

## Small RNA-divalent domains

JERZY CIESIOLKA and MICHAEL YARUS

Department of MCD Biology, University of Colorado, Boulder, Colorado 80309-0347, USA

### ABSTRACT

From a potentially completely sampled set of randomized 23-mer sequences, we selected RNAs that bind a Zn-column and also show  $K_D \approx 100\text{--}400 \mu\text{M}$  for free  $\text{Zn}^{2+}$ , probably relying on one or two direct ion coordinations. Comparison of selected sequences with previously known divalent sites suggests three or four small RNA motifs repeatedly found to interact with divalent ions. We suggest that the GC cluster, the augmented GC cluster, and the E element may be useful generalized ion-binding structures. Such structures may help identify similar divalent sites in sequenced RNAs and serve as substructures for design of functional RNA metallodomains.

**Keywords:** affinity chromatography; E element; GC cluster; RNA structure; selection; zinc;  $\text{Zn}^{2+}$

### INTRODUCTION

Divalent metal ions are required for virtually all cellular processes, and substantial deviation from homeostatic concentrations can lead to defective function. Great progress has been made in the past decade in understanding the role of protein-bound divalents (Karlin, 1993; Berg & Shi, 1996). In particular, definition of specific structural motifs (e.g., Zn fingers) now suggests hypotheses about the presence and role of bound ions in metalloproteins, even when only a primary peptide structure is known.

RNA's polyanionic character will support numerous, pleiomorphic interactions with ions. However, although divalents should be ubiquitous in the ion atmosphere around RNA structures, motifs that localize such ions are only partially characterized. Localized metal ions were observed in crystalline yeast tRNA<sup>Phe</sup> in the late 1970s (Teeter et al., 1980). However, the next 15 years did not bring any fundamentally new information on the general principles that govern the folds in specific divalent metal ion sites. Then, in the early 1980s, catalytic RNA molecules (ribozymes) were discovered that require divalents for their function. Such site-bound divalents not only stabilize the active structure of ribozymes, but also participate directly in catalysis (Pan et al., 1993; Pyle, 1993; Yarus, 1993). Thus, new RNA-divalent domains may yield new RNA structures and functions (Yarus, 1993).

Recent information on specific RNA-divalent metal ion interactions has come from several sources: in a crystal structure of the hammerhead domain, four  $\text{Mg}^{2+}$  (Scott et al., 1995) or one  $\text{Mn}^{2+}$  (Pley et al., 1994) ion were localized. In model oligonucleotides derived from the aminoacyl stem of *Escherichia coli* tRNA<sup>Ala</sup> (Limmer et al., 1993) and P1 element from the group I self-splicing intron from the large ribosomal RNA of the yeast mitochondria (Allain & Varani, 1995), NMR band shapes suggest localization of  $\text{Mn}^{2+}$ . Using  $\text{Pb}^{2+}$ -catalyzed self-cleavage, a  $\text{Pb}^{2+}$  site was isolated by selection-amplification on partially randomized tRNA<sup>Phe</sup> (Pan & Uhlenbeck, 1992).

We have used affinity selection-amplification previously to select RNA molecules that bind  $\text{Zn}^{2+}$  ( $K_D \approx 1 \text{ mM}$ ; Ciesiolka et al., 1995), and found sites possibly related to the naturally occurring E-loop motif in other RNA molecules (Wimberly, 1994).

