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T_m studies of a tertiary structure from the human hepatitis delta agent which functions *in vitro* as a ribozyme control element

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ABSTRACT

Viroids and other circular subviral RNA pathogens, such as the hepatitis delta agent, use a rolling circle replication cycle requiring an intact circular RNA. However, many infectious RNAs have the potential to form self-cleavage structures, whose formation must be controlled in order to preserve the circular replication template. The native structure of delta RNA contains a highly conserved element of local tertiary structure which is composed of sequences partially overlapping those needed to form the self-cleavage motif. A bimolecular complex containing the tertiary structure can be made. We show that when it is part of this bimolecular complex the potential cleavage site is protected and is not cleaved by the delta ribozyme, demonstrating that the element of local tertiary structure can function as a ribozyme control element *in vitro*. Physical studies of the complex containing this element were carried out. The complex binds magnesium ions and is not readily dissociated by EDTA under the conditions tested; >50% of the complexes remain following incubation in 1 mM EDTA at 60°C for 81 min. The thermal stability of the complex is reduced in the presence of sodium ions. A DNA complex and a perfect RNA duplex studied in parallel showed a similar effect, but of lesser magnitude. The RNA complex melts at temperatures ~10°C lower in buffers containing 0.5 mM $MgCl_2$ and 100 mM NaCl than in buffers containing 0.5 mM $MgCl_2$ with no NaCl (78.1 compared with 87.7°C). The element of local tertiary structure in delta genomic RNA appears to be a molecular clamp whose stability is highly sensitive to ion concentration in the physiological range.

INTRODUCTION

The human hepatitis delta agent is a novel subviral pathogen (1,2) with a circular genomic RNA (3,4) and other features suggesting a relationship to viroids (5) and circular viral satellite RNAs (6,7). This group of circular subviral RNA pathogens uses an RNA–RNA rolling circle replication pathway (8–10). In many cases replication intermediates are processed to mature length by a self-cleavage structure. Examples include the hammerhead (11,12), the hairpin/paperclip (13,14) and the delta ribozymes (15–19). All of these self-cleavage structures have been re-engineered to carry out *trans*-cleavage reactions (14,20,21). This conversion allows them to be studied in greater detail and compared with other ribozymes (22), such as the self-splicing group I (23) and group II introns (24) and the catalytic subunit of RNase P (25). In the current studies our use of a *trans*-active form of the delta ribozyme has allowed us to test the idea that ribozyme function may be regulated by a specific element in delta RNA.

Earlier studies of the self-cleavage structures in certain of the viroid-like pathogens, including the hepatitis delta agent, have shown that formation of these self-cleavage structures can be greatly reduced or eliminated by *cis*-active elements (16,26–28). Inhibitory structural features may reflect the need for these infectious RNAs to maintain a circular replication template. Several years ago we (29) identified a new element of local tertiary structure which is part of the native rod-like structure (30) of delta genomic RNA (3,4). Further investigations will be needed to determine the types and numbers of bonds present in this tertiary structure, however, it is already clear that it has certain features in common with the structural motif present in loop E of 5S rRNA and viroid RNA (31–33). All three sites contain two nucleotides which can be cross-linked to each other upon irradiation with UV light, contain a central region of bases which interact through non-Watson–Crick (tertiary) bonds and contain flanking, Watson–Crick base paired helices. We refer to the non-Watson–Crick bonds as 'tertiary interactions', in keeping with the terminology introduced by Rich and RajBhandary, who wrote 'Tertiary interactions are taken to mean the hydrogen bonds that occur between bases, between bases and backbone and between backbone residues, except for the interactions in the double helical stem regions, which are considered secondary structure' (34). We refer to the site in delta genomic RNA as a 'local tertiary structure', because in the secondary structure model of delta RNA (3) it is contained within a single stem (which has an internal 'loop'). By 'element of local tertiary structure' we

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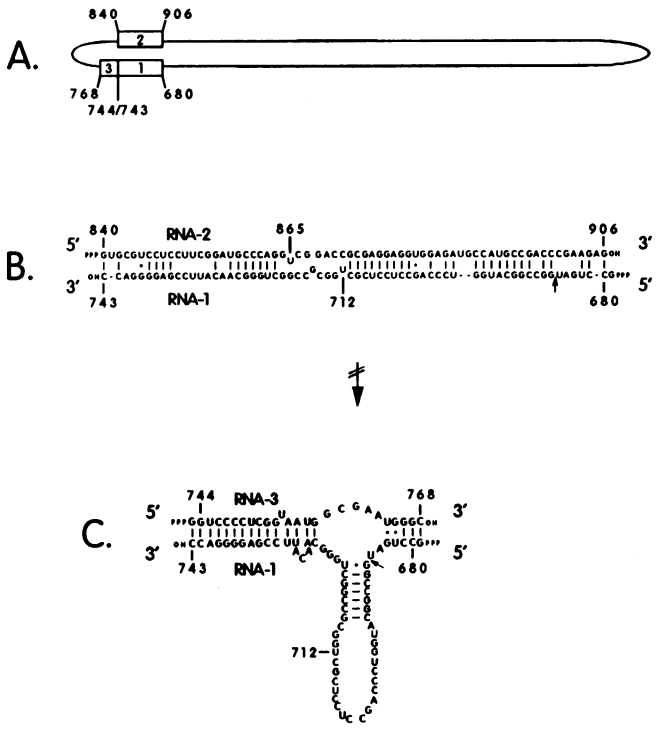

Figure 1. Delta genomic RNA and three *in vitro* transcripts. (**A**) A diagram of delta genomic RNA identifying the sequences represented by RNA-1, RNA-2 and RNA-3. These RNAs, which contain sequences from a highly conserved viroid-like portion of delta genomic RNA, were transcribed *in vitro* using the procedure introduced by Milligan *et al.* (40) at a specific activity of 300 000 d.p.m./μg and then were purified by gel electrophoresis. (**B**) The bimolecular complex between RNA-1, which contains delta bases 680–743, and RNA-2, which contains delta bases 840–906. RNA-1 contains a potential cleavage site, marked by an arrow. (**C**) A ribozyme·substrate complex between RNA-3 (delta bases 744–768) and RNA-1. To simplify the comparison between the complexes shown in (B) and (C) a possible pseudoknot structure (18,19) in the ribozyme·substrate complex has not been included in this diagram. The broken arrow between (B) and (C) indicates that formation of the ribozyme·substrate complex does not occur when RNA-1 is already associated with RNA-2 in the RNA-1·RNA-2 complex.

