Aminoacyl-RNA Synthesis Catalyzed by an RNA

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An RNA has been selected that rapidly aminoacylates its 2′(3′) terminus when provided with phenylalanyl–adenosine monophosphate. That is, the RNA accelerates the same aminoacyl group transfer catalyzed by protein aminoacyl–transfer RNA synthetases. The best characterized RNA reaction requires both Mg^{2+} and Ca^{2+}. These results confirm a necessary prediction of the RNA world hypothesis and represent efficient RNA reaction ($\geq 10^5$ times accelerated) at a carbonyl carbon, exemplifying a little explored type of RNA catalysis.

The modern protein aminoacyl–transfer RNA (tRNA) synthetases (aaR's) catalyze two essential translational reactions. They first activate the carbonyl groups of amino acids by forming aminoacyl-adenylates [aa-AMP (adenosine monophosphate); the acid anhydride of the amino acid and the 5′ AMP phosphate]:

$$\text{aa} + \text{ATP} \rightarrow \text{aa-AMP} + \text{PP}_i \quad (1)$$

(ATP, adenosine triphosphate) Then enzyme-bound adenylate is used to esterify the 2′(3′) ribose terminus of a specific adapter RNA, transferring the aminoacyl group:

$$\text{aa-AMP} + \text{RNA} \rightarrow \text{aa-RNA} + \text{AMP} \quad (2)$$

Aminoacyl-RNA formed in reaction 2 donates its amino acid to the nascent peptide carboxyl at codons cognate to the adapter. We now show that a selected RNA can catalyze reaction 2.

Phenylalanyl-AMP was synthesized and purified by established methods (*1*), ending with preparative reversed-phase chromatography (Fig. 1). Because of slow aminoacyl migration from the 5′-phosphate of AMP to its 2′(3′) hydroxyls (*2*), the adenylate is necessarily contaminated with small amounts of 2′(3′)-*O*-aminoacyl esters of AMP, a potential alternative form of activated amino acid (Fig. 1). However, controls in which the unstable adenylate was quickly hydrolyzed before introduction of the RNA, leaving the more stable ribose esters of AMP, do not give the reactions below. Thus the source of the Phe group in what follows was the adenylate, Phe-AMP, not the minor but inevitable 2′(3′)-O-aminoacyl esters.

RNA and Phe-AMP react during selection and characterization at pH 7 and 0°C (*3*), with moderate concentrations of Na^+, K^+, and Mg^{2+}. Reaction components are otherwise unexceptional, except for inclusion of divalents such as Ca^{2+}, Mn^{2+}, and Zn^{2+} (see below).

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Selection and amplification was dependent on product trapping (Fig. 2), during which the small number of catalytic RNAs is covalently linked to the desired product, thereby making the catalyst selectable. Phe-RNAs appearing after the incubation of a pool of randomized RNA sequences [50 contiguous randomized nucleotides; 1.7×10^{14} distinct initial sequences (*3*)] with Phe-AMP were trapped and stabilized to hydrolysis (*4*) with a hydrophobic naphthoxyacetyl group. The α-amino group of the amino acid was coupled to naphthoxyacetic acid with the *N*-hydroxysuccinimide (NHS) ester of naphthoxyacetic acid (*5*) to provide the activated hydrophobe. A similar selection with only the NHS ester (not shown) did not yield reactive RNAs. Accordingly, this system should select aa-RNA; even highly varied RNAs in randomized pools of this size appear inert to NHS esters.

Reactive RNAs were resolved by means of a chromatographic system that responds strongly to addition of a large hydrophobic group to a macromolecular RNA (*6*). Porous C_4-glass high-performance liquid chromatography (HPLC) (Fig. 3A) is virtually indifferent to distinctions among normal RNAs (retention time, about 14.5 minutes), and even to the addition of a mildly hydrophobic group like Phe-, as exemplified by the position of Phe-$tRNA^{Phe}$ (yeast). However, when Phe-RNA was derivatized with naphthoxyacetyl-, the RNA (exemplified by naphthoxyacetyl-Phe-$tRNA^{Phe}$) eluted decisively later, near the positions of hydrophobic small molecules like naphthoxyacetate and its NHS ester (Fig. 3A). Therefore, the trailing material behind the main peak of RNA was pooled and again amplified by complementary DNA (cDNA) synthesis and the polymerase chain reaction (PCR) (*7*) through 11 cycles of exposure to Phe-AMP and naphthoxyacetylation. The hydrophobic RNA fraction in the initial pool (Fig. 3B) was compared with the RNA after 11 rounds of selection (Fig. 3C). The comparison was based on the two identically performed chromatograms, in which the entire pool was first fractionated and then the pooled "trail" (retention time, 17.5 to 25 minutes) was concentrated by ethanol precipitation and rechromatographed. Although some sequences in the initial random pool had an affinity for the column (triangles, Fig. 3B), a new, more discrete fraction with this affinity was prominent after selection (triangles, Fig. 3C).

