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SPECIFICITY OF RIBOZYME DESIGNED FOR MUTATED *DHFR* mRNA

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Abstract—When MOLT-3 human acute leukemia cells were exposed sequentially to trimetrexate (TMQ) and then to methotrexate (MTX), the cells became resistant to antifolate. We designated this subline MOLT-3/TMQ_{800}-$MTX_{10,000}$. This cell line was found to contain two point mutations in the dihydrofolate reductase (*DHFR*) gene: a T→C transition at nucleotide 95 in codon 31, and a T→A transition at nucleotide 100 in codon 33. In an attempt to specifically inhibit these double-mutated cells, we synthesized a ribozyme which perfectly base-paired with the double-mutated *DHFR* mRNA. We found that the ribozyme for the double-mutated *DHFR* mRNA not only cleaved the mutated *DHFR* RNA, but also efficiently cleaved the wild-type RNA substrate. This observation suggests proceeding with caution when using a ribozyme against a mutated mRNA of an essential enzyme as a specific means of treatment.

Key words: dihydrofolate reductase; ribozyme; mutation; methotrexate; trimetrexate; drug resistance

MTX†, an important folate antagonist used in cancer chemotherapy, is a part of standard treatment regimens for many neoplasms, including choriocarcinoma, ALL, non-Hodgkin's lymphoma, osteosarcoma, breast cancer, and head and neck cancer [1]. Its mechanism of action is the competitive inhibition of DHFR, the enzyme responsible for maintenance of intracellular reduced folate pools. The depletion of reduced folate pools results in the cessation of DNA synthesis caused by the lack of sufficient thymidylate and purines.

The clinical usefulness of MTX is hampered by the development of tumor cell resistance. Several mechanisms of resistance identified in a cell culture system include impaired membrane transport of MTX, decreased polyglutamation of MTX, overproduction of DHFR protein as a result of gene amplification and/or overexpression, as well as alterations in the affinity of DHFR to MTX caused by mutations of the *DHFR* gene [1–4].

As a part of our project to establish and characterize MOLT-3 human ALL cell sublines resistant to various antifolates, we developed a cell line resistant to both TMQ and MTX, MOLT-3/TMQ_{800}-$MTX_{10,000}$. As described below, this subline was found to have two point mutations that were close to each other in the *DHFR* gene.

In an attempt to specifically inhibit this leukemia subline, we synthesized a ribozyme that base-paired with the mutated *DHFR* mRNA. We reasoned that such a ribozyme would be specific only for the double-mutated *DHFR* mRNA and would not influence the wild-type *DHFR* mRNA. We found, however, that the ribozyme for the mutated *DHFR* mRNA also cleaved the wild-type RNA substrate efficiently. This observation suggests that investigators should exercise caution when using a ribozyme against a mutated mRNA of an essential enzyme as a specific means of treatment.

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† Abbreviations: ALL, acute lymphoblastic leukemia; DHFR, dihydrofolate reductase; FBS, fetal bovine serum; MTX, methotrexate; TMQ, trimetrexate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; and RT-PCR, reverse transcriptase-polymerase chain reaction.

MATERIALS AND METHODS

Cell lines. The establishment and characterization of the parent MOLT-3 human ALL cell line [5] and of antifolate-resistant sublines MOLT-3/TMQ_{800} (800-fold TMQ-resistant) [6] and MOLT-3/$MTX_{10,000}$ (10,000-fold MTX-resistant) [2] were described previously. These cell lines were maintained in RPMI-1640 medium containing 10% (v/v) heat-inactivated FBS and were fed twice a week with fresh medium.

