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A Novel Acidophilic RNA Motif That Recognizes Coenzyme A†

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ABSTRACT: Specific recognition of nucleotide cofactors by RNA may be important in engineering new RNA enzymes (ribozymes). Although in vitro selections (SELEX) have identified nucleic acid motifs ("aptamers") that bind a variety of adenosine cofactors, none of these recognizes coenzyme A (CoA), the primary biological cofactor used in acyltransfer reactions. We used SELEX experiments with two random RNA pools to identify aptamers that bind CoA. Functional boundary determination and extensive comparative sequence analysis (including reselection of a mutagenized, circularly permuted RNA) led to the identification of a 52 nucleotide minimal aptamer ("min52"). The RNA structural motif contains a large internal loop with 26 unpaired nucleotides flanked by helices of any base-paired sequence. Twenty loop nucleotides are specifically required for binding activity, 12 of which are derived from the original primer binding sequences. Specificity studies with CoA analogues demonstrated that the aptamer recognizes many adenosine analogues, including ATP, and that recognition is predominantly through the Höogsteen face of adenine. Binding activity is greatest at acidic pH (optimum near 5.0), in low or no monovalent salt, and at high concentrations of either Mg^{2+} or Mn^{2+}. Strong binding activity (86% of maximum) is observed at pH 4.0, suggesting that at least some extreme conditions (acidic pH) may be compatible with RNA World theories of the origin and early evolution of life. In the presence of 10 mM Mg^{2+}, binding is unaffected by the addition of 1 mM Ca^{2+}, but it is mildly inhibited by 1 mM Zn^{2+} or Co^{2+} or by 0.1 mM Cu^{2+} or Ni^{2+}. The dissociation constant (K_d) for the association of min52 RNA with ATP in solution was measured to be 2.4 ± 0.4 μM under the conditions of the selection and 0.5 ± 0.1 μM under optimized conditions. Finally, we show that the selected CoA aptamer populations contain other RNAs at low frequencies that preferentially recognize intact CoA and are not eluted from the resin by AMP alone.

Nucleotide cofactors play a central role in much of modern metabolism, with adenosine cofactors being the most prominent. They are involved in phosphoryl (ATP), acyl (CoA),[1] and methyl (SAM) group transfers and in redox (FAD, NAD^+, $NADP^+$) and radical-mediated (SAM, CoB_{12}) reactions. The nucleotide moieties, which do not usually participate in the chemical steps of these reactions, often bind to protein enzymes through relatively generic structural motifs (e.g., the Rossman fold for redox cofactors). Many labs have engaged in the search for nucleic acid molecules (aptamers) that can accomplish similar binding functions by recognizing nucleotide cofactors and other potentially reactive molecules. Such aptamers are ideal for studies of RNA structure and host−guest recognition, for the subsequent evolution of RNA catalysts, and for developing models of biochemical evolution during the RNA World, the hypothetical evolutionary predecesor to modern, protein-mediated metabolisms in which RNA enzymes (ribozymes) catalyzed most or all of the reactions required for keeping cells alive *(1−5)*.

The connection between substrate binding and RNA catalysis has been demonstrated repeatedly in catalytic RNAs. The guanosine requirement for self-splicing group I introns can be changed into a requirement for 2-aminopurine by alterations of the G-binding site *(6)*, and a Diels−Alderase ribozyme shows exquisite substrate specificity for both the diene and dienophile substrates *(7)*. Similarly, the self-kinasing ribozymes of Lorsch and Szostak *(8)* show a 650-fold preference for ATP-γ-S over GTP-γ-S *(9)*. Competitive inhibition experiments have also demonstrated the presence of saturatable substrate binding sites in a self-aminoacylating ribozyme that uses Phe-AMP as a substrate *(10, 11)* and in an RNA amide synthase that uses biotin-AMP as a substrate *(12)*. The rapidity with which new ribozymes are being discovered suggests that a broad range of catalytic activities may exist. Including substrate binding pockets within the RNA's under selection offers a clear advantage in the search for new catalysts.

Two classes of aptamers have been shown to recognize the adenosine moieties of nucleotide cofactors. Huizenga and Szostak described a DNA element that recognizes the adenosine portion of ATP *(13)*, while three separate labs have described essentially the same RNA element that recognizes the adenosine portion of ATP *(14)*, NAD^+ *(15)*, or SAM *(16)*.

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[1] Abbreviations: SELEX, Selective Evolution of Ligands by EXponential enrichment; CoA, coenzyme A; K_d, dissociation constant; PBS, primer-binding sequence.

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The structure of this latter element has been determined independently in two labs *(17, 18)*, and its interactions with AMP were recently refined through combined mutagenesis and NMR analysis *(19)*. The bound adenosine forms the terminal A of a GNRA motif (bound AMP underlined). Other aptamer nucleotides form specific contacts with the exocyclic amine or with the ribose, while the substituent at the 5′ position is exposed to solution. Self-kinasing RNAs have been isolated from RNA pools containing variants of this motif, suggesting that it can bind ATP in a manner compatible with reactivity at the 5′ γ-phosphate. RNA aptamers that recognize other reactive moieties have also been identified, including cyanocobalamin (vitamin B_{12}) *(8)*, FAD *(15)*, various amino acids *(20−24)*, and antibiotics *(25−28)*.

Acyl-CoA is involved in primary metabolic reactions in modern biochemistry, including the citric acid cycle, fatty acid synthesis, and pyruvate metabolism. Enzymes for each of these processes must have been among the first to evolve as metabolic pathways were being established. Furthermore, acyl-CoA-dependent acyltransfers are found in a wide variety of "housekeeping" and specialized biochemistries, such as neurotransmitter synthesis (acetylcholine and serotonin), antibiotic inactivation (the CAT reaction), bacterial cell wall synthesis (NAG-NAM), and gene regulation (histone acetylation). Ribozyme catalysis of the essential chemistry at the catalytic step of acyltransfers has been demonstrated in *amino*acyltransferase and amide synthase ribozymes isolated in three labs *(12, 29, 30)*, strongly suggesting that CoA-dependent acyltransferase activity is within the RNA's catalytic potential. Finally, the possibility that CoA-dependent acyltransferase activity originated within an RNA World, as proposed by the evolutionary models of White *(31)* and Benner *(2, 32)*, adds further impetus to a search for CoA aptamers and CoA-dependent ribozymes.

We have used in vitro selections [the SELEX protocol *(33−35)*] to isolate several populations of aptamers that recognize CoA. We describe their isolation and the initial characterization of the structure and activities of the dominant sequence from one of these populations. This RNA has a high affinity for the adenosine portion of CoA, yet it is structurally and functionally distinct from previously isolated aptamers to adenosine *(14−16)*. Finally, its tolerence of extreme conditions has implications for the robustness of RNA World theory.

MATERIALS AND METHODS

Selection of CoA-Binding Aptamers. Transcripts of approximately 134 nucleotides (80CoA series) or 118 nucleotides (70CoA series) were generated by in vitro transcription from synthetic templates or PCR products using reaction conditions and templates described previously *(36)*. One nanomole of gel-purified RNA was subjected to the first cycle of selection (2−5 pool equiv), and 125 pmol of RNA was used in subsequent cycles. Transcripts were incubated with a 4% beaded agarose affinity matrix derivatized with CoA through an amide linkage at the exocyclic amine of the base (approximately 4.3 mM total CoA, Sigma). Conditions for column binding and rigorous elutions were as described previously *(28)*, except that binding buffer was 10 mM $MgCl_2$, 200 mM NaCl, 50 mM Bis-Tris, pH 6.4, throughout. Specifically bound RNAs were eluted with 7.5 mM free CoA in binding buffer. All affinity resins were purchased from Sigma.

Sequence Analysis. Specifically eluted RNA was converted to double-stranded DNA and cloned into pUC18 for sequencing using the Sanger dideoxy method and Sequenase (bacteriophage T4 DNA polymerase, U. S. Biochemicals) essentially as described by the supplier. Sequences were aligned and covariations identified manually unless otherwise noted.

