RNA aptamers that are able to complex free adenine have been isolated by a SELEX (systematic evolution of ligands by exponential enrichment) procedure. The adenine binding site was revealed by sequence alignment for a prevalent cluster of aptamers, and its structure and interactions with adenine were probed by RNase digestion studies, lead cleavage, boundary determination experiments, and truncated sequences studies. A new purine binding motif was functionally and structurally characterized and compared with other RNAs specific to purine or adenylated compounds. The affinity for adenine and the specificity for other related targets were quantified. This work suggests that the adenine binding site is composed of two independent secondary structure elements forming a bipartite binding site that interacts with adenine in a new mode of purine recognition. Such binding is of great interest because the imidazole moiety is not trapped in the binding site, and would easily be available for catalytic activity.

The RNA world hypothesis assumes that modern life forms arose from a molecular ancestor in which RNA molecules both stored genetic information and catalyzed chemical reactions. Today, DNA, from which genetic information flows, is considered a modified RNA, more stable than RNA except under acidic conditions, and therefore more capable of conserving and transferring information (1). Furthermore, the central role of RNA in contemporary metabolism still includes informational and catalytic properties; a lot of cellular RNAs are involved in key cellular functions, and some of them are catalytic RNAs. Although the RNA world hypothesis remains difficult to understand up to a point, the explanatory means remain powerful (1, 2). Contemporary transfer RNA contains a rich collection of functionalized bases created by post-transcriptional modification (3). RNA might gain chemical functionalities by using catalytic building blocks such as imidazole, and thiol, amino, and carboxylate groups, etc. as cofactors (4, 5), like many coenzymes that are nucleotide derivatives. By chemical modifications nucleotides could acquire all of the functional groups of amino acids. Moreover, primeval nucleotides were not necessarily restricted to the standard nucleotides as we know them today. Thus, modified nucleotides may have played a role in primeval catalysis (6, 7).

We have already shown that an N⁶-ribosyl adenine, which is likely to have formed under prebiotic conditions (8), is equivalent to histidine in the model reaction of p-nitrophenyl acetate hydrolysis (9). This mode of linkage leaves the imidazole portion of adenine free to react (10). In the same way we showed that when adenine is placed in a favorable microenvironment, the catalytic efficiency of adenine is heightened (11–14). Such a favorable microenvironment could be provided by an RNA adenine binding site. Of further significance is the discovery that abasic hammerhead ribozymes can be rescued by the addition of exogenous bases that restore the catalytic activity (15) or that imidazole can rescue a cytosine mutation in a self-cleaving ribozyme (16).

To this end, we have explored the possibility that regular ribonucleic acids bind adenine specifically with the aim of forming a plausible primitive equivalent of the catalytic site of an enzyme. In vitro selection or SELEX (systematic evolution of ligands by exponential enrichment) experiments using RNA have shown that nucleic acid molecules with specific molecular recognition and functional properties can be isolated from complex pools of random sequences by repeated rounds of selection and amplification (17). RNA molecules isolated by such processes can bind proteins, nucleic acids, or smaller ligands, and some of them can display biological activities with possible therapeutic significance (18, 19).

RNA aptamers that bind nucleotides, nucleosides, and nucleic bases have already been isolated. Aptamers for purine compounds, ATP, theophylline, and xanthine/guanine have their own distinctive mode for purine base recognition (20–22). Adenine is a highly plausible prebiotic molecule, and therefore adenine-binding aptamers could mimic substrate binding by early ribo-organisms that had eaten adenine “food” synthesized abiotically. With regards to the question of whether the adenine ring and specifically its imidazole moiety may have played a role in biochemical evolution of enzymes and coenzymes, it is of crucial interest to isolate RNA aptamers to adenine with high affinity and specificity and to look for specific sequences and structure patterns involved in adenine recognition. An N⁶-adenine-linked Sepharose column, which differs from the classical purine-agarose column previously used for the selection of the above mentioned RNA aptamers by linkage at position 6 of the base, was used in the present experiments.

