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Ribozyme Mimics as Catalytic Antisense Reagents

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ABSTRACT

Viral and fungal infections and some cancers may be described as diseases that are characterized by the expression of certain unwanted proteins. They could be termed induced genetic disorders, with induction provided by mutation or infection. A comprehensive method to inactivate injurious genes based on their nucleic acid sequences has the potential to provide effective antiviral and anticancer agents with greatly reduced side effects. We describe a chemical approach to such gene-specific pharmaceutical agents. Our initial efforts have been to develop new chemical reagents that can carry out catalytic destruction of specific mRNA sequences. We chose hydrolysis as a chemical means of destruction, because hydrolysis is compatible with living cells. Our sequence-specific catalytic RNA hydrolysis reagents may be described as functional ribozyme mimics. Reactivity is provided by small-molecule catalysts, such as metal complexes. Specificity is provided by oligonucleotide probes. Here we report initial results on the sequence-specific, hydrolytic cleavage of mRNA from the HIV *gag* gene, using a ribozyme mimic. The reagent is composed of a terpyridylCu(II) complex for cleavage activity and an oligonucleotide for sequence specificity.

Index Entries: Hydrolytic cleavage of RNA, sequence-directed, by ribozyme mimic, copper(II) terpyridine-catalyzed; controlling gene expression, gene-specific drugs for, antisense method of, chemoselective reagents for.

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INTRODUCTION

The Antisense Method: A Systematic Approach to Antiviral Drugs

Infectious viral diseases, such as AIDS and influenza, currently pose a great threat to society. Because of the significant differences between viral and bacterial infections, no broad-spectrum pharmaceutical agents analogous to antibiotics are presently available for viral diseases. One potentially powerful, yet clinically unrealized, method for combating viral infections is the antisense technique (*1,2*). This method involves the specific interception of viral messenger RNA by a complementary nucleic acid. Application of antisense methods to the proper RNA sequences could halt the production of viral coat proteins, viral proteases, reverse transcriptases, and other viral gene products; at the same time, normal cellular protein synthesis would proceed unimpeded. In principle, the antisense technique is applicable to any disease that may be described as the overproduction of specific undesirable proteins.

Antisense regulation of gene expression is a natural regulatory process, and it has been recognized for well over a decade that certain prokaryotic organisms synthesize antisense nucleic acids as a means of inhibiting the expression of specific genes (*3*). More recently, examples of naturally occurring antisense inhibition for eukaryotes have been discovered (*4*). Furthermore, Izant and Weintraub (*6*) found that antisense sequences could be experimentally introduced into mammalian cells and that these sequences specifically inhibited expression of the genes against which they were directed.

The Chemical Antisense Approach

Following the initial discovery of inhibition of gene expression by endogenously synthesized antisense nucleic acids, a number of groups began to use chemically synthesized oligonucleotides as specific regulators of gene expression (*see* ref. 1 for a review). Inhibition has been measured directly, by quantifying the amount of protein produced, and also indirectly, by observing the expected physiological effect when synthesis of the target protein is inhibited. Successful inhibition of viral replication was reported for Rous sarcoma virus (*6*). Similar approaches have been used to inhibit replication of HIV (*7*) and numerous other viruses, including vesicular stomatitis virus, herpes simplex virus type 1, simian virus 40, and influenza virus (*1*).

Deficiencies of the Method

Although the chemical antisense method has been successful in cell culture, it has normally required high (e.g., 50 μM) concentrations of antisense probe to be effective (*2*). This lack of efficiency precludes any

practical therapeutic applications of the technique at the present time and may be attributed to:

1. Degradation of oligonucleotides by nuclease enzymes;
2. The inefficient transport of DNA across cell membranes; and
3. The reversible nature of the duplex structure formed by the antisense probe and target sequence.

Much important chemical research has been devoted to these topics, and nucleic acid analogs have been developed that have improved stability and uptake properties. Prominent examples include the methyl phosphonates, developed by Miller et al. (*8*).

Fundamental breakthroughs are still required before the therapeutic promise of the antisense technique is realized. However, it is important to recognize that the antisense method of controlling gene expression provides a reliable set of rules, fully functional under physiological conditions, for the systematic chemical synthesis of gene-specific drugs. Given this tremendous potential, it is imperative that an effective means of utilizing antisense technology be developed. Our approach to improving efficiency is to develop catalytic antisense reagents: they combine the great specificity of a natural gene-regulation mechanism with the power of chemical catalysis.

