has been suggetsted as target, but no mutant ras genes. In another application (WO 91 / 18913) ribozymes which cleave Ha-ras mRNA at codon 12 were described. However, the described
15 ribozymes would not allow cleavage of other ras types such as N-ras. The described ribozymes are also not stable under in vivo conditions.

The present invention describes the use of enzymatic RNA molecules, especially hammerhead ribozymes, which are targeted against N-ras mRNA. The
20 enzymatic RNA molecules of this invention are directed against mutant N-ras mRNA which cleave the N-ras mRNA, preferably after codon 13. Cleavage at codon 13 has not been achieved previously for any of the ras mRNA's. Surprisingly, enzymatic RNA molecules could be obtained which are stable under in vivo conditions and which selectively cleave at codon 13 of mutant N-
25 ras mRNA. In particular, the inventions features enzymatic RNA molecules which cleave the mutant N-ras mRNA at a NUX cleavage site, where N can be any base and X can be A, C or U.

Preferred are enzymatic RNA molecules consisting of 13 to 50 nucleotides
30 which are able to cleave mutant N-ras mRNA at a NUX cleavage site, where N can be any base and X can be A, C or U. Particularly preferred are enzymatic

RNA molecules where this RNA features a hammerhead motif.

Particular mention is made furthermore of enzymatic RNA molecules where the RNA is modified as a nuclease-resistant moiety. Preferred enzymatic molecules
5 have modifications at the internucleoside phosphate residue and/or at the 2'-position of the ribose unit of nucleotides. Particularly preferred modifications at this 2'-postion are 2'-amino and 2'-fluoro groups.

Especially preferred ribozymes have the base sequence
10 5'-CCAACACCUGAUGAGCGUUAGCGAAACCUGCU-3' or
(MRE 763 C,SEQ ID NO.: 1)
5'-UCCCAACCUGAUGAGCGUUAGCGAAACACCUG-3'.
(MRE 764 U, SEQ ID NO.: 2)

15 The invention also relates to a pharmaceutical containing one or more of the enzymatic RNA molecules, where appropriate together with physiologically tolerated ancillary substances and/or vehicles. Preferred vehicles are liposomes, immuno liposomes, microparticles and nanoparticles. Furthermore, the invention relates to the use of such molecules for the preparation of
20 pharmaceuticals for the treatment of diseases which are caused or associated by the expression of N-ras involving abnormal cell growth and/or differentiation.

An "enzymatic RNA molecule" is a nucleic acid or a nucleic acid analog which binds in a predictable way to a specific region on the target mRNA and which is
25 able to cleave that mRNA at a predictable site. Enzymatic RNA molecules include Group I- and Group II-introns: Hammerhead ribozymes, hairpin ribozymes, hepatitis delta virus ribozymes ("axehead"), self-splicing introns and the subunit of RNAse P. Preferred ribozymes of these invention are those of the hammerhead type.

30

Ribozymes can be delivered in an exogenous or endogenous way into cells.

Exogenous means that chemically synthezised ribozymes or in vitro transcripts with T7 RNA polymerase are applied directly to cells. The endogenous method requires a plasmid or viral vector that produces the corresponding ribozyme via gene expression. Both types of ribozymes, synthetic or vector-encoded are
5 within the scope of this invention.

The problem which arises from exogenous delivery is the low stability of ribozymes towards degradation by nucleases occurring in cell culture supernatant. The 2'-hydroxy group plays an important role in the degradation
10 mechanism by nucleases, consequently RNA can be stabilized by 2'-modifications. Therefore, 2'-desoxyribonucleotides (Tayler, N.R., Kaplan, B.E., Seiderski, O.P., Li, H., and Rossi, J.J. (1992) Nucleic Acids Res. 20, 4559-4565; Yang, J.-H., Usman, N., Chartrand, P., and Cedergren, R. (1992) Biochemistry 31, 5005-5009), 2'-O-methylgroups (Goodchild, J. (1992) Nucleic
15 Acids Res. 20, 4607-4612), 2'-fluoro- and / or 2'-aminogroups (Heidenreich, O., Benseler, F., Fahrenholz, A., and Eckstein F. (1994) J. Biol. Chem. 269, 2131-2138) or 2'-desoxyribonucleotides together with phosphorothioat linkages (Shibahara, S., Mukai, S., Morisawa, H., Nakashima, H., Kobayashi, S., and Yamamoto, N. (1989) Nucleic Acids Res. 17, 239-252) were introduced.
20

Basically, any chemical modification can be introduced to ribozymes of the present invention which results in generic, catalytically active ribozymes (Beigelman et al. (1995) J. Biol. Chem. 270, 25702-25708; Shimayama et al. (1993) Nucleic Acids Res. 21, 2606-2611; Dong-Jing Fu and Mc Laughlin
25 (1992) Proc. Natl. Acad. Sci. 89, 3985-3989; Olsen et al. (1991) Biochemistry 30, 9735-9741; Williams et al (1992) Proc. Natl. Acad. Sci. 89, 918-921; Paolella et al. (1992) The Embo Journal 11, 1913-1919).

Ribozymes can be chemically synthesized employing known methods (see e.g.
30 Gait, M.J. in "Oligonucleotide Synthesis - a practical approach"; IRL Press, 1984; Beaucage and Iyer (1993) Tetrahedron 49, 1925 & 2223 & 6123;

Uhlmann and Peyman (1990) Chemical Reviews 90, 543; EP-A 0 552 766; EP-A 0 593 901; and references for modifications above). The construction of vector-encoded ribozymes has been described previously (Stull et al. (1995) Pharmaceutical Research 12, 465483; J. A. H. Murray (Ed.) in "Antisense RNA and DNA" 1992, Wiley-Liss, Inc., New York; R. Baserga and D. Denhardt (Ed.) in "Annals of the New York Academy of Sciences - Antisense Strategies" Vol. 660, 1992, The New York Academy of Sciences, New York).

The invention is not confined to natural L-ribofuranoside or phosphates unit in the backbone, or to the natural bases in the recognition part. Any modification in the backbone, the sugar or bases is within the scope of this invention as long as the enzymatic cleavage activity is retained. Modifications of the sugar include alpha- and D- furanosides, carbocyclic five-membered ring analogs, ring-expanded and ring-contracted sugars, and acyclic sugars. These modifications are preferably in the binding region of the ribozyme. The sugar may be modified as 2'-O-alkylribose such as 2'-O-methyl, 2'-O-butyl, 2'-O-allyl, 2'-O-methoxyethoxy, or as 2'-fluoro-2'-deoxyribose, 2'-amino-2'deoxyribose. Modifications of the phosphate internucleoside residue include phosphorothioates, phosphorodithioates, alkylphosphonates, arylphosphonates, arylalkylphosphoramidates, phosphate esters, or combinations of these modifications with phosphodiesters or themselves. The phosphate bridge may also be replaced by formacetal, 3'-thioformacetal, and methylhydroxylamine. Modifications of the bases include 5-propynyl-U, 5-propynyl-C, 7-deaza-7-propynyl-A, 7-deaza-7-propynyl-G, 5-methyl-C, 5-fluoro-U, where the base is a ribo- or deoxynucleotide. Very preferred phosphate modifications are 3'3' or 5'5'-inversions as decribed in EP- A 0 593 901. Partial substitution of the phosphate/sugar backbone by polyamide nucleic acids as described in EP-A 0 672 677 is also a preferred embodiment of this invention.

