In Vitro Evolution of a Ribozyme that Contains 5-Bromouridine

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Dedicated to Prof. *Albert Eschenmoser* on the occasion of his 75th birthday

The *Tetrahymena* group I ribozyme was modified by replacing all 99 component uridine residues with 5-bromouridine. This resulted in a 13-fold reduction in catalytic efficiency in the RNA-catalyzed phosphoester-transfer reaction compared to the behavior of the unmodified ribozyme. A population of 10^{13} variant ribozymes was constructed, each containing 5-bromouridine in place of uridine. Five successive 'generations' of *in vitro* evolution were carried out, selecting for improved phosphoester transferase activity. The evolved molecules exhibited a 27-fold increase in catalytic efficiency compared to the wild-type bromouridine-containing ribozyme, even exceeding that of the wild-type ribozyme in the non-brominated form. Three specific mutations were found to be responsible for this altered behavior. These mutations enhanced activity in the context of 5-bromouridine, but were detrimental in the context of unmodified uridine. The evolved RNAs not only tolerated but came to exploit the presence of the nucleotide analogue in carrying out their catalytic function.

Introduction. – It has been suggested that, during the early history of life on earth, RNA played a dual role as both genetic material and chief agent of catalytic function [1]. This era, usually referred to as the 'RNA world' [2], is thought to have preceded the emergence of DNA genomes and instructed protein synthesis. In the RNA world, catalytic RNA would have been responsible for replicating and maintaining an RNA genome and, ultimately, inventing protein synthesis. The discovery of RNA enzymes (ribozymes) [3] lent considerable support to the RNA-world hypothesis. Over the past two decades, a variety of RNA enzymes have been described, including several that were found in nature and a much larger number that were generated in the laboratory through test-tube evolution (for recent reviews, see [4]). Among the demonstrated catalytic activities of RNA are: polymerization of nucleoside 5′-triphosphates [5], aminoacylation of RNA [6], *Diels-Alder* cycloaddition reactions [7], peptide-bond formation [8], and glycosidic-bond formation [9].

Despite these observations, in considering the catalytic potential of RNA, especially in relation to proteins, one is struck by the limited range of functional groups that exist among the four nucleotides. RNA lacks a good general acid-base (*e.g.*, histidine), cationic group (*e.g.*, lysine), nonpolar aliphatic group (*e.g.*, leucine), and cross-linking unit (*e.g.*, cysteine). These shortcomings may be offset by RNA's ability to bind divalent metal cations and other small-molecule cofactors that can assist in catalysis [10]. Alternatively, RNA may be covalently modified to acquire additional functionality. It has been shown, for example, that RNA can catalyze a self-modification reaction that results in covalent attachment of various coenzymes and coenzyme analogues, such as NAD^+ and dephosphorylated coenzyme A [11].

Another means of achieving functional diversification of RNA involves the replacement of one or more of the component nucleotides by a nucleotide analogue. This was first demonstrated by *Eaton* and co-workers, who generated a pool of random-sequence RNAs in which uridine was replaced by a 5-imidazole derivative of uridine. They then employed *in vitro* evolution to obtain amide synthase ribozymes that contained 5-imidazole uridine residues that were essential for catalysis [12]. In another study, uridine was replaced by a 5-pyridyl derivative of uridine and *in vitro* evolution was used to obtain Cu^{2+}-dependent *Diels-Alder*ase ribozymes [13]. A similar approach was taken in developing RNA-cleaving DNA enzymes that contain 5-imidazole deoxyuridine in place of thymidine [14].

In a prebiotic context, it is difficult to envision the availability of the four standard nucleotides without the coexistence of a variety of nucleotide analogues. The nucleotide base uracil, for example, would have been accompanied by 5-(hydroxymethyl)uracil, which, in turn, would have led to a variety of 5-substituted uracil analogues [15]. Thus, unlike evolution *in vitro*, evolution in the RNA world would have occurred in the context of a complex mixture of nucleotides and nucleotide analogues. These analogues may have been excluded from the genetic material during RNA replication, or perhaps incorporated only occasionally and in a random manner. Alternatively, the incorporation of a particular nucleotide analogue, either at specific locations or throughout a RNA molecule, may have conferred some functional advantage and, therefore, been favored by natural selection.