Boundary Determinations. Transcripts were labeled at the 5′ end with [γ-^{32}P]ATP using polynucleotide kinase, or at the 3′ end with [5′-^{32}P]pCp using RNA ligase. Partially hydrolyzed, end-labeled RNA was incubated with resin, and then layered onto a bed of preformed resin. RNA from the wash fractions or specifically eluted with free CoA was precipitated and analyzed by gel electrophoresis on 12% denaturing polyacrylamide gels.

Secondary Selections. New fixed sequences were appended to the transcription template for circularly permuted aptamer "cp2" by PCR to generate template for "cp4" RNA, which has the sequence (top strand) 5′ CGGCTTAAGCT-TAATACGACTCACTATA gggttaagttagatcgaat-TCCACGAGCGAAGGGCATAAGGTATTTAATTCC-ATAAAAAGGGAATCATACACAAGACATGGTGGA-atacagtcagtcgaacccatactaatc 3′. The promoter for bacteriophage T7 RNA polymerase is doubly underlined; primers corresponding to underlined sequences were used in pool amplification; sequences shown in lower case letters correspond to primer binding sites in the RNA. Template for the mutagenized pool derived from cp4 RNA was resynthesized as bottom strand by Genemed, Inc. (South San Francisco), with 15% mutagenesis per position throughout the region shown in upper case. Transcripts from this mutated pool were subjected to additional cycles of selection as above. For the subtractive selection, 0.1 or 0.3 mM 3′AMP was included in the wash fractions prior to elution with 5 mM CoA.

Surveys of Specificity and Binding Conditions. For the survey of elution by CoA analogues, column-bound RNA was washed with 8 bed volumes of binding buffer, and then eluted with 4 bed volumes of buffer containing 0.5 mM analogue followed by 4 bed volumes of buffer containing 4 mM CoA. In the surveys of binding conditions, RNAs were folded in their respective buffers in 50 μL and layered onto columns containing a CoA resin bed volume of 125−200 μL that had been preequilibrated with the appropriate buffer. The proportion that was specifically eluted was not dependent upon resin bed volume over this range. After washing the resin with 10 bed volumes of the corresponding buffer, RNA was eluted with 6 bed volumes of normal 1× binding buffer containing 5 mM 5′ AMP. Acetate buffer was used for pH 4.0−5.0, Bis-Tris buffer for pH 5.8−7.0, and Tris buffer for pH 7.5 and 8.0.

K_d Determination. Unlabeled RNA was folded as above and incubated 5−10 min at room temperature with [α-^{32}P]-ATP (30 Ci/mmol) at a final concentration of 0.1 μM. The 300 μL binding reaction was split 5−6 ways: 3 aliquots of 90 μL each were filtered through Microcon 10 molecular weight cutoff filters (Amicon), and 2−3 aliquots of 10 μL each were counted without filtering to establish the specific activity of the label and to ensure that equal amounts of label

were used in each reaction. After spinning the filters for 100−110 s, approximately one-third of the binding reaction mixture had passed through the filter (25 ± 5 μL). The amount of radiolabel in 10 μL aliquots from above and below the membrane was determined by Cherenkov counting. The difference in counts between the two fractions was used to calculate dissociation constants (K_ds) graphically assuming a 1:1 stoichiometry. A theoretical binding curve was fit to a plot of the difference in counts between the top and bottom fractions assuming excess RNA using the expression:

$$F = \frac{[\mathrm{RNA}]}{([\mathrm{RNA}] + K_d)} M - \mathrm{Bk}$$

where F is the fraction of [α-^{32}P]ATP retained above the membrane, M is the maximal binding extrapolating to infinite [RNA], and Bk is the background signal observed in the absence of RNA. Bk was measured experimentally in both buffers used (see arrows in Figure 8), while M and K_d were optimized by the "curve fit" function of Kaleidagraph (Abelbeck Software) to give the best fit to the data. For most measurements, [α-^{32}P]ATP concentration was held constant at 5 nM. 100 nM labeled ATP was used for the two highest concentrations of min52 RNA, for all of the tRNA data, and in the competitions with 3 or 30 μM cold ATP.

RESULTS

The "ATP Aptamer" Motif Does Not Recognize CoA. We reported previously the isolation of RNA aptamers to *S*-adenosylmethionine (SAM) from an RNA pool with 80 random positions *(16)*. The motif that we identified was identical to that isolated previously in a search for NAD^+-binding RNAs *(15)*, and earlier still in a search for ATP-binding RNAs *(14)*. It is often referred to as "the ATP aptamer," even though this RNA element recognizes many adenosine cofactors that differ by the substituent at the 5′ position, and as we show below, other RNA elements also bind ATP with high affinity.

Coenzyme A (CoA) is unique among the adenosine cofactors because it contains not only the phosphopantothein chain at the 5′ position, but also a 3′-phosphate (Figure 1A). To determine whether the RNA motif in the "ATP aptamer" also recognizes CoA, radiolabeled RNA from SAM-binding isolate "S7" *(16)* was loaded onto a CoA affinity resin. None of the RNA bound this resin (not shown), but this could have been due to the nature of the linkage to the resin rather than an inability to bind CoA. The CoA was attached to the resin matrix through the exocyclic amine, and modifications to N6 disrupt binding by this aptamer. To address the question of CoA recognition directly, the same RNA was loaded onto the AMP affinity resin used during the SAM selection. RNA that remained bound to the resin after extensive washing was challenged with binding buffer containing 1 mM CoA. This treatment removed only a tiny fraction of the RNA. In contrast, subsequent elution with binding buffer containing 1 mM 5′AMP removed essentially all of the remaining RNA (Figure 1B). Therefore, this aptamer does not recognize CoA, probably because of the 3′-phosphate. These results are consistent with the inability of similar aptamers to recognize 3′-*O*-methyladenosine *(14)* and with published

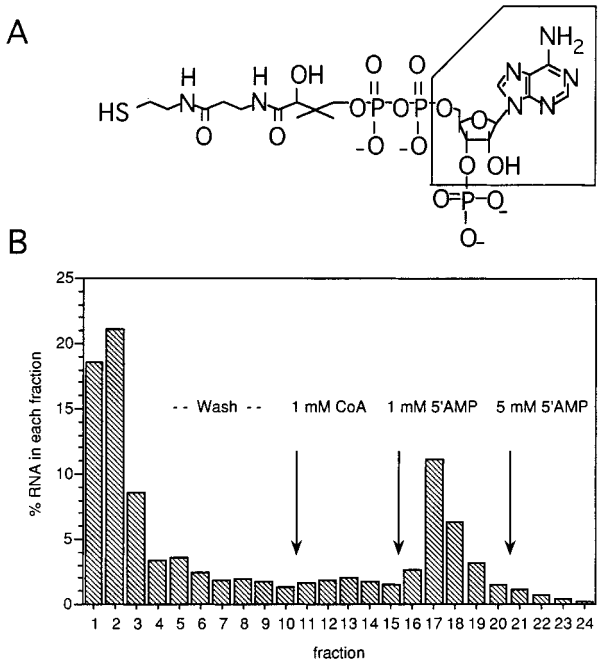

FIGURE 1: The "ATP aptamer" does not recognize CoA. (A) Structure of CoA, with the adenosine core boxed. (B) Elution profile of an "ATP aptamer," S7 RNA, originally isolated to bind *S*-adenosylmethionine *(16)*. Wash fractions (1−10) and the beginnings of each set of elution fractions (11−24) are indicated.