Prefered are also end-group modifications at eihter the 5'- or 3'(2')-terminus as described in EP-A 0 552 766. Examples of end-modifications are lipophilic

radicals such as -O-$(CH_2)_nCH_3$ (n = 6 to 18), or steroid residues, or vitamines E, A or D, or conjugates which utilize natural carrier systems such as bile acid, folic acid, mannose, or peptides. Other end-groups are intercalating moietes which enhance binding to the target such as psoralene and acridine derivatives.

5

It has to be remarked that all modifications discussed above may occur once or more in a certain RNA-molecule and that the modifications can be combined to obtain extremely stable and biologically active ribozymes.

10 In order to inhibit expression of mutant N-ras, different ribozymes against codon 13 of mutant N-ras mRNA where synthesized. The ribozyme MRE763C is directed against the point mutation [GGT (gly) → CGT (arg) transition], ribozyme MRE764U is directed against [GGT (gly) → GTT (val) transition] (Figure 1A). Furthermore, ribozymes against GUC - and GUA -tripletts in codon 64 (RE917,
15 SEQ ID No.: 14), codon 89 (RE990, SEQ ID No.: 15) and codon 103 (RE1035, SEQ ID No.: 16) of the wildtype N-ras mRNA were investigated (Figure 1B).

In one investigation, short synthetic oligoribonucleotides of 15 nucleotides chain length served as substrates. Cleavage kinetics of all ribozymes were
20 carried out under Michaelis-Menten conditions. The resulting K_m-and k_{cat}-values are shown in Table I. The analysis of the kinetic data listed in Table I reveals equal k_{cat}/K_m-values for the ribozymes MRE763C, MRE764U and RE917 of about $0.2x10^6$ $s^{-1}xM^{-1}$, whereas ribozyme RE990 posesses much less catalytic efficiency. Binding of the ribozyme MRE763C to its synthetic substrate is
25 determined by measuring the melting temperature (T_m). T_m -values of approximetly 48°C were obtained for MRE763C- and MRE764U-complexes. Ribozymes RE990 or RE1035 against the wildtype mRNA as well as their substrate complexes on the other hand reveal T_m-values of approximetly 52°C. In a further investigation, RNA containing the mutant or wildtype N-ras
30 sequence from transcription initiation site to termination site was synthesized by in vitro transcription. In order to achieve efficient mRNA cleavage, the target

sequence of the substrate (NUX-triplett, N = A,G,C,U; X = C,A,U) is not allowed to be double-stranded or part of a stable hairpin. The in vitro transcribed RNA served as substrate for five ribozymes mentioned above. The catalytic efficiency was examined under "single-turnover" conditions to detect
5 the splice products.

To demonstrate the specificity of ribozyme MRE763C the cleavage reaction was followed by polacrylamide gel electrophoresis. Both resulting cleavage products of the mRNA showed the expected size. Table II depicts the kinetic properties
10 of the ribozymes under "single-turnover" conditions. Although binding affinity for both the synthetic short substrate and the long transcribed substrate are about the same as reflected by similar K_m-values, the transcribed substrate mRNA is spliced at much slower rate than the short synthetic substrates (Table I and II). It was also found that the ribozymes MRE763C or MRE764U, directed
15 against mutant mRNA, do not cleave wildtype N-ras mRNA. Therefore, the new ribozymes are able to inhibit the expression of the N-ras oncogene without influencing the expression of the N-ras proto-oncogen. According to the k_{cat}/K_m-values the most effective ribozymes (MRE763C, MRE764U and RE917) were chosen for further investigations with different chemical modifications.
20

To prevent ribozyme degradation by RNases, they were stabilized by modification of the 2'-position of the ribose with different groups. The new ribozymes were characterized by mass spectra (MALDI). Their catalytic properties and stability in cell culture supernatant were determined. 2'-
25 Modifications in the oligoribonucleotides such as 2'-O-methyl-2'-desoxyuridine/cytidine, 2'-desoxyuridine/cytidine, 2'-fluoro-2'-desoxyuridine/ cytidine were combined with terminal phosphorothioate linkages etc. (see Table III). Ribozyme stability was examined in cell culture mediums up to a time range of about 120 h. Aliquots were taken at different times, loaded onto a gel and
30 degradation bands could be detected by silver staining. The unmodified ribozyme was digested within half a minute. Introduction of the terminal

phosphorothioate linkages at the 3'- and 5'-site resulted in a half-life time of 2-3 minutes. Further modifications (e.g. 2'-fluoro-2'-desoxyuridine) leads to an increase of stability of approximately 10 minutes. Complete substituation of all pyrimidine nucleotides (e.g. 2'-fluoro-2'-desoxycytidine) produces ribozymes
5 which are stable for of about 80 h.

The cleavage kinetics of the modified ribozymes were performed under "single-turnover" conditions with the in vitro transcribed mRNA. The results are shown in Table IV. Introduction of the three 3'- and one 5'-terminal phosphorothioate
10 groups resulted in a slight loss of catalytic efficiency. The catalytic potential of the chemically modified ribozymes changes depending on the type of chemical modifications, especially on the type of substitution of the 2'-hydroxy group of the ribose moiety. Although the catalytic activity of the modified ribozymes against codon 13 is lowered to a certain extent in the in vitro cleavage assay as
15 compared to the unmodified analogs, their overall biological activity to inhibit N-ras expression in cell culture or in vivo is much higher due to their enhanced stability against degrading nucleases.

To investigate the inhibitory effect of the modified ribozymes in cell culture, a
20 HeLa cell line containing the N-ras gene fused to a luciferase reporter gene was used (Example 8). At 10 μM extracellular concentration of the modified ribozymes MRE763C(FU,FC) or MRE764U(FU,FC) a reduction by 43-61% of expression of the N-ras-luciferase fusion gene could be obtained (Table V and VI). The effective dose could be lowered by 10 to 100-fold using uptake
25 enhancers such as lipofectamine™ or cellfectin™ (Fa. Life Technologies, Eggenstein; Germany) or by using liposomal, microparticle or nanoparticle formulations. Ribozymes containing the active hammerhead-structure, but which were not directed against N-ras mRNA (nonsense controls), did not reduce the expression of N-ras-luciferase fusion.

30

The present invention relates to the use of enzymatic RNA molecules as

pharmaceuticals. These pharmaceuticals can be used, for example, in the form of pharmaceutical products which can be administered orally, e.g. as tablets, coated tablets, hard or soft agar gelatin capsules, solutions, emulsions or suspensions. Inclusion of pharmaceuticals into liposomes, which optionally
5 contain further components such as proteines or peptides, is likewise a suitable administration form. Nano- and micro particles are also a preferred route of application. They also can be administerd rectally, for examples in the form of suppositories, or parenterally, e.g. in the form of injection solutions. Alternative administration forms are topical applications, local applications, for example in
10 form of injections. Nasal administration is also a preferred way of application.

The type of administration depends on the type of cancer to be treated. The present invention relates to the treatment of diseases which are caused or associated with N-ras expression, especially of codon 13 mutant N-ras
15 expression. N-ras specific ribozymes can for example be used to treat neuroblastoma, melanoma, acute myeoloblastic leukaemia (AML), chronic myelogenous leukaemia (CML), multiple myeloma, thyroid tumors, lymphoid disorders and liver carcinoma. For most types of cancer, such as the different types of leukemia, systemic treatment is preferred.