CATALYTIC ANTISENSE DNA

In 1986, we began to work on the idea of catalytic chemical antisense reagents, which is illustrated in Fig. 1 (*9a–k*). These reagents are designed to improve the efficiency of the chemical antisense technique, because each antisense probe is a catalyst capable of destroying many copies of the target mRNA. Stein and Cohen independently proposed that an oligonucleotide with a single pendant imidazole might function as a catalytic antisense agent (*2b*). With a catalytic drug, it should no longer be necessary to introduce large amounts of antisense probe into cells. Ultimately, we would want to couple the catalytic agents with the best advances in drug delivery and nuclease resistance to yield the most therapeutically promising molecule.

A different approach to catalytic antisense inhibition of gene expression was proposed in 1988 by Haselhoff and Gerlach (*10a*) and Cech (*10b*). Their approach is based on ribozymes, the catalytic RNA molecules discovered by Cech (*10c*) and Altman (*10d*). Ribozymes can be designed to bind and cleave target RNA sequences of interest, and the construction of ribozymes, which attack viral mRNA, may be a viable approach to antiviral therapy (*10e*). There are, however, a number of advantages that DNA- or DNA-analog-based reagents can offer. For example:

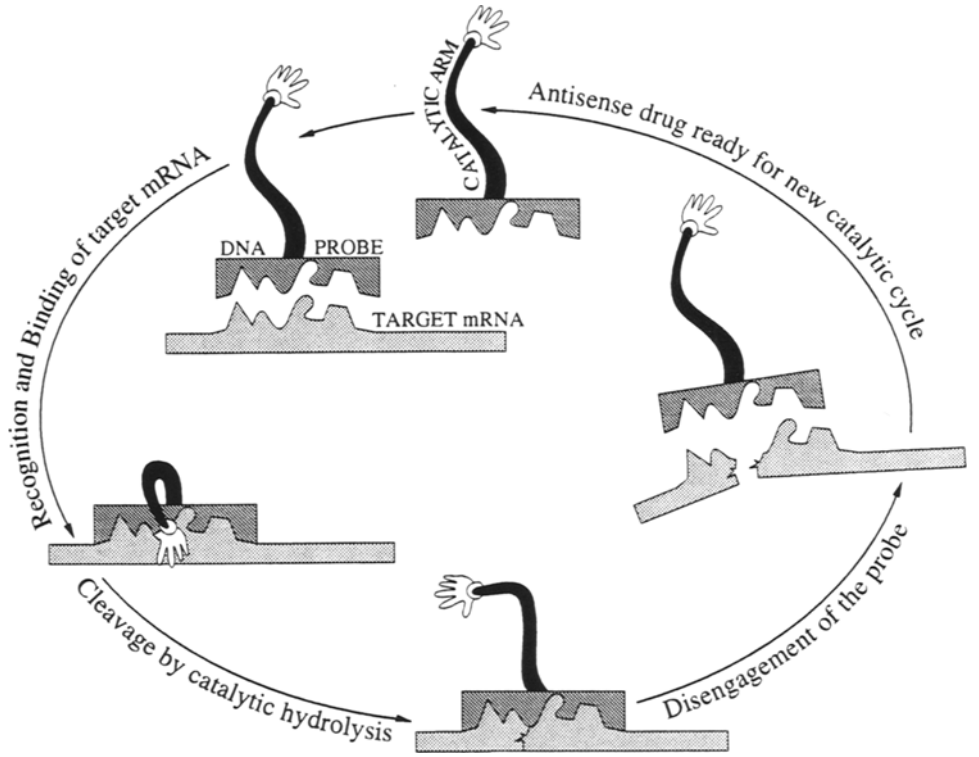

Fig. 1. Schematic view of catalytic antisense drugs. The DNA probe of the ribozyme mimic recognizes the complementary sequence in the target mRNA and binds to it. The catalyst attached to the DNA probe then cleaves the mRNA by a hydrolytic pathway. As a result of the cleavage within the duplex region, the binding of the DNA probe is lowered and the ribozyme mimic is released from the cleavage products. At this point, the ribozyme mimic is ready for a new catalytic cycle.