Table 1: Summary of SELEX Progressions[a]

				AMP counterSELEX			
cycle	70CoA	80CoA	cp4	70/100	70/300	80/100	80/300
1	1.55	1.91	0.11				
2	0.53	0.75	0.54				
3	0.96	0.12	14.0				
4	0.11	0.24	18.2				
5	0.30	0.23	21.9				
6	6.42	0.22	21.3	0.6	0.53		
7	7.29	2.46		0.52	1.03	0.34	0.20
8	27.3	37.9		0.59	1.14	0.94	0.52
9				3.32	1.42	1.67	0.33
10				6.81	1.69	2.45	1.12
11				13.34	2.55	5.46	1.41
12				8.64		9.92	6.23
13				10.14		12.32	

[a] Numbers indicate percentages of input RNA specifically eluted from CoA resin during the original selection (70CoA and 80CoA), during reselection of the mutagenized pool (cp4 reSELEX), and during counterselections of the 70CoA and 80CoA (numerator) populations with 100 or 300 μM 3′AMP (denominator).

NMR structures of the "ATP aptamer" (*17*, *18*, and Brookhaven database entry 1RAW), which show that the aptamer-binding pocket cannot accommodate the 3′ phosphate.

CoA-Binding RNA Populations. *In vitro* selections were used to isolate RNA aptamers to CoA from two independent RNA populations. Very little RNA eluted specifically during the first five to six cycles. By round 8, approximately one-third of the input RNA was specifically eluted by CoA in both populations (Table 1), and this material was cloned for sequencing. The eighth round 70CoA and 80CoA populations gave very different sequence patterns (Figure 2). Among the 48 70CoA isolates that were sequenced, 1 sequence was found in 7 isolates and 1 in 3 isolates, 7 were found in pairs, and 24 were found once only. All but two

A

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5'  gggaaaagcgaatcatacacaaga ... (70N) ... gggcataaggtatttaattccata  3'
                                                                                relative binding
(6) 70CoA#5     CATG GCAAATGGTGAAAGTGTGAGTAAG 11 nt CTT^CTTGCACAGAACATCAGCTGC GAGCGAA    +++
  ≈70CoA#46     CATG GCAAATGGTGAAAGTGCGAGTAAG 11 nt CTT^CTTGCACAGAACATCAGCTGC GAGCGAA
      "5t83"    CATG GCAAATGGTG               GGAA                 CATCAGCTGC GAGCGAA    ++
      "5t86"    CATG GTGGCAAATGGTG            GGAA              CATCA^^^GCTGC GAGCGAA    ++
(2) 70CoA#22    CATG GTACCG                   47 nt                   CGGTAC GCGCGAT    ++
     "22t75"    CATG GTACCG                   GGAA                    CGGATC GCGCGAT    ---
  ≈70CoA#42     CATG GTACCG                   47 nt                   CGGTAC GCGCGAT
(2) 70CoA#26    CATG ACCCCTGAGCG              37 nt                CGCGAAGAGGT GAGCGAT
(2) 70CoA#48    CATG GTAGGGCCT^ATTC           32 nt             GAATCAGGCATTTC NCNCNNC
(2) 70CoA#60    CATG ACCA                     51 nt                    TGGT GAGCGAC
    70CoA#14    CATG GCCAA^ACATT              38 nt                GATGTATCGGC GCGCGAA
  ≈70CoA#32     CATG GCCAA^ACATT              38 nt                GATGTATCGGC GCGCGAA
    70CoA#16    CATG ATAGTGAGGTAAG            34 nt                CTTCCTTACTAT GGGCGAT    +++
  ≈70CoA#50     CATG ATAGTGAGGTAAG            34 nt                CTTCCTTACTAT GGGCGAT
    70CoA#21    CATG GCGAAGAGGCATAGACA        25 nt          TGTCAATGAACGAACGC GAGCGAC
  ≈70CoA#56     CATG GCGAAGAGGCATAGACA        25 nt          TGTCAATGAACGAACGC GAGCGAC
    70CoA#44    CATG GAGACGGACGAT             34 nt                ATCGTGAGACTC GAGCGAC
  ≈70CoA#53     CATG GAGACGGACGGT             34 nt                ATCGTGAGACTC GAGCGAC
    70CoA#3     CATG GCATTAC^GCTTG            33 nt                CGGGTAGTGATGC GAGCGAG
    70CoA#4     CATG ACCTGACGT                41 nt                   ACGA^AGGT GCGCGAA
    70CoA#9     CATG GATGAA                   47 nt                    TTCGTC GAGCGAA
    70CoA#11    CATG ACGAG                    49 nt                     CTCGT GGGCGAT
    70CoA#12    CATG GCCCAGGG                 48 nt                   TCC^GGGC GAGCGAC
    70CoA#15    CATG GAACGCGATT               40 nt                   AGTACATCG GACCATG    +++
    70CoA#20    CAGA GCGG                     49 nt                     CCGTT GAGCGAA
    70CoA#24    CATG ACGTG                    49 nt                     CACGT GAGCGAC
    70CoA#25    CATG GGCTAGCCACCACGTAG        26 nt          CTGGGTGGGGGGC^GGTC GAGCGAC
    70CoA#29    CATG GCTTAACACAGTAGTC         26 nt           GACTA^TGTGCTAAGC GAGCGAC
    70CoA#31    CATG GCGG                     51 nt                     CCGC GAGCGAG
    70CoA#41    CATG GCAC                     51 nt                     GTGC GAGCGAC
    70CoA#45    CATG GGGAGACTCGTC             36 nt                GAC^GGACTCTC GCGCGAT
    70CoA#47    CATG GGGTTTGC                 43 nt                   GCACACCC GAGCGAC
    70CoA#49    CATG GACCG                    49 nt                     CGGTC  GAGCGAC
    70CoA#51    CATG GGCCAAAGCCA              40 nt                TGGC^ATGGTC GGGCGAC
    70CoA#52    CATG GAACACTA^CG              39 nt                CGCAGATGTTC GGGCGAA
    70CoA#57    CATG GACCTA                   47 nt                    TAGGTC GCGCGAA
    70CoA#58    CATG GTAGTG^CAGC              38 nt                GCTGATGCTAC GCGCGAC
    70CoA#59    CATG GTGAGACGA                40 nt                  TCGGGTCAC GAGCGAA
 *  70CoA#61    TGAG TACTGAGGT                41 nt                   ATCTT^GTG GTGCCGA    +++
    70CoA#62    CATG GATGCA^GGT               40 nt                  ACTGGGCATC GAGCGAA
    70CoA#66    CATG GCGCTCAGCGAATCACAGGA     18 nt       TCCT^TGACCAGC^GGGCGC GCGCGAA
    70CoA#67    CATG GGTCACCCCCG              37 nt                CGAGGGTGATC GCGCGAC
 *  70CoA#68    TAGT GAAGTAAATT^CTCCT^^GGA    27 nt             TCCCAAGGAGCAAA CTATCGC    ++++
    70CoA#69    CATG ACCTA^GTTACTCG           32 nt             CGAGTATCATAGGT GAGCGAT
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B

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5'  gggcataaggtatttaattccata ... (80N) ... ttgattcggatgctccggtagctcaactcg  3'

    80A#4   CGCTTGAATTTGTAAAACCTGAGGGTACACCGCGAAGGCTGCGCCTTGTGTCTCGAGTCATGAGTCTCACGTCCGAAC   ++++
  ≈80A#46   CGCTTGAATTTGTAAAACCAGAGGGTACATCGCGAAGGTTGCACCTTGTGTCTTGAGTCATGAATCTCATGTCCCAAAC

(2) 80A#18  ATCGACCGCCTCTACGCCAGTATATCGGACGTAGCGCTTAACCGCGTTAGGGATCGCGTCCCTTAACGGGCGACAGAA   ++++
  ≈80A#58   ATCGACCGCCTCTACGTCAGTATATGGG CGTAGGCTTTGTCTGCGTTAGGGATCGCGTCCCTTAGnnnnGAGAGAA