For the establishment of the TMQ-MTX double-resistant subline, the MOLT-3/TMQ_{800} cells were exposed continuously to increasing concentrations of MTX up to 20 μM over 1 year. Cell kill effects of the drug were determined by the MTT assay [7] as previously described [8], and a concentration–response curve was drawn on a semi-log scale. The IC_{50} values (50% inhibitory concentration of a drug for cell growth) were then determined and used for calculating the degree of drug resistance. Since this new double-resistant subline maintained TMQ resistance at about 800-fold but MTX resistance increased to approximately 10,000-fold, as compared with the parent cells, the new subline was designated as MOLT-3/TMQ_{800}-$MTX_{10,000}$. The IC_{50} values of MTX for MOLT-3, MOLT-3/TMQ_{800}, MOLT-3/

$MTX_{10,000}$ and MOLT-3/TMQ_{800}-$MTX_{10,000}$ were (mean ± SD) 10 ± 2 nM, 70 ± 20 nM, 100 ± 10 μM and 100 ± 20 μM, respectively.

All the cell lines were maintained in the appropriate concentrations of selecting agents. Drug sensitivity was tested periodically, and the degree of drug resistance was confirmed. Possible Mycoplasma contamination was also determined periodically with the MycoTest Kit (Gibco BRL, Gaithersburg, MD), and all the cell lines were found to be Mycoplasma-free. Experiments were carried out after cells were grown in the drug-free medium for more than 2 weeks. Cells in a logarithmic growth phase with a viability (trypan blue dye exclusion method) of more than 90% were used.

Southern blot analysis of *DHFR* gene and Western blot analysis of DHFR in MOLT-3 sublines have been detailed previously [9].

RT-PCR, sequencing, cloning of PCR products and creation of substrate RNA. Messenger RNA was extracted from parent MOLT-3 cells, MOLT-3/TMQ_{800}, MOLT-3/$MTX_{10,000}$ and MOLT-3/TMX_{800}-$MTX_{10,000}$ cells by using a Micro-Fast Track mRNA Isolation Kit (Invitrogen, San Diego, CA). For the RT-PCR of *DHFR* transcripts, cDNAs were synthesized by using a cDNA Cycle Kit (Invitrogen) with oligo dT primer. Based on the reported full-length *DHFR* cDNA sequence [10], the 5′ half of the cDNA was amplified by using a GeneAmp PCR Core Reagent Kit (Perkin–Elmer Cetus, Norwalk, CT) with oligonucleotide primers, 5′-AGGTCCTCCCGCTGCTGTCA-3′ (sense primer, nucleotides −19 to + 1) and 5′-GAGCTCCTTGT-GGAGGTTCC-3′ (antisense primer, nucleotides 243–262). The clear and distinct 281-bp products were identified in 2% agarose gel and 1x Tris-borate EDTA buffer.

To create *DHFR* RNA substrate, the PCR products were ligated directly to pT7 Blue T-vector (Novagen, Madison, WI). After transformation of *Escherichia coli* (Novablue, Novagen), white colonies were selected and screened by PCR for orientation using the antisense primer mentioned above and the T7 promoter primer. Plasmid DNA was prepared by using a Qiagen Plasmid Midi Kit (Qiagen, Chatsworth, CA), and double-stranded DNA sequencing was performed, directly after heat denaturation, using a Sequenase Version 2.0 Kit [11]. At least six clones were sequenced for each cell line.

The clones that could produce the sense transcript were chosen, and *in vitro* transcriptions of RNAs were carried out from plasmid templates that contained T7 RNA polymerase promoter. The transcription reaction mixture contained 5 μg linearized plasmid DNA cut with *BamH*I, 0.5 U/μL T7 RNA polymerase (New England Biolabs, Beverly, MA), 40 mM Tris–HCl (ph 7.9), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, 10 mM dithiothreitol, 0.5 mM ATP, GTP and UTP, 0.1 mM CTP, 50 μCi of [α-^{32}P]CTP (sp. act. 800 Ci/mmol; Dupont, Boston, MA), and 1 U/μL recombinant ribonuclease inhibitor (Promega, Madison, WI) in a 50-μL volume. The reactions were carried out at 30° for 3 hr. Afterwards, samples were processed in the same manner as the ribozyme synthesis as described below. The 346 base RNAs so obtained were used for cleavage reaction in a cell-free system.

