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Articles

Melting and Chemical Modification of a Cyclized Self-Splicing Group I Intron: Similarity of Structures in 1 M Na^+, in 10 mM Mg^{2+}, and in the Presence of Substrate†

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ABSTRACT: C IVS is the cyclized form of the intron from the RNA precursor of the *Tetrahymena thermophila* large subunit (LSU) ribosomal RNA. C IVS was mapped by chemical modification in 1 M Na^+, 0.05 M Na^+ and 10 mM Mg^{2+} (Na^+/Mg^{2+}), and Na^+/Mg^{2+} with CUCU substrate. The results suggest the secondary structure is similar for all three conditions. Optical melting curves were also measured for C IVS in 1 M Na^+ and Na^+/Mg^{2+} and indicate the secondary structures have similar stabilities under both conditions. Computer predictions of secondary structure and stability are in good agreement with observations. The results suggest that many of the approximations used for computer prediction of secondary structure by free energy minimization are reasonable.

Free energy minimization methods with a nearest-neighbor model predict about 70% of RNA secondary structure from sequence (Jaeger et al., 1989). To improve predictions, it is necessary to know the validity of assumptions in the current model. One assumption is that free energy parameters measured in 1 M NaCl (Borer et al., 1974; Freier et al., 1986; Turner et al., 1988) are relevant for predicting structures formed in the presence of Mg^{2+}. Another assumption is that secondary structure dominates the free energy of folding so that tertiary interactions can be neglected (Tinoco et al., 1971; Papanicolaou et al., 1984; Jaeger et al., 1989). Studies of the folding of tRNAs are consistent with these assumptions (Cole et al., 1972; Riesner & Römer, 1973; Crothers et al., 1974). Most RNAs, however, are more complex than tRNA. For example, tRNAs have no bulges, internal loops, or pseudoknots (Rietveld et al., 1982; Pleij et al., 1985). Thus, it is important to test these assumptions in other RNAs. C IVS is the cyclized form of the intron from the RNA precursor of the *Tetrahymena thermophila* large subunit (LSU) ribosomal RNA (Zaug et al., 1983). This work uses C IVS to test assumptions in free energy minimization methods by comparing chemical modification patterns and thermal melting profiles in 1 M NaCl and in 0.05 M Na^+/10 mM Mg^{2+} and by comparing these results to computer prediction. This system is also used to test whether substrate binding affects RNA folding.

The *T. thermophila* LSU intron was the first known self-splicing RNA (Kruger et al., 1982). Its structure has been studied extensively by phylogenetic analysis (Michel & Dujon, 1983; Warring & Davies, 1984; Cech, 1988), mutagenesis [see Burke (1988) for a recent review], enzymatic digestion (Cech et al., 1983; Been et al., 1987), and chemical modification (Inoue & Cech, 1985; Latham & Cech, 1989). On the basis

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of these data, the secondary structure has been refined and a tertiary structure proposed (Kim & Cech, 1987). Thus, the LSU intron provides a suitable model system for testing the assumptions used in predicting RNA folding.

Determination of the chemical reactivity of individual nucleotides in large RNAs provides an excellent method for probing RNA structure in solution (Peattie, 1979; Inoue & Cech, 1985; Moazed et al., 1986; Ehresmann et al., 1987). Calculations suggest reactivity is determined primarily by steric effects modulated by electronic environment (Lavery & Pullman, 1984). The modification reagents used in this study are dimethyl sulfate (DMS) [A(N1), A(N7), C(N3), G(N7)], diethyl pyrocarbonate (DEPC) [A(N7), G(N7)], 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) [U(N1), G(N1)], and kethoxal [G(N1+N2)] (Ehresmann et al., 1987). Modifications are detected with strand cleavage (Peattie, 1979, 1987) or reverse transcription (Inoue & Cech, 1985; Moazed et al., 1986).

Materials and Methods

Nucleic Acids. CUCU and DNA primers were synthesized by phosphoramidite methods (Beaucage & Caruthers, 1981; Matteuchi & Caruthers, 1981; Barone et al., 1984; Kierzek et al., 1986) and were purified by high-performance liquid chromatography on a PRP-1 column (Ikuta et al., 1984). Primers were chosen to provide good specificity, high RNA–DNA hybrid stability (Stern et al., 1989), and low stability for competing intra- and intermolecular DNA associations. Concentrations were determined optically with the following extinction coefficients (M^{-1} cm^{-1}) at 260 nm: IP22 (TACTTTTCCCTCC), 1.12×10^5; IP70 (AACCGATGCAATCTAT), 1.59×10^5; IP141 (AAGTTTCCCCTGAGAG), 1.60×10^5; IP274 (CATCTTCCCCGACCG), 1.31×10^5; IP350 (GCGGCTCCAGTGTTG), 1.38×10^5 (Richards, 1975). Bulk tRNA was obtained from Sigma and used without further purification.

Labeled and unlabeled C IVS was obtained as indicated previously (Sugimoto et al., 1988), except the RNA was visualized by UV shadowing rather than ethidium bromide staining and the gel elution buffer was piperazine-*N*,*N*′-bis-(2-ethanesulfonic acid) (PIPES), pH 6.5, rather than tris-(hydroxymethyl)aminomethane (Tris), pH 7.5. The reactivity of this form in reverse cyclization reactions has been reported (Sullivan & Cech, 1985; Sugimoto et al., 1988, 1989a), and it is known to bind ϵACUCU at least 1000 times tighter than expected for simple helix formation (Sugimoto et al., 1989b). Kinetics similar to those reported by Sugimoto et al. (1988) at 30 °C were observed in the 10.5 mM $MgCl_2$, 10 mM NaCl, 0.5 mM Na_2EDTA, 80 mM HEPES, pH 7.5 buffer used for much of this work. Nondenaturing 4% polyacrylamide gels run in the same buffer at 4 °C gave a single band. Concentrations were determined optically with an extinction coefficient of 3.2×10^6 M^{-1} cm^{-1} at 260 nm (Zaug et al., 1988). C IVS was stored in sterile water.

Chemicals and Enzymes. Aniline (distilled twice under reduced pressure), Tris-HCl, diethyl pyrocarbonate (DEPC), and 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate (CMCT) were from Sigma; formamide (stirred over Bio-Rad AG501-X8 resin for 2 h), ethylenediaminetetraacetic acid (EDTA), and 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) were from Aldrich Chemical Co.; dimethyl sulfate (DMS) was from Eastman Organic Chemicals; kethoxal (3-ethoxy-1,1-dihydroxy-2-butanone) was a gift from Upjohn Co.; [α-^{32}P]- and [γ-^{32}P]ATP were from New England Nuclear; avian myeloblastosis virus reverse transcriptase was from Life Sciences, Inc.; T4 polynucleotide kinase was from New England Biolabs; deoxyribo-, dideoxyribo-, and ribonucleoside triphosphates were from Pharmacia; PIPES was from Calbiochem; and ultrapure acrylamide, *N*,*N*′-methylenebis(acrylamide) (bis), boric acid, and urea were from Bio-Rad, Inc.

Melting Curves. Absorbance vs temperature melting curves were measured at 260 and 280 nm with a heating rate of 0.5 °C min^{-1} on a Gilford 250 spectrometer as described previously (Freier et al., 1983). The standard buffers were 80 mM HEPES–0.5 mM Na_2EDTA with either 1 M NaCl (1 M Na^+) or 0.01 M NaCl and 10.5 mM $MgCl_2$ (Na^+/Mg^{2+}). Buffers were adjusted to pH 7.5 with NaOH. The derivatives of melting curves were taken with a seven-point parabolic fit with binomial weighting at each temperature (Bevington, 1969).