One of the best-studied contemporary RNA enzymes is the group I ribozyme derived from the pre-ribosomal RNA of *Tetrahymena thermophila*. This ribozyme catalyzes phosphoester transfer and phosphoester-hydrolysis reactions involving RNA substrates [16]. It is an efficient catalyst that adopts a complex structure [17] and has a sophisticated catalytic mechanism [18]. It provides a good test of the biochemical and evolutionary consequences of replacing one of the standard nucleotides within a pre-existing RNA enzyme with a nucleotide analogue.

A modified form of the *Tetrahymena* ribozyme was prepared in which all 99 component uridines were replaced with 5-bromouridine. The resulting 'bromoribozyme' exhibited a 13-fold reduced catalytic efficiency (k_{cat}/K_m) in the RNA-catalyzed phosphoester-transfer reaction compared to the unmodified ribozyme. A population of 10^{13} bromoribozyme variants was constructed and five successive 'generations' of *in vitro* evolution were carried out, selecting for improved phosphoester transferase activity. The evolved molecules exhibited a 27-fold increase in catalytic efficiency compared to the wild-type bromoribozyme, even exceeding that of the wild-type ribozyme in the non-brominated form. Three specific mutations were found to be responsible for this altered behavior. These mutations enhanced activity in the context of 5-bromouridine, but were detrimental in the context of unmodified uridine. Thus, the evolved RNAs not only tolerated, but came to exploit the presence of the nucleotide analogue in carrying out their catalytic function.

Results. – The *Tetrahymena* group I ribozyme catalyzes a Mg^{2+}-dependent phosphoester-transfer reaction in which a nucleophilic guanosine 3′-OH attacks a specific phosphodiester within a ribozyme-bound oligonucleotide substrate [19]. As a

consequence of this reaction, the guanosine becomes covalently attached to the 3′-portion of the substrate (located downstream from the targeted phosphodiester), while the 5′-portion of the substrate is released. If the nucleophilic guanosine is located at the 3′-end of the ribozyme, then any ribozyme molecule that performs the reaction will become 'tagged' by attachment of the 3′-portion of the oligonucleotide substrate to the 3′-end of the ribozyme [20]. This tag provides a selectable marker, allowing amplification of only those RNAs that have undergone the phosphoester transfer reaction (*Fig. 1*).

The present study employed a 5′-truncated form of the *Tetrahymena* ribozyme that contains 393 nucleotides (*Fig. 2*). The secondary and tertiary structure of this molecule is well known [17]. In the RNA-catalyzed phosphoester-transfer reaction in which the 3′-terminal guanosine of the ribozyme serves as the nucleophile, the catalytic efficiency of the ribozyme is 1.5×10^8 M^{-1} min^{-1}, measured under single-turnover conditions (see *Exper. Part*). The ribozyme also catalyzes a site-specific hydrolysis reaction [21], whereby the attached tag sequence is released, thus restoring the 3′-terminus. The rate of RNA-catalyzed site-specific hydrolysis is 0.12 min^{-1}.

A modified form of the ribozyme was prepared in which all 99 uridine residues were replaced with 5-bromouridine. This was accomplished by *in vitro* transcription of the RNA in the presence of ATP, CTP, GTP, and 5-Br-UTP. The portion of the molecule that binds the oligonucleotide substrate (P1 helix) contains only purine residues and thus is not directly affected by bromouridine substitution. Each of the other helical elements, however, contains at least one uridine, some of which are known to be essential for catalysis [22]. Surprisingly, the brominated form of the ribozyme retained substantial phosphoester transfer activity, with a k_{cat}/K_m of 1.2×10^7 M^{-1} min^{-1}. This is reduced by *ca.* 13-fold compared to the non-brominated form. The brominated ribozyme was much less active in the site-specific hydrolysis reaction, with a catalytic rate of 0.00045 min^{-1}. This is reduced by about 270-fold compared to the hydrolysis rate of the non-brominated ribozyme.