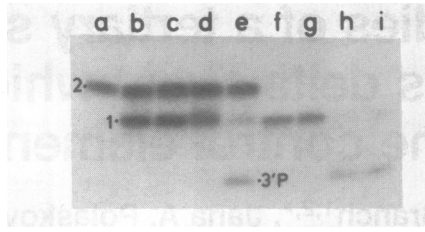

Figure 2. RNA-1 in the RNA-1·RNA-2 complex is not cleaved by a delta ribozyme, RNA-3. The RNA-1·RNA-2 complex was eluted from non-denaturing gels and either maintained on ice (lane b) or incubated at 50°C for 30 min in 5 mM $MgCl_2$, 50 mM HEPES, pH 6.5, either without RNA-3 (lane c) or with RNA-3 (lane d). A fourth aliquot of the RNA-1·RNA-2 complex was first heated in the presence of RNA-3 for 60 s at 100°C (to release RNA-1) and was then incubated in 5 mM $MgCl_2$, 50 mM HEPES (lane e). To check for a non-specific protective complex RNA-1 was incubated with a cRNA-2 (pppGCCUCUUCGGGUCGGCAUGGCAUCUCCACCUCCUCGCGGUC CGACCUGGGCAUCCGAAGGAGGACGC) for 60 min at 60°C in 2.5 mM $MgCl_2$, 5 mM HEPES. Samples were then either maintained on ice (lane f) or incubated in 5 mM $MgCl_2$, 50 mM HEPES either without RNA-3 (lane g) or with RNA-3 (lane h). Untreated RNA-1 was also incubated with RNA-3 under these conditions (lane i). Samples were denatured by heating in a formamide dye solution containing 50 mM EDTA, 50 mM HEPES and analyzed by electrophoresis in a 10% polyacrylamide gel containing 7 M urea. Lane (a) depicts RNA-2. The positions of RNA-2 (2), RNA-1 (1) and the 3′ cleavage product of RNA-1 (3′P) are identified.

mean both the nucleotides interacting through tertiary bonds and the flanking Watson–Crick base paired helices immediately adjacent to them.

As we outlined in 1990, several lines of evidence suggest that the element of local tertiary structure is part of a system which regulates formation of the delta self-cleavage structure (7). First, it contains sequences which partially overlap those in the delta self-cleavage structure and it is part of an alternative conformation, the rod-like structure, which is incompatible with formation of the self-cleavage structure, as shown by Kuo *et al.* (16). Second, it lies in a highly conserved portion of the genome that has no known coding capacity, suggesting that the site is functionally important as a structural element. Third, UV cross-linking studies suggest that it may be unusually stable and thus could provide a clamp which locally maintains the rod-like structure. Following UV cross-linking this site is so resistant to cleavage by RNase T1 that 16 of its constituent G residues are not cleaved by 1 mg/ml RNase T1 during a 45 min incubation at 37°C in 10 mM Tris, 1 mM EDTA (29,33). While UV-induced cross-links often inhibit nuclease cleavage at bonds directly neighboring cross-linked bases (35), the extensive nuclease resistance of the UV cross-linked site in delta suggests that the native form of this tertiary structure is also highly nuclease resistant.

Lazinski and Taylor recently reported that circular RNA can be a substrate for delta ribozyme cleavage and concluded that 'the ribozyme must be attenuated if the circular conformation is to be preserved' (28). The studies reported here were carried out to test the ability of the element of local tertiary structure to prevent cleavage by a *trans*-active delta ribozyme and also to measure the thermal stability of this element under ionic conditions similar to those used in studies of RNA biochemistry. For these studies we used a previously characterized, UV-sensitive, bimolecular complex (33). We found that the element of local tertiary structure reduces ribozyme-mediated cleavage to undetectable levels. This result indicates that the element of local tertiary structure can act as a ribozyme control element, providing the first example, as far as we know, of an RNA tertiary element with this function.

For thermal denaturation experiments of the bimolecular complex we employed the non-denaturing gel approach introduced by Brow and Guthrie when they determined the melting temperature of a pre-mRNA splicing complex between two yeast small nuclear RNAs, U4 and U6 (36). This technique allows reproducible side-by-side comparisons of RNA melting behavior in various buffers to be made. With this technique we found that when the concentration of magnesium ions was held constant at 0.5 mM the addition of 100 mM NaCl caused dissociation to occur at a temperature that was ~10°C lower than in the absence of added sodium ions. Our results indicate that the stability of certain RNA structures, such as the ribozyme control element from delta RNA, may be very sensitive to ionic strength in the physiological concentration range (37–39).