Ribozyme synthesis. In designing the hammerhead ribozyme for the mutated *DHFR* mRNA, we found that there were no GUC sequences, the sequence generally used for the design of a hammerhead ribozyme, in the vicinity of the two mutations. Instead, we found a UUC sequence 3 bases downstream of the second mutation. UUC was also shown to be cleaved by a hammerhead ribozyme at the same cleavage rate as that of the GUC targets [12]. The 3′ arm sequence of the ribozyme was designed to have perfect base-pairing for the two mutations (Fig. 1). The 3′ arm had 11 bases while the 5′ arm had only 8 bases.

The ribozyme RNA structure was modeled after that of Haseloff and Gerlach [13], and was synthesized by a method described by Milligan *et al.* [14]. Initially, two deoxyoligonucleotides were made by Genset (Clifton, NJ) through the Mount Sinai DNA Core Facility. The top strand contained the T7 RNA polymerase promoter region (5′-CATGTAATACGACTCACTATAGGG-3′). The bottom strand contained both the T7 RNA polymerase promoter region and the template sequence (5′-TCCAGAAATTTTTCGTCCTCAC-GGACTCATCAGCAGAGAATCCCTATA-GTGAGTCGTATTACATG-3′). Templates were prepared by heating the two DNA strands together to 80° for 2 min and then slowly cooling to room temperature. The transcription reaction mixture contained 12.5 pmol template, 1 U/μL T7 RNA polymerase, 40 mM Tris–HCl (pH 7.9), 12 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 2 mM ATP, UTP, GTP and CTP, and 1 U/μL recombinant ribonuclease inhibitor in a 50-μL volume. The reactions were carried out at 37° for 3 hr. The products were treated with RQ1 RNase-free DNase (Promega) followed by phenol-chloroform extraction and ammonium acetate ethanol precipitation.

Ribonuclease protection assay. *DHFR* mRNA was analyzed by the ribonuclease protection assay RPA II (Ambion, Austin, TX). The ^{32}P-labeled *DHFR* RNA probe used in this assay was synthesized (by *in vitro* transcription) from the opposite orientation of pT7 Blue T-vector containing RT-PCR product from MOLT-3/TMQ_{800}-$MTX_{10,000}$ cells, and then was gel purified.

In vitro *cleavage reactions.* *In vitro* cleavage reactions were carried out in the same manner as detailed previously [15]. Briefly, the ribozyme and substrate RNA were mixed in a 10-μL reaction volume containing 50 mM Tris–HCl (pH 7.5) and 1 mM EDTA. The mixture was heated to 95° for 2 min and quick-chilled on ice; various concentrations of $MgCl_2$ were added, and then the reaction tubes were incubated at 37° for various periods of time.

Formamide has been shown to increase the cleavage specificity by making mismatched pairing less stable [16]. To determine the effect of formamide, various concentrations of formamide were added to the reaction mixture, and the cleavage products were evaluated.