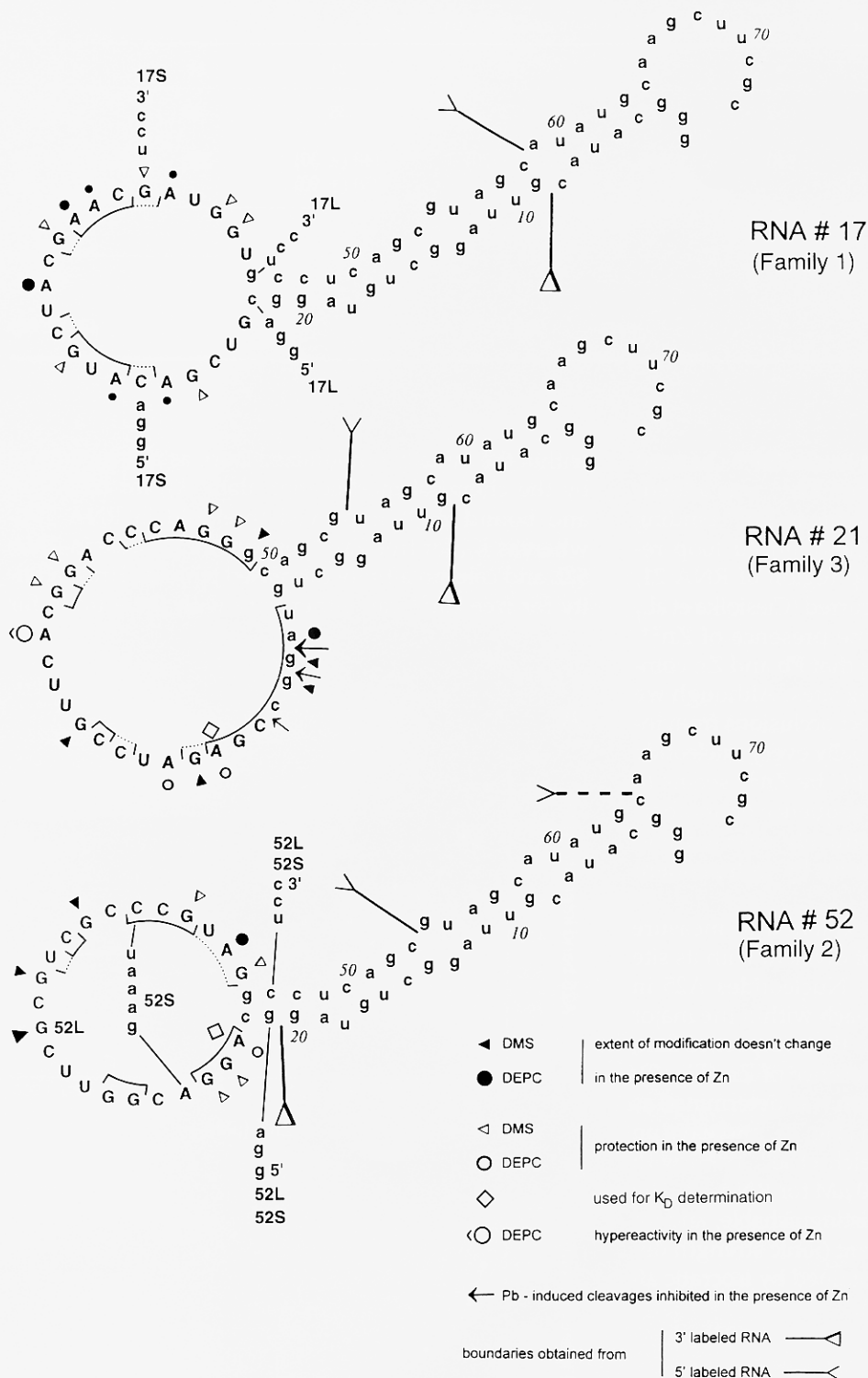
### RESULTS

#### Zinc affinity selection

For this survey, we used an RNA pool consisting of a 23-mer random region and two flanking constant regions (Fig. 1) derived from the prior selection (Ciesiolka et al., 1995); the latter form a 19-base pair stem including two mismatches and a bulged nucleotide (Fig. 2). An RNA sample was prepared by in vitro transcription of  $1.5 \times 10^{14}$  independent synthetic DNA templates, and likely contained every possible sequence of the 23-nt random region (Ciesiolka et al., 1996). The sam-

Reprint requests to: Michael Yarus, Department of MCD Biology, University of Colorado, Boulder, Colorado 80309-0347, USA; e-mail: yarus@beagle.colorado.edu.





**FIGURE 2.** Structural analysis of the selected RNA 17, 21, and 52. Results of chemical probing with diethylpyrocarbonate (DEPC), dimethylsulphate (DMS), and specific hydrolysis with Pb ions are summarized. Results of the minimal sequence requirement experiment are also shown, as are the positions of completely conserved (solid bar) and highly conserved (dotted bar) nucleotides. Lower and upper case letters indicate constant and initially randomized positions, respectively. The sequences of truncated molecules are indicated by peripheral sequences that show nucleotides added to comprise the truncate.

characterized RNAs 17, 21, and 52 to exemplify the three major families (which comprise  $30/42 = 71\%$  of the pool; Fig. 1).

The initial stem-loop sequence organization remains the thermodynamically preferred structure for all the three RNAs (Zuker, 1989) and the results of the minimal sequence experiments (Fig. 2) confirm that loop

regions are required for binding to the column and subsequent elution with free  $Zn^{2+}$ . Because  $N^7$  positions of guanine and adenine residues are preferred sites of  $Zn^{2+}$  coordination in tRNA (Rubin et al., 1983; Saenger, 1984), we probed the chemical reactivity of these positions with diethylpyrocarbonate (DEPC) and dimethylsulphate (DMS) as a function of  $Zn^{2+}$  concentration

**TABLE 1.** Dissociation constants for binding of  $Zn^{2+}$  to the selected RNAs and RNA truncates.

RNA #	9	11	17	21	24	52
$K_C$ ( $\mu M$ ) for Zn-IDA-agarose column	52	29	73	38	45	33
$K_D$ ( $\mu M$ ) for free $Zn^{2+}$ determined by:						
Isocratic elution	540	1,590	280 ( $\pm$ 70)	140 ( $\pm$ 30)	320	410 ( $\pm$ 80)
Chemical modification				250 ( $\pm$ 90)		110 ( $\pm$ 30)
RNA # (truncates)			17L			52L
$K_C$ ( $\mu M$ ) for Zn-IDA-agarose column			32			300
$K_D$ ( $\mu M$ ) for free $Zn^{2+}$ determined by:						
Isocratic elution			90 ( $\pm$ 10)			420 ( $\pm$ 60)

(Krol & Carbon, 1989). Only loop nucleotides changed susceptibility to modification when  $Zn^{2+}$  was added, indicating that structural changes due to  $Zn^{2+}$  binding are confined to the previously randomized loop regions. Moreover, within the loop region of RNA 21, strong lead-induced cleavages occurred (Ciesiolka et al., 1995) that were suppressed in the presence of  $Zn^{2+}$ , the simplest interpretation being that Pb and Zn compete for overlapping loop-binding sites. Although we cannot rigorously distinguish direct occlusions from indirect conformational effects, virtually every nucleotide protected from Pb, DMS, or DEPC by  $Zn^{2+}$  seems to be within one type of conserved sequence or another, that is, within a sequence repetitively selected and therefore implicated in zinc affinity by independent evidence (below).