MATERIALS AND METHODS

RNA complex formation and non-denaturing gel analysis

To produce the RNA-1·RNA-2 complex RNAs were sealed inside capillary tubes, incubated in water baths and then quenched

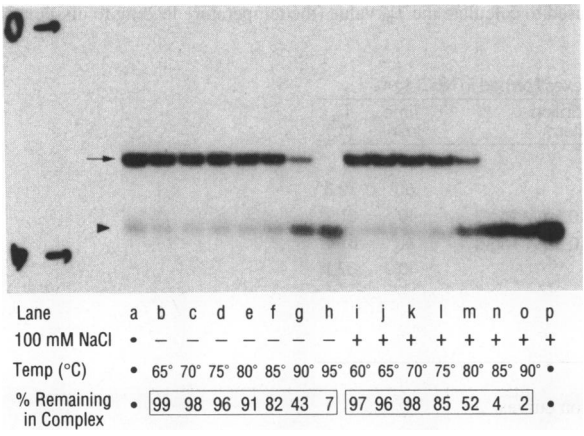

Figure 3. Non-denaturating gel analysis of the RNA-1·RNA-2 complex. An equimolar mixture of RNA-1 and RNA-2 was heated to 90°C for 1 min in water to disperse any aggregates and was then quenched on ice. One aliquot was maintained on ice (lane p), while the remainder was incubated under complex-forming conditions in a sealed capillary tube at 60°C for 60 min in 2.5 mM $MgCl_2$, 5 mM HEPES, pH 6.5, at a 330 nM concentration of RNA chains. At the end of this first incubation the sample was again quenched on ice. One sample was saved on ice to allow the extent of complex formation to be assessed (lane a), while the remainder was prepared for denaturation studies by a 5-fold reduction in the RNA concentration and adjustment of the buffer to a final concentration of either 0.5 mM $MgCl_2$, 5 mM HEPES, pH 6.5 (lanes b–h) or 0.5 mM $MgCl_2$, 5 mM HEPES, pH 7.5, with 100 mM NaCl (lanes i–o). Aliquots were then individually heated in sealed capillary tubes and incubated for 10 min at temperatures ranging from 65 to 95°C (samples with no added sodium ions) and from 60 to 90°C (samples in 100 mM NaCl), as indicated in the figure. Electrophoresis was carried out at 6°C for 18 h at a constant 80 V in a pre-chilled 8% polyacrylamide gel containing 180 mM Tris, 180 mM borate, pH 8.2 (no EDTA). An arrow identifies the position of the RNA-1·RNA-2 complex; an arrowhead indicates the position of free RNA-1 and RNA-2. The percentages of RNA remaining in the RNA-1·RNA-2 complex after the second incubation are given in the figure and were calculated using values obtained with a PhosphorImager (Molecular Dynamics). O indicates the origin of the gel.

on ice. RNA-1·RNA-2 complexs formed in 2.5 mM $MgCl_2$, 5 mM HEPES and those formed in 0.75 mM $MgCl_2$, 5 mM HEPES were incubated at 60°C; those formed in 500 mM NaCl, 5 mM HEPES and those formed in 1 M NaCl, 5 mM HEPES were incubated at 70°C. RNA duplexes formed in 2.5 mM $MgCl_2$, 5 mM HEPES were incubated at 60°C; those formed in 500 mM NaCl, 5 mM HEPES were incubated at 70°C. DNA-1·DNA-2 complexes formed in 2.5 mM $MgCl_2$, 5 mM HEPES were incubated at 50°C; those formed in 500 mM NaCl, 5 mM HEPES were incubated at 40°C. Capillary tubes were opened with a diamond pencil and samples were expelled into siliconized glass tubes. Prior to electrophoresis in non-denaturing gels samples were maintained on ice and prepared for electrophoresis by the addition of one quarter volume of a solution containing 5 mM HEPES, pH 7.0, 50% sucrose and bromophenol blue or this same solution also containing 2.5 mM $MgCl_2$. In general samples were loaded onto a pre-chilled 8% polyacrylamide gel containing 178 mM Tris, 178 mM borate, pH 8.2 (no EDTA). Electrophoresis was carried out at 6°C for 18 h at a constant 80 V. In some early experiments buffers containing 89 mM Tris and 89 mM borate were used.

Quantitation

For quantitative analysis gels were covered with Saran Wrap at the end of electrophoresis and placed in contact with Molecular Dynamics PhosphorImaging screens, usually for ~1 h. Image analysis was carried out using a Molecular Dynamics PhosphorImager and the Image Quant Software Program v.5.25. Subsequently gels were exposed to Kodak XAR film to generate autoradiograms.

RESULTS

Ribozyme inhibition by the element of local tertiary structure

The 1679 base long genomic RNA of the hepatitis delta agent (3) contains a UV-sensitive element of local tertiary structure (29). We have studied this structure using a bimolecular complex composed of two RNA molecules synthesized *in vitro* (Fig. 1A and B). This complex allows the tertiary structure to be dissected from the remainder of the delta RNA genome and studied in isolation. RNA-1, RNA-2 (the two RNAs making up this complex) and RNA-3 (a *trans*-active delta genomic ribozyme) were synthesized under conditions introduced by Milligan *et al.* (40) and then were purified from ultrathin polyacrylamide gels containing urea. RNA-1 is 65 nucleotides (nt) in length and represents sequences from the bottom strand of the rod-like secondary structure of delta genomic RNA, while RNA-2 is 67 nt in length and represents sequences from the top strand of the delta rod-like structure (see Fig. 1). As indicated by the arrow in Figure 1B and C, RNA-1 contains the phosphodiester bond broken during self-cleavage (16).