RESULTS

cDNA sequencing of MOLT-3/TMQ_{800}-

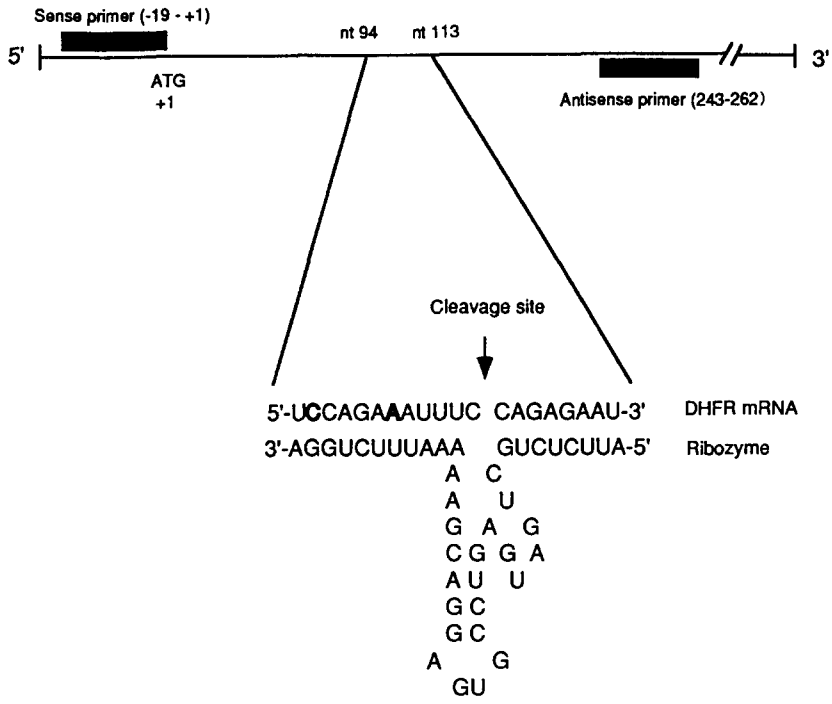

Fig. 1. Human *DHFR* cDNA and design of *DHFR* ribozyme. This hammerhead ribozyme was synthesized according to the model of Haseloff and Gerlach [13]. Two point mutations found in MOLT-3/TMQ_{800}-$MTX_{10,000}$ are indicated by bold letters.

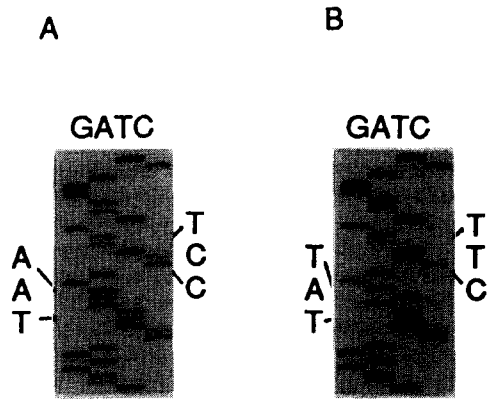

Fig. 2. cDNA sequences of the MOLT-3/TMQ_{800}-$MTX_{10,000}$ cells (A) or the MOLT-3/$MTX_{10,000}$ cells (B) in the target regions of the *DHFR* ribozyme. The sequencing template used was double-stranded pT7 Blue T-vector plasmid DNA containing the RT-PCR product of the *DHFR* mRNA (nucleotides from −19 to +262). Note two point mutations in the MOLT-3/TMQ_{800}-$MTX_{10,000}$.

$MTX_{10,000}$ cells revealed two point mutations in the *DHFR* cDNA (Fig. 2). No mutations were found in the parent MOLT-3 and MOLT-3/$MTX_{10,000}$ cells. The first mutation, a T→C base change at nucleotide 95 in codon 31, was identified previously in MOLT-3 cells resistant to TMQ [17]. The second mutation was identified as a T→A base change at nucleotide 100 in codon 33. This is a new mutation that was found only in the double-resistant subline. We observed that a T→C transition at nucleotide 95 mutation in codon 31 was present only in one allele of the MOLT-3/TMQ_{800} subline (data not shown), whereas in the double-resistant subline only the mutated allele was identified by direct sequencing of RT-PCR products, indicating that the mutant allele was dominantly expressed. These results confirmed that the double mutation was unique to the double-resistant subline.

Figure 3 shows the results of Southern blot analysis of *DHFR* gene in MOLT-3 sublines. Initially, MOLT-3/$MTX_{10,000}$ cells did not show *DHFR* gene amplification; however, they appeared to have developed amplification after prolonged exposure to MTX. MOLT-3/TMQ_{800} had an extra 7.6 kb band created by a loss of an *EcoR*I site due to a T→C transition at nucleotide 95, as previously reported [17]. Sequential development of MTX resistance in MOLT-3/TMQ_{800} cells resulted in an amplification of the extra 7.6 kb band. This observation indicates that the mutant allele of the gene was amplified in MOLT-3/TMQ_{800}-$MTX_{10,000}$ cells. Figure 4 shows the amount of the DHFR protein in MOLT-3 sublines. Both MOLT-3/$MTX_{10,000}$ and MOLT-3/TMQ_{800}-$MTX_{10,000}$ cells overproduced DHFR protein, the latter by a much higher amount than the former.