Computer Analysis. RNA secondary structures with and without chemical modification data were predicted with the program LRNA and CRNA (Zuker, 1989; Jaeger et al., 1990) with the parameters of Freier et al. (1986), Turner et al. (1988), and Jaeger et al. (1989). Enthalpy and entropy changes for terminal mismatches that have not been measured are estimated from free energy values at 37 and 0 °C, where free energies are approximated at 0 °C by making the corresponding 5′ dangling end more stable by 0.3 kcal/mol. An enthalpy of −4 kcal/mol was used for the unusually stable tetraloops listed by Jaeger et al. (1989). This value was obtained by extrapolation to zero stacking for the $\Delta H°$ of a hydrogen bond at the end of oligonucleotides (Turner et al., 1987). The results are relatively insensitive to this parameter. Optimal and suboptimal structures with $\Delta G°$'s within $2RT$ of the minimum energy were used to calculate the hypochromicity. Parameters for relative hypochromicities are those reported by Steger et al. (1984), and the relative hypochromicity for a GU pair was assumed to be equal to that of AU.

Chemical Modification Reactions. Modification reactions were carried out at 0 °C according to the procedure of Stern et al. (1989). All modification reactions were time titrated to obtain about one modification per molecule. These times were close to those of Moazed et al. (1986). Control lanes (blanks) contained no modification reagent but were otherwise treated identically. All samples were preincubated at 0 °C in the modification buffer for at least 45 min before modification reagent was added and were stirred occasionally after reagent was added. All samples contained 10 pmol of C IVS. Samples with substrate contained 50 μM CUCU.

DMS, DEPC, and Kethoxal Modification. Buffers were 80 mM HEPES–0.5 mM Na_2EDTA with either 1 M NaCl or 10 mM NaCl and 10.5 mM $MgCl_2$, pH adjusted to 7.5 with NaOH. C IVS was dissolved in 24 μL of buffer. For DMS, 1 μL of 1.5 M DMS in ethanol was added and incubated for 1.5–2 h. For DEPC, 2.5 μL of DEPC was added and incubated for 1.5 h. For kethoxal, 2.5 μL of 0.2 M kethoxal in 20% ethanol was added and incubated for 3 h.

CMCT Modification. C IVS was dissolved in 12.5 μL of 80 mM potassium borate, pH 8.2, 0.5 mM Na_2EDTA, and either 1 M NaCl or 10 mM NaCl and 10.5 mM $MgCl_2$. After preincubation, 12.5 μL of the same buffer containing 81 mM CMCT was added and incubated for 1.5 h.

Quenching of Modification Reactions. After incubation, 2.5 μL of 3 M sodium acetate, pH 7.0, and 2.5 μL of 3.8 mg/mL bulk tRNA were added, and solutions were precipitated with 3 volumes of ethanol. After centrifugation, pellets were washed with 70% ethanol. All pellets were redissolved

in 10 μL of water except for kethoxal-modified samples, which were dissolved in 10 μL of 0.2 M potassium borate, pH 7.0 (Litt, 1969; Stern et al., 1988).

Aniline Modification. Part of the DMS-modified sample was treated with a method similar to that of Peattie (1987). Dried DMS-treated C IVS was dissolved in 20 μL of fresh 0.5 M Tris-HCl, pH 8.2–0.1 M sodium borate and incubated at 0 °C in the dark for 30 min. This reaction was stopped by addition of 200 μL of 0.55 M sodium acetate, 0.55 M acetic acid, and 0.3 mg/mL bulk tRNA, and the solution was precipitated with 3 volumes of ethanol. After precipitation, the pellet was washed with 70% ethanol and dried.

This pellet was dissolved in 20 μL of 1 M aniline acetate, pH 4.5, and incubated at 60 °C in the dark for 20 min. The solution was adjusted to approximately pH 7 with ammonium hydroxide and quickly extracted five times with equal volumes of diethyl ether. After brief drying to remove ether, the solution was precipitated as described above for quenching of modification reactions.

Primer Extension and Analysis. Primer extension was used to detect all modified bases and strand cleavage sites (Inoue & Cech, 1985; Moazed et al., 1986). The hybridization solution, consisting of approximately 1 pmol of RNA and 0.6 pmol of 5′-end ^{32}P-labeled DNA primer in 2 μL of 50 mM Tris-HCl, pH 7.5, 60 mM NaCl, and 10 mM DTT, was heated to 90 °C for 1 min in a heating block; the block was quick cooled to 70 °C (IP 70, IP 141, IP 274, IP 350) or 50 °C (IP 22) in a room temperature water bath and then slow cooled to 40 °C in air. To these solutions was added 2 μL of extension mix (50 mM Tris-HCl, pH 8.2, 30 mM NaCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM each dNTP, and 0.1 unit/μL avian myeloblastosis virus reverse transcriptase) and either 1 μL of water or 1 μL of dideoxynucleotide stock (0.4 mM) (Sanger et al. 1977). These tubes were incubated 30 min at 40 °C; 1 μL of 1 mM each dNTP was added, and the samples were incubated an additional 15 min. Reactions were stopped by addition of 7 μL of loading buffer (89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA, 0.4 mg/mL each of xylene cyanol and bromophenol blue, in purified formamide). After incubation for 2 min at 90 °C, samples were loaded onto 0.4 mm × 44.5 cm, 10% acrylamide (29:1 acrylamide:bis), 7 M urea, 89 mM Tris, 89 mM boric acid, 2 mM Na_2EDTA gels and electrophoresed for 2–5 h at 60 W.

Results

Melting Curves. Melting curves and their derivatives are shown in Figure 1. There is little change in absorbance between 0 and 30 °C. The curves for Na^+/Mg^{2+} have a small transition at 37 °C that is reversible if the sample is not heated above 45 °C. This transition is absent for C IVS in 1 M Na^+ and also for the L-19 (Zaug & Cech, 1986) linear form of the intron in 1 M Na^+ or when Mg^{2+}-containing buffers are used [see supplementary material (see paragraph at end of paper regarding supplementary material)]. The main transitions in both 1 M Na^+ and Na^+/Mg^{2+} are around 70 °C. The transitions in Na^+/Mg^{2+} are more cooperative and have somewhat higher melting temperatures (T_m) than those in 1 M Na^+. When a sample in 1 M Na^+ is remelted immediately after cooling, the shapes and T_m's of the curves are identical. When a sample in Na^+/Mg^{2+} is remelted, the second melt is much less cooperative, although the initial absorbance at 0 °C and the total hypochromicity are essentially unchanged. Presumably this is due to partial strand cleavage from hydrolysis. Thus, the T_m and cooperativity observed in Na^+/Mg^{2+} represent lower limits. Melting curves were also measured at 1 mM Mg^{2+} and pH 6.5 where hydrolysis should be much slower