20

The invention is now described by examples which should not be understood as a limitation of the scope of the invention.

25 Example 1: Chemical synthesis of ribozymes

Oligoribonucleotides were prepared on an Applied Biosystems 380B DNA Synthesizer™ on a 1μmol scale. Ribonucleotide phosphoramidites and control pore glass columns were obtained from PerSeptive Biosystems®. The first 5'-
30 and the last three 3'- ribonucleosides were stabilized by phosphorothioate. Cytosine- and Uracil-ribonucleotides were replaced by the respective 2'-fluoro-,

2'-O-methyl- and 2'-desoxy-modified ribonucleotides. The oligoribonucleotides were base deprotected by incubation of the glass-support with 3 ml of aequeous concentrated ammonia / ethanol (3:1 (v/v)) at 55°C for 16 h. After complete removal of the solvent by Speed-Vac™ evaporation, the 2'-silylgroup
5 was removed by overnight incubation at room temperature in 1 M tetrabutylammonium fluoride in THF. After addition of 0.5 ml of 3 M NaOAc-solution (pH 5.2) the THF was removed on a Speed-Vac concentrator and extracted twice with 1 ml of ethylacetate. The oligoribonucleotide was precipitated by addition of 2.5 volumes of absolute ethanol. The RNA was
10 centrifuged at 13.000 U/min and the pellet was dissolved in 1 ml of water. The solution was checked for complete RNA by UV absorption at 260 nm. The RNA solution was purified on denaturing 12 or 20 % polyacrylamide gels containing 8 M urea. UV detected RNA was cut out and subsequently the referring gel pieces were eluated in 0.05 M NH_4OAc-solution (pH 7.0) overnight. The RNA
15 solution was loaded onto a Sephadex-G25® columm. Fractions of 1 ml were collected and the solution stored frozen at -20°C. The homogeneity of the ribozyme RNA and substrate RNA was checked by mass spectrometry and analytical PAGE of the 5'-^{32}P labeled oligoribonucleotides followed by autoradiography. RNA concentration was determined by assuming an extinction
20 coefficient at 260 nm of 6.6×10^3 M cm^{-1}.

Example 2: Plasmid constructions

The pcN1 plasmid (A. Hall. MRC Laboratory for Molecular Cell Biology, London)
25 contained the N-ras gene. Wildtype N-ras clones were PCR-amplified using the primers 5'-AGTGCGGATCCTAAATCTGTCCAAAGCAGAGGCAGT-3' (forward primer, SEQ ID NO.: 3) and 5'-CCGGAATTCTTACATCACCACACATGGCAATCC-3' (reverse primer, SEQ ID NO.: 4). Restriction endonuclease sites were engineered into the 5'-regions of
30 these primers (reverse primer EcoRI; forward primer BamHI). The 629 bp PCR product containing the N-ras gene product was gel purified, digested with

BamHI and EcoRI restriction endonucleases, and ethanol precipitated prior to cloning (see the cloning strategy Figure 2). In a two-part ligation the N-ras PCR product was cloned into the multiple cloning site of the pBluescript™ II KS transcription vector with the restriction enzymes BamHI and EcoRI. The ensuing
5 plasmid was named pMS1-NRAS.

In order to predict the secondary structure of the N-ras mRNA, a 200 bp fragment was cloned at the 5' translation initiation site according to the strategy of Khorana (36). For this purpose four oligodesoxynucleotides with a
10 lenght of 99-102 nucleotides containing restriction endonuclease sites XbaI and BamHI were synthesized.

Oligo1 (sense):
5'-CTAGAGAAACGTCCCGTGTGGGAGGGGCGGGTCTGGGTGCGGCTGCCGCAT
15 GACTCGTGGTTCGGAGGCCCACGTGGCCGGGGCGGGGACTCAGGCGCCT-3';
(SEQ ID NO.: 5)

Oligo1 (antisense):
5'-GCTGCCAGGCGCCTGAGTCCCCGCCCCGGCCACGTGGGCCTCCGAACCACGA
20 GTCATGCGGCAGCCGCACCCAGACCCGCCCCTCCCACACGGGACGTTTCT-3';
(SEQ ID NO.: 6)

Oligo2 (sense):
5'-GGCAGCCGACTGATTACGTAGCGGGCGGGGCCGGAAGTGCCGCTCCTTGGT
25 GGGGGCTGTTCATGGCGGTTCCGGGGTCTCCAACATTTTTCCCGGTCTGG-3';
(SEQ ID NO.: 7)

Oligo2 (antisense):
5'-GATCCCAGACCGGGAAAAATGTTGGAGACCCCGGAACCGCCATGAACAGC
30 CCCCACCAAGGAGCGGCACTTCCGGCCCCGCCCGCTACGTAATCAGTCG-3'.
(SEQ ID NO.: 8)

They were hybridized and subcloned into the multiple cloning site of pBluescript™ KS II vector applying the restriction enzymes XbaI and BamHI. The resulting plasmid was named pMS2-NRAS.

5 The plasmid pMS5-NRAS, containing the N-ras sequence from transcription initiation site to termination site was obtained by three-part ligations in which the subcloned PCR product was ligated with the synthetic gene fragment at BamHI and cloned into the pBluescript II KS (+/-) vector with the restriction enzymes XbaI and EcoRI. The correct sequence was confirmed by DNA 10 sequence analysis employing standard procedures. Transcription of pMS5-NRAS yielded a RNA of the expected length of about 900 nucleotides.

PCR technology was employed to construct two N-ras activated oncogenes (first plasmid: codon 13 GGT→GTT and second plasmid: codon 13 GGT→CGT) 15 applying two inner primer pairs:
5'-GCAGGTGTTGTTGGGAAAAGCGCACTG-3' (forward primer, SEQ ID NO.: 9);
5'-CAACAACACCTGCTCCAACCACCAC-3' (reverse primer engeeniered the first mutation , SEQ ID NO.: 10) and
5'-GCAGGTCGTGTTGGGAAAAGCGCACTG-3' (forward primer, SEQ ID NO.: 20 11); 5'-CCCAACACGACCTGCTCCAACCACCAC-3' (reverse primer engeeniered the second mutation, SEQ ID NO.: 12). For amplification of the complete fragment two outer primer
5'-AGTGCTCTAGAGAAACGTCCCGTGTGGGAGGGGCG-3' (forward primer, SEQ ID NO.: 13) and
25 5'-CCGGAATTCTTACATCACCACACATGGCAATCC-3' (reverse primer) were used. The extended PCR products were gel purified, digested with restriction enzymes XbaI and EcoRI, and ethanol precipitated prior to cloning. Subsequently each of the two PCR products were cloned into the multiple cloning site of the pBluescript II KS vector with the restriction enzymes XbaI 30 and EcoRI. The following plasmids were named pMS5A-NRAS (Introduction of the first mutation GGT→GTT) and pMS5B-NRAS (Introduction of the second

mutation GGT→CGT). Subsequent sequence analysis confirmed successful insertion of the above mutations.