1. DNA is much more stable than RNA; RNA is readily degraded by small molecule catalysts and ubiquitous ribonuclease enzymes.
2. With our catalytic DNA molecules, the binding and cleavage functions are completely independent. This gives us the possibility of infinite variation of the catalyst during optimization studies, without disrupting the selective binding.
3. Since ribozymes perform both binding and cleavage with RNA residues in a complex, interdependent fashion, it may not be possible to optimize cleavage rates sufficiently for effective therapy.
4. Rates of ribozyme reactions are highly dependent on pH and the concentration of divalent cations, such as Mg^{2+}, and many perform optimally under nonphysiological conditions (*10a*).

5. DNA probes with pendant RNA cleavage catalysts can be designed to operate optimally at pH 7, and they can be designed to be independent of metal ion concentration, as will be described.

Our DNA-based catalytic antisense reagents may be described as functional mimics of ribozymes. They present several advantages over RNA-based ribozymes, including greatly enhanced chemical and enzymatic stability, and an inherent chemoselectivity that distinguishes the DNA-based reagent from its RNA target. This chemoselectivity allows the catalytic cleavage of target RNA sequences to take place, without the danger of catalyst self-destruction.

Redox Cleavage or Hydrolysis

There has been a great deal of recent interest in the cleavage of nucleic acids by reagents termed "chemical nucleases." Current fascination with this field derives from the importance of nucleic acid processing to living cells, and from information that cleavage experiments may provide about nucleic acid structure and the nature of protein–nucleic acid interactions. Most studies on the cleavage of DNA or RNA by chemical nucleases have employed oxidative scission as the cleavage reaction. For example, in a series of novel studies, Sigman demonstrated that bis(*o*-phenanthroline) copper(II) (Cu-*o*-phen) cleaves nucleic acids in an oxidative fashion when O_2 and mercaptopropionic acid are present (*11*). Dervan (*12*), Barton (*13*), and Tullius and Dombrowski (*14*) have all reported creative studies of nucleic acid cleavage by synthetic redox methods. The natural product bleomycin (*15*), an antitumor antibiotic, has also been shown to cleave nucleic acids oxidatively, with detailed isotope labeling studies of the reaction being reported by Kozarich et al. (*15b*). Clearly, oxidative cleavage of nucleic acids has been a fruitful reaction, helping to develop the state-of-the-art picture of molecular recognition and nucleic acid structure.

Rather than rely on previously developed redox cleavage methods, we have chosen to develop novel chemical nucleases that operate by hydrolytic mechanisms (*9*). A number of distinct advantages will result from having access to both oxidative and hydrolytic chemical nucleases, because of the inherently different chemical properties of the two reactions. Some of those properties are contrasted here:

1. Oxidation tends to be destructive, especially of the sugar portion of the DNA or RNA. For example, bleomycin degrades DNA by hydrogen atom abstraction from the deoxyribose, leading to a mixture of products (i.e., base propenal) that are not simple building blocks of the parent nucleic acid (*15b*). On the other hand, the hydrolysis of the phosphodiester backbone of a nucleic acid yields intact nucleoside phosphate and

sugar-hydroxyl groups that are good substrates for enzymatic transformations, such as religation.

2. Oxidative cleavage is not generally selective for DNA or RNA. Sequence-directed redox cleavage of RNA can be accomplished by attaching Cu-*o*-phen (*11d*), Fe(II)EDTA (*12*), or similar reagents to a DNA probe. These reagents usually operate by generating free hydroxyl radicals. However, the highly reactive nature of the hydroxyl radical leads to self-destruction of the DNA probe as well as destructive cleavage of the target sequence. Furthermore, there is no current strategy for turning off OH radical production prior to hybridization, so these toxic, diffusible radicals would be produced constantly.
3. Hydrolysis provides an excellent means of chemical discrimination between RNA and DNA. RNA is considerably more susceptible to chemical hydrolysis than DNA, because the 2′-hydroxyl group of RNA promotes hydrolysis by acting as an intramolecular nucleophile. We employed these different relative reactivities to develop a rationale for constructing sequence-directed agents for the catalytic hydrolysis of RNA: catalysts that are active for RNA hydrolysis can be attached to DNA probes without danger of self-destruction, because the rate of DNA hydrolysis will be immeasurably slow.
4. Considerable cell toxicity is associated with the ancillary reagents, such as hydrogen peroxide, that are required to drive most redox cleavage. For in vivo applications, it is difficult to image that external redox equivalents could be delivered to a cell without toxic side effects. Furthermore, the high reactivity of hydroxyl radicals renders them inherently toxic. In contrast, the water required for hydrolysis is readily available, and catalytic hydrolysis of phosphate esters at pH 7 is one of the fundamental reactions of cellular biochemistry.
5. Summary. Our approach to nucleic acid cleavage maintains the advantage of high sequence specificity exhibited by "oxidative nucleases," but avoids the chemical selectivity and toxicity drawbacks of free radical chemistry. This approach uses hydrolysis instead of hydroxyl radical damage as the cleavage mechanism. Hydrolysis at pH 7 is a common enzymatic reaction, and it is completely compatible with living cells. Since it is well known that RNA is much more susceptible to chemical hydrolysis than DNA, hydrolysis also provides us with a sound chemical basis for the preparation of long-lived, catalytic ribozyme mimics. Thus, it will be possible to construct catalysts that hydrolyze RNA at an appreciable rate, but that do not hydrolyze DNA. Attaching such a catalyst to a DNA probe would result in a sequence-specific ribonuclease that cannot self-destruct.

