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A UNIQUELY MODIFIED RNA: INTRODUCTION OF A SINGLE RNA CLEAVAGE AGENT INTO THE M1 RIBOZYME

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Abstract: We describe an efficient, four-step synthesis of an N^4-modified cytidine phosphoramidite reagent (**1**) that permits nonnative functionality to be introduced into a synthetic oligoribonucleotide. This reagent was used to prepare a 377-nt *E. coli* M1 ribozyme equipped with a single RNA cleavage agent at nucleotide 11.

Many RNAs adopt discrete three-dimensional structures that are essential for their biological function. Unfortunately, our ability to decipher the structural basis of RNA function is hindered by a lack of tools to elucidate RNA tertiary folding patterns.[1] Multi–dimensional NMR methods and X-ray crystallography are revolutionizing the analysis of small elements of RNA structure but have not yet been applied generally to RNAs containing more than 50 nucleotides.[2,3] In the absence of high resolution techniques, alternative methods are needed to aid the study of both higher-order RNA structure[4-8] and the process of acquiring structure (the RNA folding problem).[1,4]

Recently we demonstrated that metal-catalyzed self-cleavage reactions performed under physiological conditions can detect alternative conformational states of proteins in solution.[9] The protein cleaving agent EDTA•Fe[10-12] was joined through a disulfide bond to a unique cysteine thiol in staphylococcal nuclease and a self-cleavage reaction was initiated with ascorbate ion. Changes in structure between the native and non-native states were characterized by comparing the self-cleavage sites observed under native and non-native conditions.[9]

Extension of this idea to the study of RNA tertiary structure and folding requires incorporation of a single cleavage agent at a precisely defined position within a large, structured RNA. The recent rediscovery[13,14] that RNA fragments can be joined by the action of DNA ligase and a complementary DNA template[15] simplifies this problem to the synthesis of a reagent that allows an RNA cleavage agent to be introduced into a synthetic oligonucleotide.[16] Here we present an efficient, four-step synthesis of N^4-(3-(*N*-(9-fluorenylmethoxycarbonyl)-amino)propyl)-5'-*O*-(4,4'-dimethoxytrityl)-2'-*O*-(*tert*-butyldimethylsilyl)-cytidine-3'-*O*-(2-cyanoethyl-*N,N*-diiso-propylphosphoramidite (**1**, Fig. 2A). This modified phosphoramidite reagent bears a masked primary amine that