Example 3: In vitro Transcription

5

For in vitro transcription pMS5-NRAS, pMS5A-NRAS and pMS5B-NRAS was linearized by EcoRI digestion, phenol extracted and ethanol precipitated. Transcription was carried out in a 100 μl mixture: containing 50 ng/μl linearized plasmid DNA, 10 mM DTT, 40 mM Tris-Cl pH 7.5, 50 mM NaCl, 8 mM $MgCl_2$,

10 2 mM spermidine, 500 μM rNTP's, 0.8 U/μl RNAse-Inhibitor, 2 μCi/μl [α-^{32}P]-ATP and 2.5 U/μl T7 RNA Polymerase. 1h incubation at 37°C, was followed by the addition of 25 Units of DNAseI and the mixture was incubated for further 30 min at 37°C. After subsequent phenol extraction the aequeous phase was transferred into a Centricon-100™ tube and centrifuged at 3.400 U/min for 30

15 min. The RNA-solution was checked for homogeneity by UV absorption and 6% analytical PAGE (8 M urea). The solution was stored frozen at -20°C.

Example 4: Kinetics with synthetic substrates

20 Kinetic constants K_m and k_{cat} were determined from Eadie-Hofstee plots carried out with 5'-^{32}P-labeled substrate. The RNA substrate was labeled by reaction with T4 polynucleotide kinase and [γ-^{32}P]-ATP. 5'-endlabeled substrate was purified from [γ-^{32}P]-ATP with Centricon-3™ tubes. Ribozyme and substrate were heated separately for 1 min at 75°C in 50 mM Tris-Cl pH 7.5. After cooling to

25 37°C for 5 min 100 mM $MgCl_2$ was added to a final concentration of 10 mM and the solution was incubated for further 5 min at 37°C. Multiple turnover reactions carried out in a volume of 100 μl with concentrations of substrate between 20-500 nM and ribozyme concentrations from 2 to 5 nM in 50 mM Tris-Cl pH 7.5 and 10 mM $MgCl_2$ at 37°C. Reactions were initiated by addition

30 of ribozyme. The reaction was stopped by mixing the ribozymes with an equal volume of stop solution (8 M urea, 25 mM EDTA). The cleavage reactions were

analyzed on 20 % denaturing polyacrylamide gels (8 M urea) and scanned on a Molecular Dynamic™ Phosphorimaging system.

Example 5: Kinetics with in vitro transcribed RNA

5

The cleavage efficiency under "single-turnover" conditions in a volume of 10 μl were determined using 20-1200 nM ribozyme, 50 mM Tris-Cl pH 7.5 and 10 mM $MgCl_2$. The reaction was initiated by addition of RNA substrate to an end concentration of 10 nM substrate over 1 h by 37°C. The reaction was stopped
10 by mixing 8 μl stoppmix to each reaction. The reaction was analyzed on a 6 % denaturing polyacrylamide gels and scanned on a Molecular Dynamics™ Phosphor Imager. The single turnover k_{cat} / K_m-values were determined as described by Heidenreich and Eckstein (1992), J. Biol. Chem. 267:1904-1909.

15 Example 6: Analysis of stability of ribozymes

NIH3T3-cells were maintained as monolayer in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS and 100 Units/ml penicillin. After attaining cell density of 2 x 10^6 cells/ml the supernatant was
20 removed from the cells. The ribozyme solution (32 μl) containing different modified ribozymes were added to the cell culture supernatant (525 μl) to reach a final ribozyme concentration of 5 μM. Aliquots (67 μl) were taken at different times and given into liquid nitrogen to stop nuclease activity. After Speed-Vac evaporation the pellets were resuspended in formamide. The different aliquots
25 were analyzed by 20 % polyacrylamide gels (8 M urea) followed by silver staining.

Example 7: Melting curves

30 UV temperature curves were measured at 260 nm using a Varian Cary-1™ UV/VIS-spectrophotometer. The target was prepared by dissolving the ribozyme

in a buffer containing 50 mM Tris-Cl, pH 7.5 and 10 mM $MgCl_2$. The sample was transferred in a cuvette with 10 mm path lenght. The ribozyme was denatured at 80°C for 5 min, subsequently the substrate RNA and substrate DNA was added and the whole mixture was renatured with 1°C/min. Melting curves at 260 nm were performed by heating the solution in the cuvette with 0.75°C/min and acquiring absorbance data every 0.5°C step until 80°C was reached. The T_m-value and thermodynamic parameters of transition were obtained by fitting data of a two transition state model with the GraFit version 2.0 from Sigma Chemicals Co. software using the method of Marky and Breslauer.

Example 8: Inhibition of N-ras in HeLa cells

To investigate the inhibitory effect of the ribozymes a N-ras-luciferase reporter gene was constructed. A 450 bp fragment of the N-ras gene, containing about 80 bp of the 5'-nontranslated sequences, the natural translation initiation codon and the nucleotides coding for 134 aminoacid of the mutant or wildtype N-ras protein, were fused in frame with the luciferase gene (Photinus pyralis). Luciferase activity (light units) of the N-ras-luciferase fusion protein is measured.

Example 9: Colony-forming agar assay

The assay is a semisolid method. Transformed NIH 3T3 cells clone easily in suspension. To keep the colonies together the cells are suspended in 0.3% agar (upper layer) and plated out over a 0.5% agar layer (lower layer) in Petri dishes. When cells are suspended in warm agar and incubated at 37°C after gelling, the individual cells at the interface between the two agar concentrations form colonies which can be easily isolated (Cowley et al., Cel 77: 841-852, 1994). Each system was set up in quadruplicate.

Preparation of the lower layer (0.5% agar)

2.5% agar was prepared by dissolution in deionized water and heating in a microwave oven. The agar was cooled to 45°C in a water bath (agar turns into
5 a gel at 37°C). An agar mix with 0.5% of agar was prepared as follows:

1 × DMEM medium	25 ml (50%)
2.5% agar	10 ml (20%)
2 × DMEM medium	10 ml (20%)
10 FCS	5 ml (10%)

1 ml samples of the agar mix were pipetted into 35 mm Petri cell culture dishes and stored at 4°C for 30 min.

15 Preparation of the upper layer (0.3% agar)

0.2 ml of agar mix was added to 1 ml of 5 x 10^4 cells/ml suspension made up to a total volume of 1.7 ml with DMEM medium. 1 ml samples were pipetted onto the set bottom layer and incubated for 14-21 days at 37°C. Agar preparations
20 in 30 mm culture dishes were placed in 100 mm Petri dishes in groups of 2, together with another Petri dish containing only sterile deionized water.

The clones were isolated from the agar under a microscope, using a pipette, into 24 well plates and then expanded.

25

Colony-forming assays

The wild type and the mutated N-ras genes (codon 13) differ only in a point mutation involving an amino acid exchange. As a result of this, protein $p21^{ras}$
30 loses the ability to change the cell metabolism, leading to malignant transformation of the cells. In contrast to normal cells, cancer cells are able to

grow in serum-free media and in soft agar (semisolid) they group into cell clusters (foci). Foci are regions in which cells proliferate completely at random. This is caused by a loss of contact inhibition of the transformed cells.

5 The cells of the constitutively N-ras expressing clones 2 and 4 (mutation in codon 13 as described above, pcDNA3-NRASC, see table VII) were subcultured in soft agar. For the soft agar assay $2.5 \cdot 10^4$ cells/ml were placed in Petri dishes. The assay is a semisolid method. The mutated N-ras clones formed isolated colonies after about 4-6 days in soft agar. NIH 3T3 cells and
10 NIH 3T3 cells transfected in a stable manner with the pcDNA3™ plasmid (commercially available from In VitroGen) were seeded out on the soft agar as controls. The two controls were unable to form foci.