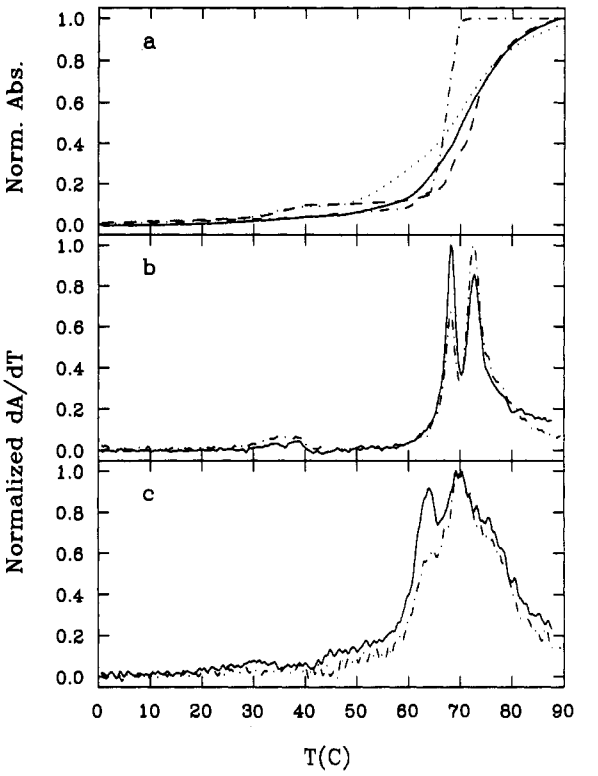

FIGURE 1: Melting curves and their derivatives for C IVS. (a) Melting curves measured at 280 nm for 1 M Na^+ (—) and Na^+/Mg^{2+} (– –) and calculated for linear (···) and circular (–·–) C IVS. (b) dA/dT curves in Na^+/Mg^{2+} at 260 (—) and 280 nm (–·–). (c) dA/dT curves in 1 M Na^+ at 260 (—) and 280 nm (–·–).

Table I: Enthalpies and Helix Composition for C IVS from Melting Curves

condition[a]	T_m (°C)	$-\Delta H°$ (kcal/mol) 260 nm	$-\Delta H°$ (kcal/mol) 280 nm	% AU pairs in transition
10.5 mM $MgCl_2$, 0.05 M NaCl	35.8	*b*	80	8
	68.1	348	263	59
	72.6	212	221	47
1 M NaCl	64.1	116	*b*	61
	69.8	*b*	*b*	61

[a] All solutions contained 80 mM HEPES, pH 7.5, and 0.5 mM EDTA. [b] Parameters could not be calculated, due to ill-defined derivative curves.

(see supplementary material). These curves had shapes similar to those shown in Figure 1a, suggesting that partial hydrolysis does not have a major effect on the initial melt. Similar results are seen by others (Puglisi et al., 1988; Ruffner et al., 1989).

Assuming independent two-state transitions, enthalpy changes in kcal/mol for each transition can be calculated from (Gralla & Crothers, 1973; Marky & Breslauer, 1987)

$$\Delta H°_{VH} = \frac{-7.0}{1/T_1 - 1/T_2} \quad (1)$$

where T_1 and T_2 are the temperatures in K at half-maximum for $d\alpha/d(1/T)$ curves derived from the melting curves. Here α is the fractional extent of melting. The calculated $\Delta H°$'s are listed in Table I. The enthalpies in 1 M NaCl are particularly unreliable, due to the presence of several transitions in each peak. The difference in calculated enthalpy between 260 and 280 nm for the 68 °C transition in Na^+/Mg^{2+} may be due to decreased resolution of transitions at 280 nm relative to 260 nm. With the parameters reported by Turner et al. (1988), the $\Delta H°$ predicted for the phylogenetic structure is

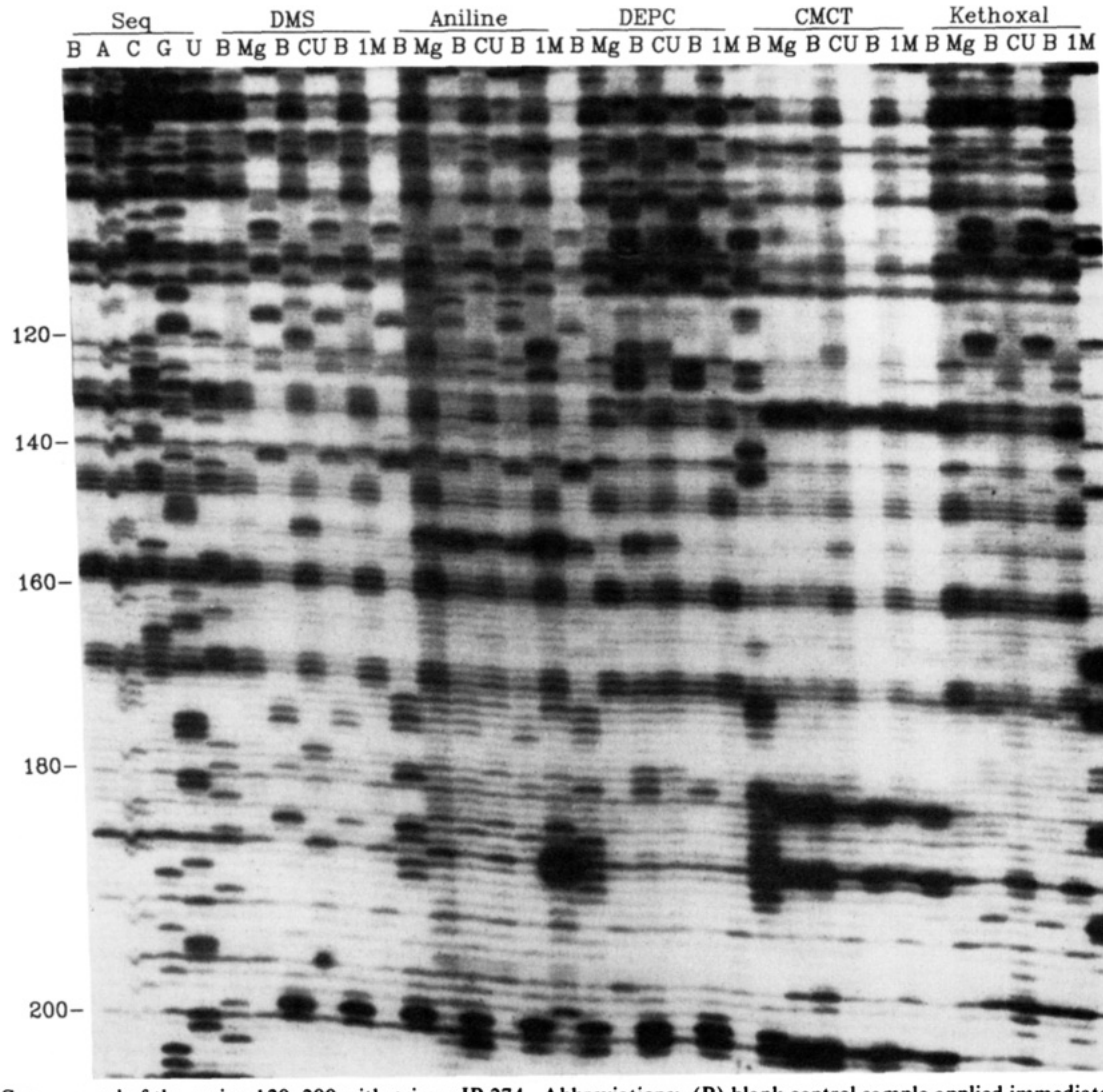

FIGURE 2: Sequence gel of the region 120–200 with primer IP 274. Abbreviations: (B) blank control sample applied immediately to the left of its corresponding experimental samples; (A, C, G, U) sequence lanes; modification in (Mg) Na^+/Mg^{2+}, (CU) Na^+/Mg^{2+} and 50 μM CUCU, and (1M) 1 M Na^+. All modifications were carried out at 0 °C (see text). Note that reverse transcriptase stops one base 3′ to a modified base and the extension is 3′ to 5′.

