THE DEVELOPMENT AND USE OF COBALT HEXAMMINE AS A TOOL TO STUDY THE TERTIARY STRUCTURE OF THE *NEUROSPORA VS RIBOZYME*

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science

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ABSTRACT

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Master of Science, 1999  
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A prerequisite to understanding how ribozymes function is an understanding of their three-dimensional structure. Until now, there was no method by which to examine the tertiary structure of the VS ribozyme precursor RNA because it self-cleaves immediately upon folding into its tertiary structure. Under most conditions, a divalent metal such as magnesium is required to induce VS ribozyme structure and function. My research shows that cobalt hexammine can induce almost the same VS ribozyme structure as magnesium does but at a 33-fold lower concentration. Moreover, VS ribozyme cleavage does not occur in the presence of cobalt hexammine alone making it a valuable tool for studying the structure of the VS ribozyme precursor RNA. I have used cobalt hexammine as such a tool and have discovered that helix Ia inhibits the formation of a long-range pseudoknot as well as a secondary structure rearrangement in helix Ib.
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INTRODUCTION

Until the early 1980s the statement “all enzymes are proteins” was considered to be one of the unshakable truths of biology. However, a paradigm shift occurred in 1982 when Thomas R. Cech and Sidney Altman discovered the first catalytic RNA – the Tetrahymena self-splicing intron (Kruger et al. 1982) and the RNA component of ribonuclease P (Guerrier-Takada et al. 1983). This finding showed that, in addition to carrying genetic information, RNA could catalyze biochemical reactions. Since then, RNA catalysts (ribozymes) have been implicated in a wide range of biological activities including tRNA and mRNA processing and the maintenance of several viral genomes (Simons and Grunberg-Mango 1998). In addition, in vitro selection techniques have facilitated the creation of novel catalytic RNAs that are able to cleave a variety of RNA sequences as well as perform other reactions. The in vitro selection of an acyl transferase ribozyme has fueled debate that ribozymes pre-date protein enzymes (Suga, Cowan and Szostak 1998).

Seven naturally occurring ribozymes have been discovered to date. They have been placed into two groups based on their size and on the reaction that they perform. The hammerhead ribozyme, hairpin ribozyme, hepatitis delta virus ribozyme and Neurospora VS ribozyme are members of a group known as the small ribozymes. These ribozymes catalyze a phosphodiester transesterification reaction in vitro to yield two RNA fragments, one containing a 5'-hydroxyl and the other a 2',3'-cyclic phosphate terminus (reviewed by Sigurdsson et al. 1998). Based on research primarily performed on the hammerhead ribozyme, this reaction is thought to proceed via in-line attack of the 2'-hydroxyl group on the 5' phosphorus in an $S_n2$-type transesterification (Figure 1). The
second group of ribozymes is called the large ribozymes. This group contains the group I intron, the group II intron and the RNA subunit of RNase P. All three large ribozymes cleave RNA to generate 5'-phosphate and 3' hydroxyl termini (reviewed by Cech 1993).

Ribozymes are powerful tools for studying the structure of RNA because they provide an easy functional assay for structure; any change that is made to the ribozyme which disrupts the structure of the RNA will also likely disrupt the catalytic function of the RNA. As a result, considerable progress has been made in elucidating elements of RNA structure that exist in ribozymes as well as in how these structural elements contribute to the catalytic function of the ribozyme.

Although RNA is only composed of four building blocks (adenine, uracil, cytosine, and guanine), it has a remarkable capacity to fold into very complex structures. It is this propensity of RNA to form such structures that endows it with catalytic properties. Understanding how RNA structure forms from primary sequence and how this structure bestows the RNA with catalytic properties is the key to understanding how ribozymes function.

I. The Role of Metal Ions in RNA Folding.

Protein folding and RNA folding are similar problems. Both protein folding and RNA folding require that a linear polymer sift through countless intermediate foldings yet consistently arrive at the correct structure. The driving force behind protein folding is the tendency for hydrophobic residues to seek refuge from aqueous solvent by burying themselves in the interior of the protein (Creighton 1993). RNA does not have hydrophobic building blocks to drive folding. RNA molecules are indeed very polar.
Each nucleotide has multiple hydrogen bond donors and acceptors as well as a net negative charge due to the phosphodiester bond which links one nucleotide to the next. The driving force behind RNA folding is the compensation of electrostatic repulsions between closely packed phosphates by positively charged metals in solution (Draper and Misra 1998).

RNA folds in several steps. The first and fastest step is the formation of the stems and loops that define the secondary structure. This occurs in milliseconds (Batey and Doudna 1998). Early polynucleotide studies revealed that the high negative charge of nucleic acids attracts a dense 'cloud' of positive metal ions (Felsenfeld and Miles 1967). This cloud of delocalized cations neutralizes the repulsive charges of the phosphate backbone and allows the formation of stems and loops that make up RNA secondary structure (Draper and Misra 1998). Both monovalent and divalent metal ions are capable of stabilizing RNA secondary structure.

A shell of coordinated water molecules surrounds metal ions in solution. Divalent magnesium, for example, has a co-ordination shell of six water molecules in an octahedral arrangement. Metal ions which are involved in stabilizing the RNA secondary structure remain fully hydrated because they are delocalized and interact with the RNA through long-range electrostatic forces (Draper and Misra 1998).

The tertiary structure of RNA forms much more slowly than the secondary structure. Recently, hydroxyl radical footprinting using synchrotron radiation has been used to determine the hierarchy of the folding pathway of the Tetrahymena group I intron (Sclavi et al. 1997). The P5abc three-helix junction forms within 1 to 2 seconds and the P4-P6 domain fully folds within 3 seconds. However, the remainder of the ribozyme, the
P3-P7 region, requires several minutes to fold (Zarrinkar and Williamson 1994; Zarrinkar and Williamson 1996).

The process by which secondary structure elements fold into a tertiary structure is presently unknown. However, high resolution X-ray crystallography has implicated divalent metal ions in the formation of RNA tertiary structure. In the X-ray crystal structures of tRNA (reviewed by Pan et al. 1993), the hammerhead ribozyme (reviewed by Wedekind and McKay 1998) and most recently the P4-P6 domain of the group I intron (Cate, Hanna and Doudna 1997), divalent magnesium ions were found bound to discrete ‘pockets’ in the RNA.

This type of discrete interaction between the RNA and a divalent metal is in contrast to the delocalized interactions that induce the formation of secondary structure. In these X-ray crystal structures, phosphate oxygens or base functional groups were found to interact with magnesium in two ways: either by removing one or more water molecules from the hydration shell and directly co-ordinating to the metal (referred to as an inner-sphere contact) or by hydrogen bonding with a water molecule that is coordinated to the metal (referred to as an outer-sphere contact). For example, five magnesium ions were found to cluster in the P5abc subdomain of the group I intron crystal structure (Cate, Hanna and Doudna 1997). Each of the ions bind the RNA through at least one inner-sphere contact. Two metals are located above and below the plane of the A-rich bulge and each forms inner-sphere contacts with three phosphate oxygens (see Figure 3a). A third metal forms outer-sphere contacts with three phosphate oxygens from helix P5c and forms an inner-sphere contact with a carbonyl oxygen of a guanosine (see Figure 3b). Two sheared G-A base pairs at the top of helix P5b co-
ordinate a fourth metal via the N7 and O6 groups of two adjacent guanosine residues (see Figure 3c). The fifth metal is coordinated to a phosphate group of an adenosine in the hairpin loop (L5c) of the three-helix junction. It is thought that this clustering of magensium ions at discrete sites in the P5abc sub-domain of the group I intron contributes to the fast folding of P5abc relative to the rest of the intron (Sclavi et al. 1998).

Divalent metal ions as opposed to monovalent metal ions are thought to play the leading role in stabilizing RNA tertiary structure (Pan et al. 1993). Divalent metals likely bind to distinct sites on the RNA because the two positive charges on a divalent metal ion allow it to interact with more than one phosphate simultaneously (Pan et al. 1993). This electrostatic attraction provides the energy to displace water from the hydration shell of a divalent metal allowing the metal to form tight, inner-sphere contacts with the RNA (Draper and Misra 1998). The weaker electrostatic attraction of monovalent metal ions makes this type of tight binding less likely and as such, monovalent metal ions are mainly delocalized.

This is not to say, however, that the discrete binding of monovalent metal ions to RNA cannot occur. In a recent crystal structure of the P4-P6 domain of the *tetrakymena* group I intron, a potassium ion was found bound to functional groups immediately under the J6/6a and J6a/6b AA platforms (Basu et al, 1998). Each site involves five direct metal co-ordinations to the RNA, including a 2’-OH and a phosphate oxygen from the backbone between the two A’s of the platform, the O6 and N7 groups of the wobble G below the platform and the O4 of a U immediately 3’ to the G. In addition, this monovalent ion binding site was found to stabilize the folding of the P4-P6 domain and
enhance the activity of the *Azoarcus* group I intron showing that specific binding of a monovalent ion has structural and functional relevance (Basu et al. 1998). Thus, monovalent metal ions can bind discretely to RNA and influence tertiary structure.

In summary, delocalized monovalent and divalent metal ions stabilize RNA secondary structure through long range electrostatic interactions. Specifically bound divalent metal ions play a major role in stabilizing the RNA tertiary structure by neutralizing regions of negative charge that accumulate as secondary structure elements condense into the tertiary structure. Monovalent metal ions can also stabilize RNA tertiary interactions although their contribution to tertiary structure is probably less significant than the contribution of divalent metal ions.

II. The Role of Metal Ions in RNA Catalysis.

The dilemma for ribozymes that catalyze proton transfer reactions is that RNA functional groups, at least in free nucleotides, have pKₐ values that lie out of the neutral range (Pan et al. 1993). Thus, RNA functional groups are not good acid-base catalysts at neutral pH. In addition, no RNA groups are positively charged at neutral pH to stabilize the negative charge that must develop on the penta-coordinated phosphate of the transition state. So how can ribozymes catalyze proton transfer reactions? One way that has been proposed is that ribozymes recruit metal ion cofactors (Dahm and Uhlenbeck 1991).

However, the distinction between metal ions that are involved in RNA folding and those that are involved in RNA catalysis is a difficult one to make. A metal ion that is involved in the catalytic mechanism of a ribozyme is probably localized to a discrete site
on the RNA just as metal ion cofactors of protein enzymes are precisely positioned (reviewed by Creighton 1993). The discrete positioning of a catalytic metal in a folded RNA surely must require several contacts between RNA functional groups and the metal. These contacts probably also play a role in stabilizing the RNA's tertiary structure. Thus, a metal ion that is involved in RNA catalysis is probably also involved in stabilizing RNA structure. Therefore, distinguishing the role of metal ions in catalysis from the role of metal ions in facilitating RNA tertiary structure is very difficult. That being said, a considerable volume of research has gone into elucidating the role that metal ions play in RNA catalysis. The majority of this work has focused on the cleavage mechanisms of the hammerhead ribozyme and the hairpin ribozyme.

Several observations on the hammerhead ribozyme are consistent with one or more divalent metal ions participating directly in the cleavage event. Firstly, the hammerhead ribozyme requires divalent metal ions for cleavage activity to occur under physiologically relevant conditions (Dahm and Uhlenbeck 1991). This is consistent with divalent metal ions being required for folding or catalysis or both. Secondly, the logarithm of the cleavage rate of the hammerhead ribozyme increases linearly with pH, with a slope near unity, suggesting that a single proton transfer is involved in the rate-limiting step (Dahm, Derrick and Uhlenbeck 1993). Thirdly, higher cleavage rates correlate with decreasing pKₐ of the solvated divalent metal that is used in the cleavage reaction (Dahm, Derrick and Uhlenbeck 1993). This has been taken as evidence of a metal being involved in the rate-limiting proton transfer step. A fourth observation which has been used in support of a divalent metal being involved in catalysis is that sulfur substitution of the Pro-Rₚ oxygen but not the Pro-Sₚ oxygen of the scissile phosphate
reduces the hammerhead cleavage rate by about 500-fold in magnesium (Dahm and Uhlenbeck 1991; Slim and Gait 1991; Scott and Uhlenbeck 1999). However, activity can be greatly restored via the addition of the thiophilic divalent metal manganese and completely restored by addition of the thiophilic divalent metal cadmium (Scott and Uhlenbeck 1999). Thiophilic divalent metals display only a slight preference for oxygen relative to sulfur inner sphere ligands. Magnesium, on the other hand, has a 30,000-fold weaker affinity for sulfur as compared to oxygen (Slim and Gait 1991). Rescue of the cleavage activity of the Pro-Rp substituted hammerhead by manganese or cadmium but not by magnesium argues that the Pro-Rp atom contributes an essential ligand to the binding of a magnesium ion at the catalytic site.

Together, these observations have been taken as evidence that one or more divalent metal ions are bound to the cleavage site of the hammerhead ribozyme and participate directly in the cleavage reaction. Several models have been proposed as to how a divalent metal bound to the Pro-Rp oxygen might participate in a rate-liming proton transfer step (reviewed by Wedekind and McKay 1998). All of the models involve the metal ion stabilizing the negative charge of the transition state as well as increasing the nucleophilicity of the 2’-OH for attack on the scissile phosphate. The divalent metal has been proposed to increase the nucleophilicity of the 2’-OH either by abstracting the 2’-OH proton via a metal bound hydroxide or through direct co-ordination of the metal to the 2’-oxygen thereby increasing the acidity of the proton and allowing an aqueous hydroxide to abstract it (reviewed by Wedekind and McKay 1998).

In contrast with experiments performed on the hammerhead ribozyme, observations on the hairpin ribozyme have suggested inner-sphere co-ordination with a metal ion is not
required for cleavage. The pivotal observation is that the hairpin ribozyme is fully functional when cobalt hexammine [Co(NH$_3$)$_6^{3+}$] is present as the only polyvalent metal (Hampel and Cowan 1997; Nesbitt, Hegg and Fedor 1997; Young, Gill and Grasby 1997). Cobalt hexammine is similar in size and geometry to magnesium hexahydrate [Mg(H$_2$O)$_6^{2+}$] and can form the same network of outer-sphere hydrogen bonds that magnesium can (Jou and Cowan 1991; see Figure 5b). However, unlike magnesium, cobalt hexammine cannot form inner sphere co-ordinations with RNA groups, as the amine ligands of cobalt hexammine do not exchange (Basolo and Pearson 1967). Thus, cobalt hexammine is incapable of forming a metal hydroxide or interacting directly with the 2'-OH or the phosphate oxygens of the scissile phosphate. Since cobalt hexammine supports hairpin ribozyme cleavage, any theory involving inner-sphere metal co-ordination in the catalytic mechanism of the hairpin ribozyme can be ruled out. Therefore, either an outer sphere metal ion co-ordination is required or divalent metal ions are not necessary for hairpin ribozyme cleavage.

Another observation that is consistent with lack of inner-sphere metal co-ordination in the hairpin ribozyme cleavage mechanism is that sulfur substitution of the Pro-R$_p$ oxygen or the Pro-S$_p$ oxygen resulted in no significant decrease in hairpin cleavage activity in magnesium. Thus, direct magnesium coordination to the phosphate oxygens at the cleavage site is not essential for hairpin ribozyme cleavage (Nesbitt, Hegg and Fedor 1997; Young, Gill and Grasby 1997).

In summary, while it appears that the hammerhead ribozyme may require the direct co-ordination of one or more divalent metal ions in its cleavage reaction, at least
one ribozyme, the hairpin, can perform site specific RNA cleavage in the absence of inner-sphere metal ion co-ordination.

