Thiophosphate interference experiments locate phosphates important for the hammerhead RNA self-cleavage reaction

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ABSTRACT

A hammerhead domain of less than 50 nucleotides is responsible for a self-cleavage reaction in the replication of plant RNA pathogens. The hammerhead is composed of three helices joining at a central conserved core of 11 single stranded nucleotides. The core is believed to fold into a tertiary structure that provides functional groups for catalysis and to coordinate one or more divalent metal ions. In this study we use a phosphorothioate substitution interference assay to identify four phosphates in the conserved core which also play a role in the self-cleavage reaction.

INTRODUCTION

The RNA genomes of several plant viroids and virusoids contain a hammerhead domain of about 60 nucleotides that is responsible for a self-cleavage reaction generating 5' OH and 2', 3' cyclic phosphate termini (1). The three RNA double helices making up the secondary structure of the hammerhead have been confirmed by phylogenetic comparison (1) and mutagenesis experiments (2–5). Eleven non-helical nucleotides make up the central core of the hammerhead. Since nine of these are conserved (1) and essential for cleavage (3,6) they presumably either participate in tertiary interactions to fold the domain into its active structure or coordinate a divalent metal ion which stimulates the self cleavage reaction (7,8). It is quite plausible that functional groups of the phosphodiester backbone also contribute to tertiary folding or metal ion binding (9). In the yeast tRNA^{Phe} crystal structure, tertiary hydrogen bonds involving 11 different phosphates and 14 different riboses have been identified (10). The four tight divalent ion binding sites all involve at least some coordination with the backbone (11–13).

The phosphodiester bonds of RNA can be replaced by phosphorothioate linkages by *in vitro* transcription using nucleoside α -phosphorothio triphosphates (NTP α S). T7 RNA polymerase specifically incorporates the Sp isomer of NTP α S with inversion of configuration to produce the Rp isomer of the phosphorothioate linkage (14). The phosphorothioate linkage has a geometry similar to the phosphodiester bond, but is more

susceptible to alkylation. This property has recently been exploited in a new method for sequencing RNA (15).

In this paper we report on the use of thiophosphate substitution to locate specific phosphodiester bonds which are important for the hammerhead self-cleavage reaction. Incorporation of several different NTP α S into relatively long transcripts containing hammerhead domains has been shown to inhibit self-cleavage (16). More recently a hammerhead containing a single phosphorothioate linkage at the self-cleavage site has been shown to have a reduced rate of cleavage (8). The inhibition of cleavage appears to result primarily from a reduced affinity of a catalytically important divalent ion. Here we show that incorporation of phosphorothioate linkages into other positions of the hammerhead also inhibit self-cleavage. Using partially substituted transcripts we are able to map positions of four phosphates within the conserved central core which inhibit self-cleavage when substituted with phosphorothioate linkages.

MATERIALS AND METHODS

Transcription Reactions

RNA was synthesized by transcription of deoxyribonucleotide templates with T7 RNA polymerase (17). Transcription reactions contained 100 nM template, 0.1 mg/ml T7 RNA polymerase, 2 mM of each of the 4 NTPs and 12 mM MgCl₂. To produce transcripts fully substituted with phosphorothioate linkages adjacent to a given nucleotide, 2 mM of the Sp isomer of the corresponding NTP α S was used. The fully substituted transcripts were internally labeled by the inclusion in the transcription reaction of [α -³²P] ATP CTP or UTP to a specific activity of $\sim 3 \times 10^5$ Ci/mole of RNA. Full length transcripts were separated from unincorporated NTPs and abortive transcription products by gel electrophoresis in denaturing polyacrylamide gels. The transcripts were localized in the gel either by autoradiography or UV shadowing and subsequently extracted by a crush and soak method. The transcripts were concentrated by ethanol precipitation and suspended in 5 mM DTT.

For the preparation of partially substituted transcripts, containing approximately one phosphorothioate linkage per molecule, each of the four NTPs was replaced separately in the transcription reaction with a mixture of 1 part NTP α S to X parts

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of the same normal NTP, where X is the total number of that nucleotide in the RNA. The partially substituted transcripts were either 5'-end labeled with [γ - 32 P] ATP and polynucleotide kinase or 3'-end labeled with [$5'$ - 32 P] pCp and RNA ligase. NTP α S were a generous gift of F. Eckstein.