The adenine ring was linked to the affinity column for selection by its N⁶-nitrogen atom to maintain the ability of the ring to interact with RNA. This column was prepared by coupling to EAH-Sepharose 4B, the N⁶-(5-carboxypentyl)adenine synthesized from 6-chloropurine and 6-aminopropionic acid. Here we
report the isolation of a new RNA aptamer motif, a bipartite RNA site that binds the adenine target.

MATERIALS AND METHODS
Preparation of the Adenine-Sepharose Column
The chemical reagents used for preparing the affinity column were from Sigma Aldrich. EAH-Sepharose 4B was obtained from Amersham Biosciences, Inc.

N6-(5-Carboxypentyl)adenine—To a solution of 6-chloropurine (1 g; 6.47 mmol) in anhydrous ethanol (15 ml) was added 6-mimocarboxylic acid (1.87 g; 14.3 mmol) and then triethylamine (1.5 ml). The solution was heated under reflux for 40 h and then evaporated to dryness. The residue was dissolved in water, and the N6-(5-carboxypentyl)adenine was precipitated by the addition of a concentrated hydrochloric acid solution to decrease the pH to 4.5. The solid was filtered off and washed with water and then with diethylether. N6-(5-carboxypentyl)adenine was finally purified by recrystallization from an ethanol-water mixture at 3:1 (0.98; 3.95 mmol; yield, 61%).

The characteristics of N6-(5-carboxypentyl) adenine are: melting point, 240–241 °C; 1H NMR (300 MHz, Me2SO-d6), δ ppm: 12.80 (1H, b s, 5-COOH or N9-H), 12.02 (1H, b s, N9-H or CO2H), 8.15 (1H, s, C2-H or C8-H), 8.06 (1H, s, C8-H or C2-H), 7.69 (1H, b s, N6-H), 3.45 (2H, s, C8-H), 2.19 (2H, t, J = 7.0 Hz, CH2), 1.54 (4H, m, 2 CH2), 1.32 (2H, d, J = 7.3, CH2).

N7-GCGAATTC—GCGAATTC (19 mg; 165 μmol) in a 1:1 mixture of dioxane:phosphate-buffered saline (100 mM, pH 7.3) followed by 10 volumes of washing buffer (100 mM NaCl, 50 mM HEPES, 5 mM MgCl2, pH 7.3). After chromatography, the column was re-equilibrated for further use with 7–10 volumes of washing buffer (100 mM HEPES, 500 mM NaCl, 10 mM EDTA, pH 7.3) followed by 10–12 volumes of binding buffer. The eluted RNAs were detected and quantified by measuring the A260 nm of the eluted fractions and by electrophoresis detection. Aliquots of all the fractions were subjected to electrophoresis on 2% agarose gel. After staining with ethidium bromide the RNA concentrations were estimated by quantifying spot intensities with an NIH Image analyzer. By this method it was possible to estimate the RNA concentration in eluted fractions and to avoid interference between the adenine of the elution buffer and the eluted RNA, because both absorb at 280 nm. The pooled RNA was concentrated by ultrafiltration with a Lida ultrafiltration unit and subjected to a reverse transcription in 100–200 μl using AMV-reverse transcriptase followed by PCR with T7 DNA polymerase (6–12 amplification cycles), both supplied by the Access reverse transcription-PCR kit from Promega. After ethanol precipitation and resuspension in water, the newly selected double-stranded DNA library produced the RNA pool for the next round of selection by in vitro transcription with T7 RNA polymerase. As soon as the eluted RNA population became detectable by electrophoresis, two-count selection rounds were performed on a Sepharose 4B column (Amersham Biosciences, Inc., 1 ml) before proceeding to the other selection rounds, to avoid the artificial amplification of Sepharose-binding RNAs. The RNA pool was applied to the Sepharose column and washed with binding buffer. The washed RNA population, without affinity for Sepharose, was recovered and amplified by reverse transcription-PCR followed by T7 RNA polymerase in vitro transcription.