III. The *Neurospora* VS Ribozyme.

The *Neurospora* VS ribozyme was isolated from an abundant noncoding single-stranded RNA, VS RNA, that is present in the mitochondria of certain natural isolates of *Neurospora* (Saville and Collins 1990). The 881 nucleotide VS RNA is capable of RNA-mediated cleavage and ligation reactions both in vivo and in vitro yielding products which contain 2',3'-cyclic phosphate and 5'-hydroxyl termini (Saville and Collins, 1991). Of the 881 nucleotides of native VS RNA, the smallest contiguous region that is required for efficient cleavage contains only 154 nucleotides (Guo et al. 1993). This construct has one nucleotide upstream of the cleavage site and 153 nucleotides downstream of it.

The most extensively characterized region of VS RNA is contained within a construct called G11Pre (Figure 2). This construct has 13 nucleotides upstream of the cleavage site and upon cleavage yields two products, a 13 nucleotide promoter-*proximal* fragment called G11P, and a 163 nucleotide promoter-*distal* fragment called G11D (Collins and Olive 1993). The cleavage rate of G11Pre does not exhibit pH dependence suggesting that, unlike the hammerhead ribozyme, a rate-limiting step, perhaps a conformational change, precedes the cleavage step or that, like the hairpin ribozyme, the rate of chemistry is not sensitive to pH (Collins and Olive 1993; Guo and Collins 1995).

A secondary structure model of G11Pre (Figure 2) has been determined which is consistent with mutational data as well as chemical modification data (Beattie, Olive and Collins 1995). The secondary structure is composed of six helices labeled I through VI
on Figure 2. Deletion analysis has shown that helices IV and VI can be considerably shortened with only a minor effect on the cleavage rate (Rastogi and Collins 1998). In addition, mutational and chemical modification data have identified an inhibitory region in helix Ia or in the adjacent region of helix II (Rastogi and Collins 1998; Beattie and Collins 1997). Disruption of this element increases the observed cleavage rate by at least 10-fold.

Stem loop I can be separated from helices II through VI to generate a substrate that can be cleaved in trans by the ribozyme (helices II through VI). Unlike all of the other small ribozymes, recognition of the VS RNA substrate by the ribozyme is through tertiary interactions since the substrate stem loop has no long regions available for Watson-Crick pairing with the ribozyme (Guo and Collins 1995). Despite the lack of extensive Watson-Crick base pairing, the substrate binds strongly to the ribozyme as indicated by the low $K_M$ of 0.13μM.

The only element of tertiary structure that has been identified in the VS ribozyme is a long-range interaction that forms between loops I and V. Mutational data as well as chemical modification data support the formation of a three base pair pseudoknot which involves base pairing between G630, U631 and C632 of loop I and C699, A698 and G697 of loop V respectively (Figure 2; Rastogi et al. 1996). This pseudoknot has been shown to be required for the self-cleavage activity of the VS ribozyme.

Recently, an in vitro selection scheme to generate catalytically active stem I variants revealed an unusual tertiary structure induced rearrangement of the secondary structure of helix Ib (Anderson and Collins, unpublished data). In the absence of divalent metal the secondary structure of helix Ib of G11Pre exists as diagrammed in Figure 2.
However, in the presence of magnesium, the functionally active secondary structure of G11Pre is thought to be rearranged with C634 bulged out of the helix and C635, C636 and C637 shifted up one base pair relative to the 5' side of the helix (Figure 4).

IV. Research Rationale.

The catalytic property of the VS ribozyme, and of ribozymes in general, makes it challenging to directly study the structure of the precursor RNA. Once the precursor RNA folds into an active structure, it cleaves to yield cleavage products. This challenge has thus far inhibited our laboratory, as well as others working on catalytic RNA, from directly studying the structure of ribozyme precursor RNA.

Researchers studying the hairpin and hammerhead ribozymes have overcome this difficulty by incorporating nucleotide analogues at the cleavage site that prevent the cleavage reaction from occurring while minimally disrupting the tertiary structure of the precursor RNA (Scott et al. 1995; Murray et al. 1998b). These ribozymes, consisting of contiguous regions of less than 50 nucleotides, are small enough to be chemically synthesized. Thus the incorporation of a nucleotide analogue at the cleavage site is relatively straightforward. The smallest functional cis-cleaving VS ribozyme is 121 nucleotides (Rastogi and Collins 1998), too large for efficient chemical synthesis. Therefore, another approach to gaining structural information on the precursor RNA must be found.

Recent studies have suggested that cobalt hexammine might be able to function as an analogue for magnesium hexahydrate in RNA folding. The observation that the hairpin ribozyme is functionally active in cobalt hexammine alone has shown that cobalt
hexammine can induce functional RNA structure in at least one ribozyme (Nesbitt, Hegg and Fedor 1997; Young, Gill and Grasby 1997). In addition, a recent NMR solution structure of the P5b stem loop of the Tetrahymena group I intron has identified a cobalt hexammine binding site that had previously been identified as binding magnesium in the crystal structure of the P4-P6 domain of the intron (Cate et al. 1996; Kieft and Tinoco 1997). These observations suggest that even though cobalt hexammine cannot make inner-sphere contacts with the RNA, it can bind to a magnesium binding site and induce at least some RNA structure.

Based on these observations, I reasoned that cobalt hexammine might be a useful tool to gain structural information on the VS ribozyme precursor RNA. Preliminary data in the lab had suggested that the VS ribozyme is not catalytically active in cobalt hexammine alone. Therefore, cobalt hexammine might fulfil the role for the VS ribozyme that nucleotide analogues played for the hammerhead and hairpin ribozymes; to prevent the cleavage reaction from occurring while minimally altering the tertiary structure of the precursor RNA.

The first aim of my research was to evaluate cobalt hexammine as such a tool. This entailed determining the extent to which cobalt hexammine can support VS ribozyme catalysis and the extent to which cobalt hexammine can facilitate the formation of the VS ribozyme tertiary structure. If cobalt hexammine could support VS ribozyme tertiary structure but not VS ribozyme catalysis, then I could use cobalt hexammine to gain structural information on the precursor RNA. The second aim of my research was, therefore, to learn about the structure of the precursor RNAs of various VS ribozyme constructs using cobalt hexammine.
MATERIALS AND METHODS

I. Synthesis of RNA by In Vitro Transcription.

RNAs were synthesized by T7 RNA polymerase transcription in vitro from clone G11 (Figure 2; Guo et al. 1993), clone ZA10 (Figure 22; Beattie 1997), or clone RS19 (Figure 17; Anderson and Collins, unpublished). Clones G11, ZA10 and H3 were linearized with SspI. Clone RS19 was linearized with EcoRI. Two hundred µl in vitro transcription reactions contained approximately 10 µg of linearized DNA, 40 mM Tris-HCl pH 8.0, 125 mM NaCl, 2 mM spermidine, 4 mM DTT, 2.5 mM MgCl₂, ribonucleoside triphosphates at 1 mM each, 400 units of RNAguard (Pharmacia), and 500 units of T7 RNA polymerase (BRL). Reactions were carried out for 1 hour at 37°C; the reaction products were extracted once with phenol/chloroform, once with chloroform, and were ethanol precipitated. Gel-purified precursor RNA was obtained by fractionation on a 4% polyacrylamide/8.3 M urea gel, located by UV shadowing, and the full length precursor was excised and eluted for 45 minutes at 65°C in water. Eluted RNA was filtered through a 0.8 µm/0.2 µm Supor (Gelman Sciences) membrane to remove polyacrylamide; it was then precipitated twice with with 1/10th volume of 3 M sodium acetate pH 5.2 and 3 volumes of ethanol, and was dissolved in water.

II. 3’-End-Labelling of RNAs.

Six µg of RNA was 3’-end-labeled with 50 µCi 5’-[³²P]-pCp in a 20 µl reaction which included 0.1 M ATP, 50 mM HEPES pH 7.5, 3.3 mM DTT, 20 mM MgCl₂, 10% DMSO, 40 units of RNAguard, and 60 units of T4 RNA ligase (BRL). The reaction was incubated for 3 hours at 4°C for G11 and ZA10 and 2 hours at 4°C for RS19. Under
these conditions G11, ZA10 and RS19 are approximately 50% cleaved. Cleavage products were gel purified from a 4% polyacrylamide/8.3 M urea gel as described above.

III. Chemical Modification Structure Probing.

Chemical Modification Reactions with diethyl pyrocarbonate (DEPC) or dimethyl sulfate (DMS) were performed as originally described by Krol and Carbon (1989) and later adapted by Beattie and Collins (Beattie 1997). Modifications were performed either under denaturing conditions at 90°C (in 200 mM HEPES pH 8.0/1 mM EDTA) or under non-denaturing conditions at 37°C (in 200 mM HEPES pH 8.0/50 mM KCl) in the presence or absence of various concentrations of Co(NH₃)₆Cl₃ or MgCl₂ (see individual figures for metal ion concentrations). The HEPES buffer was adjusted to pH 8.0 at 25°C with NaOH. For the chemical modifications approximately 0.2 µg of RNA was pre-incubated for 2-5 minutes at 37°C in the appropriate buffer with a final volume of 200 µl before the addition of modifying reagent (see below).

**DEPC modification.**

For DEPC reactions, 10 µl (non-denatured reactions) or 5 µl (denatured reactions) of DEPC was added to RNA and buffer for 15 minutes or 7 minutes. The reactions were stopped on ice, followed by the addition of 2 µg of yeast carrier tRNA and the RNA was precipitated with 1/10th volume of 3 M sodium acetate pH 5.2 and 3 volumes of ethanol. The chemically modified RNA was resuspended in 20 µl of 1.0 M aniline/acetate buffer pH 4.5 and incubated for 15 minutes at 60°C in the dark. Reactions were terminated by placing them on ice, and then were evaporated to dryness. The products were
resuspended in RNA-loading dye and were separated by gel electrophoresis. Short gel runs were performed at 70W on 12% gels pre-run for 1/2 hour with 1 M NaOAc in the bottom buffer to generate a salt gradient. The salt gradient was utilized on short gel runs to maximize separation of longer RNA fragments. Long runs were performed on 8% gels run at 70W in the absence of a salt gradient or as specified in the individual figure.

DMS modification.

For DMS reactions, 0.8 µl of DMS was added to RNA and buffer and was incubated for one minute (denatured reactions) or 5 minutes (non-denatured reactions). The reactions were stopped on dry ice/ethanol, followed by the addition of 10 µg of yeast carrier tRNA and the RNA was precipitated twice on dry ice/ethanol with 1/10th volume of 3 M sodium acetate pH 5.2 and 3 volumes of ethanol. The precipitated pellet was resuspended in 10 µl of ice-cold 50% (v:v) hydrazine:50% (v:v) water and was incubated on ice for 5 minutes. Following the hydrazine reactions, the RNA was precipitated with 100µl of 300 mM NaOAc and 300 µl ethanol on dry ice/ethanol. The pellet was rinsed with 70% ethanol, and then was treated with aniline as described for the DEPC modification reactions to induce strand scission at the site of modification. The RNA was subsequently resuspended in loading dye and visualized by denaturing polyacrylamide gel electrophoresis (as described for DEPC modification) and autoradiography.
IV. DNA Cloning.

DNA fragments for the cloning of ZA10 624/636 and 623/637 mutants were obtained by amplification of specific DNA sequences by using the polymerase chain reaction (PCR) with *Taq* polymerase and synthetic oligonucleotides essentially as performed by Beattie (1997). Primers ZA10 624/636, ZA10 623/637 and ZA10 623/637B correspond to nucleotides 617-643 of VS sequence and contain the desired mutations, as well as upstream vector nucleotides and a *HindIII* site for cloning. The sequence of ZA10 623/637 (for mutants A/T624 - A/T637 of ZA10) is 5' gggaaagcttgcaagt/TACatgcacat/Acgagcgg 3'. The sequence of primer ZA10 623/637 (for mutants C/T623-G/A637) is 5'gggaaagcttgcaagCTacatgcacac/GAgagcgg 3'. The sequence for primer ZA10 623/637B (for mutants G/A623-T/A637) is 5'gggaaagcttgcaag/GAgacatgcacacU/Agagcgg 3'. Mutant nucleotides are capitalized and underlined. Ten ng of ZA10 DNA was added to the PCR reaction and amplified with each of the above primers containing substitution mutations in stem Ib. PCR reactions (100 μl) contained 50 mM KCl, 10 mM Tris-HCl pH 9.0, 0.1% Triton X-100, 1.5 mM MgCl₂, 20 μM of either primer ZA10624/636, or ZA10 623/637, or ZA10 623/637B, 2 μM of forward sequencing primer, 25 mM each dNTP, and 0.5 u of Taq polymerase. The DNA was amplified with 25 PCR cycles consisting of a denaturing step at 94°C for 1 min., an annealing step at 55°C for 1 min, and an extension step at 72°C for 1 min.

Following the DNA amplification, PCR products were treated with Proteinase K to remove the *Taq* Polymerase. Proteinase K reactions (100 μl) contained the PCR products, 10 mM Tris-HCl pH 8.0, 5 mM EDTA, 0.5% SDS, and 8 μg of proteinase K. After 30 minutes of incubation at 37°C, the reactions were extracted once with
phenol/CIA, once with CIA, ethanol precipitated and the RNA was resuspended in 20µl water.

Following the proteinase K reaction, 8µl of the PCR products were incubated in 100µl total volume with 20U of EcoRI and 25U of HindIII in Pharmacia One-Phor-all buffer for 2 hour at 37°C. The reactions were then extracted once with phenol/CIA, once with CIA, ethanol precipitated and resuspended in 5µl water.

Fragments were cloned into the plasmid pTZ19R which had been cleaved to expose the respective HindIII and EcoRI restriction sites as performed by Beattie (1997). Ligation reactions (10 µl) contained 0.4µg of pTZ19R DNA, 2.5 µl of insert DNA, 25 mM Tris-HCl pH 7.6, 10 mM MgCl₂, 1 mM ATP, 1 mM DTT, and were incubated for 10 minutes at 65°C before the addition of 1 U of T4 DNA ligase. Ligation reactions were incubated for 1 hour at room temperature, and diluted to 50 µl. 1 µl of the diluted ligation mixture was used to transform *Escherichia coli* strain DH5αF'. Positive colonies were identified on the basis of blue/white selection and plasmid DNA was prepared by alkali lysis. Two isolates of each mutant were identified and sequenced from the T7 promoter to the SspI site, which was the 3'-end of the run-off transcripts used to measure cleavage rates, in order to confirm that only the expected mutations were present.

V. Measurement of Self-Cleavage Rates.

RNAs were synthesized by T7 RNA polymerase transcription from clone G11 (Guo *et al.* 1993), or clone ZA10 (Beattie 1997), or clones containing the ZA10 624/636 or 623/637 mutations, or clone RS19 (Anderson and Collins, unpublished). Clone G11, clone ZA10, clones containing the ZA10 mutants, and clone H3 were linearized with
Clone RS19 was linerized with EcoR1. Internally labeled radioactive precursor RNAs were obtained by 10μl in vitro transcription reactions which contained 3 μg DNA, 40 mM Tris-HCl pH 8.0, 125 mM NaCl, 2 mM spermidine, 4 mM dithiothreitol, 1 mM each NTP, 5 μCi of [α-32P]-GTP, 15 units of RNAguard, 25 units of Taq polymerase and 2.5 mM MgCl₂. Reactions were incubated at 37°C for 1 hour, brought up to 100μl with DEPC treated water, extracted once with phenol/CIA, once with CIA, ethanol precipitated and the RNA was resuspended in water.