A pool of 2×10^{13} variant ribozymes was generated, each containing 5-Br substitutions at each of the component uridine residues. Variation was provided by random mutations that were introduced at a frequency of 5% per nucleotide position over 140 nucleotide positions that encompass the catalytic core of the ribozyme (*Fig. 2*). This resulted in a population of molecules that contained an average of seven mutations relative to the wild type and included all possible 1-, 2-, 3-, 4-, and 5-error mutants [23]. The population was challenged to catalyze a phosphoester-transfer reaction involving an RNA substrate. Reacted ribozymes were selectively amplified to produce a 'progeny' population that was enriched for the desired activity (*Fig. 1*). This process was repeated for five successive rounds, each time preparing the ribozymes with 5-bromouridine in place of uridine.

By the fifth round, the population as a whole exhibited enhanced phosphoester-transfer activity compared to the starting molecule. Individuals were cloned from the population, and the nucleotide sequences of 30 of these clones were determined (*Fig. 3*). A variety of mutations were found to occur among the cloned individuals. These included three specific mutations that occurred with high frequency: an A → Y (Y = C or U) change at position 94, a C → D (D = A, G, or U) change at position 193, and an A → G change at position 210.

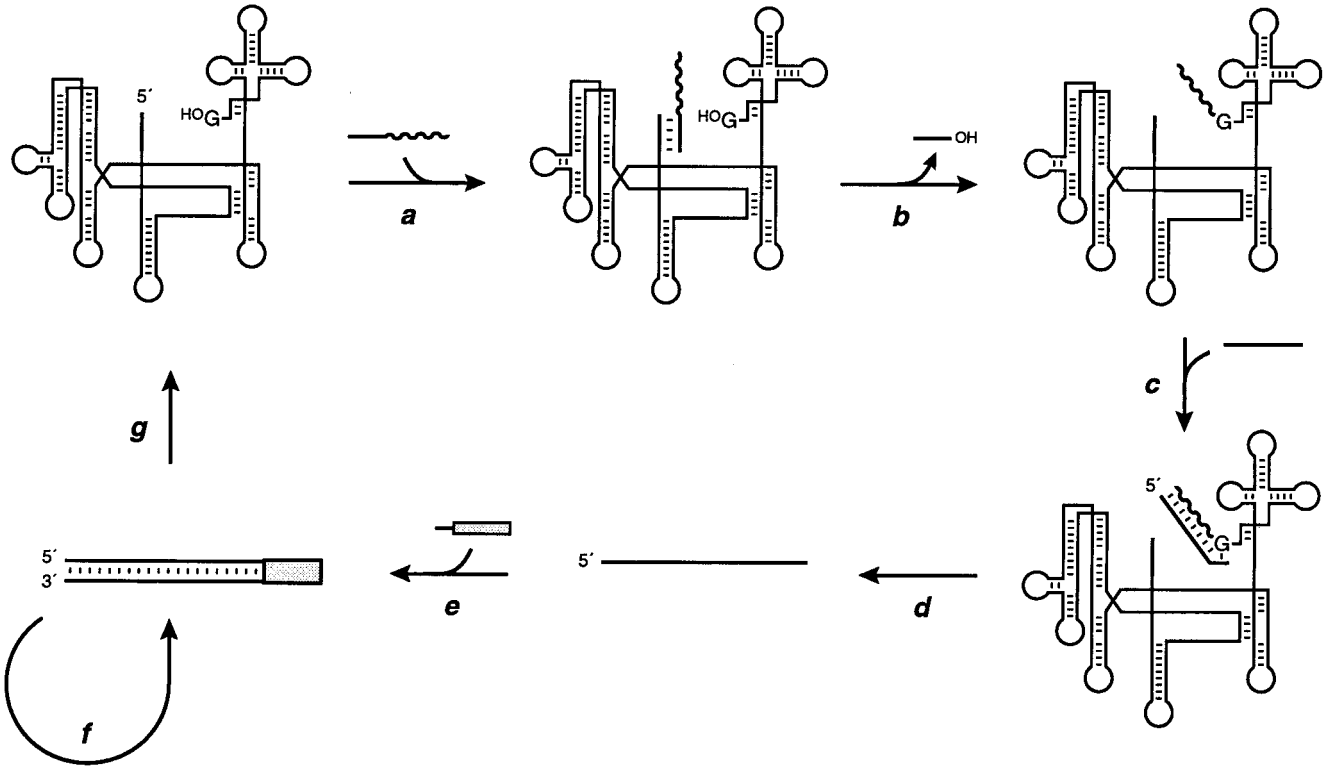

Fig. 1. *Scheme for* in vitro *evolution of ribozymes. a*) The ribozyme binds an RNA substrate through *Watson-Crick* pairing. *b*) RNA-Catalyzed phosphoester transfer results in attachment of the 3′-portion of the substrate to the 3′-end of the ribozyme. *c*) An oligodeoxynucleotide primer binds in a selective manner to the 3′-end of reacted ribozymes. *d*) The primer is extended by reverse transcriptase to generate cDNA. *e*) A second primer, containing the promoter sequence of T7 RNA polymerase (shaded box), binds to the 3′-end of the cDNA and is extended by reverse transcriptase to yield double-stranded DNA. *f*) Non-selective PCR amplification allows the introduction of random mutations and restores the 3′-terminus of the ribozyme. *g*) *In vitro* transcription generates a progeny population of ribozymes.