Cloning
To screen for RNA molecules with affinity for adenine, the RNA population eluted from the column was cloned after conversion of RNA to DNA by reverse transcription-PCR. From isolated clones, plasmids were prepared and the cloned DNAs sequenced. After plasmid purification and in vitro transcription of the PCR-amplified inserted fragment, double-stranded RNAs were detected by electrophoresis, two-count selection rounds were performed on the adenine-Sepharose column to check whether they are able to bind adenine, the function for which they had been selected.

Equilibrium Filtration
The affinity of RNAs for adenine was evaluated with unlabeled RNA and [3H]adenine (0.05 μCi, 21 Ci/mmole). The 200-μl binding reaction mixture was filtered through Microcon YM-10 molecular cut-off filters (10,000 kDa). An aliquot of 20 μl was counted without filtering to determine the total activity of the mixture. After centrifuging the filter for 3 min, 20% of the binding reaction mixture had passed through the filter. The filtrate was combined with the retentate and centrifuged again (22,000). The bound adenine was determined in counts between the applied mixture (free adenine plus complexed adenine to RNA) and the filtrate (free adenine corrected for the efficiency of filtration). The Kd was determined graphically by plotting the fraction of bound adenine as a function of bound adenine corrected for the efficiency of filtration.
function of RNA in the assay, taking $K_d$ to be equal to the RNA concentration for 50% adenine binding. It was also evaluated by comparing the distribution of the experimental values to the theoretical curve, considering the equilibrium reaction of one RNA molecule complexing one adenine molecule (Fig. 5). The 50% adenine binding method is possible when the $K_d$ is much higher than the adenine concentration; otherwise it is determined using the equilibrium constant equation.

The specificity of the RNA was studied by radioligand displacement measurements (26), using unlabeled potential competitors with [3H]adenine (18 mM) and RNA (18 or 27 mM). The fraction of adenine complexed to RNA was determined by equilibrium filtration as a function of competitor concentration, thus allowing the calculation of the competitor dissociation constants ($K_d$). The concentration of RNA in water is assumed to be 1 mg/ml for an absorbance at 260 nm = 30.

Before performing the binding measurements, the RNA (24 or 72 µM) was renatured in binding buffer by warming to 60 °C for 1 min and cooling slowly to room temperature.

**Ribonuclease Mapping**

The aim of these experiments was to probe secondary and tertiary structure elements of selected aptamers and to locate the nucleotides involved in adenine binding. Reactions were performed as described elsewhere (27, 28). The specificity of RNase V1, RNase T1, RNase T2, and RNase A served to probe RNA secondary structure. 5'-Radiolabeled aptamers were submitted to limited digestion by each enzyme, and digestion patterns were analyzed on denaturing polyacrylamide gel electrophoresis. Two enzymatic digestion conditions were performed simultaneously: one without adenine in the reaction mixture and the other in the presence of adenine (1.23 mM). Therefore, it was possible to probe RNA single- or double-stranded regions and to detect nucleotides protected from RNase hydrolysis by direct interaction with adenine or by a conformational change, thus highlighting the adenine-binding site.

**Lead Cleavage**

Adenine aptamers were also probed by lead-induced chemical cleavage in order to highlight single-stranded regions and to compare the results with the RNase mapping experiments. 5'-Radiolabeled aptamers were submitted to cleavage by various amounts (from 0 to 1 ml) of lead acetate during 1 h at room temperature in HEPES 100 mM, pH 7.3, NaCl 50 mM, and MgCl2 5 mM. The reactions were stopped by adding 1 ml of alkaline hydrolysis buffer (50 mM NaHCO3-Na2CO3, pH 9.2) and subjected to random cleavage using 20% polyacrylamide gel electrophoresis. Purified labeled RNA (1 nmol) was suspended in 400 µl of loading buffer (1 volume of formamide, 1 volume of water, 1 volume of glycerol added as carrier). Then 10,000 dpm of each sample were loaded on a 15% denaturing polyacrylamide gel, and radiolabeled fragments were detected by autoradiography.