RNA Cleavage Reactions For Rate Determinations

Substrate 1S, internally labeled with [α - 32 P] CTP for unsubstituted, α -thio ATP, GTP and UTP substituted substrate or [α - 32 P] ATP for α -thio CTP substituted substrate, was combined at a concentration of 0.2 μ M with 0.4 μ M non-labeled unsubstituted or NTP α S substituted 1R and 50 mM Tris-HCl (pH 8). The mixture was incubated at 60° for 1 minute and allowed to cool to 37°. Cleavage reactions were initiated by the addition of MgCl₂ to a final concentration of 10 mM. The reactions were incubated at 37°C. Aliquots were removed at various times and cleavage was stopped by the addition of one volume of 50 mM EDTA, 7 M urea and 0.05% xylene cyanol and bromophenol blue dyes. For rate determinations five or more time points were taken ranging from approximately 0 to 70% total cleavage. Uncleaved substrate and products were separated by electrophoresis in denaturing polyacrylamide gels and extents of cleavage were determined by quantitation of radioactivity in the substrate and product bands either using an AMBIS β particle detection system (Ambis Systems Inc., San Diego, CA) or by Cerenkov counting of the respective bands. The logarithm of the uncleaved fraction was plotted versus time and the data points were fitted to a line by a least squares method. From the cleavage half-lives ($t_{1/2}$) first order rate constants were determined using the following relationship: $k_{\text{obs}} = 0.693 / t_{1/2}$.

Phosphorothioate Substitution Interference Experiments

A solution containing 0.2 μ M of partially NTP α S substituted substrate, either 5' (1S) or 3' (2S) end labeled, 0.3 μ M of the corresponding enzyme and 50 mM Tris-Cl (pH 8) was incubated at 60° for 1 minute. After cooling, cleavage reactions were initiated by the addition of MgCl₂ to a final concentration of 10 mM and incubated at 37 or 50°C for hammerhead 1 or 2 respectively. Reactions were terminated by the addition of one volume of 50 mM EDTA, 7 M urea, and electrophoresis dyes. Uncleaved substrate and products were separated by electrophoresis in 20% denaturing polyacrylamide gels and isolated by a crush and soak method. The purified product and substrate, as well as unreacted substrate were incubated in 15% iodoethanol, 80% formamide, and 0.05% xylene cyanole and bromophenol blue dyes, at 95° for 4 minutes (15). The iodoethanol treated RNAs were separated by electrophoresis in denaturing polyacrylamide gels and subsequently visualized by autoradiography.

RESULTS AND DISCUSSION

In order to examine the effect of phosphorothioate linkages on hammerhead cleavage, the self-cleavage domain was constructed by combining two oligonucleotides of similar length. In this well characterized hammerhead (7) a 19 nucleotide ribozyme (1R) can cleave a 24 nucleotide substrate (1S) into products of 18 and 6 residues (fig 1A). *In vitro* transcription with one NTP α S and three normal NTPs was used to prepare four substrates containing phosphorothioate linkages 5' to each of the nucleotides. The cleavage of these substrates is compared to the unsubstituted substrate in figure 1B. All four substituted molecules cleaved at

Figure 1. A. Sequence of hammerhead 1 comprised of substrate (1S) and ribozyme (1R). The site of self-cleavage is indicated by an arrow and base-pairs by ellipses. Phosphates and the nucleotides 3' to them are numbered starting from the 5' end, for each molecule, therefore cleavage of 1S occurs at phosphate 19, 3' of C₁₈. B. Self-cleavage reactions of phosphorothioate substituted 1S cleaved with the unsubstituted 1R analyzed by denaturing gel electrophoresis. Unsubstituted (none) or NTP α S substituted (A, C, G, or U) 1S was incubated at 37°C in 50 mM Tris-HCl (pH 8) with (+) or without (-) unsubstituted 1R for 3, 40, 20, 20 and 120 minutes, respectively. The positions of uncleaved substrate, 5' product and 3' product are indicated. C. Kinetics of cleavage of unsubstituted (●), ATP α S (+), CTP α S (▲), GTP α S (□) or UTP α S (○) substituted 1S with unsubstituted 1R.

the expected CpU sequence as judged from the mobility of the products. Since the experiments were carried out at high RNA concentration in the presence of a molar excess of enzyme, the rate of cleavage should primarily reflect the cleavage step and not the assembly of the hammerhead or the release of the products after cleavage. While the cleavage rates of all four phosphorothioate substituted substrates were slower than the

Table I: Cleavage rates of phosphorothioate substituted hammerhead 1.