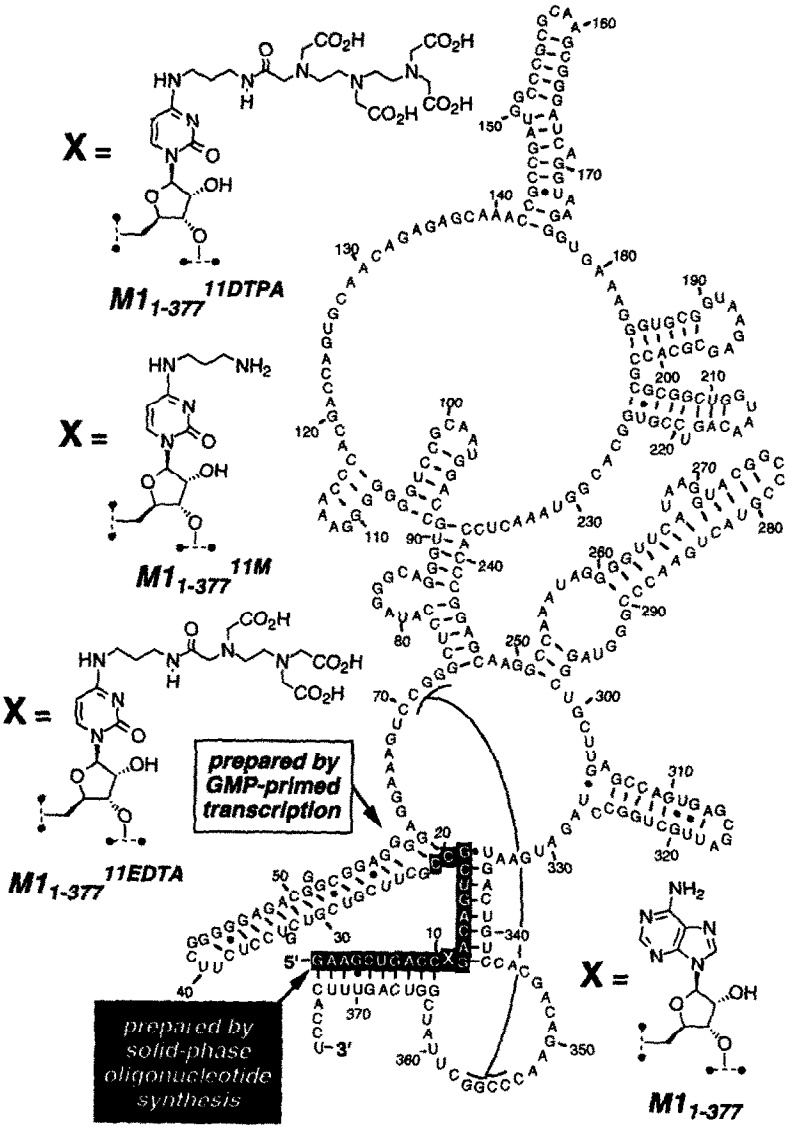

Figure 1. Nucleotides 1-377 of an *E. coli* M1 ribozyme modified site-specifically at nucleotide 11 (labeled X in figure). Nucleotides derived from $M1_{1\text{-}21}{}^{11M}$, $M1_{1\text{-}21}{}^{11EDTA}$ or $M1_{1\text{-}21}{}^{11DTPA}$ are depicted in black boxes. Nucleotides derived from $M1_{22\text{-}377}$ are shown in normal type.

is unmasked post-oligonucleotide synthesis[17-24] and may be acylated subsequently with a variety of suitably activated reporter groups. Unlike most reagents available for introducing modified bases into RNA, **1** introduces a ribonucleotide, complete with 2'-OH group.[16,25] The 2'-OH is important for RNA stability[26,27] and may contribute to RNA tertiary complexity by providing additional points of hydrogen bonding and metal binding sites.[28-36] Here we apply this reagent to generate a modified version of the 377-nucleotide (nt) M1 ribozyme of *E. coli*[37] containing a single DTPA- or EDTA-Fe RNA cleavage agent at position 11, $M1_{1\text{-}377}{}^{11DTPA}$ and $M1_{1\text{-}377}{}^{11EDTA}$ (Fig. 1).

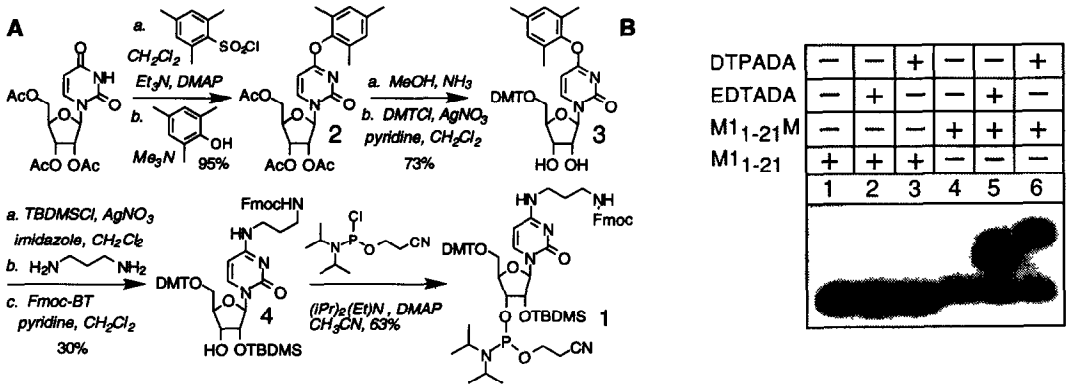

Figure 2. **(A)** Synthesis of phosphoramidite **1**. **(B)** Autoradiogram of a 20% denaturing polyacrylamide gel illustrating reaction of 83 nM ^{32}P $M1_{1\text{-}21}$ or $M1_{1\text{-}21}{}^{11M}$ with 28 mM DTPADA in DMSO:TEA:H_2O (75:5:20) or 6.5 mM EDTADA in 167 mM Na_2CO_3 (pH 8.0).