-1.1×10^3 kcal/mol when the $\Delta H°$ for loops is taken as due entirely to the 3′ dangling ends in the loops. The factor of 2 difference between the predicted and measured $\Delta H°$'s in Na^+/Mg^{2+} is expected if the transitions are not two state, but rather involve intermediates (Sturtevant, 1987). Despite this large difference, the predicted two-state $T_m = \Delta H°/\Delta S° =$ 66 °C is close to the measured T_m (see Figure 1). In this calculation, $\Delta S°$'s for loops were taken as $\Delta S° = \Delta G°_{37}$-(loop)/310.15, where $\Delta G°_{37}$(loop)'s were taken from Table I of Jaeger et al. (1989). Furthermore, the completely melted, covalently closed C IVS was assigned the entropy expected for a hairpin of 399 nucleotides relative to a completely melted, linear molecule: $\Delta S° = -\Delta G°_{37}/310.15 = -[5.8 \times 10^3 + 1.75(1.987)(310.15) \ln (399/9)]/310.15 = -31.9$ eu. This raises the T_m about 4 °C relative to that of a linear RNA with the same secondary structure. The close agreement with the measured T_m (see Figure 1a) suggests it is not necessary to know the details of RNA melting to make a reasonable prediction of T_m.

Also shown in Figure 1a are melting curves calculated from structure predictions for the circular and linear forms of the molecule. The T_m for the calculated curve for the linear form opened at the G414–A16 phosphate is about 5 °C lower than that observed for C IVS in 1 M NaCl. This is expected since the T_m for a covalently closed RNA of 399 nucleotides should be about 4 °C higher than that of the linear form, as discussed above. The calculation for the circular form, however, predicts a much more cooperative melt than observed. This is probably because the algorithm for structure prediction uses a linear approximation for multibranched loops (see Discussion).

Chemical Modification. Figures 2 and 3 show typical gels from the chemical modification experiments, and Figure 4 maps the data onto the secondary structure proposed from phylogeny (Michel & Dujon, 1983; Burke et al., 1987). Numbering and nomenclature for the structure are also shown in Figure 4. In general, most base-paired nucleotides are not modified, consistent with the phylogenetic model. Many bases in single-strand regions are also protected, however. This is true for both the base-pairing positions and N7 of purines, suggesting non-Watson–Crick interactions are also important.

Chemical Modification in Na^+/Mg^{2+} (Figure 4, Top Right). For the secondary structure helixes, strong modifications at base-pairing positions are observed only for a few bases in P7 and P9. There is little or no modification in the P5a, P5b, and P5c regions, suggesting a protected environment.

Modification in the Presence of CUCU Substrate (Figure 4, Bottom Left). CUCU causes reverse cyclization, the specific opening of C IVS at G414–A16 (Sullivan & Cech, 1985;

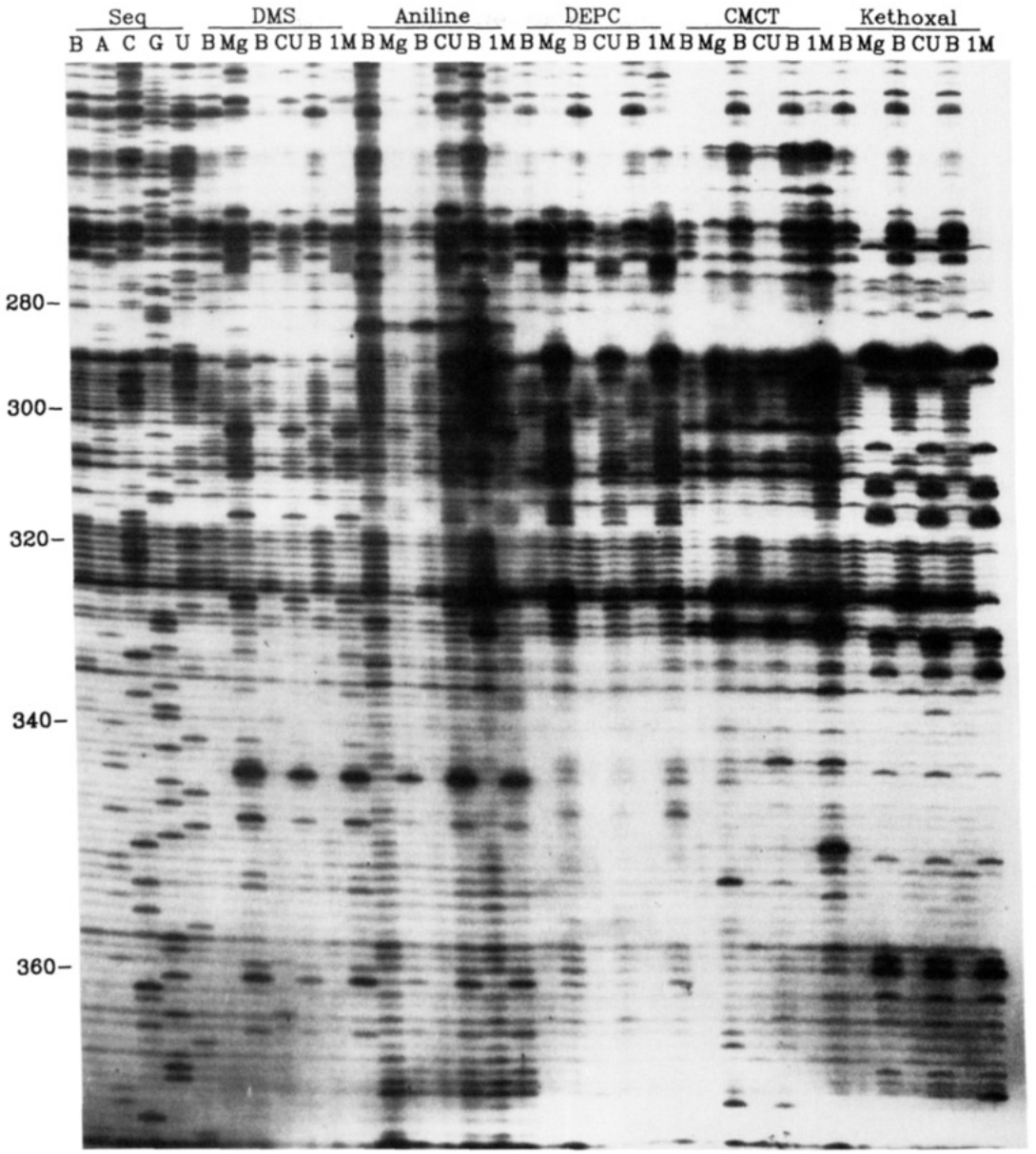

FIGURE 3: Sequence gel of the region 300–370 with primer IP 22. See caption of Figure 2 for details.

Sugimoto et al., 1988), but the predicted reactivity of CUCU at 0 °C is less than 0.1% over the modification time period of about 2 h (Sugimoto et al., 1988). This is consistent with the chemical modification data. With primer IP 22, extension occurs through the G414–A16 junction, and stops at G414 have the same intensity in the presence and absence of CUCU. Surprisingly, there is only slight protection of the internal guide sequence (IGS), nucleotides 22–27, where the substrate is expected to bind (Davies et al., 1982). This small change may be due to an alternate binding site for CUCU (Barfod & Cech, 1988), to substrate sliding on the IGS, or to the fact that CUCU binding to C IVS only involves two terminal GU pairs and one central CG pair (Been & Cech, 1987; Sugimoto et al., 1988). Most other regions show little or no change in modification pattern.

Chemical Modification in 1 M NaCl (Figure 4, Bottom Right). In general there is more and stronger modification in 1 M NaCl than in Na^+/Mg^{2+}. Nevertheless, modification patterns of only the P5a, P7, and P9 helixes disagree with the phylogenetic model.