Self-cleavage reactions were carried out either in cleavage buffer (40 mM Tris-HCl pH 8.0, 50 mM KCl) or in structure probing buffer (200 mM HEPES pH 8.0, 50 mM KCl). In addition, self-cleavage assays performed on ZA-10, ZA-10 mutants, H3 and H3ZA10 contained 2 mM spermidine. Self cleavage reactions performed on G11 or RS19 contained approximately 2 nM 3' end labeled RNA while self-cleavage reactions performed on ZA-10, ZA-10 mutants, H3 and H3ZA10 contained approximately 50 nM internally labelled RNA. Aliquots of 4 μl were removed after times specified in the individual figures and were mixed with 8 μl of RNA loading dye (for reactions in cleavage buffer) or 16 μl of RNA loading dye (for reactions in structure probing buffer; the high salt content of the HEPES buffer causes a gel running artifact unless suitably diluted). RNAs were separated by electrophoresis on 4% polyacrylamide/8.3 M urea gels and were quantified using a PhosphorImager.
RESULTS

I. Cobalt Hexammine as an Analog for Magnesium Hexahydrate.

For cobalt hexammine to be a useful tool to gain structural information about the VS ribozyme precursor RNA it must satisfy two requirements. Firstly, cobalt hexammine must not allow the VS ribozyme cleavage reaction to occur. Secondly, cobalt hexammine must not severely disrupt the tertiary structure of the ribozyme. The experiments described in this section have been performed to address these requirements.

_G11 does not cleave when cobalt hexammine is present as the only polyvalent metal._

It has been shown that 0.1 mM cobalt hexammine can facilitate efficient hairpin ribozyme cleavage (Nesbitt, Hegg and Fedor 1997). To determine whether cobalt hexammine can support VS ribozyme cleavage, we tested the VS RNA construct G11 (Figure 2) for self-cleavage activity in the presence of cobalt hexammine as the only polyvalent metal. In the presence of 100 μM EDTA, to remove any contaminating magnesium, and 50 mM KCl to aid the formation of secondary structure, no G11 cleavage activity was detectable in up to 25 mM cobalt hexammine after 52 hours (Figure 5a). Over the same period of time and under the same conditions, G11Pre is almost completely cleaved in the presence of ≥5 mM magnesium. Therefore, I conclude that cobalt hexammine alone cannot satisfy the cleavage requirements of G11.
Glodon forms almost the same tertiary structure in cobalt hexammine as in magnesium.

Since cobalt hexammine cannot make inner-sphere contacts with RNA (Basolo and Pearson 1967), the observation that GI1 does not cleave in cobalt hexammine alone suggests that cobalt hexammine fails to make one or more inner-sphere RNA contacts that are essential for cleavage activity. Inner-sphere metal coordination might be absolutely required to support VS ribozyme tertiary structure. Therefore a real possibility is that cobalt hexammine might not form VS ribozyme tertiary structure. To address the extent to which cobalt hexammine can properly fold the RNA, I have used chemical modification structure probing to compare the structure of VS RNA induced by cobalt hexammine to the properly folded structure induced by magnesium.

Ideally, I would like to compare the chemical modification structure probing pattern of the catalytically active precursor RNA, GI1Pre, in the presence of either cobalt hexammine or magnesium. But, because GI1Pre self-cleaves in the presence of magnesium, attempts to perform chemical modification structure probing on GI1Pre in magnesium have resulted in the modification of a mixture of GI1Pre and its downstream cleavage product, GI1D (Beattie 1997). As a result, a direct comparison of the chemical modification pattern of GI1Pre in magnesium relative to that in cobalt hexammine cannot easily be accomplished. However, a comparison of the chemical modification pattern of GI1D in magnesium or cobalt hexammine can be performed. The overall structure of GI1D is probably very similar to the active structure as the minimal contiguous self-cleaving VS RNA construct is only one nucleotide longer than GI1D at its 5' end.

To compare the structure of GI1D in magnesium relative to the structure of GI1D in cobalt hexammine, I have performed chemical modification structure probing on
G11D over a range of magnesium or cobalt hexammine concentrations. By performing chemical modification experiments over a range of magnesium or cobalt hexammine concentrations, metal dependent tertiary structure formation can be visualized through the change in chemical reactivity of nucleotides that participate in tertiary structure formation. This technique has allowed me to compare the metal dependent folding pathway of G11D in the presence of either magnesium or cobalt hexammine.

Chemical modification structure probing can be used to monitor the reactivity of specific nucleotides under a variety of conditions. Two chemical agents have been utilized in this study. They are diethyl pyrocarbonate (DEPC) and dimethyl sulphate (DMS).

DEPC modifies the N7 position of adenines. Adenines whose N7 positions react with DEPC are either not stacked in a helix, not involved in tertiary interactions with another base or backbone, or not involved in a direct metal co-ordination (Krol and Carbon 1989). Adenines which are reactive towards DEPC under conditions which promote secondary structure (50 mM KCl, no polyvalent metal) but show a change in reactivity towards DEPC in the presence of polyvalent metal ions are thought to be reflecting the formation of tertiary structure. There are 21 such adenines spread throughout G11D making DEPC a very useful reagent for monitoring tertiary structure formation in G11D (Beattie, Olive and Collins 1995).

DMS modifies the N3 position of cytosines and as a result, cytosines which are involved in Watson-Crick base pairing are protected from modification by DMS. Unlike DEPC reactivity, DMS reactivity is not affected by base stacking interactions (Krol and Carbon 1989). The utility of DMS comes primarily from it being able to monitor the
formation of two structural elements that are required for cleavage activity: a long-range pseudoknot that forms between loops I and V (Rastogi et al. 1996; Figure 2) and a tertiary structure induced rearrangement of helix 1b (Anderson and Collins, unpublished; Figure 4). Thus, by performing DMS modification experiments over a range of polyvalent metal concentrations, the metal dependent formation of these structural elements can be observed.

**DEPC modification of G11D in Magnesium or Cobalt hexammine**

Figure 6 shows the results of diethyl pyrocarbonate (DEPC) modification of G11D in 50 mM KCl and 0 to 10 mM magnesium or cobalt hexammine. A qualitative examination of Figure 6 reveals that adenines which become protected from DEPC with increasing magnesium also become protected from DEPC with increasing cobalt hexammine. The same is true for adenines which become more accessible to DEPC.

This suggests that the overall structure of G11D in cobalt hexammine or magnesium is very similar. Significantly, cobalt hexammine can facilitate changes in DEPC reactivity at a much lower concentration than can magnesium (compare Figure 6a and Figure 6b; see below). This indicates that cobalt hexammine is more efficient than magnesium at folding G11D.

In order to more critically compare magnesium or cobalt hexammine dependent changes in adenine reactivity towards DEPC, the experiments shown in Figure 6a and 6b were repeated at least three times and the intensity of the bands corresponding to all 21 reactive adenines were quantified. Quantification revealed that the DEPC reactivity of A676 was independent of magnesium or cobalt hexammine concentration (see Figure 7).
A676 could thus function as an internal control. Therefore, the reactivities of the remaining 20 adenines are expressed relative to the DEPC reactivity of A676 (See Appendix A).

Two examples of this quantification are shown in Figure 8a and Figure 8b. Notice that by expanding the X-axis for the cobalt hexammine curves by 33-fold, the magnesium and cobalt hexammine curves lie on top of one another (Figure 8c and Figure 8d). This indicates that the tertiary interactions which change the DEPC reactivity of A698 and A726 require a 33 times higher concentration of magnesium than cobalt hexammine to form.

To compare the folding of G11D in increasing magnesium versus increasing cobalt hexammine, the concentration of cobalt hexammine or magnesium that is required to half-maximally change the reactivity of each adenine ([M]_{1/2}) is plotted (Figure 9a). With the cobalt hexammine axis expanded 33 fold, 18 out of the 20 adenines show no significant difference in the [M]_{1/2} of cobalt hexammine or magnesium. These observations suggest that G11D folds almost identically in increasing magnesium relative to increasing cobalt hexammine. Moreover, cobalt hexammine is 33-fold more efficient at facilitating this folding than magnesium.

Adenines that display a difference in the relative [M]_{1/2} in magnesium versus cobalt hexammine are A639 and A645. A639 requires relatively more cobalt hexammine than magnesium to become half reactive. This might indicate that cobalt hexammine has a weaker relative affinity for a metal binding site near A639 than magnesium. A645, on the other hand, requires relatively less cobalt hexammine than magnesium to become half react...
reactive. Perhaps this is indicating the presence of a metal binding site near A645 which has a higher relative affinity for cobalt hexammine than magnesium.

To compare the folded structure of G11D in magnesium relative to cobalt hexammine, the reactivity of each adenine in 10 mM magnesium or 0.3 mM cobalt hexammine is plotted¹ (Figure 9b). Seventeen out of the 20 adenines display no significant difference in DEPC reactivity whether the RNA is in 10 mM magnesium or 0.3 mM cobalt hexammine. Therefore, the overall tertiary structure of G11D in magnesium or cobalt hexammine is very similar.

The three adenines which have a different reactivity to DEPC in 10 mM magnesium than in 0.3 mM cobalt hexammine likely reflect subtle structural differences. These adenines are A657, A730 and A756. A730 and A756 are located opposite each other in an asymmetric internal loop in helix VI and are more accessible to DEPC in 0.3 mM cobalt hexammine than in 10 mM magnesium (Figure 9b). These two adenines lie very close to the phosphate groups of C757 and G758 which have previously been implicated in direct co-ordination with magnesium (Sood, Beattie and Collins 1998). A657, which is located at the helix II-III-VI junction, is also more accessible to DEPC in 0.3 mM cobalt hexammine than in 10 mM magnesium. Metal binding sites have been found at helix junctions in the crystal structures of tRNA (reviewed by Pan et al. 1993) and the hammerhead ribozyme (Pley, Flaherty and McKay 1994; Scott, Finch and Klug 1995). Therefore, these differences in DEPC reactivity in the folded structure of G11D in magnesium relative to cobalt hexammine may be the result of slight structural differences very close to metal binding sites.

¹ G11D is completely folded in 10 mM magnesium or 0.3 mM cobalt hexammine (see Appendix A)
DMS modification of G11D in Magnesium or Cobalt Hexammine

To expand my analysis of G11D structure in the presence of cobalt hexammine I have used dimethyl sulphate (DMS) modification to assay the formation of two known magnesium dependent conformational changes in G11D. It has previously been shown that a long-range pseudoknot forms between loops I and V in the presence of magnesium (Rastogi et al. 1996, Figure 2). It has also been shown that the secondary structure of helix Ib rearranges upon the addition of magnesium (Anderson and Collins, unpublished data; Figure 4). I wanted to determine whether the same conformational changes occur when G11D is folded in cobalt hexammine.

Figure 10 shows the results of DMS modification of G11D in 50 mM KCl and 0 to 10 mM magnesium or cobalt hexammine. DMS modifies the N3 position of cytosines and as a result, cytosines which are involved in Watson-Crick base pairing are protected from modification by DMS (Krol and Carbon 1989). DMS structure probing of G11D in the presence of magnesium (Figure 10a, Figure 11a; Beattie, Olive and Collins 1995) reveals that C699 is protected from DMS modification in 10 mM magnesium. This protection is consistent with mutational data indicating that C699 forms a Watson-Crick base pair with G630 as part of a long range pseudoknot (Rastogi et al. 1996). In the presence of 0.25 mM cobalt hexammine, C699 is significantly protected from DMS (Figure 10b and 11a) suggesting that the pseudoknot of G11D also forms in cobalt hexammine.

DMS structure probing and mutational data have previously shown that the secondary structure of helix Ib rearranges upon the addition of magnesium (Anderson and Collins, unpublished data; Figure 4). Two indicators of helix Ib rearrangement in G11D
are the changes in DMS reactivity of C634 and C637. C634 is protected from DMS in the absence of magnesium but becomes accessible to DMS in the presence of magnesium which is consistent with the bulging of C634 out of helix Ib in the presence of magnesium (Figure 10a). C637, while accessible to DMS in the absence of magnesium, becomes protected from DMS in the presence of magnesium (Figure 10a). The protection of C637 in the presence of magnesium is consistent with mutational data that suggests pairing with G623. These changes in DMS reactivity are consistent with a magnesium dependent secondary structure rearrangement of helix Ib (Figure 4).

The structural rearrangement of helix Ib also appears to occur in the presence of cobalt hexammine. Figure 10b reveals that C634, while protected from DMS in the absence of cobalt hexammine, becomes accessible to DMS in the presence of cobalt hexammine. In addition C637, which is accessible to DMS in the absence of cobalt hexammine, becomes protected from DMS in the presence of cobalt hexammine. These changes in DMS reactivity are consistent with cobalt hexammine being able to induce the structural rearrangement of helix Ib.

Quantification of the changes in reactivity of C699 and C634 upon the addition of cobalt hexammine or magnesium is consistent with a 33-fold higher concentration of magnesium than cobalt hexammine being required to half change the reactivity of C699 and C634 (Figure 9a and Figure 11). In addition, the absolute concentrations of cobalt hexammine or magnesium that are required to half change the reactivity of C699 or C634 are in the range of concentrations required to half change the reactivity of most of the adenines (Figure 9a). These observations suggest that the formation of the pseudoknot and the rearrangement of helix Ib occur in concert with the folding of the rest of the RNA
both in magnesium and in cobalt hexammine. However, it is apparent from Figure 11 that the change in DMS reactivity of C699 and C634 is less in cobalt hexammine than in magnesium (see also Figure 9b). This observation raises the possibility that the pseudoknot might not be as stable in cobalt hexammine as in magnesium.

Mixed metal kinetic analysis

Chemical modification structure probing data suggest that cobalt hexammine can fold G1′D remarkably well. But, is the tertiary structure that is induced by cobalt hexammine functionally relevant? I reasoned that the self-cleavage rate of G1′Pre, in magnesium concentrations below that required for complete folding, should increase by pre-incubating the RNA in cobalt hexammine if cobalt hexammine can assist in folding the RNA into a functionally relevant structure. The results of such an experiment are shown in Figure 12. Pre-incubating G1′Pre in 100 μM cobalt hexammine and adding 0.75 mM magnesium to start the cleavage reaction increases the rate of cleavage by 9-fold relative to RNA pre-incubated in the absence of cobalt hexammine. This result shows that cobalt hexammine can assist in folding the RNA into a functionally relevant structure.

It is interesting that the magnitude of the cobalt hexammine dependent rate enhancement is inversely related to the magnesium concentration that was used to start the cleavage reaction. This is consistent with lower concentrations of magnesium folding G11 to a lesser extent, thus allowing cobalt hexammine to perform more folding. Cobalt hexammine can, therefore, perform at least some of the RNA folding roles of magnesium and can aid in the formation of functionally relevant structure.
The fact that this experiment works suggests that G11 has a higher affinity for magnesium than cobalt hexammine at metal binding sites which require inner-sphere co-ordination for catalytic activity. If this were not the case, then cobalt hexammine would be expected to compete with magnesium at such sites and the observed cleavage rate would decrease with increasing cobalt hexammine concentration since cobalt hexammine cannot form inner-sphere co-ordinations. Therefore, one criterion for metal binding sites which require inner-sphere co-ordination for catalytic activity is that they should have a higher affinity for magnesium than cobalt hexammine when compared to metal binding sites which do not require inner-sphere co-ordination.

II. Using Cobalt Hexammine to Gain Structural Information on G11Pre.

Chemical modification structure probing and mixed metal kinetic experiments suggest that cobalt hexammine can act as an analog for hexahydrated magnesium and can facilitate the formation of functionally relevant VS RNA tertiary structure. Since G11Pre does not cleave when cobalt hexammine is present as the only polyvalent metal (Figure 5), I reasoned that structural information about the precursor RNA (G11Pre) could be obtained by using cobalt hexammine to facilitate RNA folding.