**Boundary Analysis Experiments**

The aim of these experiments was to determine the minimal sequence requirement for adenine binding in order to localize the adenine-binding core. Adenine-binding RNA was 32P-labeled, either at its 5' terminus with T4 polynucleotide kinase and [α-32P]ATP or at its 3' terminus with T4 RNA ligase and [5'-32P]pCp, and purified on a denaturing polyacrylamide gel electrophoresis (27, 28). Purified labeled RNA (1 nmol) was suspended in 400 µl of alkaline hydrolysis buffer (50 mM NaHCO3-Na2CO3, pH 9.2) and subjected to random cleavage (100 °C, 10 min). Hydrolyzed RNA was then ethanol-precipitated and resuspended in binding buffer. The mixture was then denatured and renatured as described above under “Selection Procedure” and deposited onto the adenine-Sepharose column. Unbound RNA fragments were washed with 8 column volumes of binding buffer, and adenine-bound fragments were recovered by elution with 4 column volumes of elution buffer. Washed and adenine-bound fractions were pooled and collected for subsequent cloning. Sev-

**Isolation of RNA Aptamers—In vitro selection began with a pool of RNAs with a random sequence of 50 nucleotides flanked by two primer-binding regions. The RNAs in binding buffer were loaded onto the adenine-Sepharose column. The unbound or weakly bound RNAs were washed from the column. The RNAs that specifically bound to the adenine moiety were affinity-eluted with the binding buffer containing adenine. In the first four rounds, the amounts of RNA in the affinity-eluted fractions were too small to be detected either by spectrophotometry or by gel electrophoresis. In the fifth round, a significant amount of RNA was eluted. From the fifth selection round to the 12th and last selection round, fractions of the eluted RNAs were pooled and collected for subsequent cloning. Several individual clones were sequenced. Column washing volumes were increased from the 9th to the 12th selection round to recover RNA aptamers that bind adenine with a higher affinity. Eighteen different sequences were obtained after cloning (Fig. 2A).

The isolated RNA aptamers were classified according to their sequence similarities. As we can see in the sequence alignment (Fig. 2A), a group of aptamer sequences form a cluster in which...
At lower (pH < 4) or higher (pH > 10) values, RNA 12 E4 also shows the important role of the 6-NH$_2$ group in the interaction of adenine with the RNA 12 E4 aptamer. Such amino group effect is also found with 4-aminopyrimidine, indicating the contribution of hydrogen bonding to complex formation. This is probably why hypoxanthine, which is devoid of a 6-NH$_2$ group, has a low affinity compared with adenine. The amino group is even more effective when it is a secondary amine-NHCH$_3$ as found in 6-methylaminopurine. In this case (relative affinity to adenine is about 6), the methyl group brings an additional hydrophobic binding effect and/or a positioning effect of the molecule. However, the presence of an NH$_2$ group as in cytosine or 4-aminopyrimidine is not sufficient to provide good affinity; the purine ring is mandatory. The contribution of the purine ring of adenine to binding is low, although it is higher than that of the pyrimidine or of the imidazole moieties, in which binding alone could not be detected, in agreement with the lower hydrophobic stacking ability of each separated ring. Thus, the specificity should lie both in the structure of the overall purine ring, acting by hydrophobic stacking interactions, and in the 6-NH$_2$ group anchoring point, which increases the affinity by hydrogen bonding. The imidazole part of adenine must not be N9-substituted with a bulky group like ribose as seen in adenosine, which is not a good competitor (its ribose moiety probably sterically interferes with binding). It is interesting to note that adenine substitutions at position 8 have smaller effects; the addition of a 8-azido group increases the $K_d$ only to $28 \mu M$. The differences in RNA 12 E4 affinity between adenosine and 8-substituted adenine derivatives should be due to the differences in the position of the substitutions on the ring and/or to their volume. The 8-azido substitution is more significantly disruptive than the 8-thiol substitution, which should also be due to differences in their steric interferences and/or to the effects of the azido group on the electronic structure in the adenine ring. An additional hydrogen bond could also be formed with 8-thioadenine.