Only experimental success will demonstrate whether ribozyme mimics will provide an effective approach to gene-specific chemotherapy. However, the therapeutic promise of ribozyme mimics has led us to focus our efforts on preparation and testing of these molecules.

Sequence-Directed Hydrolysis of RNA

When our program was initiated, it was known through Breslow's extensive studies (*16*) that imidazole catalyzes the hydrolysis of RNA by a bifunctional mechanism. It was also known (*17*) that metal ions catalyze this reaction. To develop catalytic antisense reagents, we needed to identify well-defined catalyts, either metal complexes or imidazole derivatives, which could be attached to DNA. The steps we envisioned are given below.

1. Develop an assay for RNA hydrolysis.
   a. Assay under physiologically relevant conditions (pH 7, 37°C).
   b. Use true RNA substrates, not *p*-nitrophenylphosphates or other models.
   c. Develop assays that allow product isolation and characterization.
   d. Develop controls to demonstrate hydrolysis, and rule out redox or enzyme degradation.
   e. Develop site-specific cleavage assays.
2. Discover well-defined metal complexes that hydrolyze RNA.
   a. Screen likely candidate complexes for activity.
   b. Study the kinetics and mechanisms of active complexes.
   c. Develop the chemistry to attach active complexes to DNA probes.
   d. Use mechanistic results to design site-specific reagents, and test them.
   e. Develop improved catalysts with stabilities and activities suitable for in vivo work.
3. Develop synthetic methods to incorporate pendant imidazoles into oligonucleotides.
   a. Maintain compatibility with standard DNA synthesis techniques.
   b. Prepare pairs of pendant imidazoles to access bifunctional catalysis.
   c. Optimize hydrolytic activity to iterative cycles of assays and molecular design.
   d. Explore hybrid imidazole–metal complex reagents for improved activity/stability.
4. Demonstrate sequence-directed hydrolysis of RNA in three fully documented steps.