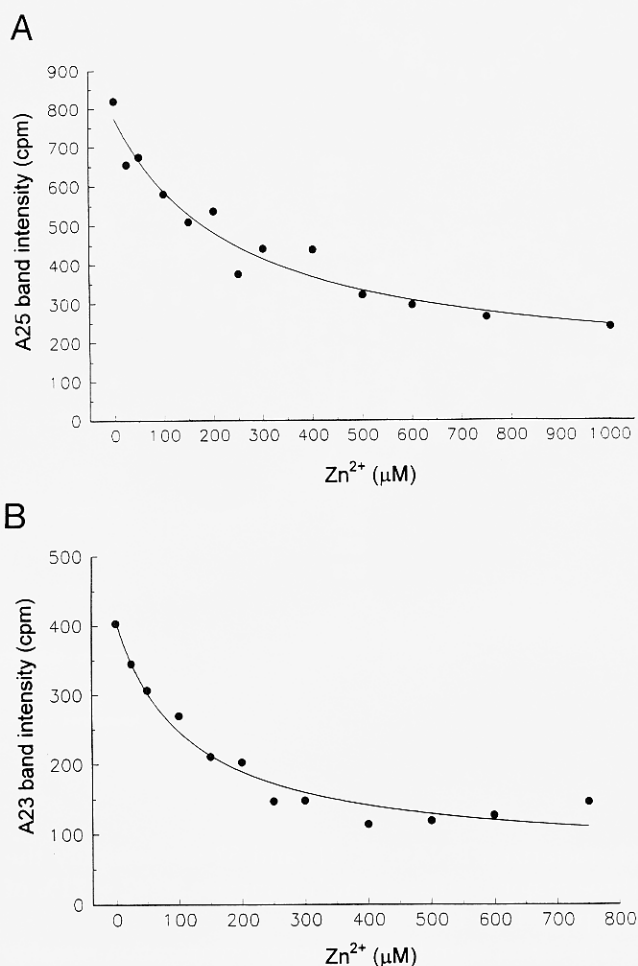
$Zn^{2+}$ -induced changes were exploited to measure the apparent  $K_D$  for association of divalent with its site in RNAs 21 and 52 in solution. Nucleotides A25 in RNA 21 and A23 in RNA 52 are each within the major block of conserved nucleotides for their respective families, and are protected in the presence of Zn (Fig. 2). Protection from DEPC modification was determined as a function of  $Zn^{2+}$  concentration (Fig. 3), and gave an apparent binding isotherm (Welch et al., 1995) with  $K_D = 250 \mu M$  for RNA 21 and  $110 \mu M$  for RNA 52. The value obtained for RNA 21 is indistinguishable, and for RNA 52 is in reasonable agreement (ionic strength differed) with independent  $K_D$ 's obtained by isocratic affinity chromatography (Table 1).

### Truncation of active molecules

To more closely delimit minimal Zn-binding domains, we synthesized four RNA truncates. The first two truncates, 17L and 52L, were derivatives of RNA 17 and 52 in which the entire loop regions were closed by arbitrary 4- and 5-base pair stems, respectively (Fig. 2). Thus, the original fixed stems are, in effect, deleted in 17L and 52L.

Both truncates bind free  $Zn^{2+}$  with undiminished affinity (Table 1). Therefore, the strong zinc sites reside

within the initially randomized loop, as also suggested by the chemical modification data.  $K_D$  for both free and bound  $Zn^{2+}$  improved threefold in RNA 17L, whereas  $K_D$  for free  $Zn^{2+}$  remained the same in RNA 52L and column affinity decreased tenfold. Thus, affinity for free  $Zn^{2+}$  can either vary with the affinity for



**FIGURE 3.** Protection of (A) RNA 21 A25 and (B) RNA 52 A23 from DEPC modification as a function of zinc concentration. Solid lines present least-squares fits to a binding isotherm.

the Zn<sup>2+</sup> column (17L) or somewhat independently (52L), suggesting that column affinity and affinity for Zn<sup>2+</sup> ion can be distinguished.

The 3' and 5' boundaries for minimal active molecules (Fig. 2) extend outside the active regions defined by truncation to 17L and 52L. The larger molecules within these experimentally determined boundaries, which refer to molecules truncated from one end only, were therefore probably mandated by correct folding rather than directly by Zn binding.

RNAs 17S and 52S are more severely truncated derivatives of 17L and 52L (Fig. 2). In RNA 17S, only the distal part of the loop of RNA 17 is preserved, closed with a new 4-base pair stem. In 52S, only the proximal loop of parental RNA 52 is preserved, closed off with a GNRA tetraloop introduced for the purpose (Fig. 2).