Characterization of the Adenine Binding Site—12 E4 RNA sequence was computed on the m-fold RNA folding server$^2$ to yield a secondary structure model (Fig. 4). As described above, a simple alignment of sequences reveals eight invariant nucleotides. They could interact directly with adenine, or they might also be involved in the binding site folding. 12 E4 RNA digestion patterns are shown in Fig. 5. They reveal that the adenine binding region is composed of two adjacent hairpin loops (hairpin loops I and II) easily attacked by single strand- or double strand-specific RNases. Three unpaired nucleotides of hairpin loop I (U31, U33, and G35) are clearly protected by adenine against single strand-specific RNase attack. They are most likely located in a loop and involved in binding with adenine, probably by a direct interaction, even if a structural stabilization effect cannot be excluded.

In addition, these two consecutive hairpin loops are flanked at their 3' side by a seven-unpaired nucleotide-long bulge (bulge III), which is also involved in adenine binding as will be discussed later in the text. As seen in Fig. 5, A–D, the cleavage pattern of each RNase is consistent with the fragment pattern observed after lead cleavage (Fig. 5E).

The minimum sequence requirements for the RNA 12 E4 aptamer were determined by boundary analysis experiments. 5' or 3' $^{32}$P-labeled RNA was subjected to partial alkaline hydrolysis, and the resulting random distribution of various length fragments was loaded on the adenine-Sepharose column. Truncated fragments able to bind adenine remained on the column, whereas the others were washed through the column.

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$^2$ On the Web at bioinfo.math.rpi.edu/~mfold/rna/form1.cgi.
umn. Their length was then obtained by denaturing polyacrylamide gel electrophoresis analysis. In the case of \( 5^{32}P \)-labeled fragments (Fig. 6A), all of the fragments of a length superior or equal to 45 nucleotides are able to bind the target. This means that critical sequence elements are located between the first and the 45th residue.

The distribution of \( 3^{32}P \)-labeled fragments that bound the adenine column (Fig. 6B) is slightly different. Fragments beginning with the 55th nucleotide from the 5\(^{32}P\) terminus are retained on the column. This indicates that the sequence requirements are located between the 55th and the 91st nucleotide from the 5\(^{32}P\) terminus. In addition, fragments of lengths varying from the 20th to the 30th nucleotide from the 5\(^{32}P\) terminus are no longer retained on the adenine column, suggesting that secondary or tertiary structures formed in the 5\(^{32}P\) end can modulate adenine binding. It is important to notice that the minimal sequence requirements for adenine binding from 5'- or 3'-labeled fragments do not overlap; this suggests that the adenine binding site is constituted of two relatively independent parts.

### Studies of Truncated and Mutated Versions of RNA 12 E4 Aptamer

When computed by the m-fold program, RNA 12 E4 [a] and RNA 12 E4 [b] sequences lead to the same secondary structure predictions as in the RNA 12 E4 sequence; they probably adopt the same folding. Their binding efficiency and their affinity constant values are also the same as RNA 12 E4. This means that the unpaired nucleotides of hairpin loop II are not involved in the interaction with the adenine target. RNA 12 E4 [46] and RNA 12 E4 [37] correspond to the minimal independent sequence elements determined by boundary analysis. Each truncated sequence alone is not able to bind the adenine target. By contrast, a 1:1 mixture of these two RNAs is retained on the adenine column with a binding efficiency of 40\%, and the value of the affinity constant is 45 \( \mu \)M. Considered with the boundary data, this indicates that the binding site requires sequence elements present in the two structures simultaneously and that the unpaired nucleotides of hairpin loop II is not needed, which is consistent with what was reported with RNA 12 E4 [a] and RNA 12 E4 [b]. RNA 12 E4 [40] is unable to bind the adenine target,