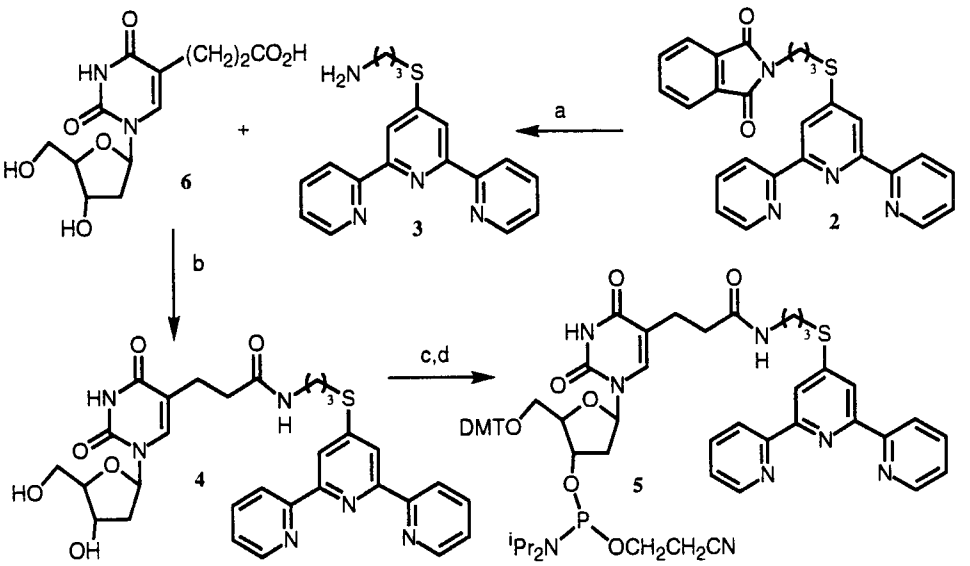

Fig. 2. Reaction scheme for synthesis of terpyridyl nucleoside phosphoramidite **5** from terpyridyl phthalimide **2.** The deprotection of **2** gave the terpyridylamine **3,** which when condensed with the nucleoside acid **6** gave the terpyridyl nucleoside **4.** The 5′OH of **4** was protected as its DMT derivative and the 3′-OH was phosphitylated. Reagents: a. Hydrazine, EtOH, 60°C, 96%; b. Ethyl-Dimethylaminopropyl Carbodiimide·HCl, DMSO, rt, 24 h, 77%; c. Pyridine, DMTCl, 24 h, 65%; d. $^{i}Pr_2NEt$, THF, 4°C, chloro-β-cyanoethyl-*N,N*-diisopropyl phosphoramidite, 86%.

a. Perform chemical assays, including mechanistic studies and product identification.
b. Optimize catalytic gene repression for in vitro translation systems. Use sequences known to be effective for viral inhibition.
c. Demonstrate and then optimize in vivo gene regulation by catalytic antisense agents.

Extensive progress has been made by our group in many of these areas (*9a–k*). Here we describe the preparation of a ribozyme mimic based on (terpyridyl)copper(II), a reagent that we had previously shown to be competent at the hydrolytic cleavage of RNA (*9c,e*). A preliminary account of this work has appeared recently (*9k*).

SYNTHESIS AND ACTIVITY OF THE RIBOZYME MIMIC

We incorporated the terpyridyl group into a DNA building block from the protected terpyridyl amine **2** (*9g*) as shown in Fig. 2. Attaching the terpyridine ligand through the C-5 position of 2′-deoxyuridine allows the modified nucleoside to remain competent for Watson-Crick base pairing (*18*). Nucleoside **4** was protected at the 5′ position and phosphitylated, and the resulting phosphoramidite **5** was used in the automated DNA synthesis of ribozyme mimic candidates.

A Sequence of RNA target

1(775)

5'-GGAGAAAUUU AUAAAAGAUG GAUAAUCCUG GGAUUAAAUA

AAAUAGUAAG AAUGUAUAGC CCUACCAGCA UUCUGGACAU

AAGACAAGGA CCAAAGGAAC **CCUUUAGAGA CUAUGUAG**AC

CGGUUCUAUA AAACUCUAAG AGCCGAGCAA GCUUCACAG-3'

159(933)

		1	20
B	Sequence of DNA target	5'-CCTTTAGAGACTATGTAGAC-3'	
C	Oligo X	5'-pCTACA**X**AGTCTCTAAAG-3'	
D	Oligo T	5'-pCTACA**T**AGTCTCTAAAG-3'	

Fig. 3. Sequence (**A**) is the 159-mer RNA fragment of a highly conserved region of the *gag* mRNA of HIV. The targeted region is highlighted in bold. Sequence (**B**) is a 20-mer DNA analog of the RNA target, which was used for the control experiments and cleavage studies. The sequence (**C**) is the ribozyme mimic, designated oligo X, with the terpyridine appended to nucleotide X. Sequence (**D**) is the control sequence analogous to oligo X, but lacking the terpyridine group. It is designated oligo T and has a thymine in place of nucleotide X.

Specific Cleavage Results

We chose as our initial RNA target a 159-mer RNA sequence derived from a conserved region of the HIV (HIVHXB2r) *gag* mRNA. This RNA sequence was prepared by transcription from a DNA template using T7 RNA polymerase. Two 17-mer oligodeoxynucleotides complementary to a region of the 159-mer RNA target were prepared for sequence-specific cleavage reactions and control studies. The ribozyme mimic 5′-pCTA CA**X** AGT CTC TAA AG-3′ (oligo X) was prepared using phosphoramidite **5,** where **X** indicates the modified nucleotide with the pendent terpyridine ligand. The **X** nucleotide is complementary to adenosine 113 of the RNA target. A control sequence (oligo T) was prepared with thymidine in place of **X.** The sequences of the RNA target and oligo's X and T are shown in Fig. 3.