The sequences represented by RNA-1 are part of two very different structures *in vivo*: a UV-sensitive element of local tertiary structure (in the native rod-like conformation) and a self-cleavage structure (active during the cleavage of replication intermediates). To test the ability of the tertiary structure to protect the potential cleavage site in RNA-1 from cleavage by RNA-3 we eluted the RNA-1·RNA-2 complex from non-denaturing gels and then incubated the complex with RNA-3 (see Fig. 2). Prior to electrophoresis in gels containing urea samples were denatured by heating them in a formamide dye solution also containing 50 mM EDTA. No detectable cleavage of RNA-1 took place when the RNA-1·RNA-2 complex was incubated with RNA-3 (Fig. 2, lane d). However, RNA-3 was active and capable of cleaving free RNA-1 (lane i). Furthermore, if RNA-1 were released from the complex by prior heating it was cleaved by RNA-3 (lane e). This result shows that RNA-1 in the complex retained the potential to act as a substrate, but did not do so because of protection conferred by the complex. Finally, to see if this protection were specific RNA-1 was first incubated with a different RNA, cRNA-2, under conditions used to form the RNA-1·RNA-2 complex and was then incubated with RNA-3. As shown in Figure 2 (lane h), cRNA-2 did not inhibit cleavage.

Thermal stability of the RNA-1·RNA-2 complex

To obtain a melting curve of the RNA-1·RNA-2 complex equimolar amounts of RNA-1 and RNA-2 were mixed together and either kept on ice (Fig. 3, lane p) or incubated in 2.5 mM $MgCl_2$, 5mM HEPES, pH 6.5, at 60°C for 60 min in a sealed capillary tube. Under these conditions a single, slowly migrating band is produced (Fig. 3, lane a, see arrow), representing the bimolecular complex. Free forms of RNA-1 and RNA-2 co-migrate, forming a single band (marked by an arrowhead in Fig. 3) which is well-separated from the bimolecular complex. Data

Table 1. T_m Values of the RNA-1·RNA-2 complex*. Thermal denaturation curves were used to calculate the T_m value (the temperature leading to dissociation of 50% of the starting complexes)

Complexes Formed in $MgCl_2$ †‡§			Complexes Formed in NaCl †‡‖		
Dissociation Buffer †	Time (min)	T_m (°C)	Dissociation Buffer †	Time (min)	T_m (°C)
0.5mM $MgCl_2$	10′	87.7°	100mM NaCl	10′	71.9°
0.5mM $MgCl_2$	60′	86.2°	100mM NaCl	60′	72.3°
0.5mM $MgCl_2$, 100mM NaCl	10′	78.1°	100mM NaCl, 0.5mM $MgCl_2$	10′	81.9°
0.5mM $MgCl_2$, 100mM NaCl	60′	78.3°	100mM NaCl, 0.5mM $MgCl_2$	60′	81.4°
0.5mM $MgCl_2$#¶	10′	86.4°	1M NaCl	10′	87.1°
0.5mM $MgCl_2$, 100mM NaCl#¶	10′	77.2°			
0.15mM $MgCl_2$#	10′	87.3°			
0.15mM $MgCl_2$, 100mM NaCl#	10′	77.0°			

*Except where noted (#), T_m values calculated from at least three independent denaturation curves.
†Reactions also contained 5.0 mM HEPES, pH 6.5.
‡Complexes were formed at an initial nucleic acid concentration of 330 nM of each chain. Samples were diluted 1:4 (one volume of sample plus four volumes of diluent) prior to dissociation studies.
§Complexes were formed in 2.5 mM $MaCl_2$ except for the complexes dissociated in 0.15 mM $MgCl_2$ and 0.15 mM $MaCl_2$/100 mM NaCl, which were formed in 0.75 mM $MgCl_2$.
‖Complexes were formed in 500 mM NaCl except for the complex dissociated in 1 M NaCl, which was formed in 1 M NaCl.
#Values calculated from two denaturation curves.
¶The dissociation buffer also included 1 mM of additional RNA phosphates (2 µg of tRNA/5 µl).

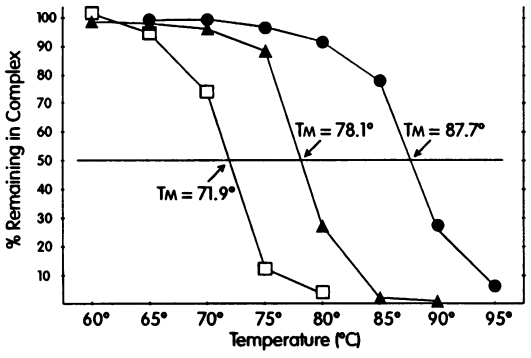

Figure 4. Thermal denaturation curves of the RNA-1·RNA-2 complex. The percentage of the RNA-1·RNA-2 complex surviving treatment at various temperatures was calculated and plotted. For this analysis the fraction of RNA in the RNA-1·RNA-2 complex was first determined for the control sample, which had been saved on ice after complex formation (see lane a in Figure 2). The analogous value was then calculated for each experimental sample, which was then divided by that of the control sample and expressed as a percentage. Complexes formed in 2.5 mM $MgCl_2$, 5 mM HEPES, pH 6.5, were subsequently heated in either 0.5 mM $MgCl_2$, 5 mM HEPES (closed circles) or in 0.5 mM $MgCl_2$, 5 mM HEPES with 100 mM NaCl (closed triangles), while complexes formed in 500 mM NaCl, 5 mM HEPES were heated in 100 mM NaCl, 5 mM HEPES (open squares). Curves are the average values from a minimum of three independent experiments.

from a Molecular Dynamics PhosphorImager were used to determine the amount of RNA in the complex compared with the amount of free RNA in each sample. The spreadsheet function of Quattro Pro 5.0 was used to calculate the percentage of RNA remaining in the complex following a variety of treatments. These results were used to generate melting curves and to algebraically calculate the mid-point of the melting curve, the T_m.