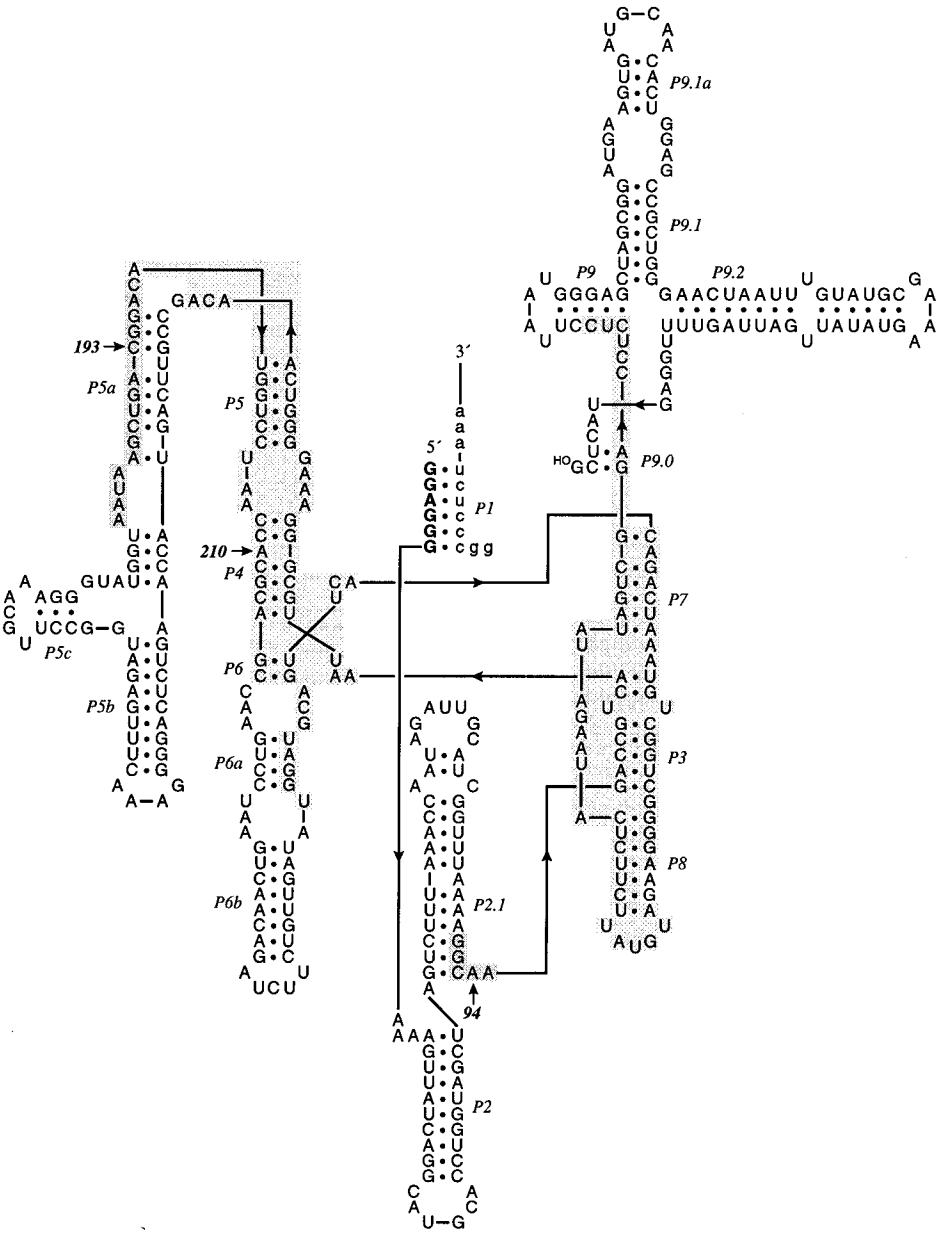

Fig. 2. *Secondary structure of the L-21 form of the* Tetrahymena *ribozyme.* The RNA substrate is shown in lowercase letters and the portion of the ribozyme that binds the substrate is shown in bold. Nucleotide positions that were partially randomized in the initial population are indicated by shaded regions. Paired structural elements are designated by *Pi*. Nucleotide positions that are discussed in the text are numbered.