To increase the number of transformed cells the individual colonies of the N-ras
15 constructs were isolated in 24-well plates and expanded. A soft agar assay was carried out again ($2.5 \cdot 10^4$ cells/ml) with clones 2 and 4 of the mutated N-ras gene. This time after 4-6 days clearly more colonies of transformed cells were discernible, and after 14 days about 70% of the cells consisted of foci. Even in the culture the cells with the mutated N-ras gene formed colonies of
20 transformed cells. It is discernible with the help of the soft agar that in the case of NIH 3T3 cells a single oncogen is sufficient to transform these into cancer cells. Transition into a malignant transformed phenotype occurred through transfection.

25 Reduction of N-ras expression by cleavage of mRNA with retrovirally mediated ribozymes in NIH 3T3 cells

The aim here was to investigate whether reversion of the malignant phenotype in soft agar occurs by infection of the N-ras transformed NIH 3T3 cells with the
30 active ribozyme MRE763C. To this end the virus populations of the previously established GP+envAm-12 producer cell lines, which constitutively express the

active ribozyme MRE763C and the pBabc-Puro plasmid, were used for gene transfer by infection into the target cells, namely NIH 3T3 cells constitutively expressing the N-ras mutants (clones 2 and 4).

5 To obtain a pure cell population expressing the ribozyme or control ribozyme (SEQ ID No.: 1 having the nucleotide A instead of G in position 10), puromycin selection (1.5 μg/ml) was started after 48 h. After 10 days of selection 3 clones from each construct were isolated in 24-well plates and expanded in T25 culture bottles.

10

1 of the 3 isolated clones and a mixed population of NIH 3T3 cells constitutively expressing ribozyme MRE763C and pBabc-Puro ((Morgenstern and Land, Nucl. Acids Res. 18:3587-3596, 1990); stably transfected with the N-ras mutants (clones 2 and 4)) were subcultured in soft agar. $2 \cdot 10^4$ cells/ml
15 were once again placed in Petri dishes. The controls used were $2 \cdot 10^4$ cells/ml NIH 3T3 cells (negative control), NIH 3T3 cells, stably transfected with the pcDNA3 plasmid (negative control), and the N-ras mutants clones 2 and 4 not infected with the retroviral constructs pBabc-PuroREC and pBabc-Puro (positive controls). The soft agar preparations were incubated for 7-14 days in an
20 incubator at 37°C and 5%CO_2.

The reduction was analysed by counting the cell clusters under a microscope. The soft agar preparations were set up in quadruplicate for each construct and the mean values were calculated, as shown in Table VII.

25

Through transduction with ribozyme a reversion of the malignant transformed phenotype has taken place, but this also occurs in the presence of the retroviral vector alone.

30

Legend to the figures:

Fig. 1: Design of chemically synthesized hammerhead ribozymes.
(A) shows ribozymes which are complexed to synthetic substrates agqainst the
5 mutant N-ras mRNA. (B) shows ribozymes which are complexed to synthetic substrates against the wildtype N-ras mRNA. The cleavage site is indicated by an arrow and the consensus sequences are boxed. Stems are numbered according to Hertel et al. (1992), Nucl. Acids Res. 20: 3252.

10 Fig. 2: Cloning strategy of the plasmid pMS5-Nras.
The pcN1 amplificat and 200 bp fragment were separately ligated in pBluescript II KS. In a three-part ligation both fragments were ligated behind the T7 promoter in the pBluescript transcription vector.

15 Table IA:

Ribozyme	k_{cat} $[s^{-1}]$	K_m [nM]	k_{cat} / K_m $[10^6 \times s^{-1} \times M^{-1}]$
RE917	0.013	78	0.2
RE990	0.004	404	0.009
RE1035	0.013	277	0.05
MRE763C	0.015	82	0.2
MRE764U	0.012	65	0.2

20 25 30

K_m and k_{cat} values of ribozymes with synthetic substrates. The k_{cat}- and K_m-values were determined under Michaelis-Menten conditions as described in

Example 4.

Table IB:

Ribozyme	Modifications	k_{cat} [min^{-1}]	K_m [nM]	k_{cat}/K_m [min^{-1} nM^{-1}]	k_{cat}/K_m (relative)
MRE763C		3.760	72	0.05100	1
	EtOMeU, EtOMeC, U_4U_7-NH_2	0.530	22	0.02400	0.470
	FU, FC, U_4U_7-NH_2	0.300	16	0.01900	0.380
	S,EtOMeU,EtOMeC,U_4U_7-NH_2	0.220	18	0.01200	0.230
	EtOMeU, EtOMeC	0.027	105	0.00026	0.005

Table IIA:

Ribozyme	k_{cat} [10^6 xs^{-1}]	K_m [nM]	k_{cat} / K_m [s^{-1}xM^{-1}]
RE917	59	63	938
RE990	72	234	307
RE1035	44	425	103
MRE763C	266	71	3752
MRE764U	137	113	1212

K_m- and k_{cat}-values of several ribozymes with transcribed N-ras mRNA as substrate. The catalytic values are determined under "single-turnover" conditions as described in Example 5.

Table IIB:

Ribozyme	Modifications	k_{react} [$10^{-6}s^{-1}$]	K_m [nM]	k_{react}/K_m [s^{-1} M^{-1}]	k_{react}/K_m (relative)
RE917		59	63	938	1
	S	88	120	733	0.78
	S, FU	60	400	150	0.16
	S, dU, dC	20	1420	14	0.015
	FU, FC	66	202	326	0.35
MRE764U		137	113	1212	1
	FU, FC	62	120	516	0.42

Tabelle IIC:

Ribozyme	Modifications	k_{react} [$10^{-6}s^{-1}$]	K_m [nM]	k_{react}/K_m [s^{-1} M^{-1}]	k_{react}/K_m (relative)
MRE763C		266	71	3748	1
	FU, FC	50	39	1266	0.340
	FU, FC, U_4U_7-NH_2	173	71	2437	0.650
	EtOMeU, EtOMeC, U_4U_7-OH	147	51	2882	0.770
	EtOMeU, EtOMeC, U_4U_7-NH_2	173	73	2370	0.630
	EtOMEU, EtOMeC, U_4U_7-F	39	135	288	0.077
	S, FU, FC	51	44	1159	0.300
	S, EtOMeU, EtOMeC, U_4U_7-NH_2	27	190	142	0.038

Table III:

Ribozyme	Stability in Supernatant of Cell Cultures (half-life-times)
unmodified	0.5 min
S	3.0 min
S, FU	10.0 min
FU, FC	50 h
FU, FC, U_4U_7-NH_2	50 h
S, FU, OMeC	80 h
S, OMeU, OMeC	80 h
S, dU, dC	80 h
S, FU, FC	80 h
EtOMeU, EtOMeC, U_4U_7-OH	30 min
EtOMeU, EtOMeC, U_4U_7-NH_2	80 h
EtOMeU, EtOMeC, U_4U_7-F	80 h
EtOMeU, EtOMeC	80 h
S, EtOMeU, EtOMeC, U_4U_7-NH_2	80 h
S, EtOMeU, EtOMeC, U_4U_7-F	80 h

Stability of chemically modified ribozymes in cell culture supernatant. Reaction conditions were carried out as described in Example 6.