The synthesis of **1** is illustrated in Fig. 2A. Reaction of 2',3',5'-tri-*O*-acetyluridine with 2-mesitylenesulfonyl chloride followed by displacement with 2,4,6-trimethyl phenol provided mesitylate **2** in 95% yield.[38] Treatment of **2** with an ammonia-saturated methanol solution,[38] followed by dimethoxytrityl chloride,[39] generated the 2',3'-diol **3** in 73% yield. Reaction of **3** with *tert*-butyldimethylsilyl chloride and silver nitrate provided a 2:1 mixture of 2' and 3'-silyl isomers.[39] Treatment of the mixture with neat 1,3-diaminopropane followed by 1-benzotriazoyl-9-fluorenylmethyl carbonate[40] afforded **4** in a three-step yield of 30%. Subsequent reaction of **4** with cyanoethyl N,N-diisopropyl chlorophosphoramidite provided **1**.[20,41] Phosphoramidite **1** was introduced into position 11 of an oligoribonucleotide (GAAGCUGACCMGACAGUCGCC, $M1_{1\text{-}21}{}^{11M}$) comprising the first 21 nucleotides of the M1 ribozyme using standard solid-phase procedures. $M1_{1\text{-}21}{}^{11M}$ was cleaved from the resin, deprotected, and gel-purified. Autoradiography of purified 5' ^{32}P end-labeled material indicated a homogeneous product of >95% purity. The presence of the modified base was verified by nuclease digestion of $M1_{1\text{-}21}{}^{11M}$ followed by HPLC analysis.[42] The chemical composition of $M1_{1\text{-}21}{}^{11M}$ was confirmed by ESI-MS.[43]

Treatment of $M1_{1\text{-}21}{}^{11M}$ with ethylenediaminetetraacetic dianhydride (EDTADA) or diethylenetriaminepentaacetic dianhydride (DTPADA) produced products with lower electrophoretic mobility on denaturing polyacrylamide gels, whereas $M1_{1\text{-}21}$, containing adenosine at position 11, showed no reaction (Fig. 2B). Reaction