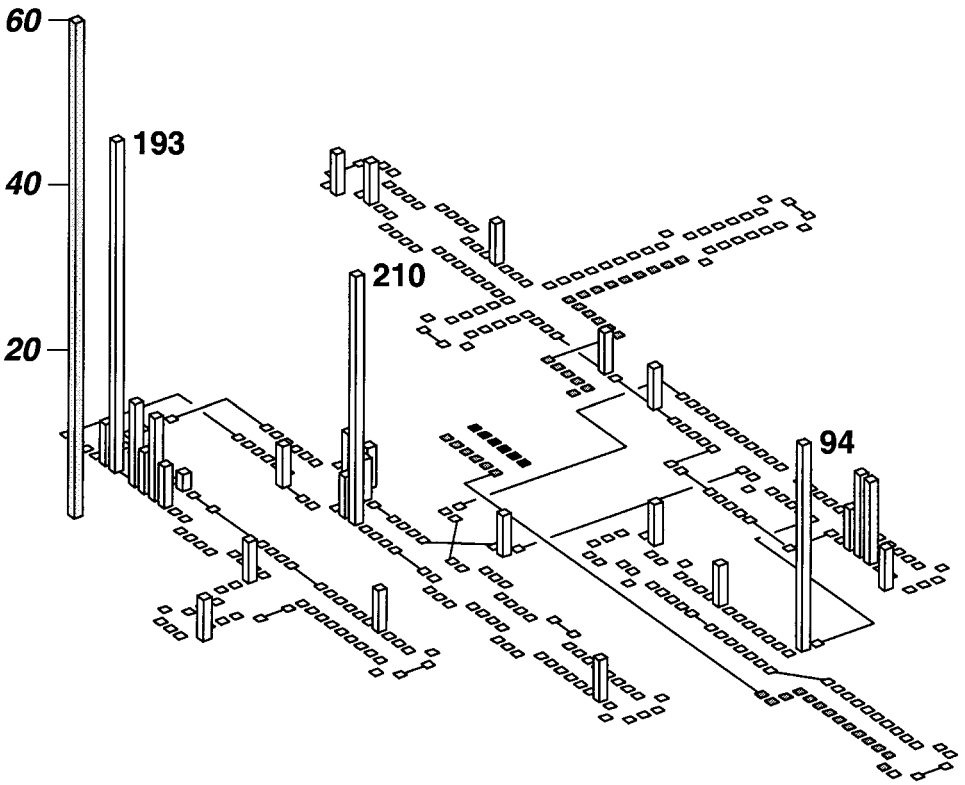

Fig. 3. *Mutations that occurred among individuals isolated from the final evolved population of ribozymes.* Box height corresponds to the frequency of mutations (%) at each nucleotide position, based on 30 clones that were sequenced. Common mutations are labeled. Nonmutable primer binding sites are shown in gray.