Figure 5 shows the results of a ribonuclease

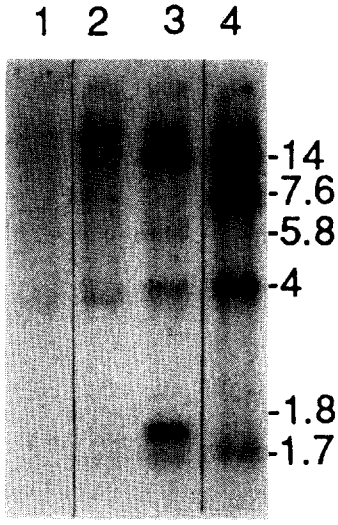

Fig. 3. Southern blot analysis of *DHFR* gene in MOLT-3 sublines. DNA (10 μg) from MOLT-3 sublines was digested with *EcoR*1 and analyzed by Southern blotting for *DHFR* gene. Lane 1, MOLT-3; lane 2, MOLT-3/TMQ_{800}; lane 3, MOLT-3/$MTX_{10,000}$; and lane 4, MOLT-3/TMQ_{800}-$MTX_{10,000}$.

protection assay using a ^{32}P-labeled *DHFR* RNA probe obtained from the double-resistant subline. This assay detected *DHFR* mRNA extracted from both MOLT-3/TMQ_{800}-$MTX_{10,000}$ and MOLT-3/$MTX_{10,000}$. Since the *DHFR* RNA probe contained two mutations, the *DHFR* mRNA from MOLT-3/$MTX_{10,000}$ was partially digested by ribonuclease at the two mismatched sites, producing three digested bands (162 bases, 114 bases and 5 bases). The smallest fragment between the two mismatches was not detected, probably due to its low radioactivity. Meanwhile, in the *DHFR* mRNA from MOLT-3/TMQ_{800}-$MTX_{10,000}$, only the 281-base band was visible, indicating that the double-mutated *DHFR* mRNA was dominantly expressed in MOLT-3/TMQ_{800}-$MTX_{10,000}$. This observation was consistent with the direct sequencing data of RT-PCR products from these cells.

In vitro transcription from the plasmid templates that contained the double mutant *DHFR* or the wild-type *DHFR* (MOLT-3/$MTX_{10,000}$) RT-PCR products generated 346-base substrates. These two substrates and the ribozyme were then used to compare the efficiency and specificity of cleavage reaction. The results of the cleavage reactions of the two *DHFR* substrates by the ribozyme in the cell-free system are illustrated in Fig. 6. Cleavage reaction was dependent on time, molar ratio of ribozyme/substrate, and Mg^{2+} concentration. The ribozyme cleaved both double-mutated and wild-type *DHFR* substrates. Comparison of the cleavage products of the two substrates revealed that the double-mutant *DHFR* substrate (the matched ribozyme–substrate complex) was cleaved a little faster than the wild-type substrate (mismatched ribozyme–substrate complex). At a 0.5 mM $MgCl_2$ concentration, which approximates physiological intracellular Mg^{2+} concentration [18], the double-mutated substrate was cleaved, while the wild-type substrate had no visible cleavage. After 5 min of incubation, the cleavage products were already seen with the double-mutated substrate, but not with the wild-type substrate. Thus, the cleavage efficiency was clearly higher with the double-mutated substrate; however, the overall differences of the cleavage reaction were not substantial.

Figure 7 shows the effect of formamide on the specificity of the cleavage reaction. The cleavage of the wild-type substrate was steadily decreased with increasing formamide concentrations, while the cleavage of the double-mutated substrate was unaffected.

DISCUSSION

In the present study, we found two point mutations of *DHFR* cDNA in MOLT-3 cells after exposure to TMQ and MTX sequentially. In our previous study, the base change (T→C) at nucleotide position 95 was found in MOLT-3 sublines resistant to TMQ [17]. This base change produces a substitution of serine (TCC) for phenylalanine (TTC). While the exact functional changes associated with this mutation have not been determined in this cell line, the same mutation developed in a human colon cancer cell line after exposure to MTX was reported to cause decreased DHFR affinity to MTX [3]. A new base change (T→A) at nucleotide position 100 produced a substitution of asparagine (AAT) for tyrosine (TAT). Since this region of DHFR enzyme appears important for the MTX affinity, the additional substitution may further influence the affinity of DHFR to MTX. The relationship between these

