Specific Hammerhead Ribozyme-mediated Cleavage of Mutant N-ras mRNA *in Vitro* and *ex Vivo*

OLIGORIBONUCLEOTIDES AS THERAPEUTIC AGENTS*

(Received for publication, November 19, 1996, and in revised form, February 24, 1997)

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Two hammerhead ribozymes targeted to point mutations in codon 13 of the N-ras oncogene were synthesized and their catalytic activity and substrate specificity evaluated *in vitro* and *ex vivo*. In *vitro* studies showed that these ribozymes were specific for the oncogenic form of N-ras, since cleavage was observed only in a 849-nucleotide-long transcript containing mutant but not wild-type N-ras sequences. For the *ex vivo* studies, the ribozymes were 2'-modified to protect them against degradation by nucleases. 2'-Fluoro-2'-deoxyuridine/cytidine-substituted ribozymes were nearly as active as their unmodified counterparts, but had a prolonged stability in cell culture supernatant containing fetal calf serum. The stability of the modified ribozymes increased by introduction of terminal phosphorothioates groups without significant influence in their catalytic efficiency. A sensitive assay based on the use of N-ras/luciferase fusion genes as a reporter system was established to detect ribozyme-mediated cleavage in HeLa cells. A reduction of nearly 60% in luciferase activity was observed in cells expressing mutant but not wild-type N-ras/luciferase fusion transcripts. Moreover, cleavage of N-ras transcripts in HeLa cells was directly confirmed by a semi-quantitative RT-PCR assay.

Catalytic RNAs include Group I and Group II introns and ribozymes of the hammerhead, hairpin, and hepatitis delta virus type and the subunit of RNase P (1–3). The presence of divalent metal ions, e.g. Mg2+ or Mn2+, is essential for their activity (4, 5). Hammerhead ribozymes are the smallest catalytic RNA found to date. They consist of three stems connected by a conserved core region. They recognize substrates containing an NUH base triplet (N can be any base; H can be A, C, or U) and cleave the phosphodiester bond on the 3’ side of H. The GUC base triplet is cleaved most efficiently (6, 7). Thus, any RNA containing this motif can be cleaved by a hammerhead ribozyme if the target sequence is accessible for ribozyme binding.

Inhibition of gene expression by trans-acting hammerhead ribozymes has been reported in, e.g., plant cells (8), mammalian cells (9–14), and Xenopus oocytes (15, 16). Ribozymes have also been successfully used against oncogenes such as *bcr-abl* (17), *c-fos*, *c-Ha-ras* (12, 18, 19), or viral RNAs (20). Thus, like other strategies, e.g. antisense and triple helix formation, ribozymes appear to be promising agents to inhibit expression of specific oncogenes.

In general, the most difficult step toward an *ex vivo* application of ribozymes is the delivery of the ribozyme into the cells. Ribozymes can be applied directly to cells (exogenous delivery), or plasmid or viral vectors can be used to express the ribozyme within living cells (endogenous delivery).

For the exogenous application, ribozymes are delivered into cells by microinjection (10) or transfection (21). In the latter case, CaCl2 or cationic liposomes have been mostly used as transfection reagents. Additional exogenous delivery methods are based on the conjugation of the oligonucleotides to polylysine compounds (22) or to lipophilic groups, like cholesterol (23, 24).

A problem that arises from exogenous delivery is the low stability of unmodified ribozymes in cell culture supernatant containing fetal calf serum. Since the 2'-hydroxyl group plays an important role in the degradation mechanism by nucleases, ribozymes have been protected against degradation by modification of the 2'-hydroxyl position (25, 26). For this 2'-deoxyribozyme (27, 28), 2'-O-methyl groups (29), 2'-fluoro- and/or 2'-amino groups (30), or 2'-deoxyribonucleotides together with phosphorothioate linkages (31) have been used.

The growth and differentiation of cells depends on a variety of parameters and signal transduction pathways. The proteins coded by the three *ras* genes (Ha-ras, N-ras, and Ki-ras) are involved in cell signal transduction and are members of the superfamly of small GTP/GDP-binding proteins (32, 33). ras mutations have been detected in a wide variety of tumors such as pancreatic carcinomas (34, 35) and tumors of the stomach and breast (36, 37). N-ras mutations have also been found in neuroblastoma, melanoma, acute myeloblastic leukemia, chronic myelogenous leukemia, and multiple myeloma (38). Studies of *ras* oncogenes in tumors have revealed several point mutations in codons 12, 13, 59, or 61, which cause structural changes in the GTP binding site and reduce GTPase activity (39). Binding of GTP leads to an active state of the Ras proteins, whereas hydrolysis of GTP to GDP causes inactivation. Mutant Ras proteins, having a reduced ability to hydrolyze GTP, remain in the active state and thus stimulate cell growth or differentiation autonomously. The inhibition of the incorrect signal transduction by ribozymes may lead to an efficient anticancer therapy.