    80A#20  CGCAGGAACGTGCGTGCAAAGAGTGGAATCGGAAAAAACGTTCCATACTGATAACGGATCCGGCTAAAGAC          +++
  ≈80A#27   CGCAGGAACGTGCGTGCAAAGAGTGGAATCGGAAAAA-CGTTCCATACTGATAACGGGTGCGGCTAGTGAAGGA       +++
  ≈80A#45   CGCAGGAACGTGCGTGCAAAGAGTGGAATCGGAAAAA-CGTTCCATACTGATAACGGGTCCGGCTAAAGAC
  ≈80A#52   CGCAGGAACGTGCGTGCAAAGAGTGGAATCGGAAAAA-CGTTGGATACTGATAACGGGTGCAGCTAAAGA
  ≈80A#68   CGCAGGAACGTGCGTGCAAAGAGTGGAATCGAAAAAA-CGTTCCATACTGATAACGGGTCCGGCAAAGAC

    80A#21  GCGAAATCGACGTTACTGAGCAACCAACGAAATTGCAAAATGGATACGTACTGAGTCGCT-CGAGATTTAGCTA        ---
  ≈80A#24   GCGAAATCGACGTTACTGAGCAACCAACGAAATTGCAAAATGGATACGTANTGAGTCGCTNCGAGATTTAGCTA

    80A#32  ATTGCCGTATATCCGCAACTAGCGAAGCTC--GTGGCGAGCACGACGTTGGAATTGAACACAACGGACCAGTCACTTCGA +++
  ≈80A#37   ATTGCCGTATATCCGCAACTAGCGAAGCTC--GTGACGCGCACGCGAT-GGGATTGAACACAACGGACCAGTCACTTCGA
  ≈80A#41   ATTGCCGTATATCCGCAACTAGCGAAGCTCGCGTGACGCGCACGCGAT-GGGATTGAACACAACGGACCAGTCACTTCGA
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FIGURE 2: Sequences of 70CoA and 80CoA populations. Constant primer-binding sequences are shown above each set in lower case letters. Numbers in parentheses indicate the number of identical sequences. Nearly identical sequences are grouped together. Sequences of truncated RNAs derived from 70CoA#5 and 70CoA#22 are shown below their parental sequences. Potential base-pairings are underlined. Gaps inserted to maintain pairing alignments are shown by "^". Asterisks denote 70CoA sequences that deviate from the consensus. Note the potential C•A pairings in isolates 70CoA15, 70CoA26, 70CoA29, and others (see Discussion for details). Relative resin binding and elution activities are indicated to the right. Not shown are 35 80CoA sequences that were isolated only once. Complete sequences are available at http://beagle.colorado.edu/~dhburke/all_seqs.html.

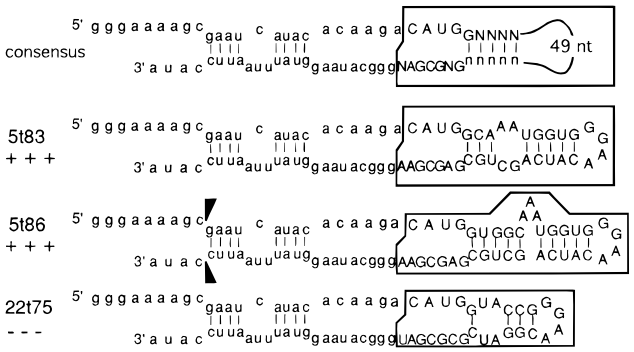

FIGURE 3: Proposed secondary structures of 5t83, 5t86, and 22t75 RNAs, with relative resin-binding and elution activities indicated, shown relative to the consensus structure (above). Functional boundaries (see Figure 4) are shown with wedges on the 5t86 structure. Boxed sequences correspond to nucleotides derived from the original 70N sequences.

of these sequences converge on an unusual sequence motif in which the first five bases of the 70N region were usually CAUGG, the last eight nucleotides of the 70N region were usually CGNGCGAN, and the sequences immediately internal to these could form complementary base pairs. In contrast, there was no obvious sequence or structural pattern in the 80CoA population, although 5 sequences (from 14 isolates) were found in 2 or more isolates with minor variations. When 12 individual aptamers from the eighth round population were screened for their abilities to bind and elute from the resin, 11 of these were active, including 2 70CoA RNAs that did not fit the above consensus sequence (70CoA61 and 70CoA68) and 4 of the 5 80CoA sequences assayed (Figure 2).

Delimiting the 70CoA Motif. An 83 nucleotide RNA ("5t83") was constructed by deleting 35 nucleotides from the initially random portion of the most abundantly isolated sequence ("70CoA5," Figure 3), leaving intact the 2 original fixed sequences and the conserved primary and secondary sequence elements from the 70N region. This RNA and a variant with altered stem sequences (5t86) were fully active in binding to and eluting from the CoA resin, but another RNA with disrupted pairings in this helix (22t75) failed to bind. These results support a requirement for the helix identified from comparative sequence analysis.

To establish the extent of participation by the primer-binding sequences in forming the binding element, we determined the 5′ and 3′ boundaries of the sequences required for binding by 5t86 using a deletion/selection experiment *(22, 28, 37)*. 3′-Labeled RNA was partially alkaline-hydrolyzed and partitioned over the CoA resin. Truncated RNAs containing all of the sequences required for binding were recovered from the column and assayed by gel electrophoresis (Figure 4A). Similar analysis was done with RNA labeled at the 5′ end (Figure 4B). Both analyses showed that no more than 5−10 nucleotides could be removed from either end without disrupting binding (Figure 3A). The observed functional boundaries are immediately across from each other in a weak potential stem formed by pairing the 5′ and 3′ primer-binding sequences. On the basis of the observed functional boundaries and the comparative sequence analysis of the original 70N sequences, we constructed a 68 nucleotide minimal model RNA ("min68," Figure 4C). Nearly twice as much min68 RNA bound to

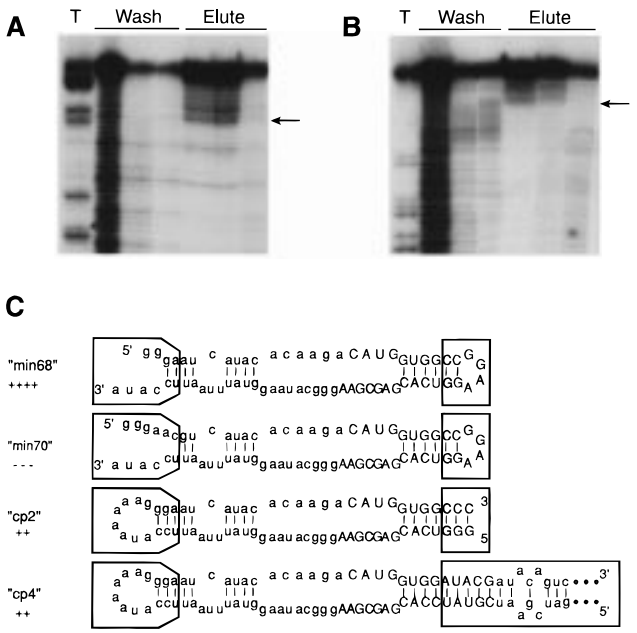

FIGURE 4: Boundary determination of 5t86 RNA using (A) 3′ or (B) 5′ end-labeled RNA. Size markers (lanes T) are RNase T1 digestions of labeled RNA. The positions of the boundaries are indicated by the arrows to the right. (C) Proposed secondary structures of min68, min70, cp2, and cp4 RNAs, with relative binding and elution activities indicated. Differences among these four RNAs are boxed.

and eluted from the CoA resin as did transcripts from the original isolates. Its smaller size may reduce the number of available alternative (inactive) conformations.

Three variations of min68 confirm the overall features of the structural model. The sequence of "min70" RNA differs from that of min68 at the 5′ end, destabilizing the terminal helix and completely eliminating activity (Figure 4C). "CoA-cp2" RNA is a circularly permuted version of min68 that retains all of the proposed secondary structural elements and internal loop sequences while joining the original 5′ and 3′ termini through a short linker of five A's (Figure 4D). CoA-cp2 RNA was equivalent to most of the individual isolates in CoA resin binding and elution activity, supporting the existence of a helix formed by base-pairing between the original fixed sequences. Appending additional sequences to either end of cp2 to create cp4 (Figure 4D) had no significant effect on its activity.