The secondary structure of G11Pre differs from G11D in that helix Ia forms in G11Pre but not in G11D where the sequence comprising the 5’ side of helix Ia is removed by self cleavage (Beattie, Olive and Collins 1995; see Figure 2). I was interested in determining how the tertiary structure of G11D might differ from the tertiary structure of G11Pre due to the presence of helix Ia.
Gl1Pre has more structure around the cleavage site than G11D.

Gl1Pre was modified with DEPC or DMS in 50 mM KCl and 0 to 10 mM cobalt hexammine (Figure 13b and Figure 14b). Almost all of the adenines in Gl1Pre exhibit the same DEPC reactivity pattern in increasing cobalt hexammine as the adenines in G11D (compare Figure 13b and Figure 6b). This suggests that the overall structure of Gl1Pre is similar to the structure of G11D. One adenine whose DEPC reactivity pattern is different in Gl1Pre relative to G11D is A639. A639 of Gl1Pre is protected from DEPC under conditions that promote secondary structure (Figure 13b - 0 mM cobalt hexammine) and remains protected with the addition of cobalt hexammine, whereas A639 of G11D is accessible to DEPC under secondary structure conditions and becomes protected from DEPC upon the addition of polyvalent metal (Figure 6a and 6b). This difference in the chemical reactivity of A639 between Gl1Pre and G11D might result from A639 stacking on helix Ia in Gl1Pre. Stacking interactions can protect the adenine N7 position from DEPC modification (Krol and Carbon 1989). In G11D, A639 cannot stack on helix Ia because helix Ia does not form in G11D (Figure 2). Alternatively A639 might base pair with G620 in Gl1Pre thus protecting it from DEPC modification in the absence of cobalt hexammine. Sheared G-A base pairs, which involve the N7 position of the adenine in hydrogen bonding, have been identified in the crystal structures of the hammerhead ribozyme (Pley et al. 1994; Scott et al. 1995) and in the internal loop of an RNA dodecamer (Beayens et al. 1996).

Another base that displays a different chemical reactivity pattern in increasing cobalt hexammine in Gl1Pre relative to G11D is C637. Unlike C637 of G11D, C637 of Gl1Pre is not accessible to DMS under secondary structure conditions (compare Figure
14b no cobalt hexammine lane with Figure 10b no cobalt hexammine lanes). This is the same trend that was observed for A639. Because DMS is not affected by stacking interactions, the protection of C637 in G11Pre in the absence of cobalt hexammine is likely due to C637 forming a non-canonical base pair with A622. Combined, the DEPC and DMS data on A639 and C637 suggest that there is more structure in the cleavage loop of G11Pre than in G11D.

Pseudoknot formation and helix IIb rearrangement are inhibited in G11Pre.

The DEPC and DMS modification reactions on G11Pre in cobalt hexammine were repeated at least three times and quantified. A summary of the quantification is shown in Figure 15a and Figure 15b. Quantification reveals four additional differences between G11Pre and G11D in cobalt hexammine. These differences occur at A645, A698, C699 and C634.

A645-G11Pre requires more cobalt hexammine to half change its reactivity than A645-G11D (Figure 15a). This suggests that a slightly different folding pathway takes place in helix I-II junction of G11Pre relative to G11D. The second difference in G11Pre is A698. Interestingly, in 0.3 mM cobalt hexammine, A698-G11Pre is more accessible to DEPC than A698-G11D (Figure 15b and Figure 16a). Mutational data indicate that in the active structure of G11, A698 forms a Watson-Crick base pair with U631 forming part of a long range pseudoknot (Rastogi et al. 1996). The metal dependent DEPC protection of A698-G11D has been interpreted by myself and others as reflecting pseudoknot formation (Rastogi et al. 1996). However, the observation that A698-G11Pre
is more accessible to DEPC than A698-G11D in 0.3 mM cobalt hexammine suggests that the pseudoknot is not as stable in G11Pre as in G11D.

C699 is another reporter of pseudoknot formation because mutational evidence has shown that it forms a base pair with G630 in making the pseudoknot (Rastogi et al. 1996). DMS structure probing of G11Pre reveals that C699 requires a much higher concentration of cobalt hexammine to become protected from DMS in G11Pre than in G11D (compare Figure 14b with Figure 10b and see the quantification in Figure 16b). Indeed, the cobalt hexammine [M]_{1/2} of C699-G11Pre is about 20 times higher than for C699-G11D (Figure 15a). Thus, the pseudoknot requires a considerably higher concentration of cobalt hexammine to form in G11Pre than in G11D. Suprisingly A698, also thought to be a reporter of pseudoknot formation, does not display this [M]_{1/2} difference between G11Pre and G11D (see discussion).

The final difference observed between G11Pre and G11D in cobalt hexammine is C634. The DMS reactivity of C634 provides a reporter for helix Ib rearrangement (Figure 4). Unlike C634 of G11D, C634 of G11Pre does not become accessible until at least 1 mM cobalt hexammine is present (compare Figure 14b and Figure 10b and see Figure 16c). Quantification shows that the cobalt hexammine [M]_{1/2} of C634 of G11Pre is at least 2 mM which is more than 20 times greater than the cobalt hexammine [M]_{1/2} of C634-G11D (Figure 15a).

It is interesting that pseudoknot formation and helix Ib rearrangement both require about 20-fold more cobalt hexammine to occur in G11Pre than G11D. In addition to suggesting that pseudoknot formation and helix Ib rearrangement are inhibited in G11Pre, this observation argues that the two phenomena may be mechanistically related.
It remains possible, however, that the observed differences between G11Pre and G11D exist in cobalt hexammine but not in magnesium. To evaluate this possibility I probed G11Pre with DEPC and DMS in 50 mM KCl and 0 to 10 mM magnesium (Figure 13a and 14a). Quantification of A698, C699 and C634 from these experiments is shown in Figure 16d, Figure 16e and Figure 16f respectively. Even though these experiments are complicated by self-cleavage (22% in 10 mM magnesium), the same differences that were observed between G11Pre and G11D at these positions in cobalt hexammine (Figure 16a, Figure 16b and Figure 16c) are also observed between G11Pre and G11D in magnesium (Figure 16d, Figure 16e and Figure 16f). Therefore, the observations that I have made on the differences between G11Pre and G11D in cobalt hexammine are reflective of relevant differences between G11Pre and G11D in magnesium.

III. Using Cobalt Hexammine to Gain Structural Information on RS19Pre.

RS19 is a circular permutation of G11 wherein the 5’ end of helix I is adjoined to the 3’ end of helix II by a 24 nucleotide linker (Anderson and Collins, unpublished; Figure 17)). Under standard cleavage conditions (see materials and methods), the cleavage rate of RS19 is nearly 100 times greater than that of G11 (Anderson and Collins, unpublished). To begin to understand the structural basis for this difference in function, I have assayed RS19 for cleavage activity in cobalt hexammine with or without limiting concentrations of magnesium. I have also performed chemical modification structure probing on RS19 in the presence of cobalt hexammine using DEPC and DMS.
RS19 does not cleave when cobalt hexammine is present as the only polyvalent metal.

To address whether RS19 is functional in cobalt hexammine alone, I assayed RS19 cleavage in the presence of 200 μM cobalt hexammine (Figure 18a). Under these conditions, no cleavage activity is detected after 60 minutes, the longest time point taken. Therefore, the cleavage rate of RS19 in 200 μM cobalt hexammine must be less 0.0005 per minute. Thus, cobalt hexammine alone cannot satisfy the cleavage requirements of RS19Pre.

Cobalt hexammine enhances the cleavage rate of RS19 10,000-fold in low magnesium.

Pre-incubating G11 in micromolar concentrations of cobalt hexammine followed by the addition of 0.75 mM magnesium increases the cleavage rate of G11 by 9-fold relative to the non-cobalt hexammine containing control (Figure 12). Can pre-incubating RS19 in cobalt hexammine followed by the addition of magnesium induce a similar rate enhancement? When RS19 was pre-incubated in 200 μM cobalt hexammine followed by the addition of 0.75 mM magnesium, 85% of it cleaved in 10 minutes (Figure 18c). This represents a rate increase of at least two orders of magnitude over the control reaction lacking cobalt hexammine (Figure 18b) in which no cleavage was detectable after 10 minutes.

To extend and confirm this result, I pre-incubated RS19 in a range of cobalt hexammine concentrations from 0.25 mM to 1.5 mM and started the cleavage reaction by adding either 0.25 mM, 0.5 mM, 0.75 mM or 1 mM magnesium. The quantification of these experiments is shown in Figure 19. As is the case with G11, the lower the magnesium concentration, the larger the cobalt hexammine dependent rate enhancement.
suggesting that cobalt hexammine and magnesium are fulfilling some of the same roles (Figure 12 and Figure 19). However, in contrast to the situation with G11, in RS19 the cobalt hexammine dependent rate increase is several orders of magnitude. Notably, the cleavage rate of RS19 in 0.25 mM magnesium is increased by at least 10,000 fold when pre-incubated in 400 uM cobalt hexammine. Thus, cobalt hexammine clearly can induce functionally relevant structure in RS19.

Another departure from G11 is that the cobalt hexammine concentration that is required to reach cleavage rate saturation in RS19 is about 400 μM while in G11 it is at most 100 μM. This observation raises the possibility that cobalt hexammine is able to fulfill folding roles that it cannot fulfill in G11.

The tertiary structure of RS19Pre is very similar to the tertiary structure of G11Pre.

In order to identify what structural differences might exist between G11Pre and RS19Pre, I have performed DEPC and DMS chemical modification structure probing on RS19Pre. DEPC modification was performed on RS19Pre over a range of cobalt hexammine concentrations up to 0.5 mM (Figure 20) and DMS modification was performed on RS19 in up to 10 mM cobalt hexammine (Figure 21). The DEPC and DMS modification patterns of RS19Pre are almost identical to that of G11Pre suggesting that RS19Pre and G11Pre have very similar tertiary structures (compare Figure 13b and Figure 14b with Figure 20 and Figure 21 respectively). Noteworthy, is the observation that in RS19Pre as in G11Pre, C699 does not get protected from DMS until at least 2 mM cobalt hexammine is added (Figure 21). Similarly, C634 does not become significantly accessible to DEPC until about 2 mM cobalt hexammine (Figure 21). Therefore, the fast
cleavage kinetics of RS19 (Anderson and Collins, unpublished data) and the very large cobalt hexammine dependent cleavage rate increase of RS19 (Figure 19) seem unlikely to be due to cobalt hexammine being better able to form the pseudoknot or shift helix I in RS19Pre than G11Pre.

*RS19Pre has less structure around the cleavage site than G11Pre.*

The RS19Pre and G11Pre chemical modification patterns differ at two positions. These nucleotides are A639 and C637 and their differences in chemical reactivity in RS19Pre relative to G11Pre suggest that RS19Pre has less structure in the internal loop of helix I than G11Pre. I have already shown that A639 in G11Pre is protected from DEPC under conditions that promote secondary structure and remains protected with the addition of cobalt hexammine (Figure 13b). I reasoned that this is either due to A639 of G11Pre being stacked on helix Ia or pairing with G620. In contrast, A639 of RS19Pre is accessible to DEPC under conditions that promote secondary structure and remains accessible to DEPC with the addition of cobalt hexammine (Figure 20). Thus, in contrast to A639 of G11Pre, the N7 of A639 in RS19Pre is not involved in secondary or tertiary structure.

A second difference in RS19 occurs at C637. C637 of G11Pre is not accessible to DMS under secondary structure conditions (Figure 14). I have proposed that this is due to C637 forming a non-canonical base pair with A622. To the contrary, C637 of RS19Pre is accessible to DMS under secondary structure conditions which argues that in the absence of polyvalent metal, the N3 position of C637 is not involved in secondary structure. Thus, the chemical reactivity patterns of A639 and C637 suggest that RS19Pre has less structure in the internal loop of helix I than G11Pre.
There are five adenines or cytosines in the internal loop of helix I in RS19 that either cannot be resolved in G11 (A621 and A622) or are not present in G11 (C639.1, A639.3 and A639.4). The chemical reactivities of these nucleotides also suggest that the internal loop of helix I in RS19 has very little structure. All of these nucleotides are accessible to chemical modification under secondary structure conditions and remain accessible to modification upon the addition of cobalt hexamine (Figure 20 and Figure 21). These observations strengthen my contention that the internal loop of helix I in RS19Pre has less structure than the internal loop of helix I in G11Pre. Possible implications of this difference will be proposed in the discussion.

IV. Structural Requirements of Helix Ib.

ZA10 is a mutant based on G11 in which four bases in helix Ib have been replaced by adenines (Figure 22a, Beattie 1997). This mutant was designed to disrupt base pairing within helix Ib while maintaining the identity of nucleotides that had been shown to be important for self-cleavage (Beattie 1997). The cleavage kinetics of ZA10 have been shown to be very similar to that of G11 (Beattie 1997). Because ZA-10 was intended to completely disrupt base pairing in stem Ib, this observation was interpreted as indicating that structure in helix Ib is not required for catalysis (Beattie 1997). However, in light of the helix Ib rearrangement that has since been shown to occur in G11 (Figure 22d), two Watson-Crick base pairs at the bottom of helix Ib appear possible in the putative native conformation of ZA10 (Figure 22b) raising the possibility that significant structure might exist in helix Ib of ZA10. To compare the structure of ZA10 to G11 and evaluate the possibility that helix Ib in ZA10 can form two base pairs, I have modified
ZA10Pre and ZA10D with DEPC and DMS in magnesium and cobalt hexammine. I have also sought mutational evidence to address the functional relevance of these putative base pairs and to understand the minimal structural requirements of helix Ib.

*The tertiary structure of ZA10D is very similar to the tertiary structure of G11D.*

To evaluate the structure of ZA10D relative to G11D, ZA10D was chemically probed with DEPC in the presence of 50 mM KCl and 0 to 10 mM magnesium (Figure 23a) or 0 to 10 mM cobalt hexammine (Figure 23b). With the exception of the four adenines in ZA10 helix Ib which do not exist in G11, the DEPC structure probing pattern of ZA10D in 0 to 10 mM magnesium is essentially the same as the DEPC pattern of G11D in 0 to 10 mM magnesium (compare Figure 6a and Figure 23a, see quantification Figure 24a and Figure 24b). Similarly, the DEPC reactivity pattern of ZA10D in 0 to 10 mM cobalt hexammine is the same as the DEPC pattern of G11D in 0 to 10 mM cobalt hexammine (compare Figure 6b and Figure 23b, see quantification Figure 24b and Figure 24c).

Not surprisingly, when the 33 fold difference in folding efficiency between magnesium and cobalt hexammine is taken into account, the same cobalt hexammine - magnesium differences observed in G11D also exist in ZA10D. In short, A639 displays a higher [M]_{1/2} for cobalt hexammine than magnesium, A645 displays a lower [M]_{1/2} for cobalt hexammine than magnesium, and A657, A730 and A756 are more reactive to DEPC in 0.3 mM cobalt hexammine than in 10 mM magnesium (compare Figure 24a and Figure 24b with Figure 24c and Figure 24d). These results indicate that the overall tertiary structure of ZA10D is very similar to the tertiary structure of G11D.
**Cobalt hexammine or magnesium induces structure in helix Ib of ZA10D.**

The four adenine nucleotides in helix Ib of ZA10 (A625, A627, A633 and A635) provide convenient reporters of stem Ib structure. In ZA10D, these adenines are relatively accessible to DEPC in the absence of polyvalent metal (see Figure 23a and Figure 23b). Thus, under secondary structure conditions, the region of helix Ib containing these adenines possesses little structure. Surprisingly, the addition of either cobalt hexammine or magnesium causes these adenines to become somewhat protected from DEPC suggesting that structure in stem Ib of ZA10D is induced by magnesium or cobalt hexammine (see quantification, Figure 25). Moreover, when the 33 fold efficiency difference between cobalt hexammine and magnesium is taken into account, these adenines require relatively more cobalt hexammine than magnesium to undergo this change in DEPC reactivity (see Figure 25 and compare Figure 24a and Figure 24c). Thus, magnesium is relatively more efficient at inducing this structure than cobalt hexammine.