The shorter truncates 17S and 52S did not bind observably to the column. We therefore sought alternative means of quantifying zinc affinity for 17S and 52S. Transitions in absorbance on addition of Zn<sup>2+</sup> were detectable, but too small for accurate affinity measurements. However, CD spectra showed substantial, reproducible Zn-dependent transitions. The amplitude of the 275-nm CD band (RNA 17S) and the 265-nm CD band (RNA 52S) were changed by 6–7% with half-transition in both cases in the range of 200–300 μM Zn<sup>2+</sup> (data not shown). Such transitions were absent in the CD of randomized RNA, or RNAs of unique but arbitrary sequence. Thus, it seems likely that zinc causes structural transitions, and these truncates therefore retain Zn<sup>2+</sup> sites even though they no longer bind to the column. Affinity for the zinc column is therefore again present in a subset of RNAs that bind Zn<sup>2+</sup>. Thus, CD spectra suggest that the core of the divalent ion site is in the distal part of the RNA 17 loop and the proximal part of the RNA 52 loop. These locations, as is plausible, also contain most of the contiguous highly conserved nucleotides for each family (Figs. 2, 4).

In Figure 4, we have collected structural data on divalent binding sites in RNA. Below we compare sequences to identify conserved features repeatedly associated with divalent binding.

## DISCUSSION

### The GC cluster

The most frequent conserved structure we term the "GC cluster," consisting most simply of two adjacent helical GC base pairs (Fig. 5). The structure likely localizes the ion in the major groove, possibly using adjacent purine N<sup>7</sup> atoms on the G's (Rubin et al., 1983; Limmer et al., 1993; Allain & Varani, 1995). A major groove location for the ion is consistent with our observed Zn<sup>2+</sup> protections at the guanine N<sup>7</sup> atom (Fig. 2).

The GC cluster is quite flexible in structure, with either the G tract 5' of the C tract, or vice versa. The

simplest site may also be expanded by adjacent GC or RY pairs, and one of the GC pairs may be a GU (Figs. 2, 4, 5). GC clusters are found in the vicinity of localized divalents in the crystal structures of yeast tRNA<sup>Phe</sup> (Fig. 4A, site 5) and the hammerhead domain (Fig. 4B, site 1), as well as in the region of Mn-dependent changes in proton resonances in model oligonucleotides (Fig. 4D,E).

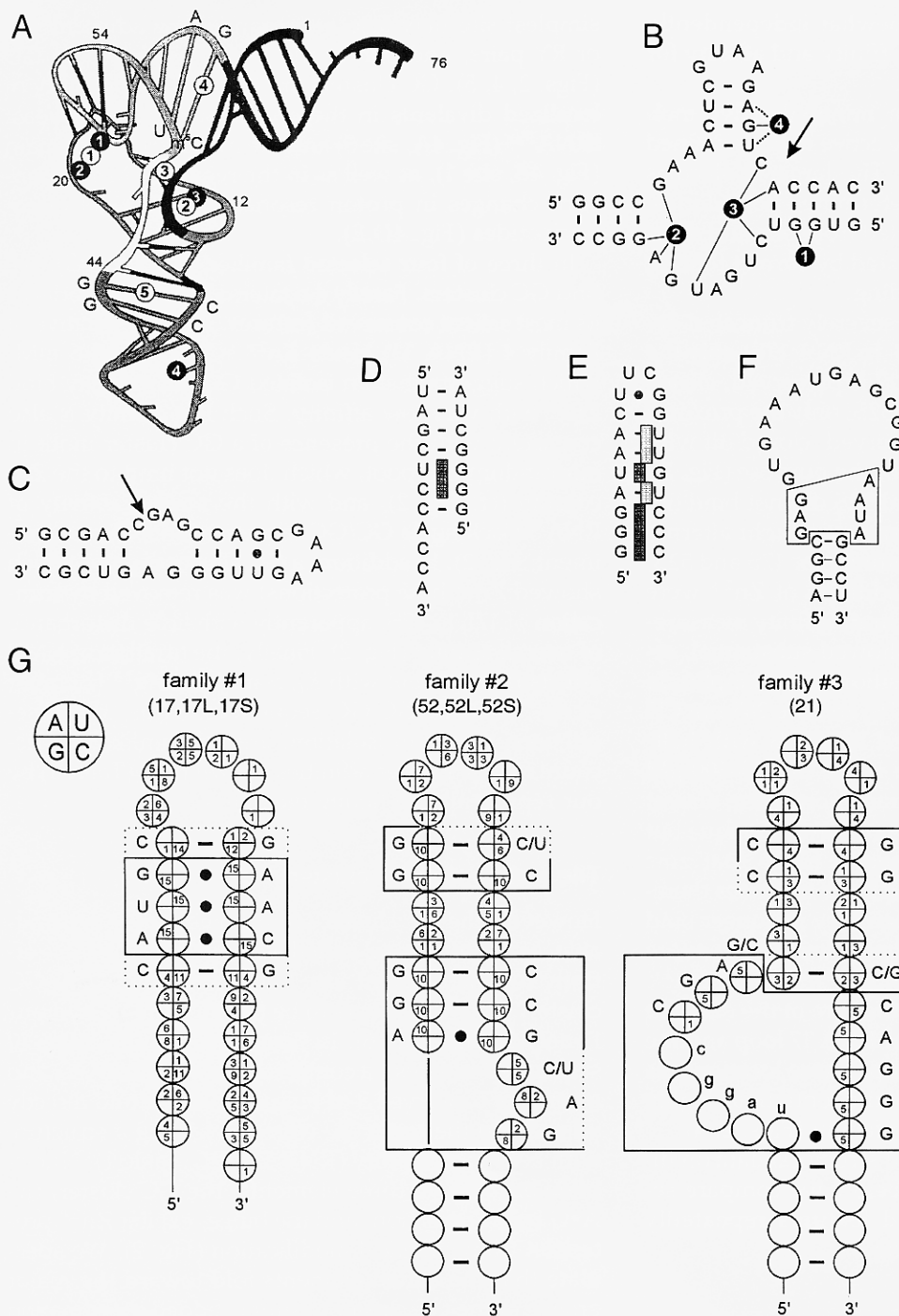
In three of four selected families (Figs. 1, 4G), we find GC clusters. Family 4 contains two such motifs as its most salient conserved features. A representative of Family 4 (RNA 11) binds Zn<sup>2+</sup> with  $K_D \approx 1.6$  mM, which suggests that an isolated GC cluster may bind Zn<sup>2+</sup> with very low affinity. Such low affinity is also consistent with the presence of a GC cluster motif in our randomized pool, which does not bind to the Zn-column (cf. Fig. 2), and with the normal activity of RNA 52 lacking its GC/GC sequence (boundary experiment, Fig. 2). In addition, a GC cluster region (which showed Zn<sup>2+</sup> base protections) was removed by truncation of 17L to 17S, apparently with little disturbance of overall affinity (Fig. 2). Finally, the titration of NMR effects is consistent with  $K_D > \text{mM}$  for Mg<sup>2+</sup> (Limmer et al., 1993) in GC/GC sequences. Thus, these sequences, acting in isolation, are very unlikely to account for the greater affinities shown here (Table 1) by our representative family members.