The design of cp4 RNA allowed us to gather covariation and conservation data for nucleotides in the original 5′ and 3′ fixed sequences, which were invisible to such analysis in the original selection. The template for cp4 was resynthesized with 15% mutagenesis per position throughout the internal region. Upon transcription, the resulting mutagenized pool was subjected to six additional cycles of affinity selection, during which complete activity returned quickly (Table 1). Covariation analysis of 21 isolates from the reselected pool strongly supports base-pairing in the helix identified in the original selection (Figure 5). The proposed pairings at the other end of the molecule were only weakly supported, although support for pairings at these positions is improved if A·C pairs are included (see Discussion). Twenty positions were invariant, more than half of which were derived from the original amplification primers. These nucleotides are assumed to be crucial for forming the active

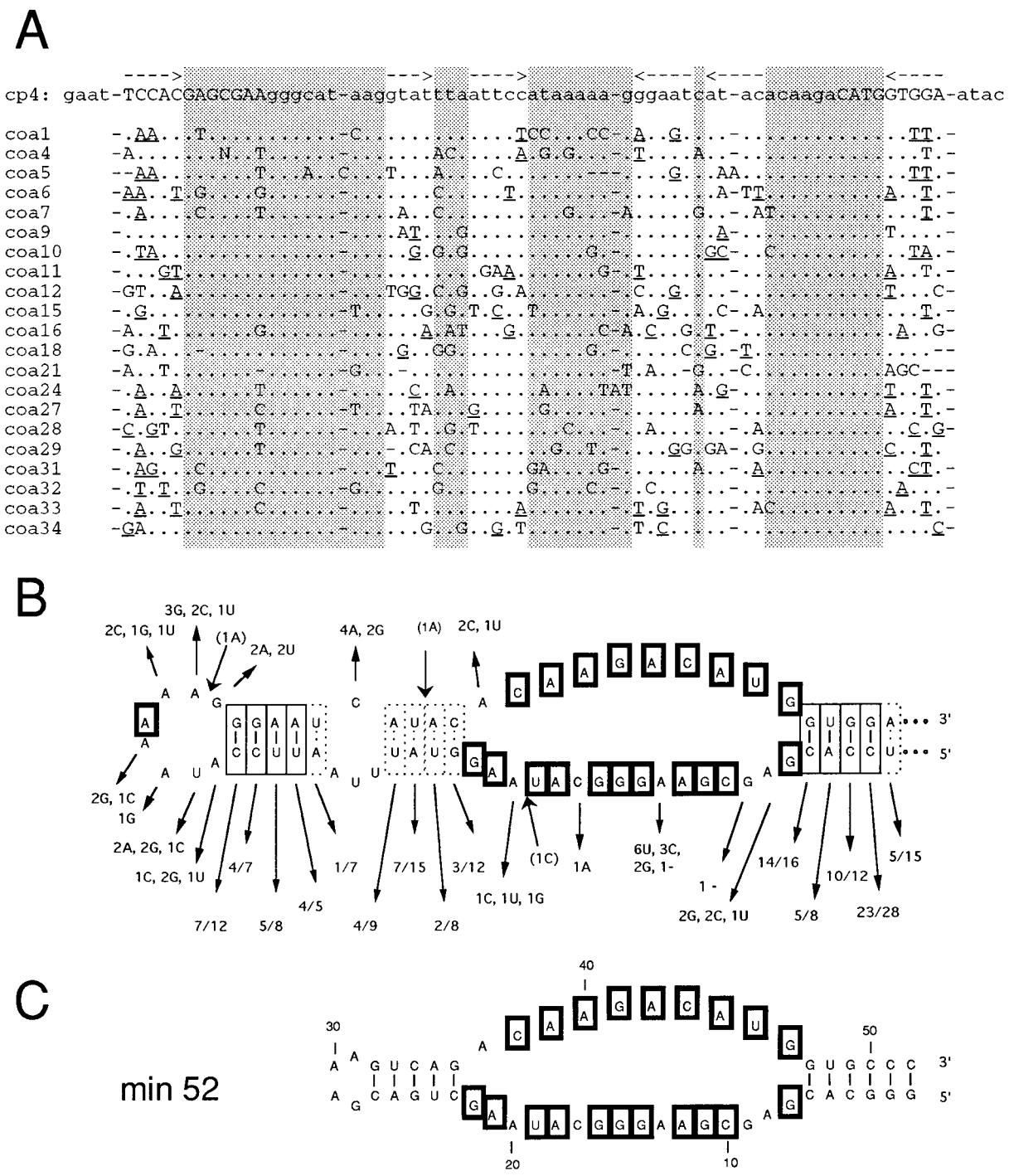

FIGURE 5: Covariation analysis. (A) Sequences of mutagenized, reselected cp4 population. Aligned nucleotides that are identical to cp4 are shown as dots. The sequence of cp4 is shown across the top, with four nucleotides from each primer-binding site shown in lower case letters, and the proposed secondary structures indicated with arrows above the sequence. Unpaired regions are shaded, covariations that support pairings are underlined (including G·U pairs; but excluding A·C pairs, see Discussion), and gaps inserted to maintain alignment are indicated with dashes. (B) Mapping the sequence data onto the proposed secondary structure. Invariant nucleotides are shown in boxes with thick lines. Covariation data are shown as fractions: numerator, total number of mutations that support pairing; denominator, total number of mutations within that pair. Base pairings that are supported by covariational analysis are boxed in solid lines; those that are not are boxed in dashed lines. Mutations at unpaired positions are enumerated, with insertions shown in parentheses and modified arrows and with gaps shown with dashes. (C) Sequence and secondary structure of min52 RNA.

binding structure or for contacting the CoA. Based on these results, we synthesized "min52" RNA, a 52 nucleotide construct containing the large internal loop flanked by helices of arbitrary sequence. Approximately 50% of the min52 RNA that was applied to the CoA affinity resin remained bound during the washes and was eluted with free CoA.

Recognition of the Höogsteen Base-Pairing Face. To define the binding specificity of the interaction, min68 RNA bound to the CoA resin was challenged with 23 different CoA analogues. Those analogues that eluted the RNA from the resin are assumed to compete with CoA for occupancy of the aptamer binding site and to contain all of the essential