### Table I

**Affinity constants of adenine analogs**

\( K_{dc} \) is evaluated by the “radioligand displacement measurement” method with adenine 18 \( \mu \)M, RNA 18 or 27 \( \mu \)M, and competitor 13.5, 18, 36, 54, 90, 180, or 270 \( \mu \)M. Prior to the binding study, RNA 72 \( \mu \)M is renatured. 6-MAP, 6-methylaminopurine; Ade, adenine.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>Formula</th>
<th>( K_{dc} ) (( \mu )M)</th>
<th>Competitor relative affinity to adenine (( K_d^{Ade}/K_d^{*} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytosine</td>
<td><img src="image" alt="Cytosine" /></td>
<td>&gt;500</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Adenosine</td>
<td><img src="image" alt="Adenosine" /></td>
<td>&gt;500</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Imidazole</td>
<td><img src="image" alt="Imidazole" /></td>
<td>&gt;500</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Pyrimidine</td>
<td><img src="image" alt="Pyrimidine" /></td>
<td>&gt;500</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td><img src="image" alt="Hypoxanthine" /></td>
<td>470</td>
<td>0.02</td>
</tr>
<tr>
<td>4-Aminopyrimidine</td>
<td><img src="image" alt="4-Aminopyrimidine" /></td>
<td>470</td>
<td>0.02</td>
</tr>
<tr>
<td>Purine</td>
<td><img src="image" alt="Purine" /></td>
<td>330</td>
<td>0.03</td>
</tr>
<tr>
<td>8-Azidoadenine</td>
<td><img src="image" alt="8-Azidoadenine" /></td>
<td>110</td>
<td>0.09</td>
</tr>
<tr>
<td>8-Thiodenine</td>
<td><img src="image" alt="8-Thiodenine" /></td>
<td>28</td>
<td>0.35</td>
</tr>
<tr>
<td>Adenine</td>
<td><img src="image" alt="Adenine" /></td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>6-MAP</td>
<td><img src="image" alt="6-MAP" /></td>
<td>1.6</td>
<td>6</td>
</tr>
</tbody>
</table>
emphasizing the importance of bulge III for adenine recognition. RNA 12 E4 [47] has the same binding efficiency as RNA 12 E4, and its $K_d = 20 \mu M$ is close to that of the full-length version of RNA 12 E4. This indicates that key sequence elements required for adenine binding are supplied by both the hairpin-loop I and bulge III.

**DISCUSSION**

**Characterization of RNA 12 E4 Adenine Binding Site**—RNA 12 E4 shares eight invariant nucleotides with a cluster of related aptamers. These similarities must be because of a functional convergence, and these nucleotides are assumed to be critical for direct adenine binding or correct folding of the RNA adenine binding site. RNA 12 E4 invariant nucleotides are U31, A32, A36, A38, A39, A42, C44, and G46 (see Figs. 2, 4, and 5F). It appears that the RNA 12 E4 secondary structure is composed of two adjacent hairpin loops, which are largely exposed to the environment as they are easily cleaved by RNases. Three unpaired nucleotides (U31, U33, and G35) of the first loop (loop I) are clearly protected by adenine against single strand-specific RNase attack, either because of a structural

**FIG. 4. Secondary structures of RNA 12 E4 and its various truncated or mutated versions.**
stabilization induced by adenine or simply because they interact directly with the adenine target (Fig. 5).

The fact that RNA 12 E4 [40] is unable to bind adenine reveals that the recognition of the target by RNA 12 E4 is complex. Hairpin loop I, carrying the three adenine-protected unpaired nucleotides, and hairpin loop II are not sufficient for binding. Other sequence elements carried by RNA 12 E4 [47] are therefore required for efficient binding (Fig. 4). These elements are supplied by bulge III. These data are consistent with those obtained by boundary analysis experiments. They reveal that crucial 5’ sequence elements are located between the 5’ terminus of 12 E4 RNA and the 3’ end of hairpin loop I and that 3’ key sequence elements are located between the 3’ terminus of 12 E4 RNA and the 5’ end of the stem flanking the 5’ side of the bulge III (Figs. 4 and 6).