Employing site-directed mutagenesis, three different ribozymes were prepared, each containing only one of the three commonly occurring mutations. The 210: A → G mutation was found to be largely responsible for the improved activity of the brominated ribozyme. The catalytic efficiency of this single-error mutant in the fully-brominated form was 3.2×10^8 M^{-1} min^{-1}. When the same mutant was prepared with unmodified uridines, its catalytic efficiency was only 1.1×10^8 M^{-1} min^{-1} (*Fig. 4*).

Discussion. – One approach to enhancing the catalytic potential of RNA involves replacing one or more of the component nucleotides with a nucleotide analogue that carries a substituent with added functionality. Previous studies involving both RNA and DNA enzymes have focused on C(5) of pyrimidines as a relatively innocuous location at which such modifications might be introduced [12–14]. A variety of substitutions can be made at this position without disrupting *Watson-Crick* pairing, although only a small subset of these are tolerated by the polymerase protein that is used to incorporate the corresponding nucleoside 5′-triphosphate analogue [24].

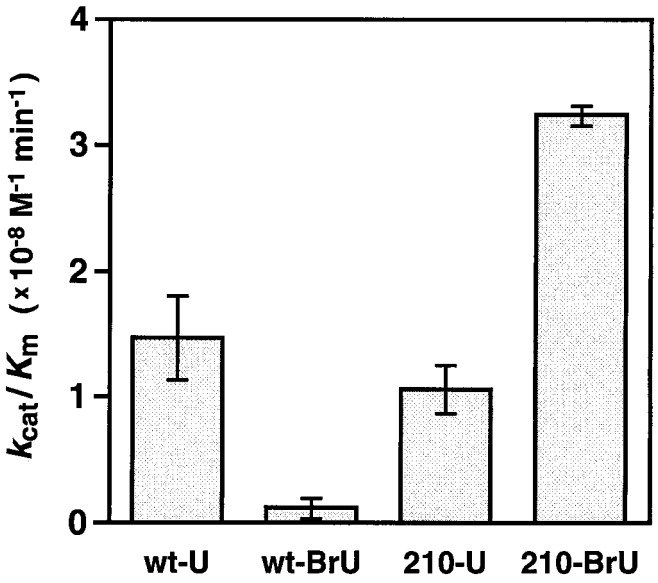

Fig. 4. *Comparison of the second-order rate constant of the wild-type and 210:A → G mutant ribozyme, transcribed in the presence of either UTP or 5-Br-UTP.* k_{cat}/K_m Values were obtained under single-turnover conditions, employing 0.05 – 1 nM ribozyme, 0.008 nM [5′-^{32}P]-labeled RNA substrate, 10 mM $MgCl_2$, and 30 mM EPPS (pH 7.5), incubated at 37°.

The present study addressed a different situation in which a pre-existing RNA enzyme, composed of the four standard nucleotides, was modified by replacing one of the component nucleotides with a nucleotide analogue. In an evolutionary context, this corresponds to a sudden change in the environment, whereby an essential natural resource is replaced by a different but related resource. For an RNA-based evolving system, the replacement of one of the four nucleotides could have both genotypic and phenotypic consequences. Alteration of the genetic material might alter the efficiency or fidelity of replication, while alteration of the corresponding catalyst might affect its structure and function.

The choice of 5-bromouridine as a replacement for uridine was a conservative one. 5-Br-UTP is incorporated readily by T7 RNA polymerase. 5-Bromouridine is copied with slightly lower fidelity compared to uridine, because the former has a greater propensity to form G·U wobble pairs [25]. However, this is not likely to make a significant difference over the modest length of the ribozyme. The effect of bromouridine substitution on RNA catalysis was more significant, resulting in a 13-fold reduction in phosphoester transfer activity compared to the behavior of the unmodified ribozyme. Yet the modified ribozyme retained substantial activity, demonstrating that none of its uridine C(5)-positions are critical for catalysis.