In the present study we report the synthesis and catalytic properties of several hammerhead ribozymes targeted against mutant N-ras transcripts. These hammerhead ribozymes were tested for efficiency and specificity *in vitro* and *ex vivo*. In
addition, the effect of 2'-modifications on the cleavage efficiency and stability of the ribozymes in cell culture media containing fetal calf serum was analyzed. Furthermore, a reporter gene system based on luciferase gene expression was established to evaluate the catalytic properties of 2'-modified ribozymes \textit{ex vivo}. A semi-quantitative RT-PCR was used to assess mRNA cleavage by the ribozymes. Our study demonstrates that ribozymes targeted against mutant \textit{N}-ras sequences are highly specific and efficient \textit{in vitro} and \textit{ex vivo}.

\textbf{EXPERIMENTAL PROCEDURES}

\textbf{Materials}

Ribonucleoside phosphoramidites and control pore glass columns were obtained from PerSeptive Biosystems. The sulfurizing reagent C-6 Thiol was purchased from Chemgens. The 2'-fluoro-modified ribonucleosides were a kind gift of Dr. T. Wittmann and Isis Pharmaceuticals. The sulfurizing reagent C-6 were obtained from PerSeptive Biosystems. The sulfurizing reagent C-6 was obtained from PerSeptive Biosystems. The sulfurizing reagent C-6 was obtained from PerSeptive Biosystems.

\textbf{Nucleoside triphosphates were purchased from Boehringer Mannheim. Radiolabeled nucleoside triphosphates [\textit{a}-32P]ATP and [\textit{y}-32P]ATP with the specific activity 3000 Ci/mmol were obtained from Amersham.}

\textbf{Chemical Synthesis of Ribozymes}

Oligoribonucleotides were prepared on an Applied Biosystems model 380B DNA Synthesizer on a 1-\mu mol scale. The oligoribonucleotides were base-deprotected by incubation of the glass support with 3 ml of aqueous concentrated ammonia (33%)/ethanol (3:1, v/v) at 55 °C for 16 h. After complete removal of the solvent by Speed-Vac evaporation, the 2'-silyl group was removed by overnight incubation at room temperature in 1 M tetrabutylammonium fluoride in tetrahydrofuran. After addition of 0.5 ml of 3 M sodium acetate solution (pH 5.2), the tetrahydrofuran was removed on a Speed-Vac concentrator and the aqueous phase was extracted twice with 1 ml of ethyl acetate. The oligoribonucleotide was precipitated by addition of 2.5 volumes of absolute ethanol, followed by centrifugation at 13,000 rpm. The pellet was dissolved in 1 ml of water. The RNA solution was purified on polyacrylamide gels (12% or 20%) containing 8 M urea. The RNA was visualized under UV light, excised from the gel, and eluted in 0.05 M ammonium acetate solution (pH 7.0) overnight. Thereafter, the RNA solution was loaded onto a Sephadex G-25 column, and fractions of 1 ml were collected and stored frozen at \textdegree C. The homogeneity of the ribozyme RNA and substrate RNA was checked by mass spectrometry and analytical PAGE. RNA concentration was determined by assuming an extinction coefficient at 260 nm of 6.6 × 10^3 M^{-1} cm^{-1} (40).

\textbf{Construction of Plasmid Containing Wild-type or Mutant \textit{N}-ras Sequences—}

A plasmid containing the full-length \textit{N}-ras cDNA sequence was constructed according to the strategy described by Khorana (42). For this purpose four overlapping oligodeoxynucleotides (99–102 nucleotides each) containing the 5'-termini sequences of \textit{N}-ras were synthesized and cloned into the CMV-promoter vector pMS1-NRAS. Thus, pMS1-NRAS contains a \textit{N}-ras sequence from the transcription initiation site to the translation termination site. The sequence was confirmed by DNA sequence analysis employing standard procedures. In \textit{vitro} transcription of pMS1-NRAS gave the expected 849-nucleotide-long RNA product.

Two point mutations in codon 13 of \textit{N}-ras were introduced into plasmid pMS1-NRAS by PCR-based technology. Plasmid pMSSS-NRAS contains a G \rightarrow T transversion at position 764, whereas plasmid pMSSB-NRAS contains a G \rightarrow C transversion at position 763. All sequences were confirmed by DNA sequence analysis.