### The augmented GC cluster

However, the GC cluster seems to be readily expandable, accommodating added elements that increase affinity for the divalent ion. Such an "augmented GC cluster" (Fig. 6) appears to be the core element in our Family 2 (Figs. 4G, 6). In Family 2 members (24% of sequenced isolates), a completely conserved A•G and a partially conserved 3-nt bulge loop are found directly adjacent to the basic GC cluster, yielding a structure with better ion affinity (RNA 52, 52L, Table 1). Nucleotide bases in all three elements, the GC cluster, the A•G base apposition, and the bulge, exhibit Zn<sup>2+</sup> protection from chemical modification (Fig. 2). We speculate that the added elements fold toward the major groove of the GC cluster to supply added coordination for the ion. The occurrence of multiple distinct motifs in Family 2 (a GC cluster plus an augmented GC cluster) is of interest in light of truncates of this family (52S), which retain only the augmented GC cluster and no longer observably bind the column, but apparently bind free Zn ion well.

### The E element

The most frequent larger conserved motif (Family 1, 36% of sequenced isolates) contains two completely conserved triads (AUG and AAC) that we suggest be apposed to form a pattern apparently related to the



**FIGURE 4.** Small RNA structural motifs associated with divalent metal binding sites. Structures of (A) yeast tRNA<sup>Phe</sup> with Mg<sup>2+</sup> sites (closed circles) and Zn<sup>2+</sup> sites (open circles); (B) hammerhead domain with Mn<sup>2+</sup> and Mg<sup>2+</sup> sites (closed circles); (C) leadzyme with point of cleavage indicated with an arrow; model oligonucleotides derived from (D) the aminoacyl stem of *E. coli* tRNA<sup>Ala</sup> with locality of the divalent indicated with a shaded bar; (E) P1 element from the group I self-splicing intron from the large ribosomal RNA of the yeast mitochondria with darker shaded bars indicating stronger ion effects; (F) previously selected Zn binding RNA with conserved sequence boxed; and (G) phylogenetic representation of three Zn-binding RNA families from the present work. In G, nucleotide composition at each position is shown (key at upper left), with empty circles representing constant nucleotides. Peripheral boxes indicate complete conservation (solid line) or incomplete but strong conservation (dotted line; as in Figs. 1, 2).

most conserved part of the E-loop motif (Wimberly, 1994), as well as to the structure we selected earlier (Ciesiolka et al., 1995; Fig. 4F). Both the triad and highly conserved flanking G:C or C:G pairs (not shown below) show Zn<sup>2+</sup> base protections (Fig. 2).

In the new motif (Figs. 4G, 6), however, a GA base pair is reversed and there is no bulged nucleotide (itself nonconserved in the consensus E-loop motif). Thus, we suggest that, subject to confirmation by structural studies, the ribosomal E-loop motif may be only the

first-discovered member of a larger family of sites that share only a subset of nucleotide elements, but may use overlapping means to bind ions. We propose that these be called E elements, in recognition of the first member of the family (Wimberly, 1995).

### A second augmented GC cluster?

The next most abundant structure we selected is Family 3 (12% of sequenced isolates), which has a unique

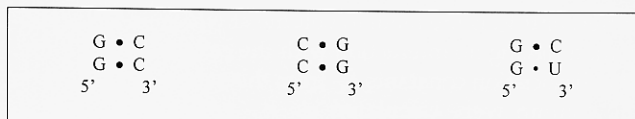


FIGURE 5. Examples of GC clusters.