After five generations of *in vitro* evolution, a 5-bromouridine-containing ribozyme was obtained that exhibited a 27-fold increase in phosphoester-transfer activity compared to the starting ribozyme. Three mutations were found to occur frequently within the evolved population of ribozymes. Interestingly, none of these was at a 5-bromouridine position or even at a position that pairs with 5-bromouridine. Based on

site-directed-mutagenesis analysis, the 210: A → G mutation was shown to be largely responsible for the improved activity of the evolved ribozyme. This is an unpaired position within the P4 stem that forms part of the catalytic core of the molecule. In the crystal structure of the P4 – P6 domain of the *Tetrahymena* ribozyme, the adenosine at position 210 is flipped out of the helix and engages in crystal packing contacts with two adenosine residues located between the P5 and P5a stems of another molecule [17]. Replacing the adenosine at position 210 by guanosine would disrupt these contacts, but is not likely to have a large effect on the structure of the ribozyme in solution.

This study demonstrates that, through *in vitro* evolution, it is possible to develop ribozymes that not only tolerate the inclusion of modified nucleotides but also exploit these modifications to achieve enhanced catalytic function. Inclusion of 5-bromouridine residues is not expected to greatly augment the catalytic potential of RNA, although it does provide potential alkylation sites, as well as heavy-atom derivatives that might be useful for crystallographic analysis. In the RNA world, faced with an ensemble of 5-substituted pyrimidines, *Darwinian* evolution appears capable of accommodating both standard and modified nucleotides while maintaining RNA-based catalytic function.

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Experimental Part

Materials. Unlabeled nucleoside 5′-triphosphates (NTPs) and deoxynucleoside 5′-triphosphates (dNTPs) were purchased from *Pharmacia*, 5-bromouridine 5′-triphosphate (5-Br-UTP) was from *Boehringer*, and dideoxynucleoside 5′-triphosphates (ddNTPs) were from *U.S. Biochemical*. [α-^{32}P]GTP, [γ-^{32}P]ATP, and [^{3}H]UTP were from *ICN Radiochemicals.* Synthetic oligodeoxynucleotides were obtained from *Operon Technologies* and purified by polyacrylamide gel electrophoresis and subsequent affinity chromatography on *DuPont NENSORB.*

Taq DNA Polymerase was purchased from *Cetus*, avian myeloblastosis virus reverse transcriptase was from *Life Sciences*, Moloney murine leukemia virus reverse transcriptase and modified T7 DNA polymerase (Sequenase) were from *U.S. Biochemical*, calf-intestine phosphatase was from *Boehringer*, and restriction enzymes and T4 polynucleotide kinase were from *New England Biolabs.* T7 RNA Polymerase was prepared from the cloned gene [26] and purified according to a procedure originally developed for SP6 RNA polymerase [27].

The RNA substrate, 5′-GGCCCUCUAAAUAAAUAAAUAAA-3′, was prepared by *in vitro* transcription of a synthetic, partially double-stranded DNA template [28]. The L-21 form of the *Tetrahymena* ribozyme was prepared by *in vitro* transcription of *Hin*dIII-digested pT7L-21 plasmid DNA [29]. The transcription mixture contained 0.1 μg/μl of linearized plasmid, 25 U/μl T7 RNA polymerase, 2 mM of each NTP, 15 mM $MgCl_2$, 2 mM spermidine, 5 mM dithiothreitol, and 50 mM Tris (pH 7.5), which was incubated at 37° for 2 h.

Preparation of the Starting Pool of Variants. Four mutagenic oligodeoxynucleotides were prepared by chemical synthesis, each containing 35 nucleotide positions that had been randomized at 5% degeneracy. These were purified by denaturing polyacylamide gel electrophoresis and 5′-phosphorylated with T4 polynucleotide kinase. Plasmid pT7L-21 was linearized with *Hin*dIII, then digested with T7 gene 6 exonuclease to remove the coding strand of the ribozyme gene [30]. Digested plasmid (10 pmol) was allowed to hybridize with 50 pmol of each of the four mutagenic oligodeoxynucleotides and 50 pmol of an oligodeoxynucleotide that was complementary to 20 nucleotides at the 3′-end of the ribozyme. The hybridization was carried out in a mixture containing 2 mM $MgCl_2$, 50 mM NaCl, and 20 mM *Tris* (pH 7.5), which was incubated at 70° for 5 min, then slowly cooled to 25° over 45 min. The annealed oligodeoxynucleotides were extended and ligated in a mixture containing 0.125 U/μl T4 DNA polymerase, 0.25 U/μl T4 DNA ligase, 0.2 mM of each dNTP, 0.4 mM ATP, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 10 mM *Tris* (pH 7.5), which was incubated at 37° for 90 min. Approximately 6 pmol of the resulting double-stranded DNA was used in an *in vitro* transcription reaction to generate the

starting pool of RNA. The RNA was purified by polyacrylamide gel electrophoresis and subsequent *Sephadex* chromatography. The final yield of RNA was 200 pmol.