\textbf{Construction of \textit{N}-ras/Luciferase Fusion Reporter Genes—}

A \textit{N}-ras/luciferase reporter gene plasmid was constructed by cloning the first 452 bp of \textit{N}-ras fused in frame with the luciferase gene. For this the translation initiation codon of the luciferase gene was mutated to ATA by insertion of a 70-bp linker containing the altered sequence at the XhoI site of plasmid pBHELuc (kindly provided by H. Hauser, Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany). Subsequently plasmids pMS5-NRAS, pMSSA-NRAS, and pMSSB-NRAS were digested with BamHI and PstI, and the so obtained 452-bp DNA fragments containing either wild-type or any of the mutated \textit{N}-ras sequences were fused in frame to the firefly luciferase gene. The \textit{N}-ras/luciferase fusion genes were cloned in the expression plasmid pCDNA3 (InVitrogen), and the resulting plasmids were named pCDNA3-LucFUW (wild-type \textit{N}-ras), pCDNA3-LucFUC (GTT \rightarrow CGT mutation), and pCDNA3-LucFUT (GTT \rightarrow GGT mutation). The sequence of the fusion genes were confirmed by DNA sequence analysis.

\textbf{Mass Spectrometry of Oligoribonucleotides}

All oligoribonucleotides (unmodified and modified ribozymes) were characterized by MALDI-TOF\textsuperscript{2} mass detection as shown in Fig. 1. The MALDI mass spectra were recorded on a VG ToFSpec. For a MALDI-TOF spectrum, the RNA solution was mixed with the matrix compound.

\textbf{Plasmid Constructions}

\textbf{Construction of Plasmid Containing Wild-type or Mutant \textit{N}-ras Sequences—}

The pcN1 plasmid (41) containing most of the \textit{N}-ras cDNA was kindly provided by A. Hall (Medical Research Council, London, United Kingdom). The \textit{N}-ras sequences were synthesized and cloned into pMS1-NRAS to generate pM1-NRAS. The pcN1 plasmid (41) containing most of the \textit{N}-ras cDNA was kindly provided by A. Hall (Medical Research Council, London, United Kingdom). The \textit{N}-ras sequences were amplified and cloned into the CMV-promoter vector pMS1-NRAS. Thus, pM1-NRAS contains an \textit{N}-ras sequence from the transcription initiation site to the transcription termination site. The sequence was confirmed by DNA sequence analysis employing standard procedures. In \textit{vitro} transcription of pM1-NRAS gave the expected 849-nucleotide-long RNA product.

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\footnote{The abbreviations used are: MALDI, matrix-assisted laser desorption ionization; TOF, time-of-flight; DTT, 1,4-dithio-di-threitol; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pair(s); FCS, fetal calf serum; PAGE, polyacrylamide gel electrophoresis; RT, reverse transcriptase.}
In Vitro Transcription

For in vitro transcription pMS5-NRAS, pMS5A-NRAS, and pMS5B-NRAS were linearized with EcoRI, phenol-extracted, and ethanol-precipitated. In vitro transcription was carried out in 100 µl of mixture containing 50 ng/µl linearized plasmid DNA, 10 mM DTT, 40 mM Tris-Cl, pH 7.5, 50 mM NaCl, 8 mM MgCl₂, 2 mM spermidine, 500 µM rNTPs, 0.8 unit/µl RNase inhibitor, 2 µCi/µl [α-32P]ATP, and 2.5 units/µl T7 RNA polymerase. After 1 h of incubation at 37 °C, DNase I (25 units) was added and the mixture incubated for another 10 min at 37 °C. After subsequent phenol extraction, the aqueous phase was transferred into a Centricon-100 tube and centrifuged at 3,400 rpm for 30 min. The RNA solution was checked for homogeneity by UV absorption and in a 6% analytical PAGE (8 M urea). The RNA was stored frozen at −20 °C.

Kinetics with Synthetic Substrates

Kinetic constants $K_m$ and $k_{cat}$ were determined from Eadie-Hofstee plots carried out with 32P-labeled substrates. Ribozyme and substrate were heated separately for 1 min at 75 °C in 50 mM Tris-Cl, pH 7.5, and for 5 min at 37 °C. Then 100 mM MgCl₂ were added to a final concentration of 10 mM and the solutions were incubated for an additional 5 min at 37 °C. "Steady state" reactions were carried out in a volume of 100 µl with substrate concentrations between 20 and 500 nM and ribozyme concentrations from 2 to 5 nM in 50 mM Tris-Cl, pH 7.5, and 10 mM MgCl₂ at 37 °C. Reactions were initiated by addition 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% polyacrylamide gels (8 M urea). Nucleic acid bands were visualized by silver staining.