Substrate	Enzyme	k_{rel}
1S	1R	(1)
ATP α S 1S	1R	0.029
CTP α S 1S	1R	0.42
GTP α S 1S	1R	0.40
UTP α S 1S	1R	0.0019
1S	ATP α S 1R	0.014
1S	CTP α S 1R	0.50
1S	GTP α S 1R	0.13
1S	UTP α S 1R	0.23

The data are normalized to a k_{obs} of 0.15 min^{-1} for the non-substituted ribozyme-substrate reaction.

Figure 2. Thiophosphate interference experiments with hammerhead 1. Substrate 1S, 5' ^{32}P labeled and partially substituted with one of the four NTP α S (as indicated) was treated with iodoethanol (Control lanes). Each partially substituted substrate was cleaved to approximately 50% completion with unsubstituted 1R. The substrate which failed to cleave (Uncleaved lanes) and the resulting 5' products (Cleaved lanes) were separated and treated individually with iodoethanol. The sequence of 1S is aligned with the NTP α S specific iodoethanol induced strand scission reactions. Circled nucleotides indicate positions which are diminished in intensity in the cleaved product and increased in intensity in the uncleaved substrate lanes relative to the remaining bands in the respective lanes. The boxed nucleotide is at the cleavage site and is increased in intensity in the uncleaved lane (lane 11) relative to the band below it, as compared to the equal intensity of these two bands in the control lane (lane 10).

unsubstituted substrate (Fig 1C, Table I), the ATP α S and UTP α S substituted molecules showed exceptionally slow rates. This suggests that a thiophosphate 5' to one or more of the five A residues and one or more of the three U residues in the substrate drastically reduces cleavage.

A similar experiment measuring the rates of cleavage with the four substituted ribozymes and unsubstituted substrate is also presented in Table I. The cleavage rate is also slower with all four substituted ribozymes, but only the ATP α S substituted molecule shows a very large reduction in reaction rate. This

suggests that substitution of at least one of the phosphates 5' to the three A residues in the ribozyme also impairs cleavage.

In order to identify the precise location of phosphorothioate linkages that reduce the rate of hammerhead cleavage, substitution interference experiments were performed. Four transcription reactions containing the four NTPs and an appropriate amount of a single NTP α S were used to prepare substrate molecules containing an average of one phosphorothioate linkage per molecule. Each of the four partially substituted substrates were then 5'- ^{32}P labeled, combined with unsubstituted ribozyme and allowed to cleave to approximately 50 percent. Denaturing gel electrophoresis was used to purify the uncleaved substrate from the larger 5'-end-labeled products. The location of phosphorothioate linkages in the starting material, the uncleaved substrate, and the larger cleaved product are detected by treating the oligoribonucleotides with iodoethanol to induce strand scission, separating the products on a sequencing gel and detecting the end labeled products by autoradiography.

The results of a thiophosphate substitution interference experiment are shown in figure 2. Reaction of the partially ATP α S substituted substrate starting material (Figure 2, lane 1) with iodoethanol reveals five products of equal intensity corresponding to the five A residues in the molecule. This indicates that the nucleoside phosphorothioates are uniformly incorporated into the transcripts during transcription and that iodoethanol induced strand scission also occurs uniformly throughout the molecule. In the uncleaved lane (lane 2) two of the five bands, corresponding to positions A₇ and A₈ (circled residues), are slightly overrepresented relative to the other bands in this lane suggesting that phosphorothioate substitution at these positions inhibits the self cleavage reaction. This conclusion is confirmed by examination of the cleaved lane (lane 3) where the A₇ and A₈ bands are substantially less intense relative to the other bands in this lane. Although the amount of enrichment in the uncleaved lane should be equal to the depletion in the cleaved lane, the result is more clearly seen in the cleaved lanes. This property of substitution interference experiments has been noted previously (18, 19).