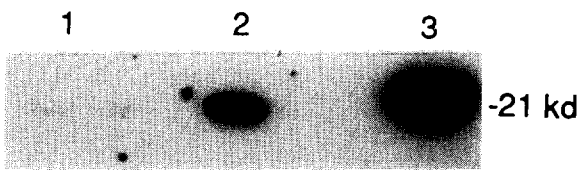

Fig. 4. Western blot analysis of DHFR in MOLT-3 sublines. Aliquots (100 μg) of protein were loaded per lane. Lane 1, MOLT-3/TMQ_{800}; lane 2, MOLT-3/$MTX_{10,000}$; and lane 3, MOLT-3/TMQ_{800}-$MTX_{10,000}$.

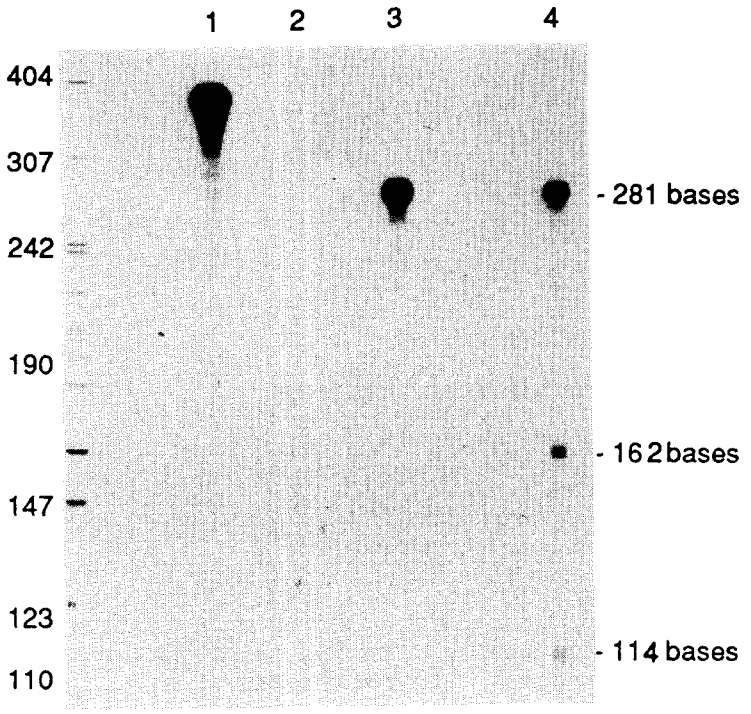

Fig. 5. RNase protection assay of *DHFR* mRNA in the MOLT-3/TMQ_{800}-$MTX_{10,000}$ or the MOLT-3/$MTX_{10,000}$ cells. mRNA (1 μg) from each cell was used. Positions of the protected fragments of *DHFR* as well as the size (nucleotides) of the molecular weight marker (radiolabeled *Msp*I digest of pBR322 DNA) are indicated. Lane 1, *DHFR* probe; lane 2, yeast RNA control; lane 3, MOLT-3/TMQ_{800}-$MTX_{10,000}$; and lane 4, MOLT-3/$MTX_{10,000}$.