Analysis of the Stability of Oligoribonucleotides

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS and 100 units/ml penicillin, 100 µg/ml streptomycin, and 1 mM L-glutamine in 96-well plates. The oligoribonucleotide solutions (32 µl) containing modified or unmodified 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 time points and shock-frozen into liquid nitrogen to stop nuclease activity. After Speed-Vac evaporation, the pellets were resuspended in formamide and analyzed on a 20% polyacrylamide gel (8 M urea). Nucleic acid bands were visualized by silver staining.

Cell Lines and DNA Transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated FCS, 100 units/ml penicillin/streptomycin (100 µg/ml), and 1 mM L-glutamine. Stable DNA transfections were performed by the calcium-phosphate precipitation method (43) with 20 µg of CoCl²⁺-purified plasmid DNA containing either wild-
In Vitro and ex Vivo Cleavage of N-ras Oncogene by Ribozymes

RESULTS

Luciferase Assay
Luciferase activity was determined by the Triton X-100 lysis method (44). Protein concentrations determined with the Bradford protein assay were used to standardize the luciferase activity (45).

Total RNA Isolation and RT-PCR
Total RNA was isolated from HeLa cells using a commercially available kit (GlassMAX RNA Microisolation Spin Cartridge System, Life Technologies, Inc.). After isolation and before the DNase I digestion step, the internal standard RNA (0.5 amol; see below) was added to the cellular RNA and the mixture was digested with DNase I for 1 h at 37 °C, followed by phenol chloroform extraction and ethanol precipitation. Reverse transcription was performed in 20 μl of reaction volume in the presence of 10 mM DTT, 50 mM Tris-HCl, pH 8.3, 3 mM MgCl₂, 75 mM KCl, 1 mM dNTPs, 0.5 unit of RNasin, 50 ng of hexamer primer, and 10 units of Moloney murine leukemia virus reverse transcriptase. The reaction mixture was incubated for 10 min at room temperature, followed by 90 min at 42 °C. The reaction was stopped by incubation at 94 °C for 7 min. An aliquot of the RT reaction was amplified under single-turnover conditions (1 min at 94 °C; 1 min at 60 °C, and 2 min at 72 °C) in a total volume of 50 μl. In parallel, a β-actin PCR was performed to control for the RNA input in the RT-PCR. The following primers were used: 5′-GGGGGGCCCCAGGCCACC-3′; 3′-β-actin, 5′-CTCCCTTAAAGTCCAGGCTGCTTT-3′; RT1-N-ras, 5′-GGAGACCCAAGCTTGGTACC-3′; RT2-N-ras, 5′-ACCTGCGCTTTACATGCCCTCTCTGAG-3′. Reaction products were separated on a 4% NuSieve agarose gel in 1× TAE and stained with ethidium bromide.

Construction of an Internal Control Plasmid for RT-PCR
To obtain an internal control for the RT-PCR, a 50-bp linker was inserted into the N-ras gene. The linker was cloned into the 5′-nontranslated region (BamHI restriction site) of the N-ras gene in the expression plasmid pcDNA3-LucFUC. The construct was named pcDNA3-LucFUCL. To generate the internal standard RNA transcript, plasmid pcDNA3-LucFUCL was linearized with BstEI, in vitro transcribed as described above, and quantified by UV spectrophotometry.

Two hammerhead ribozymes targeted against point mutations in codon 13 of the N-ras gene were synthesized. A GC transversion at position 763 generates a GUC triplet, which was targeted by the ribozyme MRE763C. An additional ribozyme, MRE764U, was targeted against a second GT transversion at position 764, which generates a GUU triplet (Fig. 2A). For comparison purposes, three ribozymes recognizing either a GUC triplet at codon 89 (RE990) or a GUU triplet at codons 64 (RE917) and codon 103 (RE1035) of the wild-type N-ras mRNA were also investigated (Fig. 2B).

To study the cleavage efficiency of the ribozymes on larger RNA substrates, transcripts containing the full-length wild-type or mutant N-ras sequence were synthesized in vitro and used under single-turnover conditions in the cleavage reaction (see “Experimental Procedures” and Table I).