Reactions were carried out with 5′[^{32}P]-end-labeled RNA, at a concentration of ca. $10^{-8}M$. Reaction products were separated by polyacrylamide gel electrophoresis under denaturing conditions, and the results are shown in Fig. 4. In the presence of Cu^{2+} ion, oligo X cleaves the RNA target at two specific sites in the region where oligo X and the RNA target are complementary (lanes 6 and 7). Cleavage occurs between U112 and A113 (major product) and between U114 and G115 (minor product). No cleavage was observed at the intervening site between A113 and U114. Nucleotide **X** is complementary to A113, and the cleavage reactions occur

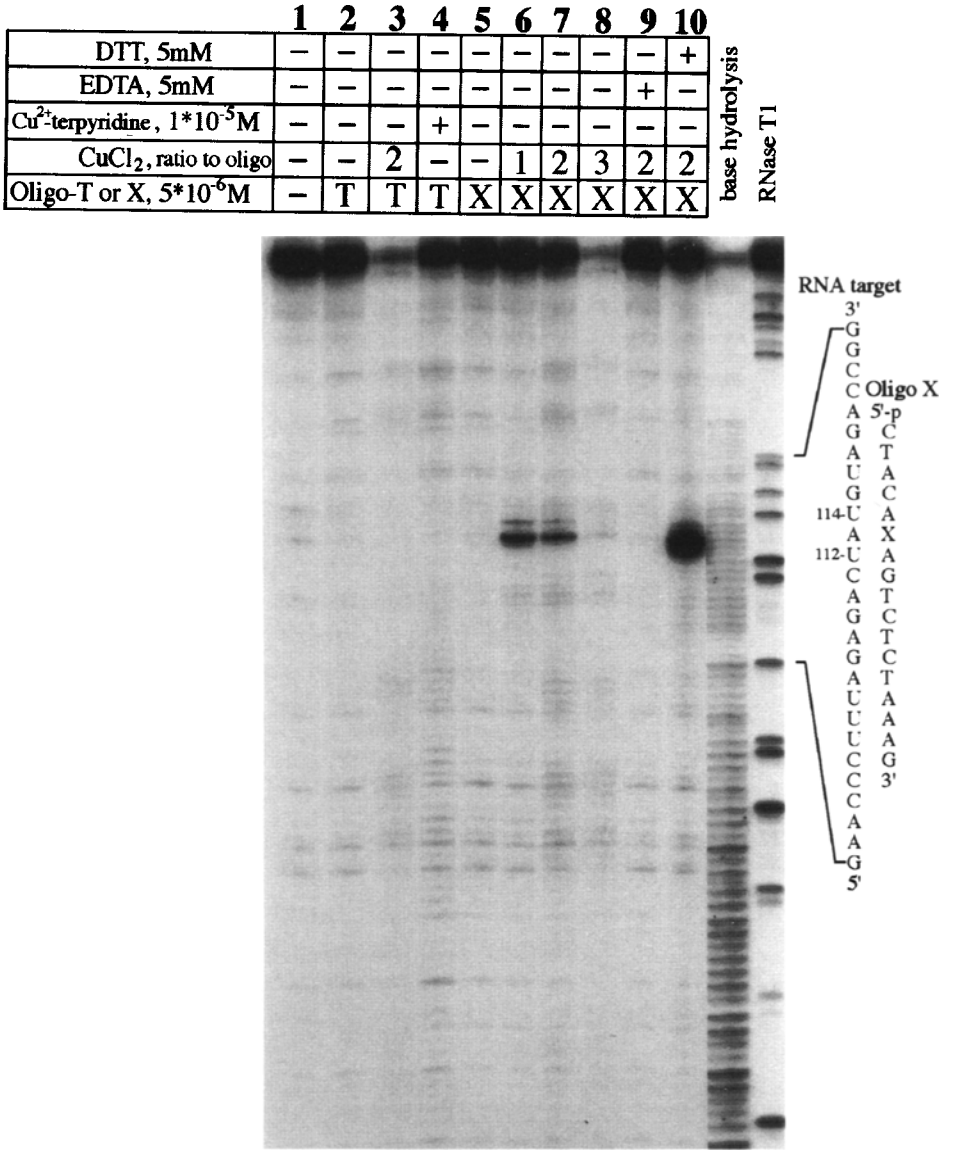

Fig. 4. Sequence-specific cleavage of the RNA target. Reactions were carried out with 5′[^{32}P]-end labeled RNA ($10^{-8}M$), and the reaction products were separated by PAGE under denaturing conditions. In control lane 1, no RNA cleavage was observed. In the presence of Oligo T, the control sequence, background cleavage owing to buffer catalysis was observed in lane 2. Lanes 3 and 4 indicate nonspecific cleavage of RNA by free aqueous $CuCl_2$ and Cu(II)terpyridine, respectively. Oligo X alone does not cleave RNA, as seen in lane 5. The addition of Cu^{2+} to oligo X resulted in cleavage at two specific sites between U112-A113 and U114-G115 (lanes 6 and 7). In lane 9, the addition of EDTA led to the suppression of cleavage. A wider range of cleavage was observed under oxidative conditions (in the presence of dithiothreitol) as shown in lane 10. The cleavage products were established by comparing them to the base hydrolysis and RNase T1 digestion lanes.