Unusual Modifications. Some unusual apparent modifications were detected. DEP modified both purines and pyrimidines (Vineze et al., 1973), while it is reported to modify only N7 of purines, normally. DMS causes a strong stop at G346 in 1 M Na^+ and a weak stop in Na^+/Mg^{2+}. CMCT modifies C260 and C262 in both 1 M Na^+ and Na^+/Mg^{2+}, and kethoxal modifies C78 in a hairpin loop. These types of modifications have not been reported previously. In all cases, the modified bases are single stranded or near the end of a helix. These unusual apparent modifications may indicate unusual environments or that modification of another nucleotide induces a stable structure that causes reverse transcriptase to pause or stop. They were not used in the computer analysis described below.

Computer Predictions of Structures. Figure 5A shows the structure predicted for C IVS at 0 °C with the algorithm of Zuker (1989) and the parameters of Freier et al. (1986) with loops treated as described by Jaeger et al. (1989). Similar structures are predicted from 0 to 37 °C. Structure predictions can also be made by incorporation of constraints based on modification data. The folding program offers two options for preventing double-stranded regions. The first option does not allow a designated base to pair. The second option, designed to incorporate nuclease sensitivity data (Zuker &

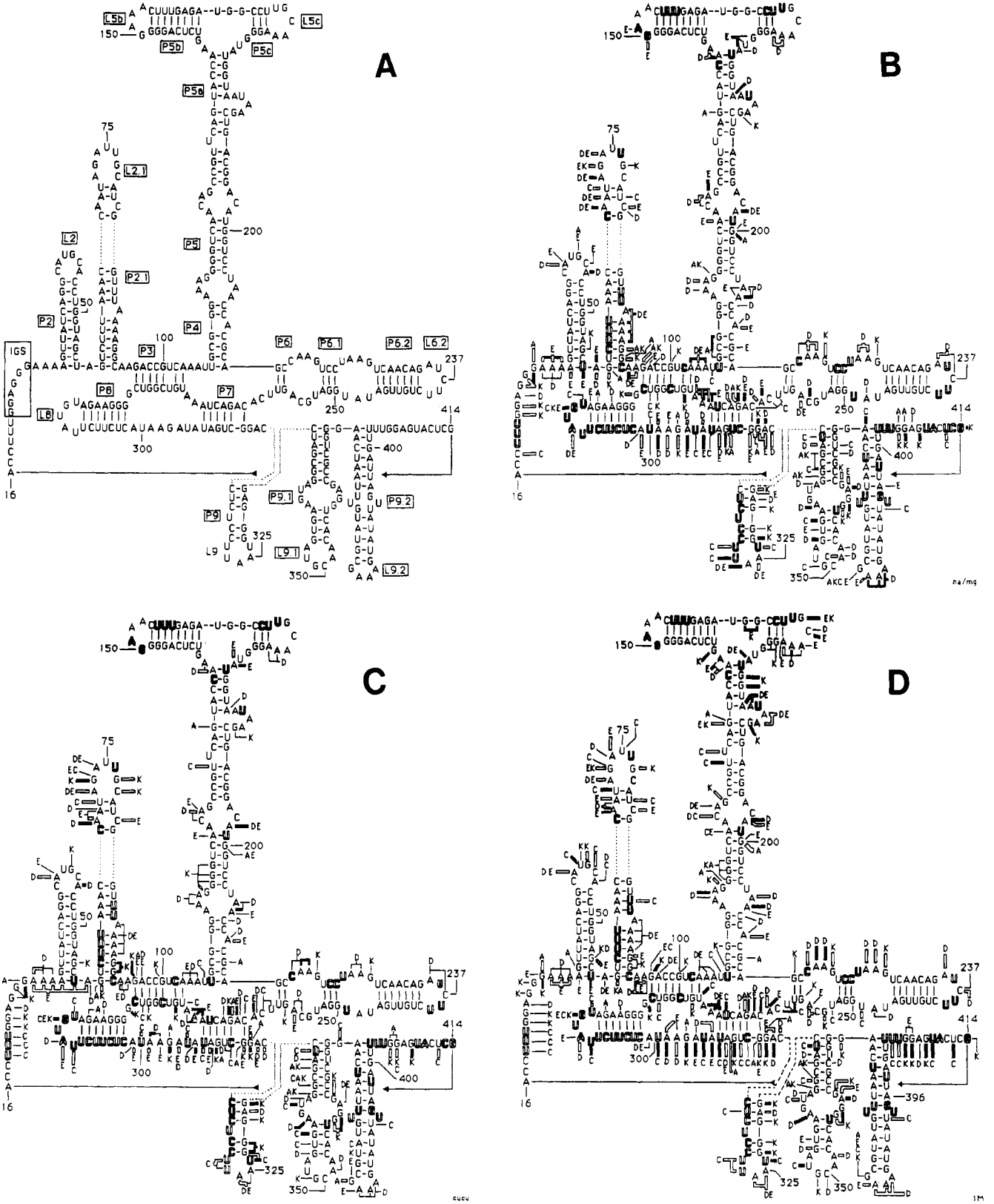

FIGURE 4: Numbering and nomenclature for C IVS (A, top left) with chemical modification data at 0 °C mapped on the phylogenetic structure (Michel & Dujon, 1983; Burke et al., 1987). The symbols show strong (solid rectangles), medium (open rectangles), or weak (—) modification by DMS (D), DMS with aniline (A), DEPC (E), CMCT (C), or kethoxal (K). Outlined bases are ambiguous due to stops in control lanes. The long, thin arrow represents the covalent bond at the cyclization junction. Buffer conditions are (B, top right) Na^+/Mg^{2+}, (C, bottom left) Na^+/Mg^{2+} and 50 μM CUCU, and (D, bottom right) 1 M Na^+.

Stiegler, 1981), allows a designated base to pair only if its 3′ neighbor is single stranded. Thus the cleaved phosphodiester bond can never be between two base pairs. This second option allows a modified base to pair at a 3′ end of a helix but not at a 5′ end. Both options were used to handle chemically modified bases and gave similar results. Figure 5B–D shows the structures predicted when strong modifications at Watson–Crick positions are used to constrain the structure prediction with option 1. In general, the predicted structures are similar to those expected from phylogeny. Minor differences