Selective Amplification Procedure. A sample of the ribozyme population (10 pmol) was added to a reaction mixture containing 50 pmol RNA substrate, 10 mM $MgCl_2$, and 30 mM EPPS (pH 7.5), which was incubated at 37° for 2 h. The ribozyme-containing products were separated from the unreacted substrate by polyacrylamide gel electrophoresis, eluted from the gel, and purified by affinity chromatography on *DuPont NENSORB*. The purified material was subjected to selective isothermal amplification, followed by cDNA synthesis and non-selective PCR amplification [23][31]. The subsequent ribozyme population was generated by *in vitro* transcription of 2 pmol of the PCR products in the presence of [^{3}H]UTP.

Cloning and Sequencing. Products of selective cDNA synthesis were amplified by the PCR using primers that contained unique *Eco*RI and *Hin*dIII sites, suitable for cloning into the pUC18 plasmid. Recombinant plasmid DNA was used to transform competent DH5α-F′ *E. coli* cells, which were grown on carbenicillin-containing plates. Colonies of successful transformants were chosen at random and grown overnight in LB media. Plasmid DNA was prepared by the boiling lysis method [32] and screened for the presence of insert by restriction digestion. The DNA was sequenced by the dideoxy chain-termination method [33]. Individual ribozymes were prepared by PCR amplification of the plasmid DNA, followed by *in vitro* transcription of 3 pmols of the PCR products in the presence of [^{3}H]UTP. The RNA was purified by polyacrylamide gel electrophoresis and subsequent affinity chromatography on *DuPont NENSORB*.

Site-directed mutagenesis was carried out on the non-coding strand of plasmid DNA obtained by T7 gene 6 exonuclease digestion and an appropriate mutagenic oligodeoxynucleotide [30], similar to the procedure used to generate the starting pool of ribozyme variants. The mutant RNAs were reverse transcribed, PCR amplified, cloned, and sequenced, as described above.

Kinetic Analysis. RNA-Catalyzed phosphoester-transfer reactions were carried out in the presence of 10 mM $MgCl_2$ and 30 mM EPPS (pH 7.5) at 37°, employing [5′-^{32}P]-labeled RNA substrate and excess ribozyme. The substrate and ribozyme were preincubated separately for 15 min at 37°, then mixed to initiate the reaction. Aliquots were withdrawn at various times and quenched by the addition of an equal volume of an ice-cold mixture containing 8M urea, 50 mM Na_2(EDTA), 0.05% xylene cyanol, 0.05% bromophenol blue, 10% SDS, 9 mM *Tris*-borate (pH 8.3), and 20% sucrose. Substrates and products were separated by denaturing polyacrylamide gel electrophoresis, visualized by autoradiography, excised from the gel, and quantified by *Cerenkov* counting. Initial rates (k_{obs}) were determined based on seven time points obtained over the first 10% of the reaction. Six different measurements of k_{obs} were obtained at three different concentrations of ribozyme.

RNA-Catalyzed site-specific hydrolysis reactions were carried out in the presence of either 2 or 5 nM ribozyme, 10 mM $MgCl_2$, 4 μg/μl bovine serum albumin, and 30 mM EPPS (pH 7.5) at 37°. Bovine serum albumin was added to prevent the RNA from adhering to the walls of the plastic centrifuge tubes, but did not affect the course of the reaction. The reactions were followed for 80 or 180 min for the uridine- or 5-bromouridine-containing ribozyme, respectively. The reaction products were analyzed as described above. Similar rates were obtained in the presence of either 2 or 5 nM ribozyme.

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