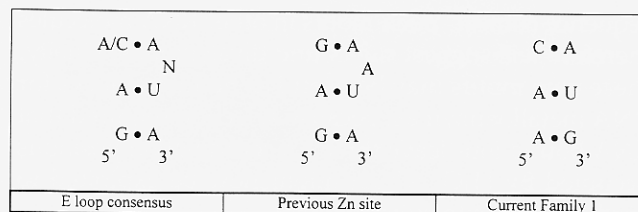


FIGURE 7. E elements.

composition, with a minimal GC cluster near an asymmetric internal loop rich in purines (8 of 11 nucleotides). This may be a second kind of augmented GC cluster, though interpretation is less obvious because of the distance between the two elements (Fig. 4G). The cluster as well as both sides of the loop show base protections in the presence of  $Zn^{2+}$  (Fig. 2). In one case, however, the loop was selected without the GC cluster (Seq. 33, Fig. 1), suggesting that it may autonomously satisfy the selection. However, the loop may perhaps serve either as an autonomous site or as an augmentation for a nearby GC cluster, depending on minor sequence changes.

### Other previously identified sites

To the selected elements above, any survey should add several structures previously known in multiple RNAs. Apposed GAG/AG is found in the crystal structures of two hammerhead ribozymes (Pley et al., 1994; Scott et al., 1995), binding an Mg or Mn ion (Fig. 4B, site 2). This motif may conceivably occur in the internal loop of the leadzyme (Fig. 4C).

The U-turn at sequence CUGA is known as an ion site from tRNA crystallography (Fig. 4A, site 4) as well as in the hammerhead structure (Fig. 4B, site 3).

A helical GU base pair was proposed as an RNA element sufficient to define a  $Mn^{2+}$  binding site responsible for NMR line broadening (Allain & Varani, 1995; Fig. 4E).

Finally, there are also ion sites unrecognizable at this level of analysis of the primary or secondary structure, which result from the proximity of widely separated nucleotides, that is, from tertiary structures (e.g., Fig. 4A, site 1). Such sites appear to be frequent in large RNAs with highly evolved tertiary structures, such as the *Tetrahymena* intron (Christian & Yarus, 1993).

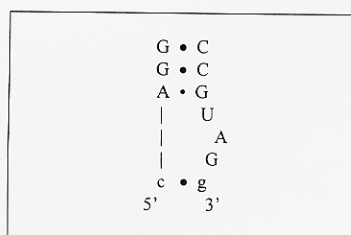


FIGURE 6. Augmented GC cluster (RNA 52).

### Zn and conformation

Zinc binding frequently may be associated with conformational change, and/or unusual RNA conformations. Secondary structures shown in Figure 4G are not strongly thermodynamically preferred. Compensatory base pair changes generally support pairings shown within selected motifs (Fig. 1), although mismatches (e.g., AC), as well as deletions or bulged nucleotides (e.g., Family 1), can replace many pairs. One result is that more than one different base pairing scheme is usually possible within 2 kcal/mol (Zucker, 1989) of the most stable conformer. Binding of a Zn ligand with  $K_D$  in the range 0.1–0.4 mM must contribute more than the net  $\Delta G^\circ$ ,  $-5.5$  to  $-4.6$  kcal/mol (from  $\Delta G^\circ = -RT \ln K$ ), to the stability of the bound conformation. Thus, it is likely that Zn ions would promote conformational change, stabilizing one of the near-isoenergetic forms of the site that is best suited to the ion. This expectation is consistent with changes in nucleotide susceptibility to modification at widely separated sites on addition of  $Zn^{2+}$  (e.g., RNAs 17 and 21, Fig. 2), and with our previously observed Zn-dependent UV cross-link (Ciesiolka et al., 1995).

### Results as a survey of sites

In view of the ease with which RNA might bind ions, perhaps the most striking finding is that a few recognizably related structures become prevalent in our selection for  $Zn^{2+}$  affinity. However, the completeness of our survey of possible sites is limited by the selection for affinity for the Zn-column, including our requirement that immobilized RNAs use only the fourth and perhaps fifth coordinations of an already-chelated Zn ion. This presumably is one factor limiting binding free energy to the range observed ( $K_D \geq 100 \mu M$  for  $Zn^{2+}$ ).

Because divalent binding sites frequently chelate several types of ions, and because this selection procedure (Ciesiolka et al., 1995) has yielded sites binding several kinds of divalents previously, it is likely that selected motifs bind divalents other than  $Zn^{2+}$ . The motifs are represented by families of 5–15 RNA molecules that differ substantially in sequence (Fig. 1). Therefore, ion binding must be somewhat independent of the RNA

sequence context. Such motifs in other RNA molecules therefore will suggest site-bound divalent ions. Finally, these new motifs may be useful as building blocks for RNA metalloenzymes, because selection required that RNA-bound ions leave three Zn coordination positions occupied by H<sub>2</sub>O, which might now be exploited to bind and activate substrates.

## MATERIALS AND METHODS

### Randomized RNA

The RNA pool with 23-mer random region was generated from a T7 promoter sequence by *in vitro* transcription (Milligan et al., 1987) of a DNA template strand of the sequence: 5'-GCG AAG CTT GCA TAT GCT ACG CTG AGG C-N<sub>23</sub>-GCC TAC AGC CTA ACG TAT GCC C << TAT AGT GAG TCG TAT TAG AGC TCG C-3', where N is equimolar nucleotides and << is the transcript start.

### Affinity selection

A mixed IDA-Sepharose-Sepharose 4B affinity column was prepared from Chelating Sepharose Fast Flow (Pharmacia) and Sepharose 4B (1 mL; capacity ca. 1.5 μmol divalent/mL gel). The column was charged with Zn ions by applying 1 mL of 20 mM ZnCl<sub>2</sub> in 0.4 M NaCl, 20 mM HEPES-Na, pH 7.0, washing out excess Zn with 10 mL of the buffer without Zn, and equilibrating the column with 5 mL of buffer A (0.4 M NaCl, 20 mM HEPES-Na, pH 7.0, 1 mM MgCl<sub>2</sub>).