While the ratio of monovalent to divalent cations within cells is ~100:1 (39), RNA structure and biochemistry are studied under a wide range of ionic conditions. At one end of the spectrum are ribozyme cleavage experiments, which are frequently performed using buffers with a comparatively low ratio of monovalent to divalent cations. In some cases, such as the endonuclease cleavage reaction carried out by a shortened form of the *Tetrahymena* self-splicing intron, 20 mM NaCl strongly inhibits the cleavage reaction in the presence of 1 mM $MgCl_2$ (41), accounting for the use of buffers with minimal monovalent cations. At the opposite end of the spectrum are thermodynamic measurements of RNA, which are almost always made in 1 M NaCl, usually with EDTA present (42,43). Monovalent cations (and no divalent cations) are also used in many hybridization protocols, such as those employing riboprobes to detect and quantify mRNAs, because of their ability to promote and stabilize RNA duplexes. With this background we compared the stability of the bimolecular RNA-1·RNA-2 complex under a variety of ionic conditions.

We first compared melting curves of complexes dissociated in magnesium ions to those dissociated in a mixture of magnesium and sodium ions. The addition of sodium ions markedly reduced the thermal stability. After incubation for 10 min at 85°C only 4% of the complexes remained when the incubation was carried out in a buffer containing 0.5 mM $MgCl_2$, 100 mM NaCl (Fig. 3, lane n), however, when the incubation was carried out in the same buffer but with no added NaCl, 82% of the complex survived incubation at 85°C (Fig. 3, lane f) and did not dissociate fully until the incubation was carried out at 95°C (Fig. 3, lane h). Thus the T_m of the complex dropped from 87.7 to 78.1°C when the incubation was carried out in a buffer containing 100 mM NaCl in addition to 0.5 mM $MgCl_2$, a reduction of 9.6°C (Figs 3 and 4 and Table 1). These results could suggest that sodium ions partially destabilize the RNA-1·RNA-2 complex.

Since the concentration of sodium ions we used, 100 mM, is only 50% less than the concentration of monovalent cations in cells (150 mM) and since 0.5 mM $MgCl_2$ equals the reported concentration of free cellular magnesium ions (37–39), these results indicate that in cells magnesium ions may have as great an impact on the stability of RNA interactions as monovalent

Table 2. T_m Values of the DNA-1·DNA-2 complex and a fifteen base pair RNA duplex*. Thermal denaturation curves were used to calculate the T_m value (the temperature leading to dissociation of 50% of the starting complexes).

Complexes Formed in $MgCl_2$ †‡§				Complexes Formed in NaCl †‡‖			
Nucleic Acid	Dissociation Buffer †	Time (min)	T_m (°C)	Nucleic Acid	Dissociation Buffer †	Time (min)	T_m (°C)
DNA-1·DNA-2	0.5mM $MgCl_2$	10'	59.5°	DNA-1·DNA-2	100mM NaCl	10'	51.3°
DNA-1·DNA-2	0.5mM $MgCl_2$, 100mM NaCl	10'	55.9°	DNA-1·DNA-2	100mM NaCl, 0.5mM $MgCl_2$	10'	56.6°
15 BP Duplex	0.5mM $MgCl_2$	10'	76.2°	15 BP Duplex	100mM NaCl	10'	64.9°
15 BP Duplex	0.5mM $MgCl_2$, 100mM NaCl	10'	71.0°	15 BP Duplex	100mM NaCl, 0.5mM $MgCl_2$	10'	73.9°

*T_m values calculated from at least three independent denaturation curves.
†Reactions also contained 5.0 mM HEPES, pH 6.5.
‡Complexes were formed at an initial nucleic acid concentration of 330 nM of each chain. Samples were diluted 1:4 (one volume of sample plus four volumes of diluent) prior to dissociation studies.
§Complexes were formed in 2.5 mM $MgCl_2$.
‖Complexes were formed in 500 mM NaCl.

cations. Experiments carried out on another complex (a ribozyme·substrate complex), which has a lower T_m than the RNA-1·RNA-2 complex and thus can be assayed at a higher ionic strength, revealed that a similar monovalent cation-induced depression of the melting curve took place when 150 mM KCl was used (unpublished observations).

Unlike the ion concentration, the incubation time had little effect on the T_m values calculated for the RNA-1·RNA-2 complex. Very similar values were obtained whether the complex was incubated under dissociating conditions for 10 or 60 min (Table 1). This result indicates that the T_m differences we measured probably reflect differences in the thermodynamic stability of the complex in the two buffers, rather than differences in the rate of dissociation. Since a 10 min incubation period appeared to bring samples close to equilibrium it was used in subsequent studies. (For comparison, Brow and Guthrie used a 5 min incubation period in their T_m studies; 36.)

The T_m values we obtained were not significantly altered by carrying out the dissociation in a buffer containing a lower concentration of magnesium ions, 0.15 mM $MgCl_2$ rather than 0.5 mM $MgCl_2$, or by decreasing the ratio of magnesium ions to RNA phosphates by adding carrier tRNA (Table 1). Taken together these studies show that this technique gives reproducible values under a variety of conditions. Our objective was to obtain information about the thermal stability of RNA prepared and used under conventional conditions. Samples were not dialyzed against buffers to reach equilibrium and no special precautions (44) were taken to remove metal ions leaching from glassware or co-purifying with the RNA. Therefore the reported values of ionic strength are not precise.

Additional experiments were carried out to explore the possible impact of the conditions used to generate the complex (the initial conditions) on subsequent melting behavior. In particular, the melting curves of complexes formed by incubation at 60°C in 2.5 mM $MgCl_2$ and then dissociated in 0.5 mM $MgCl_2$, 100 mM NaCl were compared with those of complexes formed by incubation at 70°C in 500 mM NaCl and then dissociated in 0.5 mM $MgCl_2$, 100 mM NaCl. The T_m of complexes formed in 2.5 mM $MgCl_2$ was 3.8°C lower than the T_m of complexes formed in NaCl (Table 1). Further experiments are needed to identify the factors responsible for this slight disparity. Finally, to allow a comparison with previous thermodynamic studies the T_m was measured in 1 M NaCl (with no added magnesium ions) and was found to be 87.1°C (see Table 1).