To further investigate the structure of ZA10 helix Ib, I modified ZA10D with DMS in the absence of polyvalent metal or in the presence of either 10 mM cobalt hexammine or 10 mM magnesium (Figure 26a). In the presence of 50 mM KCl and no polyvalent metal (the "No Metal" lane in Figure 26a) C626, C629, C634, C636, C637 and C641 are accessible to DMS while C632 is protected from DMS. This result, along with the observation that the ZA10 helix Ib adenines are accessible to DEPC in the absence of cobalt hexammine or magnesium suggest that helix Ib is largely single stranded in the absence of polyvalent metal (see Figure 26b). The protection of C632 is curious seeing as no helix Ib secondary structure model fits to C626, C629, C634, C636 and C641 being
accessible and C632 being protected. It is therefore unclear as to what the protection of C632 in the absence of polyvalent metal means.

In the presence of either magnesium or cobalt hexammine C636 and C637 become protected, C634 becomes hyper-reactive and the reactivity of C626 remains unchanged (Figure 26a). These results are consistent with the formation of the structure diagrammed in Figure 26c. In this model, C636 and C637 are protected from DMS by base pairing with G623 and G624 respectively. C634 attains a hyper-reactive state by perhaps attaining a structure similar to the hyper-reactive C634 of G11D in magnesium. That C626 remains reactive to DMS in the presence of cobalt hexammine or magnesium is consistent with C626 having no Watson-Crick pairing partner. While no Watson-Crick base pairing appears possible in the top part of helix Ib, the magnesium or cobalt hexammine induced protection of adenines in helix Ib (Figure 25) argue that non-Watson-Crick base pairing or base stacking interactions exist.

Cobalt hexammine or magnesium induces structure in helix Ib of ZA10Pre.

To address whether the structure of ZA10D differs from the structure of ZA10Pre I probed ZA10Pre with DEPC in the presence of 50 mM KCl and 0 to 10 mM magnesium (Figure 27a) or 0 to 10 mM cobalt hexammine (Figure 27b). Quantification of ZA10Pre DEPC modification in 0 to 10 mM cobalt hexammine (Figure 28) reveals that most adenines in ZA10Pre behave the same way towards DEPC as the adenines in ZA10D suggesting that as with G11, the overall fold of ZA10Pre is similar to that of ZA10D.

Figure 28a and 28b make it apparent that several adenines in ZA10Pre behave
differently towards cobalt hexammine than their counterparts in ZA10D. A698 of ZA10Pre is more reactive to DEPC than A698 of ZA10D in 0.3 mM cobalt hexammine (Figure 28b) suggesting that, as in G11, the pseudoknot is less stable in ZA10Pre than in ZA10D. The concentration of cobalt hexammine required to half change the DEPC reactivity of A645 is lower in ZA10D than in ZA10Pre which is also similar to the case with G11. However, A645 of ZA10Pre in the absence of polyvalent metal is more reactive to DEPC than A645 of ZA10D in the absence of polyvalent metal which is clearly different from the situation in G11.

All of the adenines probed in helix I of ZA10Pre react differently towards cobalt hexammine than their ZA10D counterparts. This suggests that helix I of ZA10Pre is in a different conformation than helix I of ZA10D. In ZA10Pre, A639 has a lower cobalt hexammine [M]_{1/2} than A639 of ZA10D (Figure 28a). Also, A639 of ZA10Pre is protected to a greater extent in the presence of 0.3 mM cobalt hexammine or 10 mM magnesium than is A639 of ZA10D (see Figure 28b and compare Figure 23a and Figure 23b with Figure 27a and Figure 27b). These results contrast the G11Pre versus G11D difference identified at A639. Recall that in G11Pre, A639 is protected from DEPC in the absence of polyvalent metal and remains protected from DEPC in the presence of cobalt hexammine or magnesium (Figure 11b). It was postulated that this protection was due to A639 either being stacked on helix Ia or forming a base pair with G620. In the absence of polyvalent metal A639 of ZA10Pre is accessible to DEPC and therefore it is not stacked on helix Ia or involved in a base pair with G620.

A627, A633 and A635 show different DEPC reactivities in ZA10Pre relative to ZA10D (Figure 28 and Figure 29). A633 and A635 are more protected from DEPC in
ZA10Pre than in ZA10D in the absence of polyvalent metal suggesting that stem Ib has more structure in ZA10Pre than ZA10D under these conditions. Perhaps the presence of helix Ia forces structure into helix Ib. Also, the cobalt hexammine [M]_{1/2} of A633 and A635 is lower in ZA10Pre than D (Figure 28a). A627 displays a very prominent difference in ZA10Pre relative to ZA10D. A627 of ZA10Pre becomes more accessible to DEPC with increasing cobalt hexammine or magnesium while A627 of ZA10D becomes protected with increasing cobalt hexammine or magnesium (Figure 29). These results indicate that the conformation of helix I of ZA10Pre is different from the structure of helix I of ZA10D in the presence and absence of cobalt hexammine or magnesium.

In an attempt to understand the structure of helix I in ZA10Pre, it was probed with DMS in the presence or absence of 10 mM magnesium or cobalt hexammine (Figure 30). In the presence of 50 mM KCl and no polyvalent metal, C626, C629, C634, C636 and C637 are relatively accessible to DMS while C632 and C641 appear relatively protected from DMS (compare the "No Metal" lane with the "Den" lane in Figure 30a). The accessibility of C626, C629, C634, C636 and C637 argue that little Watson-Crick structure exists in helix Ib in the absence of polyvalent metal (Figure 30b). The protection of C641 is consistent with C641 base pairing with C619 in helix Ia. However, as in ZA10D, in the absence of polyvalent metal, the protection of C632 is puzzling.

In the presence of 10 mM cobalt hexammine, C629 and C637 become protected from DEPC, C634 becomes hyper-reactive and the reactivity of C626 and C636 remain relatively unchanged (Figure 30a). That C636 is accessible to DMS in the presence of cobalt hexammine argues that a Watson-Crick base pair between G624 and C636 does not form in ZA10Pre. These results are consistent with the formation of the helix I
Interpretation of the DMS modification pattern of stem I of ZA10Pre in the presence of magnesium is more difficult because of the presence of greater than 50% ZA10D due to self-cleavage. The DMS modification pattern of ZA10Pre in magnesium differs from that in cobalt hexammine in that C636 and C641 are more accessible to DMS in magnesium and C634 is more hyper-reactive in magnesium than in cobalt hexammine. The accessibility of C641 in magnesium is likely due to the presence of ZA10D because C641 has no Watson-Crick pairing partner in ZA10D. The increased hyper-reactivity of C634 may also be due to the presence of ZA10D if the hyper reactive helix Ib structure is more stable in ZA10D in 10 mM magnesium than in ZA10Pre in 10 mM cobalt hexammine. Because C636 of ZA10D is protected from DMS in the presence of magnesium (Figure 26a), the reactivity of C636 in the 10 mM magnesium lane of Figure 30a must be due to C636 of ZA10Pre being quite accessible to DMS. This suggests that a Watson-Crick base pair between G624 and C636 of ZA10Pre does not form in magnesium (Figure 30c).

*The minimal structural requirement of stem Ib is one Watson-Crick base pair.*

In order to determine if the active structure or structures of ZA10Pre require the two putative Watson-Crick base pairs at the base of stem Ib (Figure 22b), I made several substitutions that would either disrupt a putative base pair or restore it (Figure 31). If a base pair between positions 624 and 636 and/or 623 and 637 is required for catalytic activity in ZA10, only those substitutions which maintain base pairing at these positions should yield catalytically active mutants.
G624 and C636 were independently mutated to either A or U (Figure 31a). All of these mutants showed decreased cleavage activity relative to wild type ZA10 suggesting some preference for a G at position 624 and/or a C at position 636. Mutants which maintain base pairing, U624/A636 and A624/U636, have a higher cleavage rate than mutants which abolish base pairing, namely A634/A636 and U624/U6361. Thus, although a mismatch between positions 624 and 636 is tolerated by ZA10, preference for a base pair exists. This suggests that if a base pair between G624 and C636 of ZA10 is forming, it is not strictly required for cleavage activity.

Eight of the fifteen possible double mutants at positions 623 and 637 were made (Figure 31b). All mutants containing a pyrimidine at position 623 have cleavage rates of less than $4 \times 10^{-5}$ per minute (the limit of detection in these experiments) indicating that a purine at position 623 is required for cleavage activity. Of the mutants containing a purine at position 623, mutants which maintain base pairing, G623/U637 and A623/U637, are the most active. That these mutants have a slower cleavage rate than ZA10 suggests some preference for the wild type G-C base pair. This preference might be due to a partiality for a stronger G-C base pair over a weaker A-U or G-U base pair. The A623/A637 mutant has a cleavage rate of less than $4 \times 10^{-5}$ per minute indicating that an A/A mismatch is not tolerated. The G623/A637 mutant has a measurable but considerably decreased cleavage rate which is consistent with the strained formation of a G/A base pair. These results strongly argue that a base pair forms between G623 and C637 of ZA10Pre and is required for catalytic activity. Thus, the native structure of helix Ib of ZA10Pre has at least one Watson-Crick base pair.
V. Other Results.

This section outlines other results that I have obtained which do not fit into the previous four sections.

*Preparation and characterization of H3ZA10.*

Structural rearrangement of helix Ib is probably not the rate limiting step of G11 cleavage since the cleavage rate of ZA10, which must have little restriction on stem Ib structure, is similar to the cleavage rate of G11 (Beattie 1997). G11 is, however, rate limited by an inhibitory element located in helix Ia (Rastogi and Collins 1998). H3 is a construct in which sequences 5' of G620 have been replaced by 2 guanosine residues such that helix Ia cannot form (Figure 32a, Rastogi and Collins 1998). The cleavage rate of H3 is approximately 10 fold higher than that of G11 yet it is not rate limited by chemistry (Rastogi and Collins 1998). I wondered whether H3 might be rate-limited by structural rearrangement of helix Ib. To test this hypothesis, I introduced the adenine substitutions of ZA10 stem Ib into an H3 background (Figure 32a). Under standard cleavage conditions (see materials and methods) H3ZA10 has a lower cleavage rate than H3 (Figure 32b) suggesting that either H3 is not rate limited by helix Ib structural rearrangement or that the ZA10 mutations don't actually facilitate Ib rearrangement. The adenine substitutions in helix Ib likely have slowed an existing rate limiting step or introduced a new one.

Perhaps H3ZA10 is rate limited by a slow folding step that might be overcome through pre-incubation. To evaluate this possibility I pre-incubated H3 and H3ZA10 RNA in 50 mM KCl with or without 2 mM spermidine and started the cleavage reaction.
with 25 mM magnesium. The results of such an experiment are shown in Figure 33. Pre-incubation in 50 mM KCl with or without 2 mM spermidine has no significant effect on the cleavage rate of H3 or H3ZA10. In short, the rate limiting step of H3ZA10 could not be overcome by these pre-incubations.

Temperature profile of H3.

The only difference in secondary structure between H3 and G11 is that H3 cannot form helix Ia. Yet H3 has a cleavage rate 10 times larger than that of G11 (Rastogi and Collins 1998). This difference in apparent cleavage activity might be due to a difference in the rate in which P, the promoter proximal cleavage product is released from D, the promoter distal cleavage product, after a cleavage event has occurred. If the rate of ligation following a cleavage event is comparable to the rate in which P falls off D, some fraction of undissociated P might ligate back on D creating precursor RNA and reducing the apparent cleavage rate. This phenomenon is more likely to occur in G11 where four base pairs hold P onto D than in H3 which lacks these interactions.

To test this hypothesis I determined the temperature dependence of the cleavage rate of H3 and G11. Assuming the rate of release of P from D in G11 increases more dramatically with temperature than the rate of release of P from D in H3, the apparent cleavage rate of G11 would be expected to increase more dramatically with temperature than the cleavage rate of H3 if the above model is correct. Time course reactions in standard cleavage buffer (see materials and methods) were carried out on G11 and H3 at 10°C, 20°C, 30°C, 40°C, 50°C and 60°C. The first order cleavage rate constants were calculated and the results were plotted in Figure 34. The temperature dependence of H3
and G11 cleavage show similar trends. One interpretation of this result is that the model described above is incorrect. Alternatively the model might be correct if the rate of dissociation of P from D in G11 does not change significantly with temperature. Or, some conformational change in G11 might slow the rate of cleavage at higher temperatures, overshadowing any increase in rate due to an increase in P dissociation with temperature.

**DISCUSSION**

Through chemical modification structure probing and mixed metal kinetic analysis, I have shown that the structure of G11D in the presence of cobalt hexammine very closely resembles the structure of G11D in the presence of magnesium. I have also demonstrated that G11Pre does not undergo self-cleavage when cobalt hexammine is present as the only polyvalent cation. As cobalt hexammine can facilitate G11 folding but not catalysis, cobalt hexammine is a very useful tool to gain structural information on precursor VS RNA.

Chemical modification structure probing on G11Pre in the presence of cobalt hexamine has revealed that a much higher concentration of cobalt hexammine is required to form the pseudoknot and rearrange the secondary structure of helix Ib in G11Pre than in G11D. Chemical modification structure probing on RS19Pre in cobalt hexamine has shown that the loop containing the cleavage site is less structured than its G11 equivalent.

I have also used cobalt hexammine to gain structural information on a stem Ib mutant, ZA10. Chemical modification structure probing of ZA10D and ZA10Pre in the presence of cobalt hexammine is consistent with the formation of two base pairs in helix
Ib of ZA10. Furthermore, mutational data confirmed that one of these base pairs, G623:C637, is required for catalysis.

I. Cobalt Hexammine as an Analogue for Magnesium Hexahydrate.

I have explored the effect that cobalt hexammine has on VS RNA tertiary structure. This has been accomplished by performing chemical modification structure probing on G11D over a range of magnesium and cobalt hexammine concentrations using the modifying agents DEPC and DMS. Chemical modification data show that, while cobalt hexammine and magnesium induce very similar tertiary structure in G11D (Figure 3 and Figure 8), cobalt hexammine is significantly more efficient than magnesium at inducing such structure. Quantification has revealed that 20 of the 22 nucleotides monitored require a 33-fold higher concentration of magnesium than cobalt hexammine to achieve the same change in chemical reactivity (Figure 5). Since changes in chemical reactivity with increasing metal concentration are reflective of tertiary structure formation (Krol and Carbon 1989), 33 times more magnesium than cobalt hexammine is required to form the tertiary structure of G11D.

A similar efficiency difference has recently been reported by another group studying the effect of cobalt hexammine on RNA structure (Hampel, Walter and Burke 1998). Hydroxyl radical footprinting experiments on the hairpin ribozyme have shown that a 50-fold higher concentration of magnesium than cobalt hexammine is required to achieve the same degree of protection of a core nucleotide from solvent. It is likely that the 50-fold efficiency difference reported by this group is not significantly different from the 33-fold efficiency difference that I have found. Because the change in solvent
accessibility of only one hairpin nucleotide was reported in this study, the 50-fold efficiency difference between magnesium and cobalt hexammine could very well be an overestimate. Had I, for example, only looked at the change in chemical reactivity of A645, I would have reported a 120-fold efficiency difference between cobalt hexammine and magnesium since A645 is one of two outliers to the 33-fold efficiency difference trend. If future studies should demonstrate that the 50-fold difference observed for the hairpin ribozyme and the 33-fold difference observed for the VS ribozyme are indeed significantly different, then the tertiary structure of the hairpin ribozyme likely forms more readily in cobalt hexammine than does the tertiary structure of the VS ribozyme. As the hairpin ribozyme is functionally active in cobalt hexammine and the VS ribozyme is not, an increased ability of the hairpin ribozyme to form structure in cobalt hexammine might contribute to its activity in cobalt hexammine.