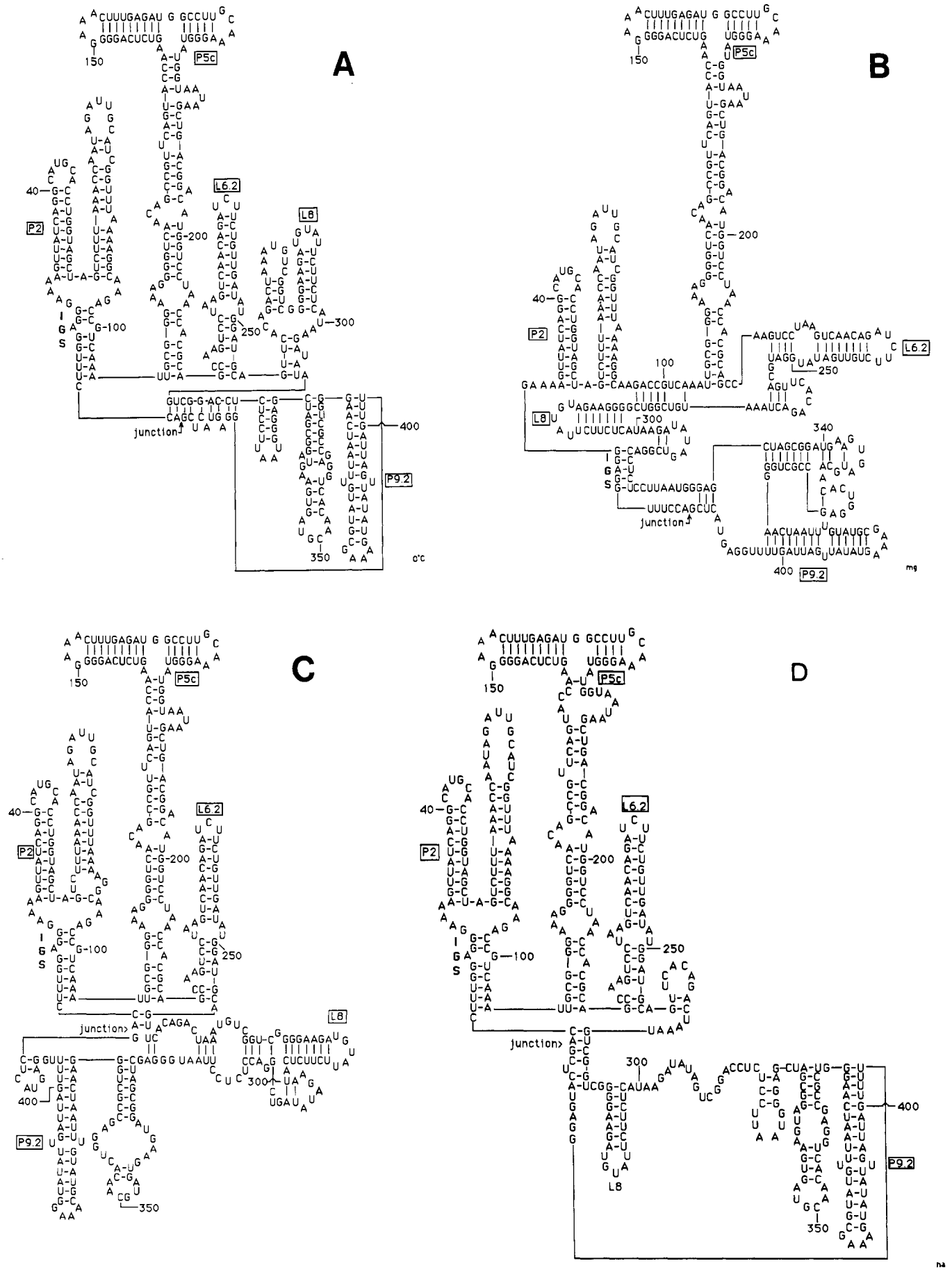

FIGURE 5: Computer predictions of secondary structure for C IVS at 0 °C, using the program of Zuker (1989) with the parameters of Freier et al. (1986) and Jaeger et al. (1989). Predicted structure (A) without modification data and (B–D) forcing strongly modified Watson–Crick sites single stranded in (B) Na^+/Mg^{2+}, (C) Na^+/Mg^{2+} and 50 μM CUCU, and (D) 1 M Na^+.

are observed for P5c in which the helix is slipped relative to the phylogenetic model.

Discussion

The purpose of this work is to provide insight into interactions important for predicting RNA secondary and tertiary structure from sequence. The LSU intron provides a model system of convenient size that contains all the motifs associated with RNA secondary structure (Michel & Dujon, 1983; Warring et al., 1983; Cech et al., 1983; Inoue & Cech, 1985; Been et al., 1987; Latham & Cech, 1989). The circular form provides an additional constraint that is also inherent in some computer algorithms that predict suboptimal folding (Steger et al., 1984; Zuker, 1989).

Comparison of Results in 1 M Na^+ and 0.05 M Na^+/10 mM Mg^{2+}. Most methods for structure prediction use parameters measured with oligonucleotides in 1 M NaCl (Tinoco et al., 1973; Freier et al., 1986; Turner et al., 1988; Jaeger et al., 1989). An important question is whether 1 M Na^+ is a reasonable approximation for buffers containing Mg^{2+} or if the stabilities of certain motifs are different in the presence of Mg^{2+}. Thus the structure of C IVS was probed in buffers containing either 1 M Na^+ or 0.05 M Na^+/10 mM Mg^{2+}. These experiments were done at 0 °C where weak interactions will be stabilized and single structures will most likely predominate.

Figure 4 presents the modification data mapped onto the phylogenetic structure. Figure 6A highlights positions where chemical reactivity in 1 M Na^+ differs from that in Na^+/Mg^{2+} by at least two levels, e.g., weakly to strongly reactive. For both 1 M Na^+ and Na^+/Mg^{2+}, the reactivity is largely in agreement with the accepted structure. In both cases, P7, potentially part of a pseudoknot (Rietveld et al., 1982; Pleij et al., 1985; Puglisi et al., 1988), is attacked, suggesting that if this helix forms at 0 °C, it is in dynamic equilibrium with single strands. This has also been suggested by a dramatic increase in k_{cat}/K_m when this helix is mutated to create mismatches (Williamson et al., 1987). For 1 M Na^+, the reactivity of helix P5a is also inconsistent with the phylogenetic structure. Computer predictions of structure constrained with strongly modified nucleotides single stranded suggest these local changes do not affect the other helixes (see Figure 5). Evidently, only 1 of 17 phylogenetically determined secondary structure helixes is different when Na^+/Mg^{2+} is replaced by 1 M Na^+. Here a helix is defined as a region containing three or more base pairs with no bulge or interior loops containing three or more nucleotides. The helix P5a contains a large bulge (Collins, 1988), suggesting large bulges may be preferentially stabilized by Mg^{2+}. Thus, comparison of the chemical modification data in 1 M Na^+ and Na^+/Mg^{2+} suggests a limited effect of Mg^{2+} on secondary structure.

Comparison of predicted structures for 1 M Na^+ with those determined from phylogeny and from chemical mapping in Na^+/Mg^{2+} also suggests that Mg^{2+} does not have a major effect on secondary structure. Panels A and D of Figure 5 show structures predicted without and with constraints from chemical modification data in 1 M Na^+. Considering secondary structures helixes and allowing slipped helixes, these structures contain respectively 16 and 15 of the 17 helixes determined phylogenetically. For comparison, 15 phylogenetic helixes are predicted correctly when constraints are included from the chemical modification data in Na^+/Mg^{2+}. Thus the results with 1 M Na^+ and Mg^{2+} are again similar.

One aspect of the structure prediction algorithm may give misleading results when chemical modification data are included. Because the algorithm was designed to incorporate nuclease sensitivity rather than chemical modification data, it either prohibits pairing of a modified nucleotide or permits a modified nucleotide to be base paired on the 3′ side of a helix but not on the 5′ side (Zuker, 1989; Jaeger et al., 1990). To determine if this limitation affects the comparisons of predicted and phylogenetic structures, nucleotides that are base paired on the 3′ and 5′ sides of phylogenetic helixes were removed from the constraints based on modification data. Structures predicted for 1 M Na^+ and Na^+/Mg^{2+}, in the absence of these constraints, each contained one additional phylogenetic helix, P3 and P9, respectively. Thus, although this limitation affects the details of the comparison, 1 M Na^+ and Na^+/Mg^{2+} still give similar results.

The melting curves shown in Figure 1 are also consistent with a relatively small effect of Mg^{2+} on stability. Two major transitions are observed in both 1 M Na^+ and Na^+/Mg^{2+}, and the melting temperatures in Na^+/Mg^{2+} are only about 5 °C higher than in 1 M Na^+. The presence of two major transitions in both buffers and the large $\Delta H°$'s in Na^+/Mg^{2+} suggest the transitions involve disruption of secondary rather than tertiary structure. This suggests that C IVS melts either as two separate domains or to an intermediate secondary structure as observed for viroids (Riesner et al., 1979; Steger et al., 1984; Riesner, 1987). The good agreement between the observed melting curve and that predicted for a linearized form of C IVS is consistent with secondary structure dominating stability, since only secondary structure interactions are included in the algorithm. It had been suggested that secondary structure interactions are not sufficient to rationalize the high-temperature stability of IVS (Szostak, 1986).