Table IV:

Ribozyme	k_{cat} [10^6 x s^{-1}]	K_m [nM]	k_{cat}/K_m [s^{-1} x M^{-1}]
RE917	59	63	938
RE917 (thioat)	88	120	733
RE917 (thioat, FU)	60	400	150
RE917 (FU, FC)	66	202	326
RE917 (thioat, dU, dC)	20	1420	14

25

Ribozyme	k_{cat} [10^6 x s^{-1}]	K_m [nM]	k_{cat}/K_m [s^{-1} x M^{-1}]
MRE763C	266	71	3752
MRE763C (thioat, FU, $OMeU_4U_7$)	53	61	869
MRE763C (thioat, FU, FC)	51	44	1159
MRE763C (FU, FC)	50	39	1266
MRE764U	137	113	1212
MRE764U (FU, FC)	62	120	516

5

10

15 k_{cat}- and K_m-values of different modified ribozymes with transcribed N-ras mRNA. Reactions conditions were as described in Example 5.

Table V:

Inhibition of N-ras-luciferase gene expression by 10 μM MRE763 in HeLa cells.

20

ribozyme	light units (10^5)	reduction (%)
nonsense	2.58	0
MRE763C(FU,FC)	1.0-1.1	57-61

25

Table VI:

Inhibition of N-ras-luciferase gene expression by 10 μM MRE764 in HeLa cells.

30

ribozyme	light units (10^5)	reduction (%)
nonsense	4.4	0
MRE764U(FU,FC)	2.2-2.5	43-50

35

Table VII: Reduction in transformed N-ras NIH 3T3 cells by ribozymes

Construct	Number of clones [mean] clone 2/clone 4	Reduction [%]
NIH 3T3 (negative control)	3	
NIH 3T3 (pcDNA3) (negative control)	5	
NIH 3T3 (pcDNA3-NRASC) (positive control)	91/102	0
NIH 3T3 (pcDNA3-NRASC) infected with ribozyme	42/49	54/52
NIH 3T3 (pcDNA3-NRASC) infected with pBabc-Puro	65/60	29/41

5

10

15

20

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Hoechst Aktiengesellschaft
(B) STREET: -
(C) CITY: Frankfurt am Main
(D) STATE: -
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(F) POSTAL CODE (ZIP): 65926
(G) TELEPHONE: 069-305-3005
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(I) TELEX: 041234-700 hod

(ii) TITLE OF INVENTION: Reagent and method for inhibition of N-ras expression

(iii) NUMBER OF SEQUENCES: 13

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(C) CITY: Toronto
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(E) COUNTRY: Canada
(F) ZIP: M5H 3Y2

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: CA
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(C) TELEX:

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..32

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

CCAACACCUG AUGAGCGUUA GCGAAACCUG CU 32

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 32 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: RNA

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..32

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

UCCCAACCUG AUGAGCGUUA GCGAAACACC UG 32

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..36

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

AGTGCGGATC CTAAATCTGT CCAAAGCAGA GGCAGT 36

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..33

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

CCGGAATTCT TACATCACCA CACATGGCAA TCC 33

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 100 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..100

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

CTAGAGAAAC GTCCCGTGTG GGAGGGGCGG GTCTGGGTGC GGCTGCCGCA TGACTCGTGG 60

TTCGGAGGCC CACGTGGCCG GGGCGGGGAC TCAGGCGCCT 100

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 102 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..102

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

GCTGCCAGGC GCCTGAGTCC CCGCCCCGGC CACGTGGGCC TCCGAACCAC GAGTCATGCG 60

GCAGCCGCAC CCAGACCCGC CCCTCCCACA CGGGACGTTT CT 102

(2) INFORMATION FOR SEQ ID NO: 7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 101 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..101

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

GGCAGCCGAC TGATTACGTA GCGGGCGGGG CCGGAAGTGC CGCTCCTTGG TGGGGGCTGT 60

TCATGGCGGT TCCGGGGTCT CCAACATTTT TCCCGGTCTG G 101

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 99 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..99

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

GATCCCAGAC CGGGAAAAAT GTTGGAGACC CCGGAACCGC CATGAACAGC CCCCACCAAG 60

GAGCGGCACT TCCGGCCCCG CCCGCTACGT AATCAGTCG 99

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..27

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

GCAGGTGTTG TTGGGAAAAG CGCACTG 27

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 25 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..25

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CAACAACACC TGCTCCAACC ACCAC 25

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..27

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

GCAGGTCGTG TTGGGAAAAG CGCACTG 27

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..27

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

CCCAACACGA CCTGCTCCAA CCACCAC 27

(2) INFORMATION FOR SEQ ID NO: 13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(ix) FEATURE:
(A) NAME/KEY: exon
(B) LOCATION: 1..35

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

AGTGCTCTAG AGAAACGTCC CGTGTGGGAG GGGCG 35

We claim:

1. An enzymatic RNA molecule that cleaves mutant N-ras mRNA at a NUX cleavage site, wherein N can be any base and X can be A, C or U, and which comprises the base sequence 5'-CCAACACCUGAUGAGCGUUAGCGAAACCUGCU-3' (SEQ ID NO: 1) or 5'-UCCCAACCUGAUGAGCGUUAGCGAAACACCUG-3' (SEQ ID NO: 2).

2. The enzymatic RNA molecule of claim 1, wherein the RNA has a hammerhead motif.

3. The enzymatic RNA molecule of claim 1 or 2, wherein said enzymatic RNA molecule further comprises a chemical modification.

4. The enzymatic RNA molecule of claim 3, wherein said enzymatic RNA molecule comprises at least one chemical modification selected from the group consisting of (1) a modification of at least one of the 2'-hydroxy groups of said enzymatic RNA molecule, (2) a modification of at least one sugar residue in the phosphate/sugar backbone of said enzymatic RNA molecule, (3) a modification of at least one phosphate internucleoside residue of said enzymatic RNA molecule, (4) a replacement of at least one phosphate bridge of said enzymatic RNA molecule with a compound selected from the group consisting of formacetal, 3'-thioformacetal and methylhydroxylamine, (5) a partial substitution of the phosphate/sugar backbone of said enzymatic RNA molecule with polyamide nucleic acid, (6) a modification of at least one of the bases of said enzymatic RNA molecule, and (7) a substitution of at least one end of said enzymatic RNA molecule.

5. The enzymatic RNA molecule according to claim 4, wherein said chemical modification is a modification of at least one of the 2'-hydroxy groups of said enzymatic RNA molecule.

6. The enzymatic RNA molecule according to claim 5, wherein the 2'-

hydroxy modification is selected from the group consisting of 2'-deoxyribo-, 2'-O-methyl-, 2'-fluoro- and 2'-amino-.

7. The enzymatic RNA molecule according to claim 4, wherein said chemical modification is a modification of at least one sugar residue in the phosphate/sugar backbone of said enzymatic RNA molecule.

8. The enzymatic RNA molecule according to claim 7, wherein the modified sugar is selected from the group consisting of alpha-furanoside, D-furanoside, carbocyclic five-membered ring analogs, ring-expanded sugars, ring-contracted sugars, and acyclic sugars.

9. The enzymatic RNA molecule according to claim 8, wherein the modified sugar is located in the binding region of said enzymatic RNA molecule.

10. The enzymatic RNA molecule according to claim 7, wherein said modified sugar is selected from the group consisting of 2'-O-alkylribose, 2'-fluoro-2'-deoxyribose and 2'-amino-2'-deoxyribose.