It is tempting to speculate about the structural reasons that underlie the two outliers to the 33-fold efficiency difference between magnesium and cobalt hexammine. One outlier, A645, requires a 120-fold higher concentration of magnesium than cobalt hexammine to reach the same degree of protection from DEPC. The efficiency difference displayed by A645 may indicate that there is a metal binding site involving the N7 position of A645 that has a particularly high affinity for cobalt hexammine. Because chemical modification structure probing monitors the consequences of metal binding, this efficiency difference could also indicate that cobalt hexammine induces the formation of tertiary structure involving the N7 position of A645 much more efficiently than magnesium. The second outlier to the 33-fold efficiency difference between magnesium and cobalt hexammine is A639 which requires just 12 times more magnesium than cobalt.
hexammine to reach an equivalent degree of protection from DEPC. Since A639 is situated on the opposite side of the internal loop that contains the cleavage site, it is intriguing to speculate that cobalt hexammine might bind to a metal binding site near the cleavage site with a lower relative affinity than magnesium. The lower relative affinity of cobalt hexammine for such a metal binding site could be the result of cobalt hexammine failing to form an inner sphere contact with the RNA that is required for catalysis. Alternatively, cobalt hexammine might induce a slightly altered structure around the cleavage site that is incompatible with catalysis.

In addition to the two nucleotides which do not share the 33-fold efficiency difference between cobalt hexammine and magnesium, a few nucleotides which do share the 33-fold efficiency difference show subtle quantitative differences in chemical reactivity towards cobalt hexammine or magnesium. The structural basis of such small differences in chemical reactivity is difficult to interpret as there is little presently in the literature linking small differences in chemical reactivity to specific structural changes.

We can, however, speculate about what some of these chemical reactivity differences mean based on the positions of the nucleotides that display them. For example, A730 and A756, two nucleotides located opposite each other in an asymmetric loop in helix VI, are both more protected from DEPC in 10 mM magnesium than an equivalent effective concentration of cobalt hexammine, 0.3 mM (Figure 6). These two nucleotides are very close in secondary structure to the non-bridging phosphate oxygens of C757 and G758 which have been implicated in forming inner-sphere contacts with magnesium (Sood, Beattie and Collins 1998). As cobalt hexammine cannot make inner-sphere contacts, inner-sphere co-ordination with the phosphate oxygens of C757 and
G758 cannot occur in cobalt hexammine. Cobalt hexammine would, therefore, be expected to bind less tightly than magnesium to this metal binding site, if at all. It is likely that the differences in DEPC reactivity at A730 and A756 in 10 mM magnesium relative to 0.3 mM cobalt hexammine are reflective of this.

What is the functional significance of the subtle differences in chemical reactivity that are displayed by certain nucleotides in cobalt hexammine relative to magnesium? I have shown that G11 does not undergo self-cleavage when cobalt hexammine is present as the only polyvalent cation. It is therefore possible that the observed differences in structure are detrimental to the RNA's ability to self-cleave. To evaluate this possibility, self-cleavage experiments on G11Pre in the presence of both cobalt hexammine and magnesium were performed. In the presence of 0.75 mM magnesium, a 9-fold increase in cleavage activity was detected over the range of cobalt hexammine concentrations where significant changes in chemical reactivity were observed (Figure 10). This shows that cobalt hexammine can aid in the formation of functionally relevant structure and that differences in the chemical reactivity of certain nucleotides in G11D are not reflective of structure that is detrimental to catalytic function.

One explanation for the lack of VS ribozyme catalysis in cobalt hexammine is that the cleavage mechanism requires the direct co-ordination of a divalent metal-ion to an RNA group. It has been postulated for the hammerhead ribozyme that such an interaction might be required to activate the 2' oxyanion nucleophile or stabilize the transition state (reviewed by Wedekind and McKay 1998). If these interactions are required for G11 cleavage, cobalt hexammine would be unable to form them and G11 would be inactive in cobalt hexammine alone. However, this possibility has been made
less likely by the recent observation that a *trans*-acting VS ribozyme construct, along with the hammerhead and hairpin ribozymes, are catalytically proficient in 4 M sodium, lithium or ammonium ions (Murray et al. 1998a). These are all monovalent ions. The simplest interpretation of these results is that under conditions of high ionic strength, the direct co-ordination of a divalent metal ion to the RNA is not required for catalysis by these ribozymes. Whether or not this is also the case under less extreme conditions is, however, unclear.

II. Using Cobalt Hexammine to Gain Structural Information on G11Pre.

Through DEPC and DMS modification as well as mixed metal kinetic assays, I have shown that cobalt hexammine is able to induce nearly the same structure in G11D that magnesium is able to form. This, in conjunction with my observation that G11Pre does not cleave in the presence of cobalt hexammine alone makes cobalt hexammine a useful tool to gain structural information on the precursor RNA, G11Pre.

Chemical modification structure probing on G11Pre in the presence of cobalt hexammine has yielded several interesting findings that indicate that structural differences between G11Pre and G11D exist. Firstly, DMS modification data show that the pseudoknot, as assayed by C699 protection, requires a 20-fold higher concentration of cobalt hexammine to form in G11Pre than in G11D (Figure 15a). Similarly, helix Ib rearrangement, as assayed by C634 accessibility, also requires a 20-fold higher concentration of cobalt hexammine to occur in G11Pre than in G11D (Figure 15a). That both of these phenomena require significantly more cobalt hexammine to form in G11Pre
than in G11D suggests that either pseudoknot formation is required for helix Ib rearrangement or vice versa.

If the 33-fold efficiency difference between cobalt hexammine and magnesium holds for pseudoknot formation and helix Ib rearrangement in G11Pre, then about 50 mM magnesium (33 x 1.5 mM cobalt hexammine; which is the approximate $[\text{Co}]_{1/2}$ of C634 and C699 of G11Pre) would be required to half-complete these tertiary structure events. If this were the case, then it would explain why the magnesium concentration that is required to saturate the cleavage rate of G11 is well above 50 mM (Olive and Collins 1998). Attempts to directly detect pseudoknot formation or helix Ib rearrangement in G11Pre in the presence of magnesium have been unsuccessful because self-cleavage begins to occur at a lower magnesium concentration than pseudoknot formation and helix Ib rearrangement can be detected (Figure 14a, Figure 16e and Figure 16f). Changes in the reactivity of C634 and C699 were only detectable above 5 mM magnesium owing to the accumulation of G11D. Thus, consistent with our findings in cobalt hexammine, helix Ib rearrangement and pseudoknot formation require a higher concentration of magnesium in G11Pre than in G11D.

The second interesting finding that chemical modification structure probing of G11Pre in cobalt hexammine has revealed is that A698 requires roughly the same concentration of cobalt hexammine to become half protected from DEPC in G11Pre as in G11D (Figure 15a). As mutational evidence has shown that A698 is involved in the pseudoknot (Rastogi et al. 1996), this result is in apparent conflict with the 20-fold higher cobalt hexammine concentration that is required to form the pseudoknot as monitored by C699. One interpretation of these conflicting results is that in low concentrations of
cobalt hexammine, the pseudoknot base pair involving A698 forms while the pseudoknot base pair involving C699 does not. A more plausible explanation, however, is that protection of A698 is not only due to pseudoknot formation but is also due to local structure in loop V such that even if the pseudoknot does not form, local structure protects the N7 of A698 from DEPC. The U696, G697 and A698 residues of loop V likely form a uridine turn (Rastogi et al. 1996). The crystal structures of tRNA<sup>Pre</sup> and the hammerhead ribozyme have revealed that one of the two stabilizing interactions of a uridine turn is a hydrogen bond between the 2'-OH of the uridine and the N7 of the adenine (reviewed by Quigley and Rich 1976; and Wedekind and McKay 1998). If such a uridine turn forms in the presence of cobalt hexammine in G11Pre, DEPC would be prevented from reacting with the N7 position of A698 even in the absence of a formed pseudoknot.

It is tempting to speculate as to why pseudoknot formation and the rearrangement of helix Ib require a higher concentration of cobalt hexammine or magnesium in G11Pre than in G11D. The only difference in secondary structure between G11Pre and G11D is the presence of helix Ia. Helix Ia might therefore constrain the formation of the pseudoknot and the rearrangement of helix Ib. If this were true, then disrupting helix Ia would be expected make it easier to form the pseudoknot and rearrange helix Ib which might allow these phenomena to occur at a lower magnesium or cobalt hexammine concentration. As a consequence of this, disruption of helix Ia might lower the concentration of magnesium that is required to achieve maximal cleavage activity (henceforth referred to as the magnesium optimum). This, in fact, has been observed. Mutations that disrupt helix Ia lower the magnesium optimum to less than 10 mM.
(Rastogi and Collins 1998). My results may therefore provide a structural basis for the inhibitory element that has been identified in helix Ia.

III. Using Cobalt Hexammines to Gain Structural Information on RS19Pre.

RS19 is a circular permutation of G11 in which the 5' end of helix I is joined to the 3' end of helix II by a 24 nucleotide linker (Anderson and Collins, unpublished; Figure 17). RS19 and G11 have very different cleavage activities. Under standard conditions, the cleavage rate of RS19 is 100-fold higher than that of G11 (Anderson and Collins, unpublished). In addition, mixed cobalt hexammine and magnesium experiments have shown that cobalt hexammine can increase the cleavage rate of RS19 by at least 10,000-fold (Figure 19). This is in stark contrast to the 9-fold increase in cleavage rate observed for G11 under similar conditions (Figure 12). As well, the cobalt hexammine dependent RS19 cleavage rate increase is saturated by about 400 μM cobalt hexammine (Figure 19) while the rate increase of G11 is saturated by at most 100 μM cobalt hexammine (Figure 12).

In an attempt to understand the structural differences between RS19 and G11 that contribute to their different cleavage kinetics, I performed chemical modification structure probing on RS19Pre over a range of cobalt hexammine concentrations using the modifying agents DEPC and DMS. The only significant structural difference that I observed between RS19Pre and G11Pre was that RS19Pre appeared to have less structure in the internal loop of helix I than was observed for the helix I internal loop of G11Pre. Chemical modification of RS19Pre showed that with the exception of C637, which became somewhat protected from DMS in the presence of cobalt hexammine, every
nucleotide that was probed in the internal loop of helix I was accessible to chemical modification both in the absence and presence of cobalt hexammine (Figure 20 and Figure 21). This result argues that the internal loop of helix I in RS19Pre has very little structure. In contrast, chemical modification of G11Pre revealed that both nucleotides that were probed in the internal loop of helix I, C637 and A639, were protected from chemical modification in the absence and presence of cobalt hexammine, arguing that the internal loop of helix I of G11Pre contains significant structure.

It is possible that there are differences between RS19Pre and G11Pre that I have not detected through DEPC and DMS modification which contribute to the different cleavage activities of these constructs. Nevertheless, it is interesting to consider how the relaxed structure around the cleavage site of RS19Pre relative to G11Pre might contribute to the cleavage activity differences of RS19 and G11. One way that structure around the cleavage site could prevent G11Pre from displaying the fast kinetics of RS19Pre is if a conformational change at the cleavage site is required prior to cleavage. In this case, structure around the cleavage site of G11Pre might inhibit the conformational change, limiting its cleavage rate, while the relaxed structure of RS19Pre could allow the conformational change to occur unrestrained.

In the hammerhead ribozyme and the in-vitro selected leadzyme, a conformational change at the cleavage site is thought to take place prior to cleavage (Pley et al. 1994; Scott et al. 1995; Scott et al. 1996; Mckay 1996; Hoogstraten, Legault and Pardi 1998). The original X-ray crystal structures of the hammerhead ribozyme (Pley et al. 1994; Scott et al. 1995; Scott et al. 1996) and the recent NMR solution structure of the leadzyme (Hoogstraten, Legault and Pardi 1998) have revealed the ground state.
structures of these ribozymes. In each of these structures, the cleavage site phosphate is positioned incorrectly for in-line nucleophilic attack by the 2’OH, which is thought to be the first step of the cleavage reaction (Pan et al. 1993; Figure 1). For in-line attack to occur, a conformational change is thought to take place prior to cleavage. A recent X-ray crystal structure of a modified hammerhead ribozyme, which is proposed to represent an intermediate in the reaction pathway, has revealed a structure in which the active site more closely resembles an in-line conformation (Murray et al. 1998b). This suggests that indeed, a conformational change does take place. It is therefore reasonable to propose that a similar conformational change might be necessary for cleavage by the VS ribozyme.

Two pieces of data implicate the structure of the internal loop of helix I in the rate limiting step of G11. The first is that carboxyethylation of the N7 position of A639 by DEPC enhances the cleavage rate of G11 (Beattie and Collins, 1997). This modification of A639 would be expected to alter the structure of the stem I internal loop. Because this alteration of structure increases the cleavage rate, the structure of the stem I internal loop must be involved in the rate determining step of G11. The second piece of evidence that points to the stem I internal loop structure being involved in the rate limiting step of G11 is that the insertion of any nucleotide between A639 and G640 increases the cleavage rate of G11pre by at least 100-fold (Zamel and Collins, unpublished data). Such an insertion might very well disrupt an inhibitory structure in the internal loop and allow a conformational change at the cleavage site to occur more readily.

The results of the mixed metal experiments are consistent with the hypothesis that structure around the cleavage site of G11 inhibits a conformational change at the
cleavage site. In G11Pre, cobalt hexammine would likely stabilize structure around the cleavage site, making a cleavage site conformational change rate limiting at higher cobalt hexammine concentrations. This would limit both the concentration of cobalt hexammine that is required to reach cleavage rate saturation and the extent of the rate increase. In RS19Pre, due to its relaxed cleavage site structure, cobalt hexammine would be less able to stabilize structure around the cleavage site and might enable RS19Pre to reach a faster rate-limiting step, requiring a higher concentration of cobalt hexammine.

IV. Structural Requirements of Helix Ib.

ZA10 was created in order to completely disrupt base pairing in helix Ib by replacing four nucleotides in stem Ib with adenines (Figure 22a). The cleavage kinetics of ZA10 were shown to be very similar to the cleavage kinetics of its parent construct G11 (Beattie 1997). In order to evaluate if the adenine substitutions of ZA10 have completely disrupted base pairing in helix Ib, I performed chemical modification structure probing on ZA10D and ZA10Pre using the modifying agents DEPC and DMS. Chemical modification of ZA10D has suggested that, in the presence of magnesium or cobalt hexammine, stem Ib forms a structure with C634 being in a hyper-reactive orientation and C637 and C636 being protected from chemical modification, probably because of Watson-Crick pairing with G623 and G624 respectively (Figure 26a). Thus, in ZA10D, two closing base pairs at the bottom of helix Ib likely form (Figure 26c).

To evaluate if these base pairs also form in ZA10Pre, I performed chemical modification structure probing on ZA10Pre in cobalt hexammine or magnesium. The DMS modification pattern of stem Ib of ZA10Pre in 10 mM cobalt hexammine or 10 mM
magnesium is consistent with a Watson-Crick base pair forming between G623 and C637 but inconsistent with a base pair forming between G624 and C636 (Figure 30a and Figure 30c). Thus, in contrast to ZA10D, in ZA10Pre the base pair between G624 and C636 likely does not form in the presence of cobalt hexamine or magnesium.