T_m measurements of an RNA duplex and a DNA-1·DNA-2 complex

Having found that 100 mM NaCl reduced the thermal stability of the RNA-1·RNA-2 complex we examined nucleic acids lacking the tertiary interactions present in the RNA-1·RNA-2 complex by studying a perfect RNA duplex and a DNA-1·DNA-2 complex. For the RNA duplex studies two complementary RNAs, pppGUCGCGCGUAGCAUC and pppGAUGCUACGCGC-GAC, were transcribed *in vitro* (40), purified and then incubated in 5 mM HEPES, 2.5 mM $MgCl_2$ to form duplexes. Dissociation was then measured in either 0.5 mM $MgCl_2$ or in 0.5 mM $MgCl_2$, 100 mM NaCl. The T_m values were 76.2 and 71°C respectively (Table 2). Thus the T_m values of duplexes incubated in the absence of added sodium ions and in the presence of 100 mM NaCl differed by 5.2°C. This result indicates that helical regions, as well as more complex structures, show a sodium-associated T_m decrease.

DNA-1 and DNA-2 contain thymidine but otherwise have the sequences of RNA-1 and RNA-2. DNA-1 and DNA-2 were labeled at their 5′-ends by incubation with [γ-^{32}P]ATP and polynucleotide kinase. Like the T_m of the RNA-1·RNA-2 complex and that of the 15 bp RNA duplex, the T_m of the DNA-1·DNA-2 complex was reduced (from 59.5 to 55.9°C) by the addition of sodium ions. In this case the 3.6°C temperature difference, like that of the perfect RNA duplex, was smaller than the reduction observed in studies of the RNA-1·RNA-2 complex.

Resistance of the RNA-1·RNA-2 complex to dissociation by EDTA

To test the accessibility of bound magnesium ions the RNA-1·RNA-2 complex was incubated with EDTA. In these experiments complexes were formed in 2.5 mM $MgCl_2$. After quenching on ice the buffer was adjusted so that it contained either 0.5 mM $MgCl_2$, 4 mM NaCl or these same components plus 1 mM EDTA. Samples were then incubated at a range of temperatures for times varying from 1 to 81 min. Analysis by non-denaturing gel electrophoresis revealed that complexes were not as quickly dissociated by incubation with EDTA as we had expected (Fig. 5A). Complete dissociation at 65°C required incubation for >9 min. To investigate this effect control experiments were carried out. They indicated that the dissociation observed in samples treated with EDTA was caused by the EDTA

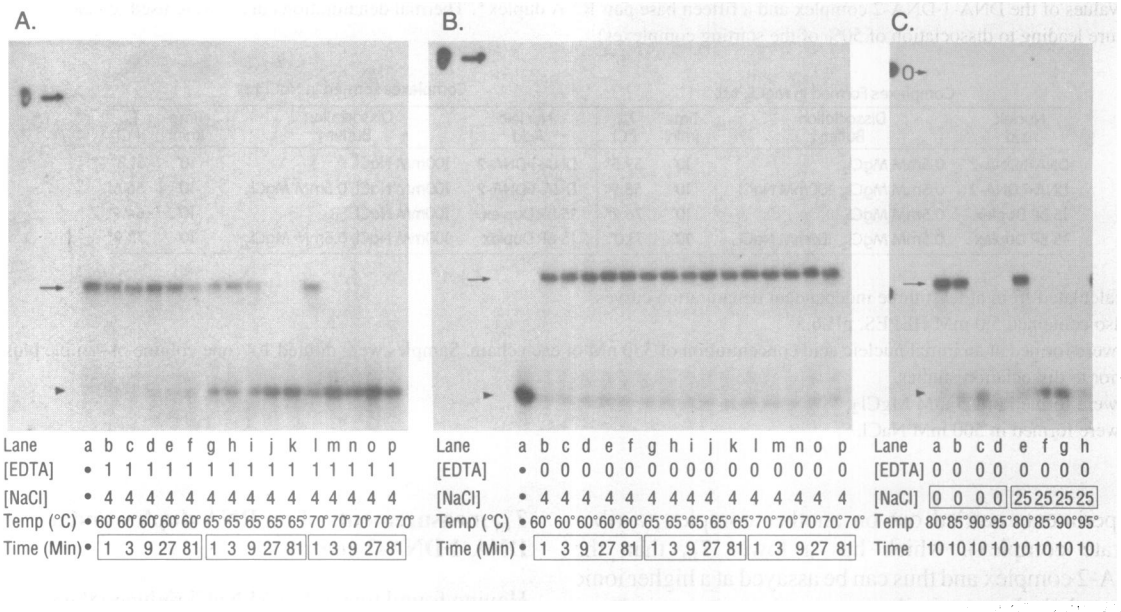

Figure 5. Treatment of the RNA-1·RNA-2 complex with EDTA. RNA-1·RNA-2 complexes were produced as described in the legend to Figure 3. After quenching on ice RNAs were diluted and buffers were adjusted to the following concentrations: 0.5 mM $MgCl_2$, 5 mM HEPES, 1 mM EDTA, 4 mM NaCl (**A**); 0.5 mM $MgCl_2$, 5 mM HEPES, 4 mM NaCl (**B**); 0.5 mM $MgCl_2$, 5 mM HEPES (**C**, lanes a–d); 0.5 mM $MgCl_2$, 5 mM HEPES, 25 mM NaCl (**C**, lanes e–h). Individual aliquots were then incubated at the temperatures specified for times ranging from 1 to 81 min. An aliquot of the RNA-1·RNA-2 complex is shown in (**A**), lane a. The position of this complex is marked by an arrow. An aliquot of the disaggregated equimolar mixture of RNA-1 and RNA-2 appears in (**B**), lane a. The position of the free RNAs is identified by an arrowhead. O indicates the origin of the 8% non-denaturing polyacrylamide gels, which contained 89 mM Tris, 89 mM borate, pH 8.2.

and not by the sodium ions present in the EDTA solution. Complexes incubated at 70°C in 0.5 mM $MgCl_2$, 4 mM NaCl did not dissociate (Fig. 5B). The concentration of sodium ions needed to cause a detectable shift in the dissociation curve (in the absence of EDTA) was ~25 mM (Fig. 5C), a level exceeding that of the magnesium ions by ~50-fold.