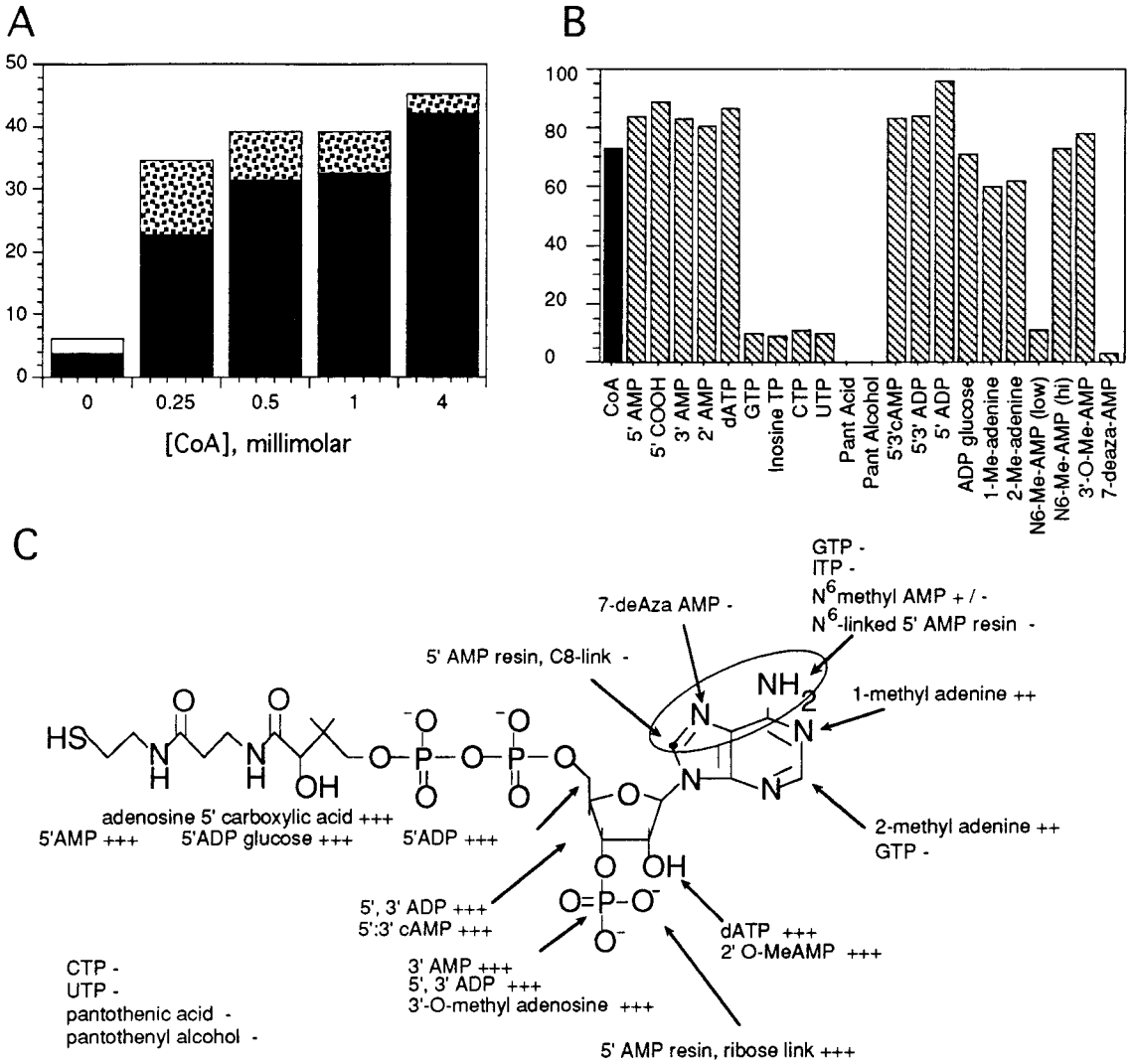

FIGURE 6: Specificity of the interaction. (A) Minimal CoA concentration required to elute min68 RNA from CoA resin. Percentages of RNA eluted in 4 volumes of binding buffer containing CoA at the concentration indicated (solid bars); additional RNA eluted with 4 volumes of buffer containing 4 mM CoA (stippled bars) or no CoA (white bar). (B) Relative percentages of RNA eluted by 4 volumes of 0.5 mM CoA analogues. (100% is defined as the sum of RNA eluted in both the four analogue fractions and four additional volumes containing 4 mM CoA.) For *N*6-methyl-AMP, "hi" and "low" refer to elution with 4.0 and 0.5 mM analogue, respectively. Elutions with pantotheinic alcohol and pantotheinic acid were at 10 mM analogue. (C) Relative elution activities of CoA analogues are mapped onto the structure of CoA.

recognition elements seen by the aptamer. To minimize possible nonspecific effects that could come from using high analogue concentrations, we first determined the minimal concentration of CoA required to elute most of the bound RNA. Min68 RNA bound to the CoA resin was challenged with 4 volumes of 0.25, 0.5, 1.0, 2.0, and 4.0 mM CoA. Any RNA not removed by this treatment was eluted with 4 volumes of CoA at 4 mM in order to make unambiguous the total amount of elutable RNA. Of the total removed by this two-step elution, nearly three-quarters (73%) was eluted by the 4 volumes of 0.5 mM CoA (Figure 6A), so this concentration was chosen for use with the analogues following a similar two-step protocol. All adenosine derivatives with unmodified bases eluted more than 60% of the elutable RNA in 4 column volumes, regardless of modifications of the sugar (Figure 6B). Thus, the sugar does not appear to be required for recognition, nor are there obvious steric constraints on the sugar environment. The pantothein arm of CoA was insufficient for recognition by the aptamer, as neither pantotheinic acid nor pantotheinyl alcohol eluted any RNA, even at 10 mM concentration. 1-Methyl- and 2-methyladenine also eluted the RNA with slightly reduced efficiency. Only modifications at the N6, N7, and C8 positions interfered significantly with elution. 7-Deaza-GTP failed to elute the RNA, as did GTP and ITP, which contain keto groups in place of the exocyclic amine. These three also failed to elute when used at 4 mM (not shown). *N*6-Methyl-AMP did not elute min68 RNA appreciably when assayed at 0.5 mM, although it did elute well when used at 4 mM. We postulate that the methyl group reduces the affinity of the analogue for the aptamer to the point that higher concentration is required for binding to occur, but not so much as to eliminate binding (for example, by requiring a conformational change in the RNA to accommodate the methyl group). In separate assays, min68 RNA was retained on an AMP-derivatized resin linked through the ribose hydroxyls (periodate-oxidized), but not on an AMP-derivatized resin linked through the N6 position by an aliphatic chain, nor on an AMP-derivatized resin linked through the C8 position. We conclude that min68 RNA recognizes CoA