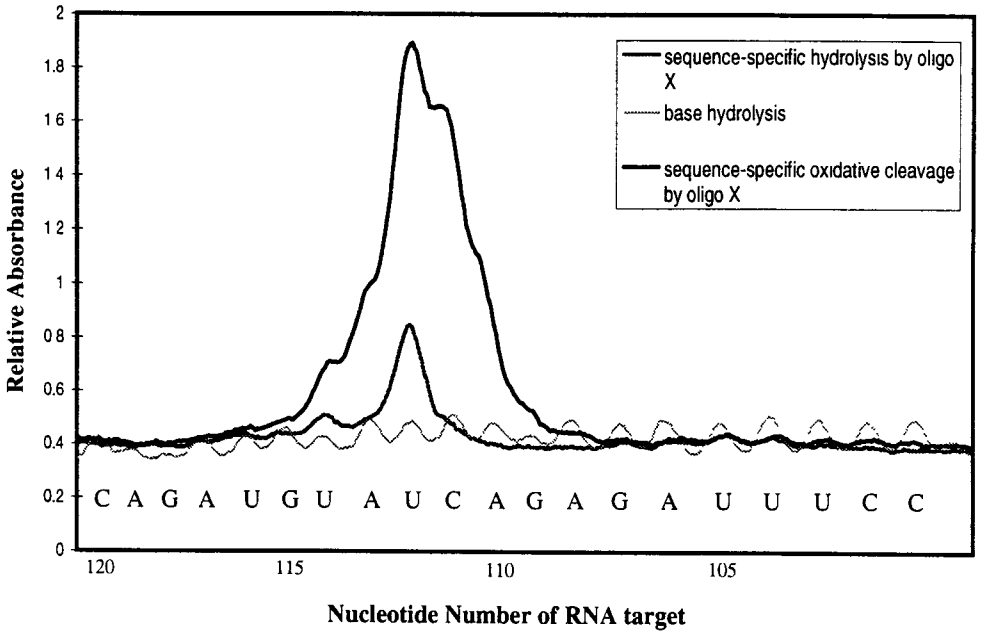

Fig. 5. Densitometry traces of key lanes from Fig. 4 showing sequence-specific hydrolytic cleavage, sequence-specific oxidative cleavage, and uniform base hydrolysis of RNA. *See* key below for the identity of products. The predominant hydrolytic cleavage of RNA takes place between U112 and A113. Oxidative cleavage takes place over a six-base region from G109 to G116.

immediately adjacent to this position. Densitometry traces of the cleavage lanes are given in Fig. 5.

No sequence-specific reaction occurred when oligo T and Cu^{2+} ion were added to the target RNA (lane 3), which indicates that the incorporated terpyridine (the X nucleotide) is necessary for the specific cleavage shown in lanes 6 and 7. Other control studies showed that oligo X failed to cleave RNA in the absence of Cu^{2+} ion (lane 5), and when both Cu^{2+} ion and excess EDTA were present (lane 9).

Sigman has shown that Cu(II) reagents can cleave nucleic acids by an oxidative mechanism (*11*). If an oxidative mechanism were operating, we would expect both DNA and RNA to be cleaved under the same conditions (*19*). Since, however, the hydrolytic cleavage of RNA occurs 10^6 times faster than DNA hydrolysis (*20*), we would expect no DNA hydrolysis to occur under conditions where RNA undergoes extensive hydrolytic cleavage.