Ribozyme MRE763C showed the best catalytic activity. A computer-assisted folding analysis of the RNA substrate revealed a number of mismatches (bulges) at the binding region of ribozyme MRE763C. The same applies for the binding region of ribozyme MRE764U. In contrast the cleavage site of ribozyme RE1035 is embedded within a region that folds in a stable hairpin structure. Thus, the differences in the catalytic efficiency of the enzyme and the large RNA substrate correlate well with the predicted secondary structure of the 849-nucleotide-long transcript. According to the kcat/Km values, the most effective ribozymes (MRE763C, MRE764U, and RE990) were chosen for further investigations. The specificity of one of the ribozymes (MRE763C) to cleave only N-ras sequences containing a point mutation at position 763 was examined next. Incubation of MRE763C with a 849-nucleotide-long mutant N-ras transcript resulted in the expected cleavage products of 534 and 315 bases (Fig. 4A). The same result was obtained for ribozyme MRE764U (data not shown). In contrast, incubation of MRE763C with transcripts containing the wild-type N-ras sequences did not result in any detectable substrate cleavage, demonstrating the absolute specificity of the ribozyme for its substrate in vitro.
Since the final goal of our experiments was to demonstrate the efficiency of these ribozymes in cell culture, pyrimidine ribonucleotides containing 2'-OH substitutions were used for the synthesis of ribozymes to increase the stability of the oligoribonucleotides against degradation by RNases. Several 2'-modified ribonucleotides such as 2'-O-methyl-2'-deoxyuridine/cytidine, 2'-deoxyuridine/cytidine, or 2'-fluoro-2'-deoxyuridine/cytidine were combined with terminal phosphorothioate linkages (see Table III). The modified ribozymes were incubated in cell culture media at 37 °C for up to 120 h. At specific time points, aliquots were taken from the supernatant, frozen, and dried. The pellets were subsequently resuspended in formamide and analyzed on a polyacrylamide gel. While the unmodified ribozyme was degraded within 30 s, the introduction of the three phosphorothioate linkages at the 3'-end increased the half-life of ribozymes to 2–3 min. Other modifications (e.g. 2'-fluoro-2'-deoxyuridine) led to an additional increase in stability (Fig. 5A). Finally, complete substitution of all pyrimidine nucleotides (e.g. 2'-fluoro-2'-deoxyuridine) prevented ribozyme degradation for up to 80 h (Fig. 5B). Most of the modified ribozymes were still capable of cleaving the N-ras transcripts. The introduction of the three 3'- and one 5'-terminal phosphorothioate groups led only to a minor decrease in catalytic potency (Table IV). However, the catalytic efficiency of ribozymes containing 2'-fluoro-2'-deoxyuridine nucleotides and phosphorothioate groups was very low compared with the unmodified ones (k_{react}/K_m = 150 s^{-1} M^{-1} versus 938 s^{-1} M^{-1} for the modified and unmodified ribozymes, respectively). The catalytic potency of ribozymes containing phosphorothioate groups and 2'-deoxyuridine or 2'-deoxyuridine decreased 50–60-fold compared with the unmodified ones. Substitution of the 2'-hydroxy group by 2'-O-methyl-2'-deoxyuridine/cytidine led to a complete loss of activity. In contrast, additional introduction of 2'-fluoro-2'-deoxyuridine into ribozymes containing 2'-fluoro-2'-deoxyuridine led to a 2-fold increase in catalytic activity independently of the presence or absence of terminal phosphorothioate groups (compare RE917 (S, FU) and RE917 (FU, FC) in Table IV). According to these results, modified ribozymes containing 2'-fluoro-2'-deoxyuridine and 2'-fluoro-2'-deoxyuridine with or without phosphorothioate groups were used for the studies in cell culture.

To examine the cleavage properties of the modified ribozymes ex vivo, a N-ras/luciferase fusion minigene was constructed as shown in Fig. 6. A 452-bp N-ras DNA fragment, containing 452 bp 5'-untranslated sequences, the N-ras translation initiation codon, and sequences coding for the first 134 amino acids of wild-type or mutant N-ras, was fused in frame with the firefly luciferase gene. In addition the AUG translation initiation codon of the luciferase gene was mutated to ATA. In this construct the expression of the luciferase gene depends on an intact AUG on the N-ras/luciferase fusion mRNA. Thus, cleavage of N-ras sequences by ribozymes can be assessed by the reduction in luciferase activity.

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**Table II**

Catalytic efficiencies of different ribozymes with in vitro transcribed full-length N-ras transcripts

| Ribozyme     | k_{react} | K_m | k_{react}/K_m |
|--------------|-----------|-----|--------------|
| RE917        | 10^{-6} M^{-1} s^{-1} | 63 ± 20 | 938 |
| RE990        | 72 ± 7.3  | 234 ± 28 | 307 |
| RE1035       | 44 ± 11   | 625 ± 41 | 103 |
| MRE763C      | 266 ± 25  | 71 ± 13  | 3752 |
| MRE764U      | 137 ± 12.2| 113 ± 21 | 1212 |

HeLa cells were transfected with plasmids containing either wild-type or mutant N-ras/luciferase fusion gene under the transriptional control of a cytomegalovirus promoter/enhancer element. Neomycin-resistant clones were isolated and tested for luciferase activity. The HeLa cell clones C#3 (GGT → CGT mutation at position 763), T#4 (GGT → GTT transversion at position 764), and W#2 (wild-type N-ras sequence) showed the highest luciferase activity and were chosen for all subsequent experiments.