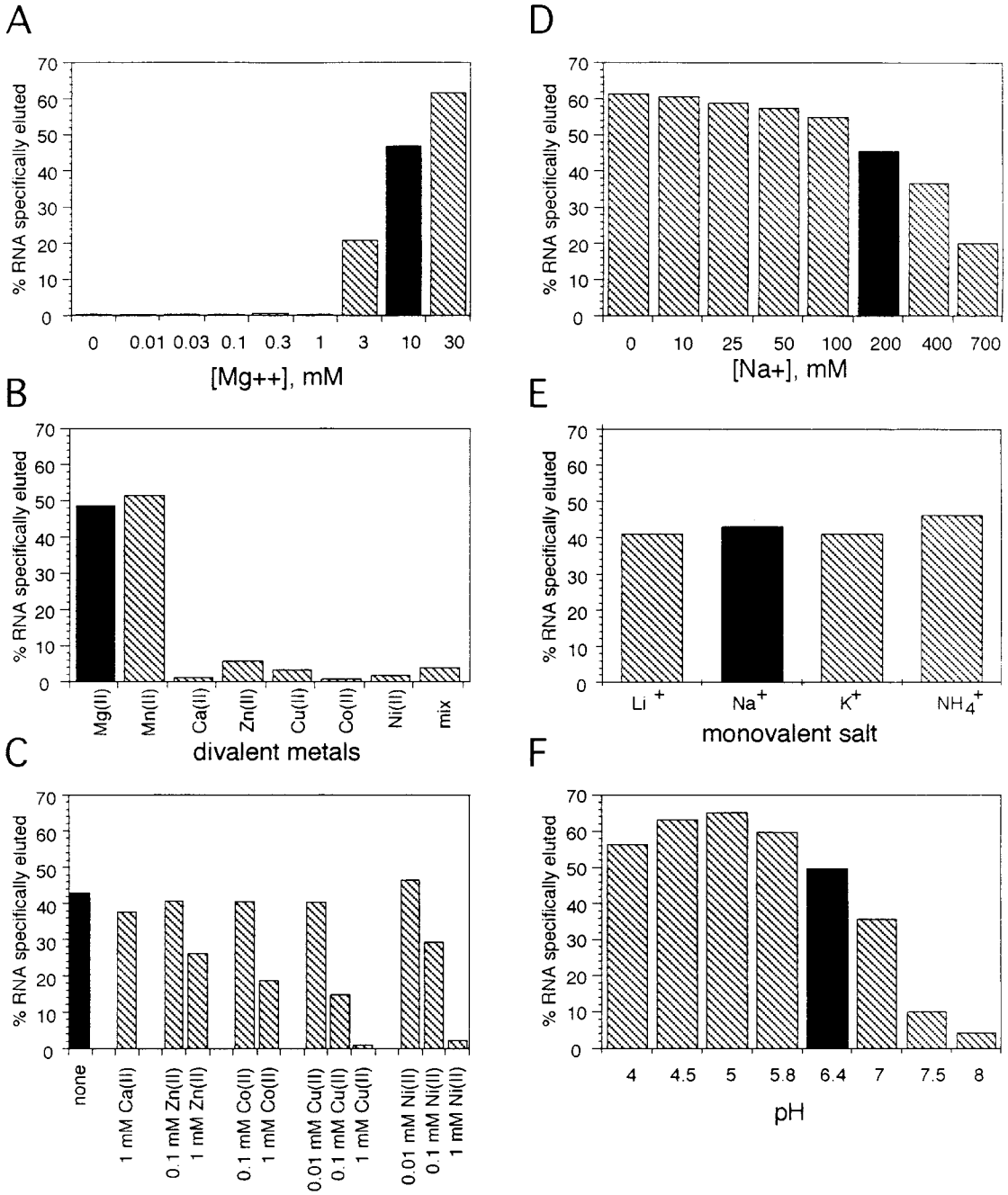

FIGURE 7: Survey of binding activity as a function of (A) magnesium ion concentration, (B) divalent metal identity, (C) divalent metals in addition to 10 mM Mg^{2+}, (D) sodium concentration, (E) monovalent ion identity, and (E) pH. RNA was folded in buffer that differed from normal in the manners indicated under each bar, loaded onto preequilibrated resin, washed in the same buffer, and eluted from the affinity resin by 6 volumes of normal binding buffer containing 5 mM 5′ AMP. Black bars indicate the activity of min52 RNA under conditions used during the original selection.

predominantly through the Höogsteen face of the base, with likely steric constraints near the C8 and N6 positions and hydrogen bonds to N7 and N6.

Optimization of Binding Conditions. The selections for CoA binding were performed in a buffer containing moderate salt and Mg^{2+} concentrations at slightly lowered pH. To investigate the effects each of these components had on determining the outcome of the selection, we performed column retention and elution assays with min52 RNA under a variety of buffer conditions (Figure 7). The relative percentages of RNA that bind the resin under each condition are probably a combination of effects on folding and affinity. Binding was very sensitive to magnesium ion concentration. Activity was rapidly lost as Mg^{2+} concentration decreased from 10 mM to 3 mM, and disappeared at 1 mM and below, while raising it to 30 mM stimulated the observed activity (Figure 7A). Mn^{2+} could substitute readily for Mg^{2+}, but Zn^{2+}, Ca^{2+}, Cu^{2+}, Co^{2+}, and Ni^{2+} could not (Figure 7B). Combinations of 10 mM Mg^{2+} with each of the inactive metals at 1 mM showed that binding activity was unaffected by added Ca^{2+}, was slightly inhibited by added Zn^{2+}, more so by added Co^{2+}, and was eliminated by Cu^{2+} or Ni^{2+}. 100 μM Zn^{2+} or Co^{2+} were tolerated, as was 10 μM Cu^{2+} or Ni^{2+} (Figure 7C). Monovalent salts had the opposite effect. Activity rose as Na^{+} concentration decreased below 200 mM, and fell sharply at higher concentrations (Figure

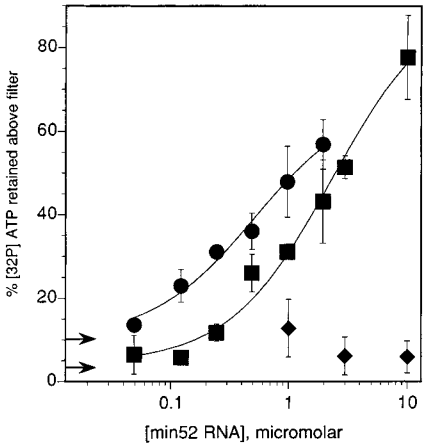

FIGURE 8: K_d determination in solution. Differences in radioactivity between the retentate and filtrate in equilibrium filtration assays are plotted as a function of added min52 RNA concentration in the buffer used for the selection (circles) or in optimized buffer (30 mM $MgCl_2$, 50 mM sodium acetate, pH 5.0) (squares). Average values from three trials at each RNA concentration are shown, with error bars indicating the standard deviations among those trials. Arrows to the left indicate the RNA-independent signal, which was 10.4% for the optimized buffer (top) and 4.4% for the selection buffer (bottom). Binding reactions with tRNA (diamonds) were done at 25, 75, and 250 μg/mL, which is roughly equivalent in total concentration to 1, 3, and 10 μM min52 RNA.

7D). It is not clear whether the high salt is stabilizing an inactive alternative RNA conformation, competing directly with the Mg^{2+}, or otherwise interfering with the aptamer−target interaction. The observed activity is compatible with the presence of other monovalent ions, since replacing the Na^+ with 200 mM K^+, NH_4^+, or Li^+ had little effect (Figure 7E). Binding and elution activity was stimulated by increasing the acidity of the medium, but it fell sharply as the pH approached neutrality (Figure 7F). Binding activity is surprisingly high at very acidic pH (86% of the maximal observed activity at pH 4.0). For acetate buffer, activity increased slightly as the pH rose from 4.0 to 5.0, while for Bis-tris buffer, activity decreased as the pH rose from 5.8 to 7.0. Taken together, these results suggest that the optimum pH is near 5.0.

Quantitation of Binding in Solution. To demonstrate whether the CoA aptamers recognize their targets in solution and to quantify the strength of the interaction, min52 RNA was incubated with [α-^{32}P]ATP and partitioned in triplicate through microconcentrator filters *(38)*. The size fractionation membranes of these filters retain RNA·ATP complex above the membrane, while free ATP partitions freely into both the retentate and the filtrate. Alpha label was used so that ATP hydrolysis would not result in free label that could not participate in the binding reaction. The difference in radioactivity in the two fractions, which is taken to be an indication of the amount of complex formed, increased with RNA concentration (Figure 8). A best-fit theoretical binding curve yielded a dissociation constant (K_d) of 2.4 ± 0.4 μM when the assay was performed in the buffer used during the selection. Nearly 5-fold-stronger binding (K_d = 0.5 ± 0.1 μM) was observed when this buffer was replaced with an optimized buffer based on the above survey of binding conditions (30 mM $MgCl_2$, 50 mM acetate, pH 5.0). [Reported errors in K_d values reflect the precision with which the optimized curve fit the available data. If the bound complex reequilibrates rapidly during the course of the assay, the amount of complex formed will be underestimated *(28)* and K_d will be overestimated. The K_d values reported here can therefore be considered as upper limits.] A 30- to 300-fold excess of unlabeled ATP added prior to the labeled ATP eliminated this signal, consistent with there being a saturatable binding site in the RNA. No increase in counts above the membrane was observed for tRNA over the same concentration range as that used for min52, consistent with the observed effects being the result of specific binding, rather than a nonspecific effect of added RNA.

Aptamers Specific for CoA. Because the dominant functional motif in the 70CoA pool elutes equivalently with 13 different adenosine analogues, the same RNA motif probably would have been recovered if any of these molecules had been the target of selection rather than CoA. To determine whether the selected pools contained any RNAs that required intact CoA for elution, the products of Rnd 5 (70CoA) or Rnd 6 (80CoA) from the original selection were allowed to bind CoA resin and were washed with 10 column volumes of buffer containing either 100 or 300 μM 3′ AMP (approximately one-thirtieth and one-tenth, respectively, the concentration of immobilized CoA on the resin). These rounds were chosen because their products gave the first detectable signal in the subsequent round upon elution with CoA when AMP was not included in the wash (Table 1). Reliable signal returned in the third or fourth round of 100 μM counterselection (nine total rounds of selection) and a few rounds later for the 300 μM counterselection. After eight rounds of counterselection (thirteenth or fourteenth round total), approximately one-tenth of the RNA survived the 100 μM AMP counterselection and was eluted with CoA (Table 1). For both the 70/100 and the 80/100 sets, approximately 20% of the input RNA was eluted by CoA when the wash steps did not include an AMP counterselection (not shown). Sequences from the two populations with the 100 μM counterselection (not shown) were highly diverse and did not yield any discernible sequence or structural motifs (L. Heyer and G. Stormo, personal communication), and they are presumed to be unrelated to each other. Thus, even though the initial 70CoA population appeared to be uniformly comprised of aptamers that recognized the Höogsteen face of adenine, there were also many other sequences present at low frequency that required intact CoA for elution. Some of these may make contacts with the pantothein arm, whose several potential H-bond donors and acceptors offer many opportunities for interaction with the RNA.