The densitometry results in Fig. 6 show the results of cleavage studies on the DNA target whose sequence is given in Fig. 3B. Under the conditions where oligo X + $CuCl_2$ effectively and specifically cleaves RNA, the DNA target is not cleaved. Additionally, if the cleavage reaction is forced to proceed oxidatively by the introduction of DTT, the DNA is cleaved, giving a range of products. A similar result was found for the RNA target, as

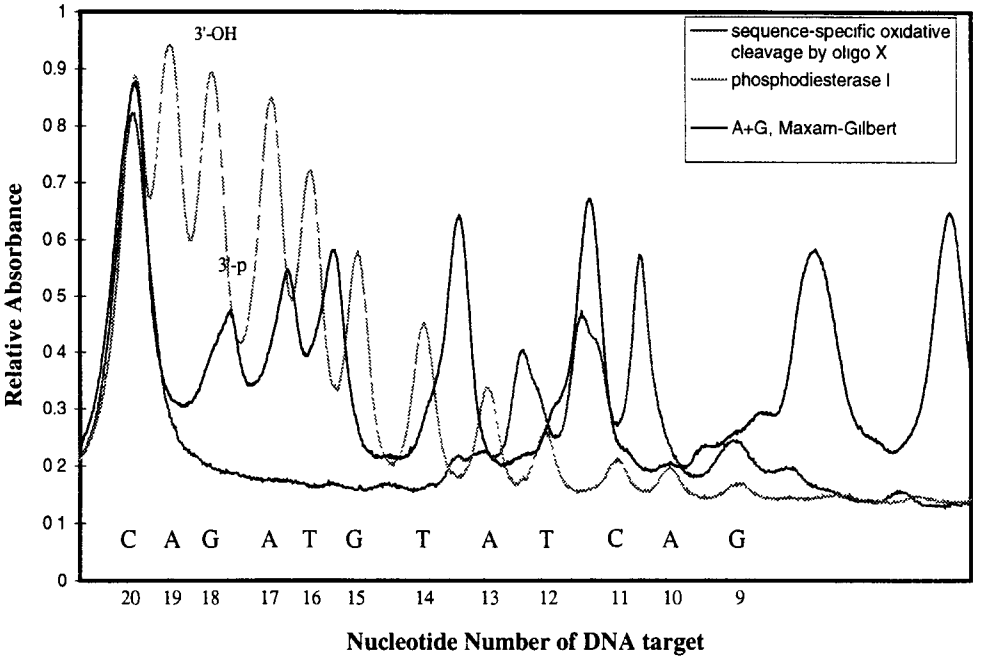

Fig. 6. Densitometry of the autoradiogram for reactions of the DNA target with oligo X under hydrolytic and oxidative conditions. The DNA target was cleaved by oligo X with Cu^{2+} in the presence of DTT, a reducing agent. The DNA target was not cleaved in the absence of DTT (data not shown). Thus, the DNA is inert under the same conditions that lead to sequence-specific hydrolytic cleavage of RNA.

shown in Fig. 5: when oxidative cleavage of RNA is forced to occur by the introduction of DTT, cleavage occurs over a range of five nucleotides. This contrasts with the precise cutting that occurs under hydrolytic conditions.

CONCLUSIONS

Our design of ribozyme mimics is based on the concept that a reagent capable of hydrolytic RNA cleavage can be linked to or incorporated in an oligodeoxynucleotide, thereby combining reactive and molecular recognition domains within one synthetic construct. Sequence-specific cleavage of the RNA target was observed for our initial ribozyme mimic, within the duplex region. Controls showed no evidence of oxidative cleavage and confirmed that the covalent incorporation of copper terpyridine into the oligodeoxynucleotide was required for activity. The new class of designed, synthetic enzyme reported here is characterized by the ability to control independently:

1. The active-site metal coordination (*21*) environment;
2. The identity of the active-site metal;

3. The distance between reactive center and recognition element; and
4. The level of complementarity between the target and probe.

The control afforded by chemical synthesis will be used to develop a detailed picture of the role of metals in ribozyme activity. Ribozyme mimics may have utility in RNA structure mapping, and as catalytic reagents for damaging injurious genomic or messenger RNA.

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21. We have reported the preparation of bipyridine building blocks for the construction of ribozyme mimics with bidentate instead of tridentate ligands. *See* ref. (*5*).

Note added in proof: Part of this study was recently communicated in: Bashkin, J. K., Frolova, E. I., and Sampath, U. (1994), *J. Am. Chem. Soc.* **116,** 5981–5982.