The absence of significant differences in intensity in any of the bands in the CTP α S and GTP α S experiments in Figure 2 indicate that phosphorothioate substitution in these positions do not greatly affect cleavage. For the UTP α S experiment one of the bands, corresponding to the phosphate at the cleavage site (P₁₉), is slightly overrepresented in the uncleaved substrate lane (lane 11) relative to the band below it, and as compared to the equal intensity of these two bands in the control lane (lane 10). This is expected if substitution at P₁₉ is inhibitory to self-cleavage. However the large amount of cleavage product in the cleaved lane (lane 12) makes it impossible to look for the more diagnostic reduction in intensity. The inhibitory effect of phosphorothioate substitution of P₇, P₈ and P₁₉ in the substrate was confirmed by substitution interference experiments performed on another hammerhead (2) which differed in the sequence of all three helices (data not shown).

In order to locate the one or more thiophosphates responsible for the low rate of cleavage of the fully substituted ribozyme, it was necessary to design a new hammerhead (Figure 3A). This hammerhead, similar in configuration to one recently described (20), is constructed from two oligoribonucleotides of dissimilar length. The 32 nucleotide substrate (2S) contains the entire stem I, closed by a four nucleotide loop, and can anneal to the 15 nucleotide ribozyme (2R) through stems II and III. Cleavage of 2S results in a 5' product of 8 nucleotides and a 3' product of

Figure 3. A. Sequence of hammerhead 2 comprised of substrate (2S) and ribozyme (2R). Phosphates and the nucleotides 3' to them are numbered starting from the 5' end. The site of self-cleavage is indicated by an arrow. The 3' terminal C of 2S (in parenthesis) is not present in the nascent transcript. It was added using RNA ligase. **B.** Thiophosphate Interference experiments with 3'-³²P labeled 2S. The sequence of 2S is aligned with control lanes. The circled nucleotide identifies the position in the ATP α S experiment where there is an increased intensity in the uncleaved substrate and a diminished intensity in the cleaved product. The boxed nucleotide corresponds to the cleavage site.

Figure 4. Essential phosphates in the hammerhead domain. Shown is the hammerhead consensus as recently refined (6). N indicates any nucleotide, Y any pyrimidine and H any nucleotide except G. Ellipses indicate Watson and Crick base pairs. Arrows indicate positions of phosphates (dots) which when substituted with phosphorothioate linkages inhibits the self-cleavage reaction.

24 nucleotides. Thiophosphate substitution interference experiments were performed by partial substitution of 2S with each of the four NTP α S, 3' end labeling with [5'-³²P] pCp, cleavage with unsubstituted 2R and, isolation of the uncleaved substrate and 3' product. Iodoethanol cleavage of the unreacted substrates produced uniform strand scission at each substituted phosphodiester bond (Figure 3B, lanes 1, 4, 7 and 10). For the ATP α S substituted substrate the band corresponding to A₂₇ is slightly overrepresented (circled) in the uncleaved substrate (lane 2) and completely absent in the product lane (lane 3) indicating that phosphorothioate substitution of P₂₇ in 2S is inhibitory to the self cleavage reaction. No differences are observed in the intensities of bands with the GTP α S and UTP α S substituted molecules. In the CTP α S experiment a single band at position 9 (boxed), corresponding to the phosphate at the cleavage site, is overrepresented in the uncleaved substrate lane (lane 5) but cannot be examined in the cleaved lane (lane 6) due to the presence of product. Again this is suggestive but not conclusive that substitution at P₉ reduces cleavage. For the CTP α S substituted experiment additional bands in the control and uncleaved substrate lanes, relative to the product lane, are presumably the result of nuclease contamination. These bands are not present in other experiments and do not correspond to the position of cytosine residues in this molecule. The experiments with hammerhead 2 therefore identify one additional phosphate in the hammerhead that is sensitive to thiophosphate substitution. This phosphate, P₂₇ in 2S and P₁₄ in 1R, is 5' to a conserved A residue.