Thirty micrograms of internally labeled <sup>32</sup>P RNA (7.4 × 10<sup>14</sup> molecules transcribed from 1.5 × 10<sup>14</sup> DNA templates) in 250 μL of buffer A was heated at 65 °C for 5 min, cooled to room temperature over 15 min, and loaded onto the affinity column. The column was washed with 20 volumes of buffer A and subsequently with 9 mL of the same buffer containing 2 mM ZnCl<sub>2</sub>. The Zn-eluted pool (0.15, 0.09, 0.03, 1.54, 8.5, 16, and 29% of the applied RNA was recovered in the 1–7 round of selection) was recovered by ethanol precipitation, reverse transcribed, amplified by PCR, and then transcribed into RNA for the next round of selection (Tuerk & Gold, 1990).

The column was washed with 20 column volumes of the start buffer. This requires that retained RNAs have dissociation constant  $K_C$  (for fixed ligand; Ciesiolka et al., 1996) roughly ≤ 0.05 the concentration of fixed ligand, e.g., ≤ 0.075 mM. The first nine column volumes after the start of the Zn<sup>2+</sup> elution were pooled. This condition approximately constrains the  $K_D$  (for free ligand; Ciesiolka et al., 1996) of the eluted RNA to be ≤ 0.7 the free ligand concentration, e.g., ≤ 1.5 mM.

### $K_D$ from isocratic affinity elution

The  $K_D$  for binding Zn ions to the RNA in solution was determined by isocratic elution (Dunn & Chaiken, 1974; Connell et al., 1993) from the equation:  $K_D = L\{(V_{el} - V_n)/(V_e - V_{el})\}$ , where  $L$  is the free ligand concentration used to isocratically elute RNA loaded onto the affinity column,  $V_{el}$  is the median elution volume of RNA eluted in the continuous presence of free ligand,  $V_e$  is the median elution volume measured in the absence of free ligand with the same column

buffer, and  $V_n$  is the median volume at which an RNA population having no interaction with the column would elute. The dissociation constants  $K_C$  from the small ligand fixed on the column were calculated as:  $K_C = L_C\{V_n/(V_e - V_n)\}$ , where  $L_C$  is the concentration of ligand within the column bed. Internally labeled <sup>32</sup>P RNA (ca. 3 × 10<sup>5</sup> cpm, 0.125 μM RNA in total volume of 250 μL) was heated at 65 °C for 5 min in buffer A and cooled to room temperature over 15 min. Zn ions were added to an appropriate concentration, samples incubated at room temperature for additional 10 min, and loaded onto the 1-mL Zn-column that had been equilibrated in buffer A containing the same concentration of Zn ions as in the sample.

### Minimal sequence experiments

The minimal sequence required for binding to the Zn column and subsequent elution with Zn was determined as follows: the RNA was labeled at the 3'- or 5'-end with <sup>32</sup>P, partially hydrolyzed under alkaline conditions, and precipitated with ethanol. The digestion products were resuspended in buffer A, subjected to renaturation, and loaded onto the Zn-column. The column was washed with 6 mL of buffer A (first 3 mL were collected, flow-through fraction) and subsequently with 6 mL of the same buffer, but containing 2 mM Zn<sup>2+</sup> (RNA bound fraction). RNA from the both fractions was precipitated with ethanol, resuspended in the loading buffer, and electrophoresed through a denaturing 12% polyacrylamide gel, along with alkaline and RNase T1 hydrolysates.

### DEPC and DMS reactions

For probing N<sup>7</sup> of adenine and guanine bases with DEPC and DMS, 3'-end labeled RNA (ca. 100,000 cpm, 5 pmol of RNA/200 μL reaction volume) was heated at 65 °C for 5 min in 80 mM HEPES-Na, pH 7.0, 40 mM NaCl, 1 mM MgCl<sub>2</sub>, and cooled slowly (1 °C/min) to room temperature. Zn ions were added and the samples were incubated at room temperature for 10 min. In the reaction with DMS, 10 μL of the reagent in ethanol (1:12 dilution, v/v) was used for 30 min at 25 °C. Modification with DEPC was performed using 10 μL of DEPC at 25 °C for 1 h. After modification, the RNA was ethanol precipitated and treated with sodium borohydride and aniline (Krol & Carbon, 1989). Zinc-protected nucleotides were determined with 0.5 mM and 2 mM added ion. The samples were analyzed on denaturing 12% polyacrylamide gels.

To determine zinc affinity in Figure 3, radioactive bands were excised and intensity ( $I$ ) measured by Cerenkov counting. Dissociation constants ( $K_D$ ) for Zn<sup>2+</sup> are determined from least-squares fitting to protected bands using  $I = I_{sat} + I_0/(1 + [Zn]/K_D)$ , where  $I_{sat}$  is the intensity at saturation with Zn, and  $I_0$  is the incremental intensity (over  $I_{sat}$ ) without Zn (cf. Welch et al., 1995).