DISCUSSION

CoA Aptamers. In vitro selections with two random pools identified many CoA-binding RNAs, including uncharacterized RNAs whose elution from the resin is dependent on intact CoA. RNA "min52" contains all of the required binding elements of the dominant motif from the 70CoA pool within a 52 nucleotide transcript that contains 2 stems separated by a large internal loop. All 20 specifically required nucleotides are located within the loop, as shown by mutagenesis and reselection experiments. A Blast search did not identify any matches to this motif in the available genomic sequence databases. Assuming a requirement for four Watson−Crick base pairs of any sequence at each end of the large internal loop, only one in $4^{28} = 7 \times 10^{16}$ random

sequences (0.004 copy in the starting 80N pool) would be expected to contain this element. Since 1 of the stems and 12 of the specifically required nucleotides are provided by the fixed sequences, the initial 70N population contained 1 of these motifs in approximately every $4^{12} = 2 \times 10^7$ sequences ($>10^7$ copies in the starting 70N pool). Many of the 70CoA isolates had stems that contained considerably more than four base pairs. Their added stability likely increased the fraction that was properly folded and allowed them to be enriched to a greater degree than less stable RNAs. For example, the most frequently encountered sequence, that of 70CoA5, contains the longest stem of all 70CoA isolates. Binding is sensitive to pH, high salt, and the concentration and identity of divalent metal ions.

An Acidophilic Aptamer. The in vitro selections were performed slightly below neutrality (pH 6.4) to prevent oxidation of the CoA sulfhydryl. The resulting 70CoA aptamer has a pH optimum more than 1 pH unit lower still, and it is more active at pH 4.0 than at the pH of the selection. These results suggest that protonation of one or more titratable groups with pK_a's between 6 and 7 in either the aptamer or the bound target is required either in the binding interaction or for formation of the binding site. The pK_a for the N1 of free 5′ AMP is 3.88 and for the N3 of 5′CMP is 4.54 *(39)*, but the immediate environmental context can greatly alter the pK_a's of individual protons. pK_a's near neutrality have been observed by NMR for protonated A·C and A·G base pairs *(40−42)*. There are many potential A·C and A·G pairings within the two helices established through covariation analysis both in the original selection (Figure 2) and in the mutagenic reselection (Figure 5), and the large internal loops could also be stabilized by such pairings. Nevertheless, we cannot ascribe the pH dependence of this aptamer to any particular residue or set of residues at this time.

Jayasena and Gold (1997) recently described a novel self-cleaving ribozyme that does not appear to require divalent metal ions and has a pH optimum around 4.0 *(43)*. Their ribozyme and our aptamers are the only in vitro selected RNAs we are aware of that operate 3 or more pH units from neutrality, even though neither was isolated under conditions that were intended to favor acidophily. The self-capping ribozyme isolated by Huang and Yarus has a pH optimum near 5.5, but it rapidly loses activity at lower pH and is nearly inactive at pH 4.5 *(44, 45)*. Proponents of the RNA World theory for the origin and early evolution of life have pointed to in vitro selected aptamers and ribozymes as evidence for the versatility of nucleic acid function. The fact that nearly all of these results have been obtained under mild laboratory conditions would seem to limit their applicability to the potentially unfriendly conditions of early earth chemistry. Our results suggest that at least some extreme conditions (acidic pH) may also be compatible with an RNA World, thereby making the theory more robust than it would be if all of the supporting evidence came from mild conditions.

Influence of Experimental Conditions on the Outcome of SELEX. There is an adage among practitioners of SELEX that "you get what you select for," in recognition of the fact that every element of an experiment can become a selective criterion, whether or not it is intended as such. If we had carried out the selections using more monovalent salt, less Mg^{2+}, a different divalent metal ion, a higher pH, or a different linkage between the CoA and the resin, we would likely have isolated some other RNA motif, despite the overrepresentation of min52-like sequences in the starting 70N pool. Indeed, the same starting pool could yield several nonoverlapping solutions to a particular biochemical problem if selected in parallel under several different sets of conditions. Experimental conditions often influence the outcome of in vitro selections. For example, ribozymes and aptamers selected in the presence of complex mixtures of metals often require one or more of the "odd" metals or other nonstandard conditions, as is the case for an isoleucine aptamer that requires Zn^{2+} for binding activity *(23)*, a self-capping ribozyme that requires Ca^{2+} *(44, 45)*, a self-aminoacylating ribozyme that requires Ca^{2+} and Mg^{2+} *(11)*, a Diels−Alderase ribozyme that requires Cu^{2+} *(7)*, and a cyanocobalamin pseudoknot aptamer that requires Li^{+} *(8)*.

Long random regions are often used in selections for RNAs with complex catalytic functions, based in part on the rationale that they can explore a broader range of sequence and conformational space *(46, 47)*, and in part because the longer random regions generally decrease biases introduced by the design of primer binding sequences (PBS's). Indeed, where PBS's have been recruited as required elements in SELEX-derived motifs, the random regions have been short (30−50N), often comprising less than half of the total transcript *(10, 48, 49)*. The prominent role of PBS's in forming the active structure of min52 RNA forced the motif to localize immediately adjacent to the PBS, such that only nucleotides proximal to the PBS participated significantly in determining the outcome. In the 70CoA selection, the length of the random region is largely irrelevant beyond the minimal 23 or so nucleotides needed to fill out the motif.

Adenosine Binding by the "ATP" and "CoA" RNA Aptamers. Both the "CoA" aptamer motif represented in min52 RNA and the "ATP" aptamer recognize ATP and other adenosine cofactors, and so their nomenclature reflects their intended selection targets rather than functional differences. Nevertheless, there are many differences between them. The "ATP" aptamer functions both above neutrality (pH 7.6 in the ATP and NAD^+ selections) *(14, 15)* and in the absence of magnesium *(17)*, while the "CoA" aptamer requires acid conditions and high Mg^{2+} or Mn^{2+} concentrations ($\geq$10 mM). The "ATP" aptamer occurs much more frequently than the "CoA" aptamer in random pools (approximately 1 in 10^{11} sequences). Even though both bind adenosine in solution, neither could have arisen in the other's selection because of constraints imposed by the method of attaching the targets to the affinity resins. For the ATP, NAD^+, and SAM selections, the respective targets were all attached through the C8 position, which blocks binding by the "CoA" aptamers. The CoA was attached through the exocyclic amine (N6), which blocks binding by the "ATP" aptamer (unpublished observation). The "ATP" aptamer interacts with the Watson−Crick base-pairing face of the adenine and with the 2′- and 3′-hydroxyls of the sugar and cannot bind CoA because of the 3′-phosphate, while the "CoA" aptamer recognizes primarily the Höogsteen face of the adenine. Nevertheless, both give dissociation constants in the low micromolar range. We also note that mutants of the "ATP" aptamer could, in principle, open up the binding pocket in order to accommodate the 3′-phosphate of CoA. The "CoA" aptamer is capable of binding a broader range

of adenosine cofactors, since it recognizes all sugar modifications. Thus, it may be feasible to isolate several classes of cofactor-dependent ribozymes (redox, methyltransfer, acyltransfer) from pools containing the "CoA" aptamer motif.

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