11. The enzymatic RNA molecule according to claim 10, wherein the 2'-O-alkylribose is selected from the group consisting of 2'-O-methyl, 2'-O-butyl, 2'-O-allyl and 2'-O-methoxyethoxy.

12. The enzymatic RNA molecule according to claim 4, wherein said chemical modification is a modification of at least one phosphate internucleoside residue of said enzymatic RNA molecule.

13. The enzymatic RNA molecule according to claim 12, wherein said modified phosphate internucleoside is selected from the group consisting of phosphorothioate, phosphorodithioate, alkylphosphonate, arylphosphonate, arylalkylphosphoramidate, phosphate ester, 3'-3'-inversion and 5'-5'-inversion.

14. The enzymatic RNA molecule according to claim 4, wherein said chemical modification is a replacement of at least one phosphate bridge of said enzymatic RNA molecule with a compound selected from the group consisting of formacetal, 3'-thioformacetal and methylhydroxylamine.

15. The enzymatic RNA molecule according to claim 4, wherein said chemical modification is a partial substitution of the phosphate/sugar backbone of said enzymatic RNA molecule with polyamide nucleic acid.

16. The enzymatic RNA molecule according to claim 4, wherein said chemical modification is a modification of at least one of the bases of said enzymatic RNA molecule.

17. The enzymatic RNA molecule according to claim 16, wherein the modified base is selected from the group consisting of 5-propynyl-U, 5-propynyl-C, 7-deaza-7-propynyl-A, 7-deaza-7-propynyl-G, 5-methyl-C and 5-fluoro-U.

18. The enzymatic RNA molecule according to claim 4, wherein the chemical modification is a substitution of at least one end of said enzymatic RNA molecule, wherein said substituted end is selected from the group consisting of 5'-end, 3'-end and 2'-end.

19. The enzymatic RNA molecule according to claim 18, wherein said substituted end comprises a compound selected from the group consisting of lipophilic radical, steroid residue, vitamin E, vitamin A, vitamin D, a conjugate which utilizes natural carrier systems and an end-group with intercalating moieties which enhances binding to said mutant N-ras mRNA.

20. The enzymatic RNA molecule according to claim 19, wherein the lipophilic radical is $-O-(CH_2)_nCH_3$, wherein n is an integer from 6 to 18.

21. The enzymatic RNA molecule according to claim 19, wherein the conjugate which utilizes natural carrier systems is selected from the group consisting of bile acid, folic acid, mannose and peptide.

22. The enzymatic RNA molecule according to claim 19, wherein the end-group with an intercalating moiety is a psoralene derivative or an acridine derivative.

23. A pharmaceutical containing one or more of the enzymatic RNA molecules as claimed in any one of claims 1 to 22 and a physiologically tolerated ancillary substance or vehicle.

24. A use of the enzymatic RNA molecule according to any one of claims 1 to 22 for the preparation of a pharmaceutical for the treatment of a disease which is caused or associated by the expression of N-ras involving abnormal cell growth or differentiation.

25. A use of the enzymatic RNA molecule according to any one of claims 1 to 22 for the treatment of a disease which is caused or associated by the expression of N-ras involving abnormal cell growth or differentiation.

Fig. 1: Design of chemically synthesized hammerhead ribozymes.