These clones were transiently transfected with the ribozymes MRE763C and MRE764U using LipofectAMINE™ as described under “Experimental Procedures.” As a control for unspecfic cleavage, a ribozyme containing an active catalytic site but no homology to the target N-ras sequence was used (nonsense ribozyme). Similarly, catalytically inactive ribozyme containing an adenosine residue instead of guanosine at position 5 (iMRE763C and iMRE764U) were used to estimate the reduction in luciferase activity caused solely by the hybridization of the ribozymes to the target sequences (antisense effect).

Treatment of HeLa cells with LipofectAMINE™ alone caused a significant reduction in luciferase activity. This effect was attributed to the toxicity of cationic liposomes on HeLa cells since a large number of cells died shortly after the addi-
occurred during the RNA isolation procedure (47, 48), an internal dinitro-isothiocyanate method. Since cleavage of target RNA may For this total RNA was isolated from clone C#3 by the guani-
mRNA, a semi-quantitative RT-PCR reaction was established. 
investigate the reduction in the amount of N-
cleaved by the ribozymes is not possible by this assay. To 
mRNA efficiently, a quantitative assessment of RNA molecules 
the ribozymes MRE764U and MRE763C cleave the fusion 
mRNA. 

The reduction in luciferase activity caused by MRE763C was specific. Treatment of the HeLa cell clone W#2, which expresses wild-type N-ras sequences, with MRE763C led to a 20% reduction in luciferase activity (mean value of 4 experiments, Fig. 7). This value is within the range of the luciferase activity obtained from HeLa cells treated with the inactive form of the ribozyme (iMRE763C) and thus is probably due to an antisense effect. For this study eight independent experiments were evaluated, whereas the deviation of the mean was calculated for each value from six parallel reactions. 

The reduction in luciferase activity caused by MRE763C was specific. Treatment of the HeLa cell clone W#2, which expresses wild-type N-ras sequences, with MRE763C led to a 20% reduction in luciferase activity (mean value of 4 experiments, Fig. 7). This value is within the range of the luciferase activity obtained from HeLa cells treated with the inactive form of the ribozyme (iMRE763C) and thus is probably due to an antisense effect. For this study eight independent experiments were evaluated, whereas the deviation of the mean was calculated for each value from six parallel reactions. 

TABLE III

Stability of chemically modified ribozymes in cell culture supernatant containing FCS

| Ribozyme | Cell supernatants (half-lives) |
|----------|-------------------------------|
| Unmodified | 0.5min |
| S | 3.0min |
| S, FU | 10.0min |
| FU, FC | 50 h |
| FU, FC, U<sub>6</sub>U<sub>7</sub>NH<sub>2</sub> | 50 h |
| S, FU, OMeC | 80 h |
| S, OMeU, OMeC | 80 h |
| S, dU, dC | 80 h |
| S, FU, FC | 80 h |

Fig. 5. Stability of modified ribozymes in cell culture media containing FCS. A, ribozyme MRE763C stabilized by introduction of three 3'-terminal and one 5'-terminal phosphothioate linkages and 2'-fluoro-2'-deoxycytidine was incubated in the presence of FCS for 5, 15, 30, 45, 60, 240, and 480 min (lanes 2–8). Aliquots were taken at the indicated time points and analyzed on a polyacrylamide gel (lane 1, control before addition of FCS). B, ribozyme MRE763C containing (in addition to the modifications mentioned in A) 2'-fluoro-2'-deoxycytidine groups and incubated with FCS for 4, 8, 24, 48, 72, 96, and 120 h (lanes 2–8).

TABLE IV

Catalytic efficiencies of different modified ribozymes with in vitro transcribed full-length N-ras RNA

| Ribozyme | Modification | \( K_m \) (relative) | \( k_{\text{cat}}/K_m \) (relative) |
|----------|-------------|---------------------|---------------------|
| RE917 | S | 59 ± 8.2 | 63 ± 20 |
| | S, FU | 88 ± 13.3 | 120 ± 38 |
| | S, dU, dC | 60 ± 7.4 | 400 ± 58 |
| | S, FU, OMeC | 20 ± 8.1 | 1420 ± 253 |
| | S, OMeU, OMeC | ND | ND |
| | FU, FC | 66 ± 8.1 | 202 ± 33 |
| MRE763C | FU, FC | 266 ± 25 | 71 ± 13 |
| | FU, FC, U<sub>6</sub>U<sub>7</sub>NH<sub>2</sub> | 173 ± 15.3 | 71 ± 21 |
| | S, FU, FC | 51 ± 9.8 | 44 ± 17 |
| MRE764U | FU, FC | 137 ± 12.2 | 113 ± 21 |
| | FU, FC, U<sub>6</sub>U<sub>7</sub>NH<sub>2</sub> | 173 ± 15.3 | 71 ± 21 |
| | S, FU, FC | 51 ± 9.8 | 44 ± 17 |