To evaluate whether the active conformation of ZA-10Pre requires base pairing between G623 and C637 or G624 and C636, I created a set of mutants to disrupt or restore each of these putative base pairs. Disruption of pairing potential between positions 634 and 636, only reduced activity by a maximum of 10-fold. Restoration of pairing via non wild-type Watson-Crick base pairs did not significantly restore activity. These two observations suggest that base pairing between G624 and C636 is not required for activity (Figure 31a). On the contrary, base paring between G623 and C637 is required for activity. Disruption of this base pair reduced cleavage activity by at least 500-fold. Restoration of a substantial amount of activity was possible through non wild-type base pairs, provided that a purine was present at position 623 (Figure 31b). The purine requirement at 623 might result from a pyrimidine not being able to form one or more tertiary structure contacts that are required for activity. Or, the requirement might be the ramification of a metal binding site that is disrupted by the presence of a pyrimidine. In either case, a base pair between positions 623 and 637 is required for cleavage activity and is consistent with DMS structure probing data on ZA10Pre. Therefore, this base pair defines the minimal Watson-Crick requirement of stem Ib.
V. Future directions.

My finding that cobalt hexammine facilitates almost the same tertiary structure in G11D as magnesium does and that G11Pre is not catalytically active in cobalt hexammine alone has allowed me to use cobalt hexammine as a tool to gain access to the structure of the precursor ribozyme. Chemical modification structure probing of G11Pre, RS19Pre and ZA10Pre has yielded a considerable amount of relevant information on the tertiary structure of these precursor RNAs. Cobalt hexammine will be useful in the future to study the structures of these RNAs in more detail as well as to study the structures of other precursor RNAs. In addition, other transition metal complexes may prove valuable. Cobalt pentammine [Co(NH₃)₅]⁺⁺, in contrast to cobalt hexammine, can form inner-sphere contacts with RNA (Tinoco and Kieft 1997). Cobalt pentammine may therefore be able to induce even more VS ribozyme structure than cobalt hexammine. Cobalt pentammine might even be able to support VS ribozyme catalysis if only one inner-sphere co-ordination is required for catalytic activity.

More specific future directions follow from some of the interesting findings of my research. Firstly, the correlation between the high cobalt hexammine concentration that is required to form the pseudoknot and rearrange helix Ib of G11Pre and the high cleavage rate magnesium optimum of G11 suggest a link between these two phenomena. H3, a mutant that completely lacks helix Ia (Figure 32a), has a considerably lower magnesium optimum than G11 (Rastogi and Collins 1998). Thus, a testable prediction of the correlation between magnesium optimum and the concentration of cobalt hexammine that is required to form the pseudoknot and rearrange helix Ib is that H3 should form the pseudoknot and rearrange helix Ib at a significantly lower cobalt hexammine
concentration than G11. Other helix Ia mutants might display intermediate magnesium optima. If pseudoknot formation and helix Ib rearrangement are responsible for the magnesium optimum, then these mutants would be expected to require intermediate concentrations of cobalt hexammine to form the pseudoknot and rearrange helix Ib.

Secondly, it is interesting that H3, in addition to having a lower magnesium optimum, cleaves much faster than G11 in saturating magnesium (Rastogi and Collins 1998). The fast cleavage rate of H3 in saturating magnesium is not necessarily caused by the same phenomenon that is responsible for the low magnesium optimum of H3. In addition to lacking helix Ia, H3 likely has more flexibility around the cleavage site than G11. In light of the theory that structure around the cleavage site is an inhibitory element, it is plausible that the enhanced cleavage rate of H3 is due, not specifically to the absence of helix Ia, but to relaxed structure around the cleavage site that is a consequence of the absence of helix Ia. The insertion of any nucleotide between A639 and G640 dramatically enhances the cleavage rate of G11 (Zamel and Collins, unpublished data). This observation could be used to test whether cleavage rate and magnesium optimum are independent variables. These mutants probably do not disrupt helix Ia, although chemical modification structure probing could verify this. If these mutants do not disrupt helix Ia, a testable prediction is that they should display a high magnesium optimum, like G11, and thus require a high concentration of cobalt hexammine to form the pseudoknot and rearrange the secondary structure of helix Ib. If this prediction should turn out to be true, then fast cleavage kinetics on one hand and low metal requirements for pseudoknot formation and helix Ib rearrangement on the other are separate phenomena.
Thirdly, my finding that the minimal secondary structure requirement of stem Ib is one closing base pair raises the question of why this is the minimal requirement. Two explanations are plausible. Either the ribozyme cannot bind to stem I without a closing base pair in stem Ib or the ribozyme can bind such a stem I but cannot form a structure that is compatible with catalysis. To determine if the ribozyme can bind a stem I lacking a closing base pair in stem Ib, binding studies, as developed by Zamel and Collins (unpublished data), could be carried out using an isolated stem loop I containing or lacking a closing base pair in stem Ib. If stem loop I lacking a closing base pair in stem Ib displays very weak binding to the ribozyme then it is likely that the reason that such mutants are inactive is because stem loop I is not recognized by the ribozyme. On the other hand, if a stem loop I lacking a closing base pair in stem Ib can bind efficiently to the ribozyme then the mutant stem loop I likely cannot form a structure that is compatible with catalysis. Chemical modification structure probing experiments could be carried out to identify structural reasons for the lack of activity.
Figure 1. Chemistry of Small Ribozyme Cleavage

The 2'-proton is removed either by abstraction by a metal hydroxide or by a direct metal co-ordination with the 2'-oxygen to increase the acidity of the 2'-proton allowing its abstraction via an aqueous hydroxide. The 2'-oxyanion attacks the 5'-phosphorus via S_n2 in-line attack resulting in a 2'3'-cyclic phosphate with inversion of configuration at the phosphorus.
Cleavage
Figure 2. Secondary Structure Model of G11Pre

VS nucleotides are in uppercase and are numbered as in Saville and Collins (1990). Vector nucleotides are in lowercase. Nucleotides that are present in G11Pre but not in G11D are boxed. Adapted from Beattie, Olive and Collins (1995).
Figure 3. Three Magnesium Binding Sites in The Group I Intron Metal Ion Core

The RNA uses a combination of inner-sphere and outer-sphere co-ordination to bind divalent metal ions. (a) A tri-hydrated magnesium directly coordinated to three phosphate oxygens within the A-rich bulge. (b) Binding site at the top of the three-helix junction involves direct co-ordination between the carbonyl oxygen of G188 and outer-sphere hydrogen bonds to three phosphate groups. (c) Magnesium binding to tandem G-A base pairs adjacent to the three-helix junction involves direct co-ordination with the N7 of G163 and the O6 of G164 and outer-sphere hydrogen bonding to the G163 phosphate. Adapted from Tinoco and Kieft (1997).
Figure 4. The Helix Ib Rearrangement Model

Mutational data and chemical modification data support a rearrangement in the secondary structure of helix Ib upon the addition of magnesium. Under conditions that promote the formation of secondary structure, C634 is paired with G625, C635 is paired with G624, C636 is paired with G623 and C637 is unpaired. Upon addition of magnesium and tertiary structure formation, C634 is bulged out of helix Ib, C635 is paired with G625, C636 is paired with G624 and C637 is paired with G623.
Cleavage Site

Magnesium
Figure 5. G11 is Not Active in Cobalt Hexammine Alone

(a) $^{32}$P-internally-labeled G11Pre was incubated under structure probing conditions (200 mM HEPES pH 8.0 and 50 mM KCl at 37°C) with various concentrations of MgCl$_2$ or Co(NH$_3$)$_6$Cl$_3$. After 52 hours, aliquots were subjected to denaturing polyacrylamide gel electrophoresis and visualized via autoradiography. The upper band is the precursor RNA (G11Pre) and the lower band is the downstream cleavage product (G11D). (b) Schematic representations of hexahydrated magnesium (left) and cobalt hexammine (right) are displayed. Both metals have similar size and geometry but the water ligands of hexahydrated magnesium can exchange while the amine ligands of cobalt hexammine cannot. One hundred $\mu$M EDTA was included in the self-cleavage reaction to remove trace divalent contaminants.
### 52h Time Course

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**a**

Pre-D

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Hexahydrated Magnesium

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Hexammine

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Cobalt Hexamminine
Figure 6. DEPC Modification of G11D in Magnesium or Cobalt Hexammine

3'-end-labeled G11D was modified with DEPC under denaturing conditions (den) and under conditions containing 0 to 10 mM (a) MgCl$_2$ or (b) Co(NH$_3$)$_6$Cl$_3$. Control lanes in which the RNA was not modified with DEPC were also included (unmod). Sites of modification were detected by cleavage with aniline, subjected to denaturing electrophoresis on 8% polyacrylamide gels (top) or 12% polyacrylamide gels containing a salt gradient (bottom), and visualized by autoradiography. Band intensities corresponding to bases which are indicated by number were quantified using a PhosphorImager (see Appendix A).
DEPC Modification of G11D
Figure 7. A676 DEPC Reactivity is Independent of Magnesium or Cobalt Hexammine Concentration

The intensity of bands corresponding to DEPC modification of G11D at A676 and A698 over an increasing concentration of (a) MgCl₂ or (b) Co(NH₃)₆Cl₃ are plotted. The values are expressed as a fraction of full length unmodified RNA to correct for slight differences in loading among the lanes. The data represent the mean values over at least three independent experiments. Error bars represent one standard deviation.
a. DEPC Reactivity vs. [MgCl$_2$]

b. DEPC Reactivity vs. [Co(NH$_3$)$_6$Cl$_3$]
Figure 8. Quantification of A698 and A726 DEPC Reactivity

The intensity of bands corresponding to DEPC modification of G11D at A698 (a and b) and A726 (c and d) over an increasing concentration of MgCl₂ (Mg) or Co(NH₃)₆Cl₃ (Co) are plotted. Band intensities are expressed as "relative counts" since they are normalized to the intensity of A676 which serves as an internal control (see figure 7). MgCl₂ and Co(NH₃)₆Cl₃ concentrations are plotted on the same scale (a and b) or the Co(NH₃)₆Cl₃ scale is expanded 33-fold relative to the scale of MgCl₂ concentrations (c and d). Band intensities are averaged over three experiments with error bars representing one standard deviation.
Figure 9. A Comparison of the Chemical Reactivity Pattern of G11D in Magnesium Relative to Cobalt Hexammine

(a) The Co(NH$_3$)$_6$Cl$_3$ or MgCl$_2$ concentrations ([Co]$_{1/2}$ or [Mg]$_{1/2}$ respectively) that are required to half change the chemical reactivity of many nucleotides in G11D are plotted. As in figure 8(c) and figure 8(d), the Co(NH$_3$)$_6$Cl$_3$ scale is expanded by 33-fold relative to the MgCl$_2$ scale. The values are an average of three experiments with error bars representing one standard deviation. (b) The band intensity of many nucleotides in G11D in no polyvalent metal (“No Metal”), 10 mM MgCl$_2$ or 0.3 mM Co(NH$_3$)$_6$Cl$_3$ are plotted. Band intensities are normalized to the intensity of A676 or C677 and are referred to as “relative counts” to reflect this. The values are an average of three experiments with error bars representing one standard deviation.
Reactivity of G11D to DMS and DEPC

Part a: [metal] at ΔReactivity_{1/2}

Part b: Reactivity of G11D to DMS and DEPC

Legend:
- ○: G11D in Co
- ●: G11D in Mg

Graphs show the reactivity of G11D in different conditions.
Figure 10. DMS Modification of G11D in Magnesium or Cobalt Hexammine

3'-end-labeled G11D was modified with DMS under conditions containing (a) 0 to 10 mM MgCl₂ or (b) 0 to 10 mM Co(NH₃)₆Cl₃. Sites of cytosine modification by DMS were detected by hydrazine treatment followed by strand cleavage with aniline. RNA fragments were visualized via denaturing polyacrylamide gel electrophoresis on 8% gels run at 70W for 3 hours (top) or 6 hours (bottom). Background U residues are a result of the hydrazine treatment. Control lanes were run containing either the input RNA (Input) or RNA which was not modified with DMS but was treated with hydrazine and aniline to yield a U ladder. To demarcate the position of cytosines, a C/U ladder was also run. Band intensities corresponding to bases which are indicated by number were quantified using a PhosphorImager.
DMS Modification of G11D
Figure 11. Quantification of C699 and C634 DMS Reactivity

The intensity of bands corresponding to DMS modification of Gl1D at (a) C699 and (b) C634 over an increasing concentration of MgCl₂ (Mg) or Co(NH₃)₆Cl₃ (Co) are plotted. Band intensities are expressed as “relative counts” since they are normalized to the intensity of C677 which serves as an internal control since its reactivity is independent of MgCl₂ and Co(NH₃)₆Cl₃ concentration (see C677 quantification in Appendix A). MgCl₂ and Co(NH₃)₆Cl₃ concentrations are plotted on different scales with the Co(NH₃)₆Cl₃ scale expanded 33-fold relative to the MgCl₂ scale. Band intensities are averaged over three independent experiments with error bars representing one standard deviation.
Figure 12. G11 Cleavage in Mixed Cobalt Hexamine and Magnesium

$^{32}$P-internally-labeled G11 Pre was pre-incubated in 40 mM Tris-HCl pH 8.0 and 50 mM KCl at 37°C with various concentrations of Co(NH$_3$)$_6$Cl$_3$ up to 100 µM. After 5 minutes MgCl$_2$ was added to a final concentration of 0.75 mM, 1 mM, 1.5 mM, 2 mM or 5 mM. Aliquots were removed at various times, analyzed by gel electrophoresis and quantified. The first order rate constant for self-cleavage in each combination of Co(NH$_3$)$_6$Cl$_3$ and MgCl$_2$ was calculated from the slope of a plot of the log of fraction uncleaved versus time. The first order cleavage rate constant is plotted against the Co(NH$_3$)$_6$Cl$_3$ concentration for each concentration of MgCl$_2$. 
G11 Cleavage in Mixed Metal

Cleavage Rate (per hour)

[Co(NH₃)₅Cl] μM

- ▲ 0.75 mM Mg
- ● 1 mM Mg
- • 1.5 mM Mg
- □ 2 mM Mg
- ■ 5 mM Mg
Figure 13. DEPC Modification of G11Pre in Magnesium or Cobalt Hexamine

3'-end-labeled G11Pre was modified with DEPC under denaturing conditions (den) and under conditions containing 0 to 10 mM (a) MgCl₂ or (b) Co(NH₃)₆Cl₃. Control lanes in which the RNA was not modified with DEPC were also included (unmod). Sites of modification were detected by cleavage with aniline, subjected to denaturing polyacrylamide electrophoresis on 8% polyacrylamide gels (top) or 12% polyacrylamide gels containing a salt gradient (bottom) and visualized by autoradiography. Band intensities corresponding to bases that are indicated by number were quantified using a PhosphorImager (see Appendix A).
DEPC Modification of G11Pre
Figure 14. DMS Modification of G11Pre in Magnesium or Cobalt Hexammine

3'-end-labeled G11Pre was modified with DMS under conditions containing (a) 0 to 10 mM MgCl₂ or (b) 0 to 10 mM Co(NH₃)₆Cl₃. Sites of cytosine modification by DMS were detected by hydrazine treatment followed by strand cleavage with aniline. RNA fragments were visualized via denaturing polyacrylamide gel electrophoresis on 8% gels run at 70W for 3 hours (top) or 6 hours (bottom). Background U residues are a result of the hydrazine treatment. Control lanes were run containing either the input RNA (Input) or RNA which was not modified with DMS, but was treated with hydrazine and aniline to yield a U ladder. To demarcate the position of cytosines, a C/U ladder was also run. Band intensities corresponding to bases which are indicated by number were quantified using a PhosphorImager.
DMS Modification of G11Pre
Figure 15. A Comparison of the Chemical Reactivity Pattern of G11Pre Relative to G11D in Cobalt Hexammine