Data from gel electrophoresis experiments of EDTA-treated samples are summarized in Figure 6. When incubated in 1 mM EDTA at 50°C >85% of the complexes formed in the presence of magnesium ions survived for 81 min, while >55% survived incubation for this time when incubated in 1 mM EDTA at 60°C. At 70°C ~35% of the complexes survived incubation for 3 min and ~5% survived for 9 min. These studies with EDTA suggest that the magnesium ions bound to the RNA-1·RNA-2 complex are relatively inaccessible (but not necessarily tightly bound). Because the dissociation constant for the EDTA–magnesium ion complex is strongly pH dependent and because the pH of the (HEPES) buffer used for these experiments changes with temperature, further experiments are needed to determine the relative affinity for magnesium ions of EDTA and the RNA-1·RNA-2 complex.

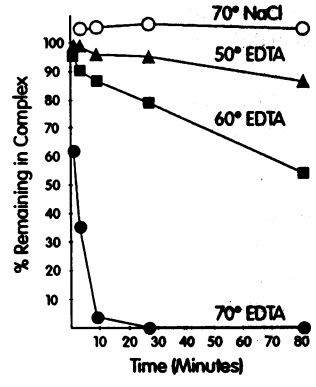

Figure 6. Dissociation curves of complexes in EDTA. The RNA-1·RNA-2 complex, formed by incubation at 60°C for 1 h in 2.5 mM $MgCl_2$, 5.0 mM HEPES, pH 6.5, was then incubated in either 0.5 mM $MgCl_2$, 5 mM HEPES, 4 mM NaCl or this same buffer augmented with 1 mM EDTA. RNA-1·RNA-2 complexes were resolved from free RNA-1 and RNA-2 by electrophoresis in non-denaturing gels. Products were analyzed as described in the legends to Figures 3 and 5. Results are shown for samples incubated in 1 mM EDTA for 1, 3, 9, 27 or 81 min at three temperatures, 50 (closed triangles), 60 (closed squares) and 70°C (closed circles), and for samples incubated in 4 mM NaCl at 70°C (open circles).

DISCUSSION

A ribozyme control element in hepatitis delta RNA

Our studies of a bimolecular complex indicate that genomic RNA of the human hepatitis delta agent may contain an element of local tertiary structure which acts as a ribozyme control element. Complexes containing this element are strongly protected from cleavage by a *trans*-active delta ribozyme. Thus *in vitro* this element can function independently and does not require stabilizing forces provided by the remainder of the circular genome. However, *in vivo* it may be part of a larger system used to control ribozyme formation. In fact, Lazinski and Taylor report that ribozyme attenuation can result from base pairing of a variety of sequences complementary to the ribozyme, indicating that the tertiary interactions are not essential for inhibition under the conditions they analyzed (28). Ribozyme control elements may exist in the genomic (and antigenomic) RNAs of additional circular RNA pathogens which, like delta RNA, have the potential to form

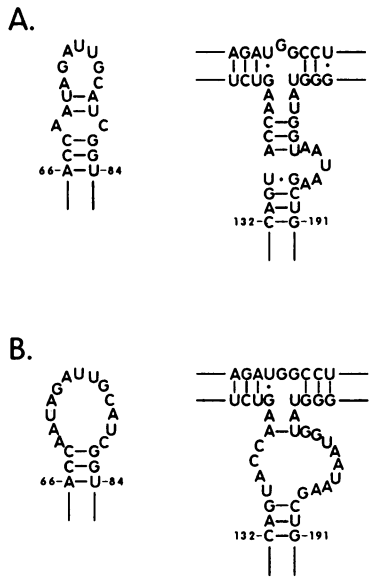

Figure 7. RNA secondary structures of the *Tetrahymena* self-splicing intron in 50 mM NaCl and 10 mM $MgCl_2$ (**A**) and 1 M NaCl (**B**), redrawn from Jaeger *et al.* (58).

self-cleavage structures. More work will be needed to determine the relative importance of secondary and tertiary structural features in the regulation of ribozymes and in the conformational switching carried out by infectious circular RNAs.

Thermal denaturation studies of a naturally occurring RNA sequence: reduced T_m in buffers containing sodium and magnesium ions

In our studies of the melting behavior of the bimolecular complex from delta RNA we compared the thermal stability of a natural sequence RNA over a wide range of ionic conditions with varying ratios of monovalent to divalent cations. We included buffers with ion concentrations near the physiological range. Although studies of RNA thermodynamics are usually carried out in 1 M NaCl (42,43), we explored the effects of other ionic conditions, because we wished to know the thermal stability of our complex in the much lower ionic strength buffers commonly used to study RNA biochemistry and present in cells.