control for the RT-PCR was generated to estimate the degree of N-ras/luciferase mRNA cleavage during the extraction proto-
col. For this, a 50-bp oligonucleotide was cloned within the ras sequences in the expression vector pEAD3-LUCFUC. From this construct an in vitro transcribed RNA, 50 nucleotides longer than the N-ras/luciferase mRNA, was generated and served as internal control in the RT-PCR. The amplified region of the internal standard thus can be easily distinguished from the RT-PCR product originated from the N-ras/luciferase mRNA on agarose gels.

Total RNA obtained from the HeLa cell clone C#3 was mixed with the internal standard RNA at a molar ratio of approximately 1. The samples were treated with DNase I to destroy
any residual DNA remaining in the RNA preparation. At this stage any ribozyme-mediated cleavage of RNA will affect equally the internal standard transcript and the N-ras/luciferase target sequences. Upon reverse transcription and PCR, the internal standard control should generate a 450-bp-long PCR product, while amplification of a segment of the N-ras/luciferase fusion mRNA should generate a 394-bp DNA fragment. The RT-PCR done on RNA obtained from clone C#3 treated with the nonsense ribozyme gave the expected product of 394 bp (Fig. 8, lane 5). When the ethidium bromide intensity of this band was compared with that obtained from untreated cells (Fig. 8, lane 4), no significant reduction was observed, suggesting that treatment of the cells with a nonsense ribozyme does not reduce the amount of N-ras/luciferase transcripts. In addition, cleavage of the internal control was not observed (compare the ethidium bromide intensity of the 450-bp PCR product in Fig. 8, lanes 2 and 5). In contrast, treatment of cells with the active ribozyme MRE763C caused a significant reduction in N-ras transcripts. A densitometric scanning analysis of the PCR products observed in the agarose gel revealed at least a 10-fold reduction in the amounts of N-ras/luciferase mRNA. A reduction of the internal control was also observed, confirming the observations of others that during RNA extraction a cleavage of target RNA occurs (47, 48). However, only a 3-fold reduction in the amounts of control RNA were observed, suggesting that most of the N-ras/luciferase mRNA cleavage occurred inside the cells and not during the extraction procedure.

**DISCUSSION**

In the present study we examined the catalytic efficiencies, specificities, and intracellular activities of hammerhead ribozymes targeted against N-ras transcripts containing wild-type sequences or point mutations at codon 13. For short synthetic substrates, the $K_\text{m}$ values of MRE763C and MRE764U ranged between 65 and 82 nM and the $k_{\text{cat}}$ values between 0.7 min$^{-1}$ and 0.9 min$^{-1}$, respectively, resulting in catalytic efficiencies ($k_{\text{cat}}/K_\text{m}$) of 0.2-10$^6$ s$^{-1}$M$^{-1}$. The catalytic data obtained with the larger RNA substrate correlates with predictions of
FIG. 8. RT-PCR analysis of N-ras transcripts after treatment of HeLa cell clone C#3 with ribozyme MRE763C. A, schematic representation of the RT-PCR analysis of ribozyme MRE763C-mediated cleavage of transcripts in HeLa cells. B, RT-PCR analysis of RNA isolated from HeLa cells. Total RNA was isolated from HeLa cell clone C#3, mixed with the in vitro transcribed RNA (internal control), and digested with DNase I. Thereafter the RT-PCR was done as described under “Experimental Procedures.” Lane 1, size marker (pBlue-script DNA digested with HaeIII); lane 2, RT-PCR product obtained from 0.5 amol of internal standard RNA; lane 3, control reaction with cellular RNA in which reverse transcriptase was omitted from the RT-PCR; lane 4, RT-PCR product obtained from total cellular RNA before ribozyme treatment; lane 5, RT-PCR products obtained from RNA isolated from HeLa cell clone C#3 after treatment with the nonsense ribozyme; lane 6, RT-PCR products obtained from RNA isolated from HeLa cell clone C#3 after treatment with ribozyme MRE763C. The 450-bp DNA fragment correspond to the length of the product expected from the internal standard RNA after RT-PCR, while the 394-bp fragment correspond to the product of the RT-PCR of the cellular N-ras/luciferase mRNA transcript. The products of the RT-PCR were analyzed in a 4% NuSieve agarose gel after ethidium bromide staining.
the FOLD program, which suggests that the N-ras RNA folds into a secondary structure with a number of hairpins, double-stranded regions, bulges, and mismatches, which may hinder the binding of ribozymes to the target. The ribozymes RE917, MRE764U, and MRE763C, which show best catalytic properties under single-turnover conditions, bind to a region of the N-ras transcript composed of double-stranded structures interrupted by unpaired ribonucleotides. These mismatches produce particularly single-stranded regions where riboyme binding is facilitated. RE990 and RE1035, in contrast, show low cleavage efficiency, probably because their target sites are embedded within the stem of stable hairpin structures.