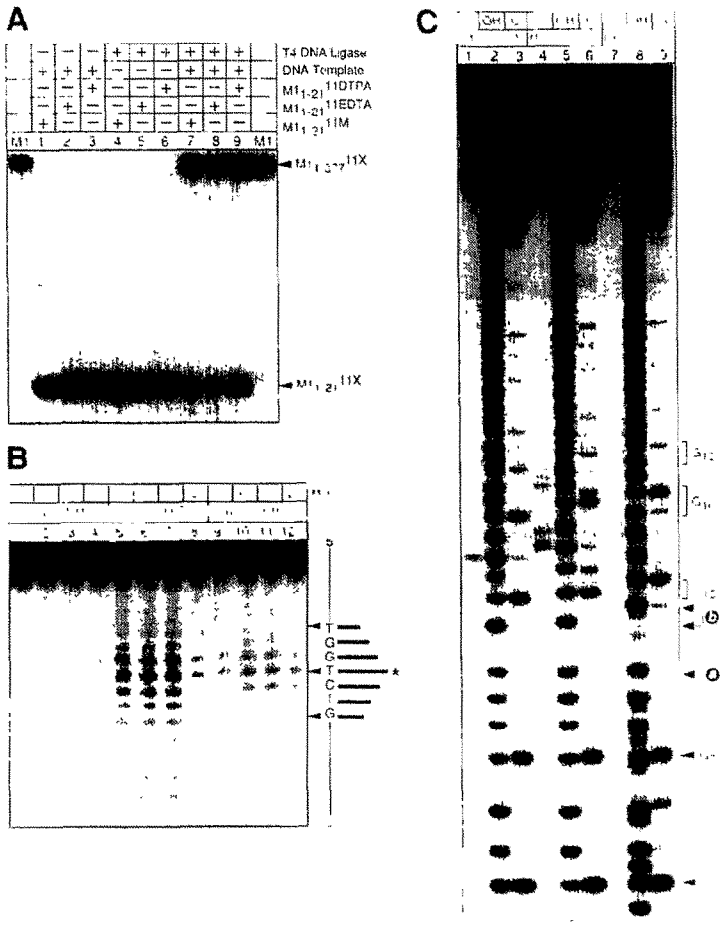

Fig. 3. **(A)** Autoradiogram of a 10% denaturing polyacrylamide gel illustrating ligation of 5' ^{32}P $M1_{1\text{-}21}{}^{11M}$, $M1_{1\text{-}21}{}^{11EDTA}$, or $M1_{1\text{-}21}{}^{11DTPA}$ (0.5 μM) to $M1_{22\text{-}377}$ (0.5 μM) in the presence of template DNA (0.5 μM) and T4 DNA ligase.[13] Lanes labeled M1 contain ^{32}P $M1_{1\text{-}377}$ prepared by transcription. Ligation yields (lanes 7-9) approach 60%. **(B)** Autoradiogram of a 20% denaturing polyacrylamide gel illustrating cleavage of 5' ^{32}P CCCCGAAGAGGACGACGACGAAGCGGCGACTGTCTGGTCAGCTTC by $M1_{1\text{-}21}{}^{11M}$, $M1_{1\text{-}21}{}^{11EDTA}$, or $M1_{1\text{-}21}{}^{11DTPA}$ in the presence of 1 μM Fe(III), 1 μM Cu(II), or 100 μM Zn(II) in a buffer containing 20 mM potassium phosphate, 20 mM KCl (pH 8.0) and 10 mM DTT. Histograms correspond to data from lane 6. The cleavage seen in lane 5 is likely due chelation of adventitious iron by $M1_{1\text{-}21}{}^{11EDTA}$ since it is suppressed by excess Zn(II). **(C)** Autoradiogram illustrating that EDTA (lane 6) and DTPA (lane 9) are tethered uniquely to position 11 in $M1_{1\text{-}377}$ RNAs prepared by ligation. 5' end-labeled ligation products were subjected to base-catalyzed hydrolysis (pH 9.5, labeled OH) and G-specific sequencing (T1 endonuclease, labeled G) reactions and fractionated on a 20% denaturing gel. Cleavage products with 10 nucleotides or fewer (position **a** or lower) comigrate independent of the base at position 11, whereas those with 11 nucleotides or more do not (position **b** and above). Both EDTA (lanes 4-6) and DTPA (lanes 7-9) retard the mobilities of cleavage products greater than 10 nucleotides in length.

with EDTADA proceeded readily in 167 mM Na_2CO_3 (pH 8.0), whereas reaction with DTPADA was most efficient in DMSO:TEA:H_2O (75:5:20). The reactions were performed on a preparative scale (5 nmol) and the gel-purified products identified as $M1_{1\text{-}21}{}^{11EDTA}$ (52% yield) and $M1_{1\text{-}21}{}^{11DTPA}$ (69% yield) by ESI-MS[43] and by a base-specific sequencing assay (not shown). Both $M1_{1\text{-}21}{}^{11EDTA}$ and $M1_{1\text{-}21}{}^{11DTPA}$ promote cleavage of a complementary DNA oligonucleotide upon incubation with Fe(III) or Cu(II) (but not excess Zn(II)) and DTT (20 mM potassium phosphate pH = 8.0, 20 mM KCl, 25 °C, 14h) (Fig. 3A). Maximal cleavage occurs over a 7 base range centered about the complement of the modified base (★). Cleavage is more efficient with the EDTA derivative than with the DTPA derivative.[6]

To complete the modified ribozyme syntheses, $M1_{1\text{-}21}{}^{11M}$, $M1_{1\text{-}21}{}^{11EDTA}$ and $M1_{1\text{-}21}{}^{11DTPA}$ were ligated to $M1_{22\text{-}377}$, which contains the remaining 355 nucleotides of M1. $M1_{22\text{-}377}$ was prepared by T7 RNA polymerase catalyzed transcription of pPR22A-T7.[44] Addition of GMP to the transcription reaction produced RNA transcripts containing a 5'-monophosphate and avoided the capricious two-step procedure necessary to convert the 5'-triphosphate obtained in GTP primed transcriptions into the 5'-monophosphate required by DNA ligase.[45] Ligations were performed in the presence of T4 DNA ligase and a DNA template complementary to the 5' 45 nucleotides of M1 RNA (Fig. 3B lanes 7-9) and proceeded with efficiencies that approached 60%. No ligation was observed in the absence of template or ligase (lanes 4-9), and neither modified base had a significant effect on the ligation yield. A base-specific digestion assay was used to verify that the full length ribozyme carried a site-specific modification at position 11 (Fig. 3C). Ligation reactions were performed with either $M1_{22\text{-}377}$ or the oligonucleotide as the labeled component; however, ligation of 5' ^{32}P oligonucleotides to $M1_{22\text{-}377}$ yields modified 5' ^{32}P M1 RNA of high specific activity suitable for further study. Experiments to determine the self-cleavage sites of $M1_{1\text{-}377}{}^{11EDTA}$ are in progress.

Summary We describe an efficient synthesis of a modified phosphoramidite reagent that permits nonnative functionality to be introduced into a synthetic oligoribonucleotide. The modified base described here differs little from cytosine and causes minimal destabilization within a model RNA hairpin (ΔT_m = 0.3 kcal•mol^{-1}). Thus, it may be positioned within both single-stranded and duplex regions and should participate in many tertiary interactions. We note that a variety of different reporter groups can be joined to a single oligonucleotide containing this base with subsequent ligation to another RNA molecule. This method represents an alternative to that of Tor and Dervan,[46] which requires synthesis of a deoxyribonucleoside phosphoramidite and a ribonucleotide triphosphate to direct the enzymatic incorporation of an isomeric base into an RNA oligomer, and to that of Nolan *et al.*[47] which places the modification at the 5' end of a circularly permuted RNA.

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