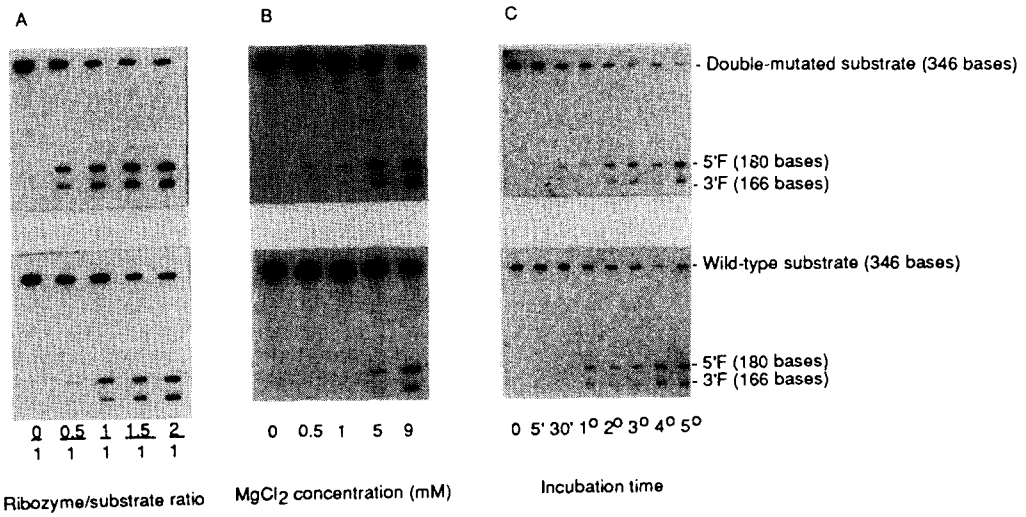

Fig. 6. Cleavage of *DHFR* RNA substrate (346 bases) by *DHFR* ribozyme to two cleavage products [180 bases 5′ fragment (5′F), and 166 bases 3′ fragment (3′F)]. Only the substrate was labeled with [α - ^{32}P] CTP (sp. act. 800 Ci/mmol). Panel A: Cleavage reaction with increasing proportions of the *DHFR* ribozyme. The ribozyme/substrate RNA ratio is shown on a molar basis. The reactions were run for 3 hr at 37° in the presence of 10 mM $MgCl_2$. Panel B: Mg^{2+} concentration vs cleavage activity. Equimolar amounts of *DHFR* ribozyme and substrate were incubated with various concentrations of $MgCl_2$ for 3 hr. Panel C: Reaction time vs activity profiles of the *DHFR* ribozyme. *DHFR* RNA substrate and a double molar amount of *DHFR* ribozyme were incubated for the indicated period of time (0 min to 5 hr) in the presence of 9 mM $MgCl_2$.

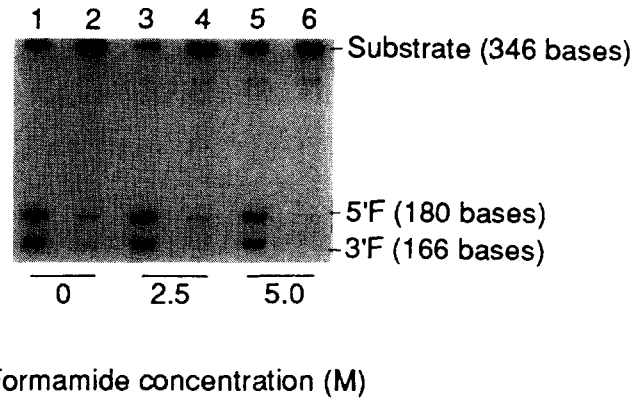

Fig. 7. Increased specificity of cleavage in the presence of formamide. *DHFR* RNA substrate and double molar amount of *DHFR* ribozyme were incubated for 3 hr at 37° in the presence of 9 mM $MgCl_2$ and the indicated concentrations of formamide. Lanes 1, 3 and 5: the double-mutated substrate (matched ribozyme–substrate complex); lanes 2, 4 and 6: the wild-type substrate (mismatched ribozyme–substrate complex).

amino acid substitutions and the affinity of DHFR protein to antifolates is currently under investigation.

MOLT-3/TMQ_{800}-$MTX_{10,000}$ cells dominantly expressed the double-mutated *DHFR* mRNA and overproduced the DHFR protein, which was a higher amount than that in MOLT-3/$MTX_{10,000}$ expressing only normal DHFR. Furthermore, our previous study showed that the uptake of MTX in MOLT-3/$MTX_{10,000}$ was decreased as compared with the parent cells [2], while the uptake of MTX in the double-resistant subline was not decreased (unpublished data). Thus, the overproduction of the double-mutated DHFR seems to be the major mechanism of MTX resistance in MOLT-3/TMQ_{800}-$MTX_{10,000}$ cells.