Ribozyme MRE763C was found to be extremely specific in vitro. A single ribonucleotide change at position 763 (C→G transversion) abolished completely cleavage by the ribozyme. Thus, riboyme MRE763C could be a very useful in the context of a clinical application, since it will cleave exclusively mutated N-ras transcripts but will dispense the wild-type counterparts.

For a clinical application of ribozymes, high catalytic efficiency, stability, and good availability has to be achieved. Rapid degradation of oligoribonucleotides in living cells diminishes availability of the ribozyme with a concomitant decrease in efficiency. Modifications such as terminal phosphorothioate or rapid degradation of oligoribonucleotides in living cells diminish these observations reveal that the 2'-O-methyl substitution causes complete catalytic inactivation of riboyme RE917 (Table IV). In contrast, replacement of 2'-O-methyl groups by amino or fluorine groups reduce the catalytic efficiency only 2-3-fold. Similarly, in a previous study Heidenreich et al. (30) found that a riboyme containing 2'-amino groups at positions 4 and 7 of the catalytic core was almost as active as its unmodified counterpart. This was explained by proton-donor and acceptor properties of the amino group, which are similar to those of the hydroxyl group. Thus, these observations reveal that the 2'-position of U₄ plays an essential role in the riboyme catalysis.

For the in vitro studies, modified ribozymes containing 2'-fluoro-2'-deoxyuridine/cytidine groups alone or in combination with terminal phosphorothioate linkages were used. The modified ribozymes were active ex vivo. The N-ras/luciferase reporter system used in our studies provides a very sensitive assay to detect riboyme activity, since the expression of the luciferase reporter gene depends on an intact N-ras sequence. This cell experiments revealed a reduction in luciferase activity of up to 55% in HeLa cells treated with MRE763C. A low but detectable antisense effect was observed since incubation of HeLa cells expressing mutant N-ras/luciferase fusion transcripts with the inactive forms of MRE763C or MRE764U resulted in a 20% reduction in luciferase activity. The cleavage activity of riboyme MRE763C was restricted to mutated N-ras sequences, as the luciferase activity in HeLa cells expressing a wild-type N-ras/luciferase transcript was not reduced above the levels expected from an antisense effect.

Estimates of riboyme activity ex vivo based on reporter assays have to take into account the half-life of the protein being measured, in our case a Ras/luciferase fusion protein. For example, a reduction in enzyme activity will not reflect faithfully the decrease in the amounts of the corresponding mRNA if the half-life of the protein is high. Since the half-life of a Ras/luciferase protein was not known, we estimated the cleavage efficiency at the RNA level by analyzing directly the amount of mRNA molecules cleaved by the riboyme MRE763C. For this type of analysis an internal RNA standard is required, since cleavage of the substrate may also occur during the RNA extraction protocol (47, 48). The amount of RT-PCR product obtained from the standard RNA can be estimated by densitometric evaluation of the ethidium bromide bands in the agarose gel and thus can be correlated to the amounts of internal standard RNA. At a standard concentration of 0.5 amol (3·10⁹ RNA molecules), equivalent RT-PCR signals from total cellular RNA and standard RNA were observed, suggesting that the amount of ras/luciferase mRNA expressed in this cell clone was roughly 3·10⁵ molecules. After treatment of the cells with riboyme MRE763C, no visible RT-PCR product was observed (Fig. 8, lane 6). Since the sensitivity of the RT-PCR reaction was 3·10⁵ RNA molecules, the amount of ras/luciferase transcripts in the riboyme-treated cells was reduced from 3·10⁵ to less than 30,000 molecules.

The usefulness of riboyme MRE763C for clinical purposes, for example within purging strategies for leukemic cells in the context of autologous bone marrow transplantation, still remains to be demonstrated. The experiments presented here provide a starting point on which clinical application of MRE763C could be based.

**Acknowledgments**—We thank O. Heidenreich, S. Klein, P. Marschall, and M. Schnee for critical reading of the manuscript and helpful discussions and H. Brill for the measurement of the mass spectra. The Georg-Speyer-Haus is supported by the Bundesministerium für Gesundheit and the Hessisches Ministerium für Wissenschaft und Kunst.

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