### Lead hydrolysis

Lead hydrolysis was performed essentially as described in Ciesiolka et al. (1995). Briefly, <sup>32</sup>P-end labeled RNA (0.25 μM RNA concentration) was heated at 65 °C for 5 min in the following buffer: 40 mM NaCl, 20 mM HEPES-Na, pH 7.0,



1 mM MgCl<sub>2</sub>, and cooled slowly (1 °C/min) to room temperature. In experiments performed in the presence of Zn, 0.1 mM or 0.25 mM metal ions were added and the samples were incubated at room temperature for 10 min further. Subsequently, lead acetate solution to 0.25 mM or 1 mM was added and the reactions proceeded at 25 °C for 20 min. The reactions were quenched with EDTA (≥twofold excess over divalent ions) in the loading buffer and the samples were analyzed on denaturing 12% polyacrylamide gels.

### CD titration

CD spectra were measured using an Aviv model 62DS spectropolarimeter in a 0.1-cm-pathlength cuvette. Samples were prepared in buffer A at RNA concentration 300 µg/mL. Binding curves of Zn were determined by measuring the change in ellipticity at 265 or 275 nm, with 30 s averaging times at each Zn concentration.

### ACKNOWLEDGMENTS

We thank Dr. Mark Manning of the University of Colorado Health Sciences Center for the use of his CD spectrometer, and the NIH for support of this research.

*Manuscript accepted without revision May 24, 1996*

### REFERENCES

- Allain FHT, Varani G. 1995. Divalent metal ion binding to a conserved wobble pair defining the upstream site of cleavage of group I self-splicing introns. *Nucleic Acids Res* 23:341-350.
- Berg JM, Shi Y. 1996. The galvanization of biology: A growing appreciation for the roles of zinc. *Science* 271:1081-1085.
- Christian EL, Yarus M. 1993. Metal coordination sites that contribute to structure and catalysis in the group I intron from *Tetrahymena*. *Biochemistry* 32:4475-4480.
- Ciesiolka J, Gorski J, Yarus M. 1995. Selection of an RNA domain that binds Zn<sup>2+</sup>. *RNA* 1:538-550.
- Ciesiolka J, Illangasekare M, Majerfeld I, Nickles T, Welch M, Yarus M, Zinnen S. 1996. Affinity selection-amplification from randomized oligonucleotide pools. *Methods Enzymol* 267:315-335.
- Connell GJ, Illangasekare M, Yarus M. 1993. Three small ribooligonucleotides with specific arginine sites. *Biochemistry* 32:5497-5502.
- Dunn M, Chaiken IM. 1974. Quantitative affinity chromatography. Determination of binding constants by elution with competitive inhibitors. *Proc Natl Acad Sci USA* 71:2382-2385.
- Karlin KD. 1993. Metalloenzymes, structural motifs, and inorganic models. *Science* 261:701-708.
- Krol A, Carbon P. 1989. A guide for probing native small nuclear RNA and ribonucleoprotein structures. *Methods Enzymol* 180:212-227.
- Limmer S, Hofmann HP, Ott G, Sprinzl M. 1993. The 3'-terminal end of tRNA determines the structure and stability of the aminoacyl acceptor stem. *Proc Natl Acad Sci USA* 90:6199-6202.
- Milligan J, Groebe DR, Witherell GW, Uhlenbeck OC. 1987. Oligoribonucleotide synthesis using T7 RNA polymerase and synthetic DNA templates. *Nucleic Acids Res* 15:8783-8798.
- Pan T, Long DM, Uhlenbeck OC. 1993. Divalent metal ions in RNA folding and catalysis. In: Gesteland RF, Atkins JF, eds. *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 271-302.
- Pan T, Uhlenbeck OC. 1992. A small metalloribozyme with a two-step mechanism. *Nature* 358:560-563.
- Pley HW, Flaherty KM, McKay DB. 1994. Three-dimensional structure of a hammerhead ribozyme. *Nature* 372:68-74.
- Pyle AM. 1993. Ribozymes: A distinct class of metalloenzymes. *Science* 261:709-714.
- Rubin JR, Wang J, Sundaralingam M. 1983. X-ray diffraction study of the zinc binding sites in yeast phenylalanine tRNA. Preferential binding of zinc to guanines in purine-purine sequences. *Biochim Biophys Acta* 756:111-118.
- Saenger W. 1984. *Principles of nucleic acid structure*. New York/Berlin/Heidelberg/Tokyo: Springer-Verlag.
- Scott WG, Finch JT, Klug A. 1995. The crystal structure of an all-RNA hammerhead ribozyme: A proposed mechanism for RNA catalytic cleavage. *Cell* 81:991-1002.
- Teeter MM, Quigley GJ, Rich A. 1980. In: Spiro T, ed. *Nucleic acid-metal ion interactions*. New York: John Wiley & Sons. pp 146-177.
- Tuerk C, Gold L. 1990. Systematic evolution of ligands by exponential enrichment: RNA ligands to bacteriophage T4 DNA polymerase. *Science* 249:505-510.
- Welch M, Chastang J, Yarus M. 1995. An inhibitor of ribosomal peptidyl transferase using transition state analogy. *Biochemistry* 34:385-390.
- Wimberly B. 1994. A common RNA loop motif as a docking module and its function in the hammerhead ribozyme. *Nature Struct Biol* 1:820-827.
- Yarus M. 1993. How many catalytic RNAs? Ions and the Cheshire Cat conjecture. *FASEB J* 7:31-39.
- Zuker M. 1989. On finding all suboptimal foldings of an RNA molecule. *Science* 244:48-52.