(a) The Co(NH$_3$)$_6$Cl$_3$ concentrations ([Co]$_{1/2}$) that are required to half change the chemical reactivity of many nucleotides in G11Pre and in G11D are plotted ([M]$_{1/2}$). The values are an average of three experiments with error bars representing one standard deviation. (b) The band intensity of many nucleotides in G11Pre and in G11D in the presence or absence of 0.3 mM Co(NH$_3$)$_6$Cl$_3$ are plotted. Band intensities are normalized to the intensity of A676 or C677 and are referred to as “relative counts” to reflect this. The values are an average of three experiments with error bars representing one standard deviation.
Reactivity of G11 Pre and G11 D to DMS and DEPC
Figure 16. Quantification of the Reactivity of A698, C699 and C634 in G11Pre and G11D in Cobalt Hexamine or Magnesium

The intensity of bands corresponding to the modification of G11Pre or G11D at A698 in (a) Co(NH₃)₆Cl₃ or in (d) MgCl₂, at C699 in (b) Co(NH₃)₆Cl₃ or in (e) MgCl₂ and at C634 in (c) Co(NH₃)₆Cl₃ or in (f) MgCl₂. Band intensities are expressed as “relative counts” since A698 is normalized to the intensity of A676 while C699 and C634 are normalized to the intensity of C677. Band intensities are averaged over three independent experiments with error bars representing one standard deviation.
Figure 17. Secondary Structure of RS19Pre

VS nucleotides are in uppercase and are numbered as in Saville and Collins (1990). Vector nucleotides are in lowercase and inserted linker nucleotides are either in lower case or are represented by dotted lines. Adapted from Anderson and Collins, unpublished data.
Figure 18. Preliminary Mixed Metal Experiment on RS19

(a) $^{32}$P-internally-labeled RS19Pre RNA was pre-incubated in 40 mM Tris-HCl pH 8.0 and 50 mM KCl at 37°C for 1 minute followed by the addition of 200 µM Co(NH$_3$)$_6$Cl$_3$. Aliquots were removed at various times, and analyzed by denaturing gel electrophoresis and autoradiography. (b) RS19Pre RNA was pre-incubated as in (a) except that after 1 minute 0.75 mM MgCl$_2$ was added instead of Co(NH$_3$)$_6$Cl$_3$. Aliquots were removed at various times and analyzed. (c) RS19Pre RNA was treated identically as in (a) except that 5 minutes after the Co(NH$_3$)$_6$Cl$_3$ addition, 0.75 mM MgCl$_2$ was added. Aliquots were removed at various times and analyzed. The upper band is RS19Pre and the lower band is RS19P, the upstream cleavage product.
a. Time (min)

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Pre -  P -

200μM Co(NH₃)₆Cl₃

b. Time (min)

| 0  | .08 | 1  | 10 |

Pre -  P -

0.75mM MgCl₂

c. Time (min)

| 0  | .08 | 1  | 10 |

Pre -  P -

200μM Co(NH₃)₆Cl₃

0.75mM MgCl₂
Figure 19. RS19 Cleavage in Mixed Cobalt Hexammine and Magnesium

$^{32}$P-internally-labeled RS19Pre was pre-incubated in 40 mM Tris-HCl pH 8.0 and 50 mM KCl at 37°C for 1 minute followed by the addition of various concentrations of Co(NH$_3$)$_6$Cl$_3$ up to 600 μM. After 5 minutes MgCl$_2$ was added to a final concentration of 0.25 mM, 0.5 mM, 0.75 mM, or 1 mM. Aliquots were removed at various times, analyzed by gel electrophoresis and quantified. The first order rate constant for self-cleavage in each combination of Co(NH$_3$)$_6$Cl$_3$ and MgCl$_2$ was calculated from the slope of a plot of the log of fraction uncleaved versus time. The first order cleavage rate constant is plotted against the Co(NH$_3$)$_6$Cl$_3$ concentration for each concentration of MgCl$_2$. 
RS19 Cleavage in Mixed Metal

![Graph showing cleavage rate vs. [Co(NH$_3$)$_6$Cl$_2$] concentration for different Mg concentrations. The graph includes lines for 0.25mM Mg, 0.5mM Mg, 0.75mM Mg, and 1mM Mg. The y-axis represents cleavage rate (per min) on a log scale, and the x-axis represents [Co(NH$_3$)$_6$Cl$_2$] concentration (µM).]
Figure 20. DEPC Modification of RS19Pre in Cobalt Hexamine

3'-end-labeled RS19Pre was modified with DEPC under denaturing conditions (den) and under conditions containing 0 to 10 mM Co(NH₃)₆Cl₃. Control lanes in which the RNA was not modified with DEPC were also included (unmod). Sites of modification were detected by cleavage with aniline, subjected to denaturing polyacrylamide electrophoresis on 8% gels run for (a) 4.5 hours at 32.5W or for (b) 6 hours at 65W and visualized by autoradiography.
DEPC Modification of RS19 Pre
Figure 21. DMS Modification of RS19Pre in Cobalt Hexammine

3’-end-labeled RS19Pre was modified with DMS under conditions containing 0 to 10 mM Co(NH$_3$)$_6$Cl$_3$. Sites of cytosine modification by DMS were detected by hydrazine treatment followed by strand cleavage with aniline. RNA fragments were visualized via denaturing polyacrylamide gel electrophoresis on a (a) 8% gels run for 6 hours at 70W or (b) 12% gels run with a 1 M NaCl gradient for 2.5 hours at 70W. Background U residues are a result of the hydrazine treatment. Control lanes were run containing either the input RNA (Input) or RNA which was not modified with DMS, but was treated with hydrazine and aniline to yield a U ladder. To demarcate the position of cytosines, a C/U ladder was also run.
Figure 22. ZA10

(a) ZA10 was designed to disrupt base pairing in helix Ib (Beattie 1997). The 4 adenines that replace G11 nucleotides are unfilled. (b) If the helix Ib rearrangement model holds for ZA10 Pre, two base pairs might form in helix Ib in the functionally relevant secondary structure. (c) G11 Pre secondary structure in the absence of polyvalent metal. (d) The functionally relevant secondary structure of G11 Pre in the presence of Co(NH$_3)_6$Cl$_3$ or MgCl$_2$. 
Figure 23. DEPC Modification of ZA10D in Magnesium or Cobalt Hexammine

3'-end-labeled ZA10D was modified with DEPC under denaturing conditions (den) and under conditions containing 0 to 10 mM (a) MgCl$_2$ or (b) Co(NH$_3$)$_6$Cl$_3$. Control lanes in which the RNA was not modified with DEPC were also included (unmod). Sites of modification were detected by cleavage with aniline, subjected to denaturing electrophoresis on 8% polyacrylamide gels (top) or 12% polyacrylamide gels containing a salt gradient (bottom) and visualized by autoradiography. Band intensities corresponding to bases which are indicated by number were quantified using a PhosphorImager (see Appendix A).
DEPC Modification of ZA-10D
Figure 24. A Comparison of the Chemical Reactivity Pattern of ZA10D Relative to G11D in Magnesium or Cobalt Hexammine

The (a) MgCl₂ [M]₁/₂ or (c) Co(NH₃)₆Cl₃ [M]₁/₂ of adenines in ZA10D are plotted against adenines of G11D. The band intensity of ZA10D adenines in the presence or absence of (b) 10 mM MgCl₂ or (d) 0.3 mM Co(NH₃)₆Cl₃ are plotted against G11D adenines. Band intensities are normalized to the intensity of A676 and are referred to as “relative counts” to reflect this. The values are an average of three experiments with error bars representing one standard deviation.
[MgCl₂] at ΔReactivity₁/₂

DEPC Reactivity of G11D and ZA10D

- ZA10D No Metal
- G11D No Metal
- ZA10D 10mM Mg
- G11D 10mM Mg

Relative Counts
DEPC Reactivity of ZA10D and G11D

- ZA10D No Metal
- G11D No Metal
- ZA10D 0.3mM Co
- G11D 0.3mM Co

[Graph showing reactivity data for ZA10D and G11D with different concentrations of Co]
The intensity of bands corresponding to DEPC modification of ZA10D at (a) A625, (b) A627, (c) A633, and (d) A635 over an increasing concentration of MgCl₂ (Mg) or Co(NH₃)₆Cl₃ (Co) are plotted. Band intensities are expressed as “relative counts” since they are normalized to the intensity of A676 which serves as an internal control. MgCl₂ and Co(NH₃)₆Cl₃ concentrations are plotted on different scales with the Co(NH₃)₆Cl₃ scale expanded 33-fold relative to the MgCl₂ scale. Band intensities are averaged over three independent experiments with error bars representing one standard deviation.
Figure 26. DMS Modification of ZA10D in Cobalt Hexamine or Magnesium

(a) 3'-end-labeled ZA10D was modified under denaturing conditions (Den), under non-denaturing conditions in the absence (No Metal) or presence of 10 mM Co(NH$_3)_6$Cl$_3$ or 10 mM MgCl$_2$. A mock reaction in which DMS was not added is also included (Mock). Following modification, the RNA was treated with hydrazine to detect modified cytosine residues. Sites of modification were identified by aniline cleavage, electrophoresis on 8% polyacrylamide gels and autoradiography. DMS chemical modification data from (a) are consistent with the formation of these secondary structures in the absence (b) or presence of polyvalent metal (c).
DMS Modification of ZA10D

b
ZA-10D
No Metal

\[
\begin{align*}
C & \quad C \\
U & \quad U \\
A & \quad A \\
C & \quad C \\
G & \quad G \\
A & \quad A \\
A & \quad A \\
G & \quad G \\
G & \quad G \\
\end{align*}
\]

Cleavage Site

Mg\(^{2+}\) or Co(NH\(_3\))\(^{3+}\)

c

\[
\begin{align*}
C & \quad G-C \\
U & \quad U-A \\
A & \quad A \\
C & \quad C \\
G-C & \quad G-C \\
G-C & \quad G-C \\
G & \quad A \\
G & \quad A \\
G & \quad A \\
\end{align*}
\]

Cleavage Site
Figure 27. DEPC Modification of ZA10Pre in Magnesium or Cobalt Hexammine

3'-end-labeled ZA10Pre was modified with DEPC under denaturing conditions (den) and under conditions containing 0 to 10 mM (a) MgCl₂ or (b) Co(NH₃)₆Cl₃. Control lanes in which the RNA was not modified with DEPC were also included (unmod). Sites of modification were detected by cleavage with aniline, subjected to denaturing electrophoresis on 8% polyacrylamide gels (top) or 12% polyacrylamide gels containing a salt gradient (bottom) and visualized by autoradiography. Band intensities corresponding to bases which are indicated by number were quantified using a PhosphorImager (see Appendix A).
DEPC Modification of ZA-10Pre
Figure 28. Comparison of the DEPC Reactivity Pattern of ZA10Pre Relative to ZA10D in Cobalt Hexammine

(a) The Co(NH$_3$)$_6$Cl$_3$ [M]$_{1/2}$ for adenines in ZA10Pre and in ZA10D are plotted. (b) The band intensity of adenines in ZA10Pre and in ZA10D in the absence of polyvalent metal and in 0.3 mM Co(NH$_3$)$_6$Cl$_3$ are shown. Band intensities are normalized to the intensity of A676 and are referred to as “relative counts” to reflect this. Values are an average of three experiments with error bars representing one standard deviation.
Figure 29. Quantification of the DEPC Reactivity of ZA10Pre Stem Ib Adenines in Cobalt Hexammine

The intensity of bands corresponding to DEPC modification of ZA10Pre at (a) A625, (b) A627, (c) A633 and (d) A635 over increasing concentrations of Co(NH₃)₆Cl₃ are plotted. Band intensities are expressed as “relative counts” since they are normalized to the intensity of A676 which serves as an internal control. Band intensities are averaged over three independent experiments with error bars representing one standard deviation.
Figure 30. DMS Modification of ZA10Pre in Cobalt Hexamine or Magnesium

(a) 3’-end-labeled ZA10Pre was modified under denaturing conditions (Den), under non-denaturing conditions in the absence (No Metal) or presence of 10 mM Co(NH$_3$)$_6$Cl$_3$ or 10 mM MgCl$_2$. A mock reaction in which DMS was not added is also included (Mock). Following modification, the RNA was treated with hydrazine to detect modified cytosine residues. Sites of modification were identified by aniline cleavage, electrophoresis on 8% polyacrylamide gels and autoradiography. DMS chemical modification data from (a) are consistent with the formation of these secondary structures in (b) the absence of polyvalent metal or (c) in the presence of MgCl$_2$. 
DMS Modification of ZA10Pre

b

ZA-10Pre
No Metal

C
U
A
C
A
G
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Figure 31. Self-Cleavage Rates of ZA10 Stem Ib Mutants

Internally labeled ZA10 stem Ib mutant RNA and wild type ZA10 RNA were incubated under conditions that allow for self-cleavage of the wild type molecule (40 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM spermidine and 25 mM MgCl₂ at 37°C). Aliquots were removed at various times, analyzed by gel electrophoresis and quantified. The fraction of cleaved RNA versus time for ZA10 position 624 and 636 mutants is shown in (a) and for ZA10 position 623 and 637 mutants is shown in (b). The first order rate constant for ZA10 wild type and ZA10 mutant RNA was calculated from the slope of the log of fraction uncleaved versus time plot. The first order cleavage rate constant for the ZA10 624/636 mutants is shown in (c) and for the ZA10 623/637 mutants in (d).
Figure 32. Self-Cleavage Rate of H3 versus H3ZA10

(a) The secondary structure of H3 with the ZA10 stem Ib adenine substitutions indicated with arrows. VS nucleotides are in uppercase and are numbered as in Saville and Collins (1990). Nucleotides that are not part of H3D are boxed. (b) Cleavage kinetics of H3 relative to H3ZA10. Self-cleavage reactions were performed as in Figure 31.
a.

b.

H3 vs. H3ZA10 Timecourse

- - H3
- - H3ZA10

Fraction Cleaved

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1

Fraction Uncleaved

0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1

Time (minutes)

0 2 4 6 8 10 12 14 16 18 20
Figure 33. H3ZA-10 and H3 cleavage reactions with or without pre-incubation

“P + S” indicates pre-incubation in 50mM KCl and 2mM spermidine; “P - S” indicates pre-incubation in 50mM KCl only; “No P + S” indicates no pre-incubation but the cleavage reaction contained spermidine; “No P - S” indicates no pre-incubation and the cleavage reaction did not contain spermidine. After pre-incubation, 25mM magnesium was added to start the self-cleavage reactions. Aliquots were taken at various times, analyzed by denaturing gel electrophoresis and quantified.
Figure 34. Temperature Dependence of H3 and G11

Internally labeled H3 or G11 RNAs were incubated at various temperatures under conditions that allow for self-cleavage (40 mM Tris-HCl pH 8.0, 50 mM KCl, 2 mM spermidine and 25 mM MgCl₂). Aliquots were removed at various times, analyzed by gel electrophoresis and quantified. The first order rate constant for self-cleavage at each temperature was calculated from the slope of a plot of the log of fraction uncleaved versus time. The first order cleavage rate constant is plotted against temperature.
Temperature dependence of H3 & G11

Temperature (°C)

Cleavage Rate (per minute)

- H3
- G11
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