The substitution interference experiments locate four phosphates in the hammerhead (Figure 4) that are responsible for the large reductions in cleavage rates for the fully substituted RNAs in Table 1. The 35-fold reduction in cleavage rate for ATP α S substituted 1S results from phosphorothioates at P₇ and P₈. The 73-fold reduction for ATP α S substituted 1R results from the phosphorothioate at P₁₄. Although the data is less clear, the 520-fold reduction in cleavage rate of UTP α S substituted 1S is most likely to be due solely to the thiophosphate at the cleavage site. Experiments with another hammerhead containing a single phosphorothioate linkage at the cleavage site give similar large reductions in cleavage rate (8). Finally, the 2 to 8 fold reductions in cleavage rate observed for fully CTP α S and GTP α S 1S and CTP α S, GTP α S and UTP α S 1R are too small to reveal differences in the substitution interference experiments.

A substantial reduction in the hammerhead cleavage efficiency has been reported for a hammerhead present in the plus strand

RNA of satellite tobacco ringspot virus (sTobRV) when substituted with ATP α S, but not with the other NTP α S (16). In this study the hammerhead was present on a single transcript and only complete substitution with one or more NTP α S was examined. Since an A residue occurred 3' to the cleavage site of sTobRV, the reduced cleavage was attributed solely to substitution of that bond. Using complete substitution of this hammerhead contained on a single transcript it is not possible to distinguish between the effect on cleavage of substitution of just one or multiple essential NpA bonds. Our work indicates that in addition to the phosphate at the cleavage site, substitution of three additional phosphates 5' to essential A residues are also expected to contribute to the reduced cleavage efficiency observed for the hammerhead of sTobRV.

How do phosphorothioate linkages reduce the rates of hammerhead cleavage? In an extensive study of another hammerhead, the introduction of a single thiophosphate at the cleavage site reduces the affinity of a catalytically important ion (8). The reasons why substitution of the other three phosphates reduce cleavage efficiency are less well understood. In tRNA functional groups on the sugar-phosphate backbone are known to participate in H-bond interactions and / or coordinate divalent metals in order to stabilize the tertiary structure. Since the four phosphates identified by phosphorothioate substitution interference are all in the conserved central core of the hammerhead and lie between essential nucleotides, it is possible that they are involved in tertiary folding of the molecule. The altered charge localization and bond order of the phosphorothioate linkage (21) would be expected to alter the hydrogen bonding potential of this internucleotide linkage. Alternatively, as in tRNA, the phosphates may be involved in the coordination of a divalent metal ion. In this case the substitution of sulfur for oxygen could alter or eliminate entirely a coordination site for an essential metal ion. The relatively small reductions in cleavage rate observed when thiophosphates are present in other positions may be the result of slightly decreased duplex stability and subtle changes in helix geometry as was observed in deoxynucleotides (22).

The thiophosphate substitution assay has general application for the localization of important phosphodiester bonds in other RNAs. Its utility has been demonstrated in the recent identification of phosphates important for the self-splicing reaction of the *Tetrahymena* rRNA (19). The substitution of phosphorothioate for phosphodiester linkages is almost an ideal chemical modification. Since the two linkages are virtually identical structurally, the modification is not expected to significantly disrupt the RNA structure. However, the substitution of sulfur for oxygen is potentially sufficient to alter the interaction of that phosphate with other atoms. These could be intramolecular interactions involving RNA folding as discussed here or intermolecular bonds between an RNA and a protein. Phosphorothioate substitution has been shown to either increase or decrease the affinity of a small RNA hairpin for R17 coat protein (23). By using the substitution interference protocol described here, one could map phosphates essential for RNA-protein interactions.

Presently the only useful structural probe for phosphates is the strong alkylating agent ethyl-nitroso-urea which reacts with accessible phosphates and produces a strand break (24). It should also be possible to use phosphorothioate containing RNA in structure mapping experiments. By using partially substituted RNAs and small alkylating agents such as dimethylsulfate (25)

or I₂ (F. Eckstein, personal communication), internal phosphorothioate linkages would be expected to show a reduced rate of alkylation.

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