The mutated *DHFR* mRNA was found only in antifolate-resistant sublines. Since *DHFR* is an essential enzyme for normal as well as tumor cells and mutated *DHFR* appears to be present in tumor cells only, this appeared to be an ideal target for a specific blocking. One means to intercept target RNAs is to use a ribozyme, or a catalytic RNA. These RNAs possess the dual properties of RNA sequence-specific recognition, analogous to conventional antisense molecules, and site-specific cleavage of RNA substrate [13, 19–21]. The cleavage reaction is catalytic in that more than one substrate molecule is processed per ribozyme molecule. Ribozyme against HIV-1 is being developed as a possible drug for the treatment of AIDS [22]. Ribozyme against *ras* oncogene was shown to reverse malignant phenotype [23]. Ribozyme against *bcr-abl* mRNA retarded cell growth in a Philadelphia chromosome positive cell line [24]. These efforts are aimed at elevating ribozyme to such a level that it could be used as a specific therapeutic agent.

In an effort to make the double-mutated *DHFR* mRNA disabled, we created the hammerhead ribozyme designed to cleave the UUC sequence of the amino codon 34 of *DHFR* mRNA. The cleavage site is close to the two point mutations found in MOLT-3/TMQ_{800}-$MTX_{10,000}$. The 3′ arm sequence of the ribozyme had perfect base-pairing with the mutations.

Our results show that although the *DHFR* ribozyme designed against the double-mutated *DHFR* RNA cleaved the double-mutated *DHFR* RNA more efficiently than the normal *DHFR* RNA, the cleavage reaction was found to be of low specificity. Zang *et al.* [16] reported that by adding formamide or decreasing the divalent metal ion concentration from 10 to 2 mM, the substrate specificity was reversed, thereby allowing the *Tetrahymena* ribozyme to discriminate against the mismatched substrate. We observed that at low Mg^{2+} concentrations, or in the presence of formamide, the ability of the hammerhead ribozyme to distinguish the matched from the mismatched substrate definitely improved. Thus, our results are in accord with their report. The same authors also showed that at high Mg^{2+} concentrations in the absence of formamide, the *Tetrahymena* ribozyme cleaved the mismatched short oligonucleotide substrate more efficiently than the matched substrate. They speculated that mismatches destabilized ribozyme–substrate complex, which might result in the faster cleavage reaction in the mismatched short oligonucleotide substrate as compared with the matched substrate. Herschlag [25] observed, however, that adding more bases ultimately reduced this discrimination as cleavage occurred essentially every time the target RNA or a mismatched RNA bound ribozyme. Since the length of the flanking sequences in our ribozyme was 19 bases, our ribozyme may be classified as a case of long recognition sequence in terms of its low specificity at high Mg^{2+} concentrations in the absence of formamide.

He also reported that the maximum discrimination that could be obtained was expected to be greater with an A-U-rich recognition sequence than with a G-C-rich recognition sequence. This is because the weaker A-U base pairs (relative to G-C base pairs) allowed recognition to be spread over a larger

number of bases while preventing binding that was too strong [25]. While our ribozyme had A-U-rich recognition sequences, one of the mismatches was a U-G pair. Since the free-energy change for internal U-G pair is minimal [26], the discrimination against the wobbly U-G mismatch appears to be low. Another important factor to determine the specificity of a ribozyme is how close the mismatch is to the cleavage site. Joseph *et al.* [27] found that for the hairpin ribozyme, when the mismatches were close to the cleavage site less than 3 bases away, the specificity of the cleavage reaction was high. In contrast, when the mismatches occurred 4 or more bases away from the cleavage site, specificity was reduced significantly. In our hammerhead ribozyme, the mismatches occurred 6 and 11 bases away from the cleavage site, respectively, and the specificity was found not to be substantial.

Our results cast doubt as to the future of a ribozyme designed for a mutated mRNA as a therapeutic agent. Every attempt should be made to characterize the specificity of a ribozyme before it can be claimed as a specific drug. New efforts are needed to improve the specificity of ribozyme-mediated cleavage reaction.

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