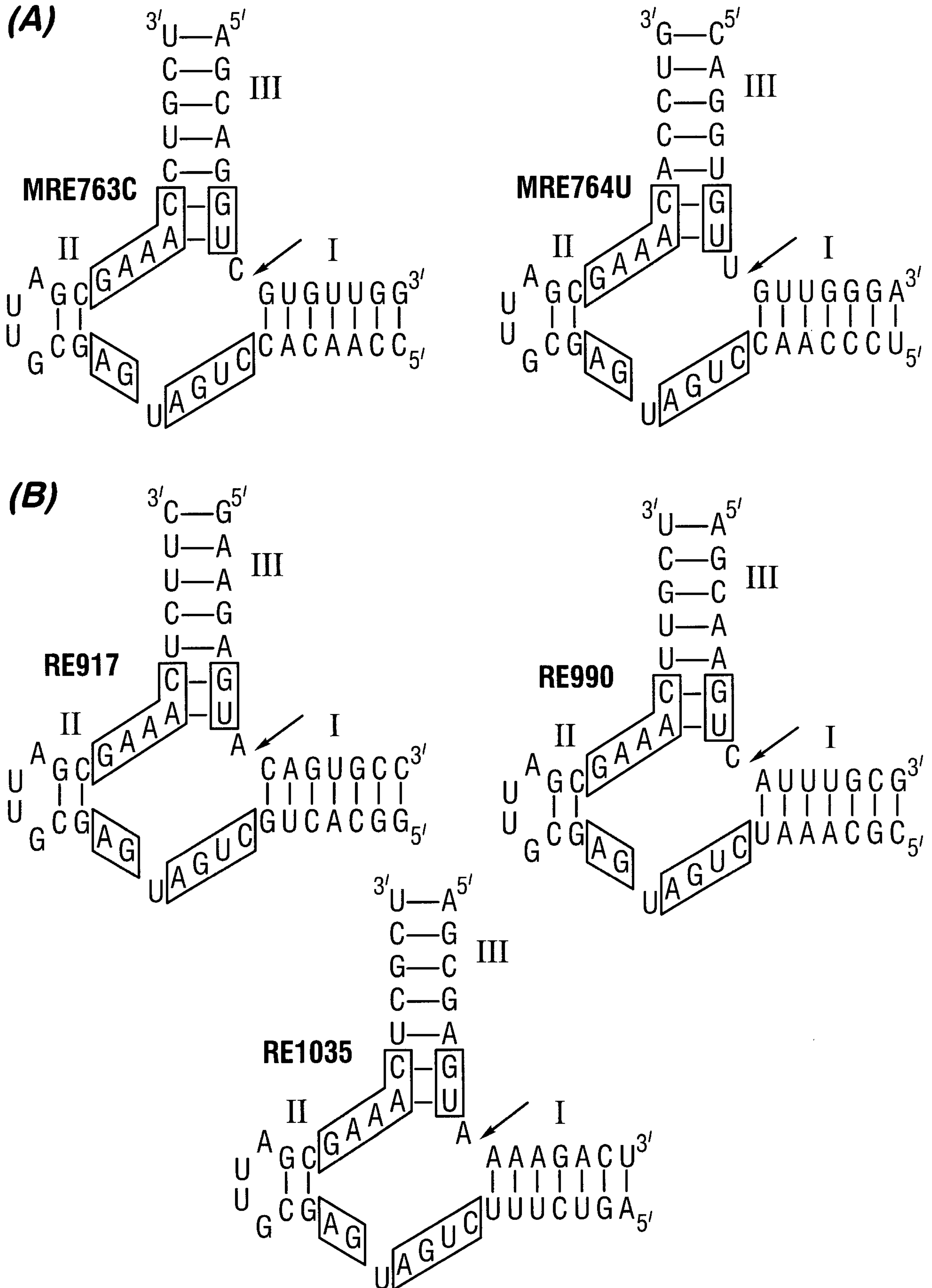
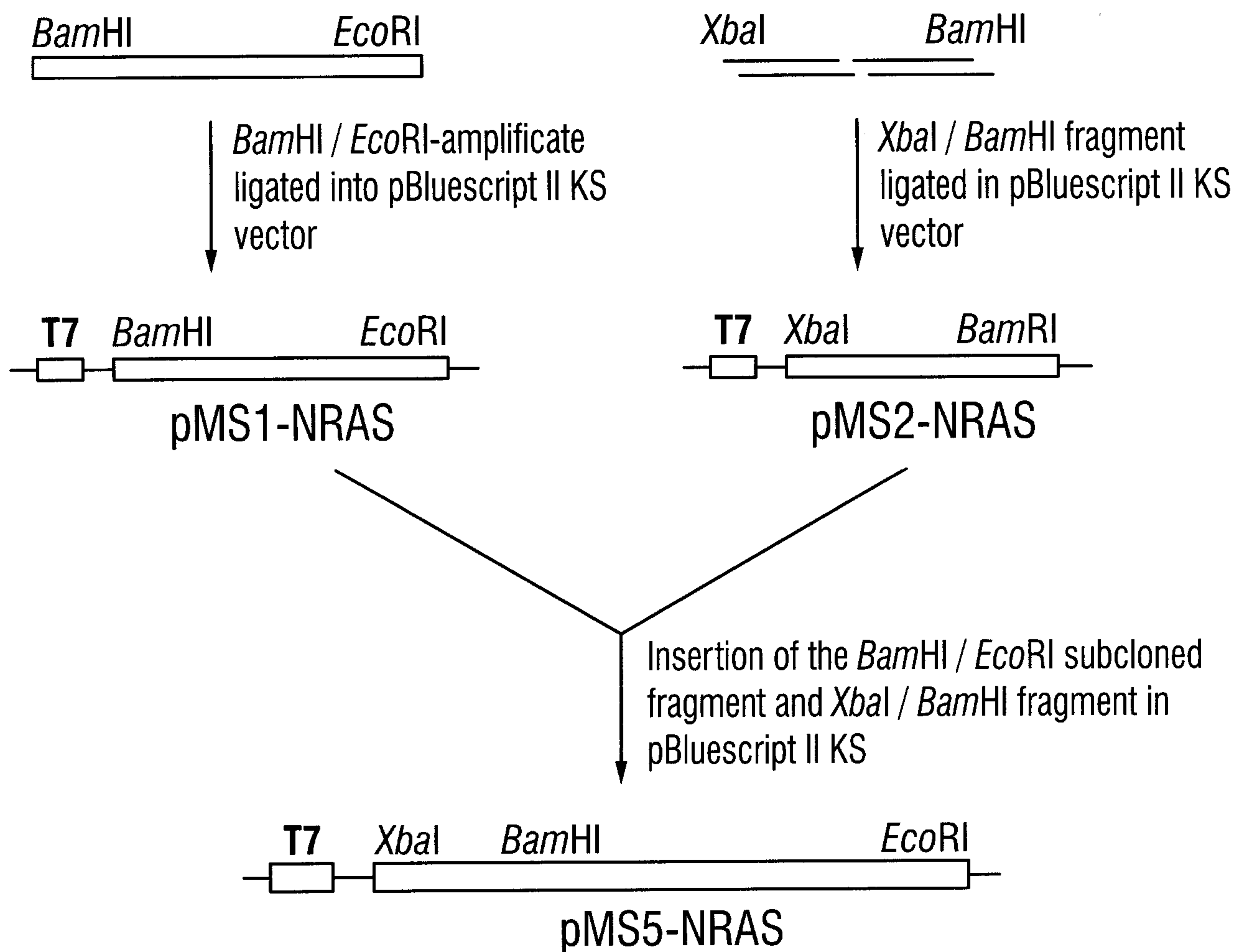
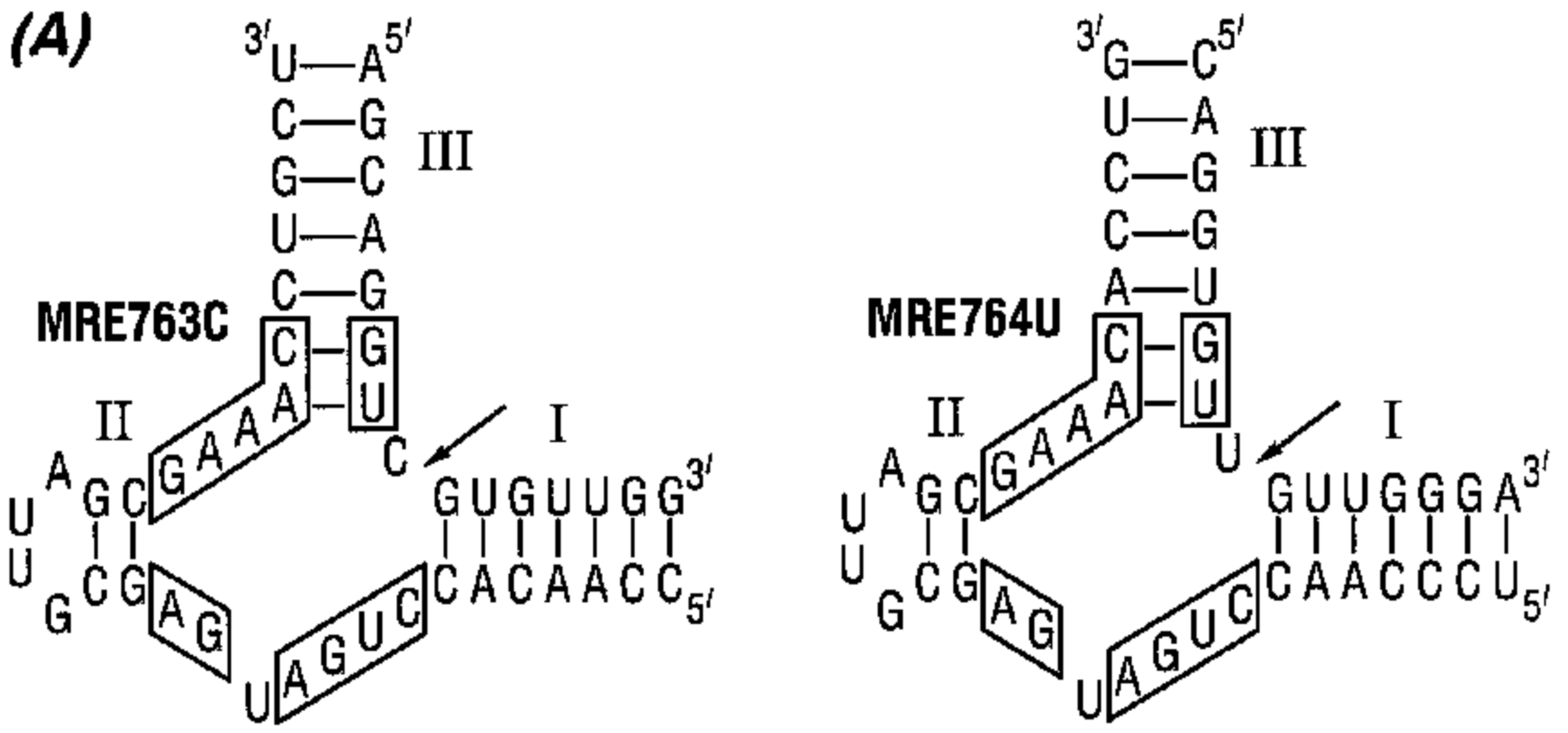


Fig. 2: Cloning strategy of the plasmid pMS5-NRAS.



Design of chemically synthesized hammerhead ribozymes.



(B)

RE917

RE990

RE1035