Taken together, RNase mapping experiments, lead cleavage, boundary determination analysis, and the study of truncated and mutated versions of 12 E4 RNA suggest that the adenine binding site is composed of two relatively independent secondary structure elements. The 5’ element is a hairpin loop that carries six unpaired nucleotides, three of which are protected by adenine against RNase attack, and the 3’ element is a seven-nucleotide-long bulge for which interactions with adenine have not yet been evidenced. These two RNA elements

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**FIG. 5. RNase and lead probing of RNA 12 E4 adenine binding site.** E4 aptamer was subjected to digestion by RNases T1, T2, A, and V1 or cleaved by lead acetate. RNase V1 cleaves RNA specifically in double-stranded regions, whereas RNases T1, T2, and A preferably cleave in single-stranded regions (RNase T1 cleaves downstream of G, RNase A cleaves downstream of pyrimidines). In each digestion experiment, a constant amount of RNA 12 E4 was digested during 20 min at room temperature by increasing amounts of each RNase (from 0 to 0.2 units/μg RNA for RNase T1, 0 to 0.02 units/μg RNA for RNase T2 and V1, and 0 to 20 units/μg RNA for RNase A). Lead cleavage studies were performed in the presence of 0 to 1 mM lead acetate during 1 h at room temperature. Then 10,000 dpm of each digestion reaction was deposited onto 10 or 15% denaturing polyacrylamide gels. After migration, labeled digestion patterns were revealed by autoradiography. H, alkaline-hydrolyzed RNA. RNase T1 digestion (A), RNase T2 digestion (B), RNase A digestion (C), RNase V1 digestion (D), lead cleavage (E), and RNase and lead mapping (F) of RNA 12 E4 aptamer are shown. Cleavage sites were obtained experimentally with RNases T1, T2, A, and V1 in the absence and presence of adenine, showing the protective effect of adenine against RNase cleavage. The eight invariant nucleotides mentioned in the text are underlined (F).
must constitute a bipartite RNA structure able to bind the adenine target. The way in which such a bipartite binding site recognizes adenine is not yet clearly understood. Do the two parts of the binding site pinch the adenine target, or do these two elements pinch themselves to form a structure that recognizes adenine as a whole? Even if this question remains unanswered, such a bipartite structure would therefore constitute a new mode of binding purine or adenylated compounds (20–22, 29–31). The RNA aptamer to adenine shows some differences from other purine aptamers, such as theophylline (21) or xanthine/guanine (XBA RNA) aptamers (22). The secondary structure of the RNA 12 E4 binding site, with a terminal hairpin loop and a bulge, differs from theophylline and XBA RNA aptamers, which have internal loop. The recognition mode is also different; the 6-NH₂ group of adenine plays an important role in the case of RNA 12 E4, whereas a 6-carbonyl group (in xanthine, guanine, or hypoxanthine) is important for XBA RNA aptamer recognition. Indeed, adenine does not bind to the XBA RNA aptamer (Kₐ = 2900 μM) as opposed to the RNA 12 E4 (Kₐ = 10 μM). On the other hand, RNA 12 E4 displays a low affinity for hypoxanthine (Kₐ = 470 μM), which is a target of XBA RNA aptamer.

Structural studies suggest that a terminal hairpin loop (hairpin loop I) with three unpaired bases, probably interacting with the target, and a seven-nucleotide-long bulge (bulge III) at the opposite side of hairpin loop I strongly contribute to the formation of the adenine binding site, which can be viewed as a kind of claw. Coupled with analyses of the RNA aptamer specificity, our studies suggest a novel mode of purine recognition with a bipartite site involving weak hydrophobic stacking interactions with the entire purine ring and stronger hydrogen bonding with the 6-NH₂ group of adenine.

This new purine binding mode is of great interest because the imidazole moiety is not trapped in the binding site and would easily be available for catalytic activity. This mode of adenine recognition should also be considered in relation to different binding modes of RNA aptamer motifs involved in adenylated compounds recognition (20, 29–31). These works have evolutionary implications, as adenine and adenylation compounds may have played crucial roles in early evolution. It has been shown recently that a single adenosine is involved for peptide bond formation within the ribosome (32) and that imidazole alone can rescue mutations in ribozymes (16). Adenine is itself a prebiotic analog of histidine, which is well represented at the active site of numerous enzymes (9), and cofactor-assisted ribozymes could thus be considered remnants of RNA world ribozymes handling exogenous small prebiotic reactive ligands as cofactors.

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