Our most striking result was the finding that addition of 100 mM sodium ions decreased the T_m of the complex by almost 10°C when melting experiments were carried out in 0.5 mM $MgCl_2$. Qualitatively similar reductions in the T_m measurements, but of lesser magnitude, were found in melting studies of a 15 bp RNA duplex and a bimolecular complex composed of DNA, in which the T_m dropped by 5.2 and 3.6°C respectively. These results indicate that both the helical regions and the tertiary interactions of the RNA-1·RNA-2 complex are subject to sodium ion-induced loss of thermal stability. Although the bimolecular complex we studied has a unique structure, it shares features with other RNAs. Our results suggest that many other RNAs also have a lower T_m in buffers with a mixture of magnesium and sodium ions than they do in buffers with magnesium alone. Although we did not do a complete titration of the effect, we detected a reduction in thermal stability at a sodium:magnesium ratio of 50:1 (using 25 mM NaCl and 0.5 mM $MgCl_2$). This is close to the 20:1 ratio which inhibited ribozyme function in studies by Grosshans and Cech (41), suggesting that reduced stability might underlie the loss of endonuclease activity they reported. We wish to consider two different mechanisms which might contribute to the sodium ion-induced decrease in T_m we observed: counterion condensation effects and specific RNA–magnesium interactions.

Several investigators have identified specific conditions in which the T_m of a DNA duplex was lowered by an increase in the concentration of sodium ions (at a constant concentration of magnesium ions). Dove and Davidson (45) and Record (46) observed this effect, but only when the concentration of sodium ions was low. At concentrations above 25 mM the effects of Mg^{2+} were 'swamped', because of the stabilizing effects of the added sodium ions (46). Williams *et al.* found that the T_m of a DNA hexamer in 500 mM $MgCl_2$ was ~2°C lower in the presence of 150 mM NaCl than in 12 mM NaCl, but only at the highest concentration of $MgCl_2$ tested, 500 mM (47). In previous studies of RNA the extremely complex melting profiles of poly(A)·2 poly(U) were determined by Krakauer, who concluded that interactions between RNA polynucleotides and metal ions are consistent with the notion that 'those with Na^+ are qualitatively different from those with Mg^{2+}, the latter, but not the former resulting in a definite complex' (48). Manning has proposed a theory of counterion condensation to account for the lower T_m of DNA and RNA helices in certain buffers containing a mixture of divalent and monovalent cations (49,50). More studies are needed to determine how this theory applies to the melting of RNA molecules such as the RNA-1·RNA-2 complex, which is a composite of helical regions and more complex features (see Fig. 1).

Divalent cations stabilize certain RNA structures

It is clear from many lines of evidence that divalent cations are important for RNA structure and function. For example, divalent cations stabilize regions of tRNA in which phosphate groups are particularly close to each other, helping to generate the functional conformation (51–53). In addition, both M1 RNA, the catalytic subunit of RNase P (54), and the self-splicing intron from *Tetrahymena thermophila* (55,56) have structural, as well as catalytic, requirements for divalent cations. Furthermore, certain hairpin structures can be converted to pseudoknot structures by the addition of magnesium ions (57). Jaeger and colleagues have identified some of the specific features stabilized by magnesium ions by comparing the structure of the *Tetrahymena* intron in 1 M NaCl and in 50 mM NaCl, 10 mM $MgCl_2$ (58). As illustrated in Figure 7, which is redrawn from their study, magnesium ions stabilize large bulge loops and GU base pairs. They also yield more cooperative melting transitions of this intron and slightly raise the melting temperature compared with that in 1 M NaCl (58).

Our results indicate that stability of the RNA-1·RNA-2 bimolecular complex is very sensitive to the ratio of divalent to monovalent cations and that magnesium ions greatly enhance stability. Similar effects may be widespread. By stabilizing large bulge loops and non-Watson–Crick base pairs the divalent cations present in cells may promote certain RNA–RNA interactions, such as binding between small nuclear RNAs in the spliceosome (59), which would not otherwise be thermodynamically favorable. In addition, RNA conformational switching may be facilitated by the evolution of RNA structures that convert from one form to another

depending upon the local availability of multivalent cations and cationic proteins.

It will be important to learn whether magnesium ions not only differentially stabilize bulge loops and non-Watson–Crick base pairs, but also influence the configuration of duplex regions. Crystallographic studies provide little direct information concerning the position of magnesium ions in RNA duplexes. A high resolution crystal structure of an RNA helix composed of 14 AU base pairs was recently described by Dock-Bregeon and colleagues (60), who indicated that 'the clear identification of cations proved surprisingly difficult', although 'this crystal structure provides the best information on an RNA helix at the atomic level'. However, it is clear that these crystals, which were grown in 0.4 M magnesium ions, have a very deep and narrow major groove. The resistance of the RNA complex to dissociation by EDTA observed in our studies could be explained if the magnesium ions associated with these molecules were in a protected environment, such as a deep major groove or the interior of a tertiary structure.

SUMMARY

We have identified a possible ribozyme control element from delta RNA and studied the thermal denaturation profiles of a bimolecular complex containing this element. We found a reduction in thermal stability that occurs in buffers containing a mixture of sodium and magnesium ions. A qualitatively similar effect, but of lower magnitude, was observed in two other nucleic acid complexes, suggesting that it is a widespread phenomenon and may be an important factor when experiments are carried out in buffers using physiological salt concentrations.

The approach we used in these thermal denaturation studies is based on a method introduced by Brow and Guthrie (36). This technique has several useful features. It allows reproducible side-by-side comparisons of thermal stability to be made in various buffers using very little RNA and no specialized equipment. Furthermore, with this method RNA breakdown is kept to a minimum and any degradation products generated during the experiment can be readily identified and accounted for. In future studies we hope to define the relative importance of tertiary and secondary structure in stabilizing the ribozyme control element and to identify the changes taking place as this stable structure opens up for replication, perhaps through the binding of host proteins, as we suggested previously (7). Its unusual physical properties may allow this element to act as molecular clamp, with a built-in release mechanism.

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