The extreme cooperativity predicted for the circular form probably reflects the unrealistic linear approximation for multibranch loops. This approximation is required to assure the algorithm finds the lowest free energy structure (Zuker, 1989). Unfortunately, it makes large multibranch loops much more unfavorable than more reasonable approximations (Jacobson & Stockmayer, 1950; Chan & Dill, 1990). Since melting of a covalently closed circular RNA requires formation of large multibranch loops, this leads to predicted melting behavior that is unrealistically cooperative. The program of Zuker and Stiegler (1981) used a more realistic treatment of multibranch loops, and when modified to accept circular RNAs, it was able to predict the melting of circular viroids quite well, although an unusual combination of thermodynamic parameters was required (Steger et al., 1984).

The results discussed above are all consistent with a model in which secondary structure interactions dominate the folding stability of C IVS. There is no evidence to suggest tertiary interactions play a major role in stability, even though they are known to be important for substrate binding (Sullivan & Cech, 1985; Sugimoto et al., 1988, 1989a,b; Danenberg et al., 1989; Herschlag & Cech, 1990). Similar conclusions have been reached for tRNA (Cole et al., 1972; Crothers et al., 1974; Riesner & Römer, 1973) and for viroids (Riesner, 1978; Steger et al., 1984). With the possible exception of large bulge loops, the evidence also indicates the presence of Mg^{2+} does not strongly affect the stabilities of secondary structure interactions. This is also consistent with the measured effects of Mg^{2+} on the stability of the fully paired duplex $(dGCATGC)_2$ (Williams et al., 1989), and with recent correlations of thermodynamic parameters with in vivo expression of the MS2 coat gene (de Smit & van Duin, 1990) and with in vivo splice site selection of the sunY intron of phage T4 (Michel et al., 1990).

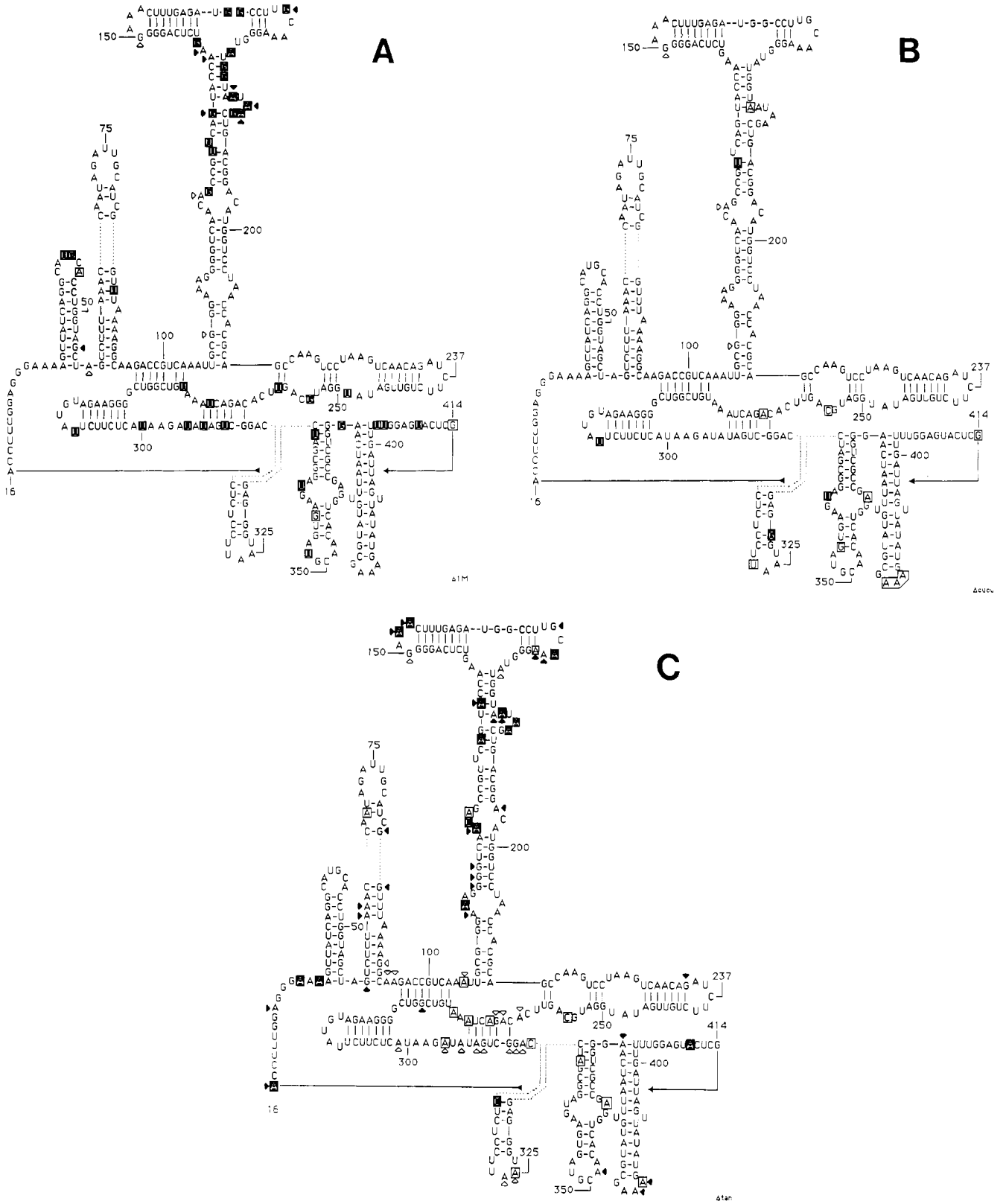

FIGURE 6: Chemical modifications of C IVS under several conditions, as compared to the modifications in Na^+/Mg^{2+}. Marked bases are modified less (base code in open square or marked by open arrowhead) or more (base code in solid square or marked by solid arrowhead) at Watson–Crick (squares) or N7 (arrowheads) sites. Differences shown are at least 2 orders (for example, weak vs strong modification). (A) 1 M Na^+; (B) Na^+/Mg^{2+} with 50 μM CUCU; (C) results of Inoue and Cech (1986) (150 mM NaCl/10 mM $MgCl_2$, 50 mM Na cacodylate, pH 7.5, 30 °C).

Comparison of Chemical Modification Results with the Energy Model for Prediction of Structure. Recently, several changes have been made in the energy model used for predicting RNA structure from sequence (Turner et al., 1987, 1988; Jaeger et al., 1989). Two of these involve stacking energies of nucleotides at the ends of helixes. Specifically, GU pairs and unpaired nucleotides adjacent to helixes were given favorable free energies. These changes were suggested by the increased stabilities of oligonucleotide duplexes with terminal GUs and unpaired nucleotides (Petersheim & Turner, 1983;

Table II: Percentage of Terminal Nucleotides That Are Strongly or Moderately Reactive in C IVS and 16S rRNA[a]

	C IVS (%)				
loop	Na^+/Mg^{2+}	Na^+/Mg^{2+} + CUCU	1 M Na^+	16S (%)[b] K^+/Mg^{2+}	av
	Strongly Stacking Sequences				
hairpin	33	0	33	17	19
internal	25	25	14	28	27
multibranch	50	50	100	33	38
	Weakly Stacking Sequences				
hairpin	67	33	67	50	51
internal	78	44	90	38	54
multibranch	56	11	56	51	46

[a] Nucleotides were considered modified if they were strongly or moderately reactive at a base-pairing position. Total number of terminal nucleotides (not including those with control stops): (C IVS) Hairpins, 3 strong, 3 weak; internal loops, 4 strong, 9 weak (10 for 1 M Na^+); multibranch loops, 4 strong, 9 weak. (16S) Hairpins, 18 strong, 28 weak; internal loops, 23 strong, 30 weak; multibranch loops, 27 strong, 36 weak. See text for definition of strong and weak stacks. The pseudoknots were split into two multibranched loops. [b] Moazed et al. (1986).

Hickey & Turner, 1985; Freier et al., 1985, 1986; Sugimoto et al., 1987a,b). Since unstacked nucleotides are expected to be more reactive than stacked nucleotides, it is interesting to compare the results from chemical modification with expectations from the energy model.

There are seven potential terminal GU pairs in C IVS that were probed: G116U205, G141U162, G215U258, G220U253, G227U247, G368U403, and G378U393. In Na^+/Mg^{2+}, Na^+/Mg^{2+} with CUCU, and 1 M Na^+, respectively, one, two, and seven of these GUs had at least one moderate hit. There were no strong hits at any of these nucleotides. The chemical modification data on Moazed et al. (1986) on 16S rRNA in a buffer with 0.4 M K^+ and 20 mM Mg^{2+} show that 14 of 23 terminal GU pairs are not modified. The results suggest terminal GUs form pairs as suggested by Crick (1966) and that Mg^{2+} may stabilize this interaction.

Free energy increments for unpaired terminal nucleotides are very sequence dependent, ranging from 0 to −1.7 kcal/mol (Freier et al., 1986; Turner et al., 1988). It has been suggested that sequences with increments more favorable than −1 kcal/mol are strongly stacking sequences, while those with increments less favorable than −0.3 kcal/mol are weakly stacking (Sugimoto et al., 1987; Turner et al., 1987, 1988). All strongly stacking sequences are at the 3′ side of helixes, while most weakly stacking sequences are at the 5′ side of helixes. Table II lists the sum of moderate and strong chemical modifications observed for strongly and weakly stacking sequences in various motifs in C IVS and in 16S rRNA (Moazed et al., 1986). For internal and hairpin loops, weakly stacking sequences are roughly twice as likely to be modified as strongly stacking sequences. For multibranch loops, however, there is little difference between weakly and strongly stacking sequences. The results suggest that terminal stacking energies are important determinants of structure for internal and hairpin loops but that multibranch loops are more complex.

The energy model for bulges can also be compared with modification data. For example, bases A87, A88, A89, and A90 form a single bulged A and three AU pairs, but all bases have equal probability of being modified. This is expected from the energy model for bulges and from studies of deoxyoligonucleotides with similar possibilities for bulge migration (Woodson & Crothers, 1988). Phylogeny suggests A89 or A90 is the preferred bulge (Nielson & Engberg, 1985). A similar pattern is found for the G925, G926, G927, and G928 region of *Escherichia coli* 16S rRNA (Moazed et al., 1986). All these bases are modified equally, whereas phylogeny suggests only G926 is bulged. In contrast, chemical modification of P5a suggests U131 is more likely to be bulged than U130. In Na^+/Mg^{2+} with CUCU, only U131 is modified, and in 1 M NaCl, U131 is modified more than U130. The results suggest the energy model for single bulges is reasonable, although there may be additional subtle effects of sequence.

Possible Tertiary Interactions. In the crystal structure of tRNA, tertiary interactions create regions of high charge density that are stabilized by Mg^{2+} (Kim et al., 1974; Robertus et al., 1974). Thus, differences in chemical modification in the absence and presence of Mg^{2+} may suggest regions of tertiary structure. The results in Figures 4 and 6 suggest two such regions: P5 and the termini of P9.1 and P9.2.

As discussed above, P5 is modified more strongly in the absence than in the presence of Mg^{2+}. This may be due to stabilization of the bulges in P5a by Mg^{2+}. In Na^+/Mg^{2+}, however, there is relatively little modification of the entire P5 region relative to other regions with similar numbers of unpaired nucleotides (see Figure 4). For example, the GA_3 loop L5b is modified much less than the GA_3 loop L9.2. This suggests P5 may be protected by tertiary interactions. Latham and Cech (1989) found that the ribose groups of many of the single-stranded nucleotides in P5 were also protected from cleavage. Tertiary interactions involving P5 are also suggested by sequence conservation in this region (Collins, 1988) and by the effects on activity of deleting P5 (Joyce et al., 1989).

A second cluster of nucleotides that are modified more in the absence of Mg^{2+} are U333, G368, U403, and U404 (see Figure 6B). It has been suggested that weakly stacking nucleotides permit turns in the sugar–phosphate backbone (Sugimoto et al., 1987; Turner et al., 1987, 1988). Either U403 or U404 is a weakly stacking nucleotide, depending on whether G368 and U403 are paired. Thus, this is a region where a turn is thermodynamically favorable. Such a structure would be stabilized by Mg^{2+}, so that the enhanced modification in the absence of Mg^{2+} is consistent with a turn at either U403 or U404.

Modification in the Presence of Substrate. Figure 6B highlights positions where reactivity changes by at least two levels when CUCU is added to the Na^+/Mg^{2+} buffer. No major changes are observed. This is consistent with the minor changes observed by Inoue and Cech (1984) and Latham and Cech (1989) on slightly different forms of the intron: increased methylation of A28 as L IVS is converted to C IVS (Inoue & Cech, 1984) and a change in the sugar cleavage of U75 (Latham & Cech, 1989) upon substrate binding to L-21 ScaI IVS. One potentially important change is the enhanced reactivity of G328 in P9, since P9 potentially provides an alternate binding site for CUCU. In general, however, the results suggest the structure of C IVS does not change drastically upon substrate binding.

Comparison to Inoue and Cech (1986). Inoue and Cech (1986) studied the chemical modification of C IVS made in situ at 30 °C. Walstrum and Uhlenbeck (1990) recently showed that the in situ form of C IVS reacts much faster than the form studied here. Thus, a comparison with the data of Inoue and Cech (1986) provides an opportunity to compare the two different forms. Figure 6C shows positions where chemical reactivity differs by at least two levels for the two forms. The only interior base-paired nucleotides that change are A70, A133, A136, A265, and A334, all of which appear in weak helixes. Inoue and Cech (1986) did not use as many probes as this study, so other differences may exist. Never-

theless, the comparison suggests the secondary structures of the two forms are similar.

Conclusions

These results have several implications for predicting RNA secondary structure from sequence. The secondary structures and stabilities of C IVS in 1 M Na^+ and in 0.05 M Na^+/10 mM Mg^{2+} are similar, indicating thermodynamic parameters measured in 1 M Na^+ are useful for prediction of secondary structures in Mg^{2+}. The results also support inclusion of favorable free energy increments for terminal GU pairs and for stacking interactions in internal and hairpin loops. Multibranch loops, however, appear more complicated. A linear approximation for multibranch loops is not adequate for large loops. Thus, algorithms with this linear approximation will have difficulty predicting structures with large multibranch loops. Finally, incorporating chemical modification data into structure prediction algorithms will be most effective when both 5′- and 3′-terminal base-paired nucleotides are allowed to be modified.

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Supplementary Material Available

Two figures showing melting curves for C IVS and L-19 IVS (2 pages). Ordering information is given on any current masthead page.

Registry No. Na, 7440-23-5; Mg, 7439-95-4.

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