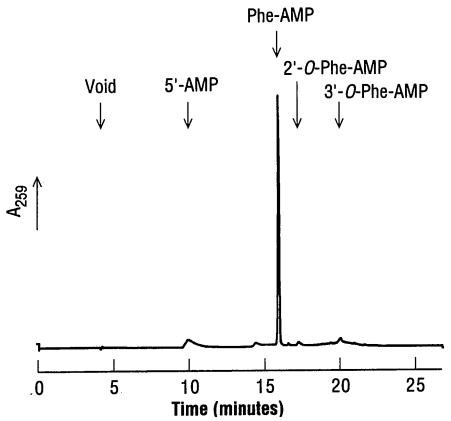

Fig. 1. Fractionation of purified Phe-AMP by analytical HPLC. The profile shows A_{259} after application of Phe-AMP to a C_4 porous glass column eluted with a gradient of acetonitrile and 0.1 M ammonium acetate, pH 4.5. Some impurities are marked.

The 17.5- to 25-minute trail from cycle 11 (triangles, Fig. 3C) was converted to cDNA, cloned, and sequenced by dideoxynucleotide extension (*7*). Of 42 sequences, 35 were each different from the other, 3 were a set of identical sequences, and 4 comprised sets of two. The three sets represent three single parental sequences because the variety in the original pool was grossly insufficient to contain sequences identical over 50 randomized nucleotides. Therefore RNA at cycle 11 was diverse in structure and in origin, with 42 sampled individuals derived from 38 initial molecules.

One in five clones, transcribed and tested individually by HPLC, was highly reactive with Phe-AMP. Isolate 29, which is characterized below and shown in Fig. 4, was one of the triplet of identical clones in the sequenced set.

Isolate 29's calculated stable secondary structure (Fig. 4) shows two hairpin domains with small bulge and loop defects. This distribution of loops, bulges, and helices is in excellent agreement with the RNA's kethoxal and lead susceptibilities; therefore the calculated secondary structure is realistic. The 3′ domain that bears the amino acid resembles the TΨ-CCA domain of a tRNA in some respects. But this sequence represents a single parent; accordingly, the relative importance of isolate 29's

substructures is not deducible from the original selection.

We have characterized the product of the reaction of phenylalanyl-adenylate and isolate 29 RNA transcript. The hydrophobic product from this singular transcript was clearer than for the heterogeneous pool (Fig. 5). In particular, the reactive fraction was evident in the first chromatogram, and did not require refractionation (Fig. 3). After longer incubation, more than 90 percent of isolate 29 RNA (gel-purified to contain only the transcript of canonical length) was recovered as product. Because the characterization experiments below gave similar data, they can be represented by the data from one of them. A periodate experiment was chosen (Fig. 5) and is described below.

Product formation required the reactions of Fig. 2, in the expected order. The hydrophobic product required successive exposure to Phe-AMP, then naphthoxyacetyl-NHS. Neither Phe-AMP reaction alone, nor naphthoxyacetyl-NHS alone, produced the new species. Most telling, successive exposure to naphthoxyacetyl-NHS, then to Phe-AMP in the absence of naphthoxyacetyl-NHS also failed to generate the product. It appears that (Fig. 2) RNA must first be acylated, then the aminoacyl group can be naphthoxyacetylated to produce the chromatographic shift.

The initial reaction product had the buffer sensitivity that is characteristic of aminoacyl esters of ribose (*4*). Mild alkaline hydrolysis after exposure to Phe-AMP (37°C for 1 hour, 0.2 M tris-HCl, pH 8) prevented appearance of the reaction product.

The initial product appeared to be subsequently converted to *N*-blocked aminoacyl-RNA, as we intended (Fig. 2). Exposure to 20 mM Cu^{2+} at pH 5.2 after exposure to Phe-AMP prevented formation of the product, but 20 mM Cu^{2+} was without effect if Phe-AMP had been followed by naphthoxyacetyl-NHS. This corresponds to the sensitivity of aa-RNA to Cu^{2+}-facilitated hydrolysis, and the expected resistance to the same treatment after the α-amino group was blocked (*8*).

Formation of the product required the guanosine terminal 2′(3′) ribose hydroxyls. No result thus far distinguished between internal (2′) aminoacylation and terminal

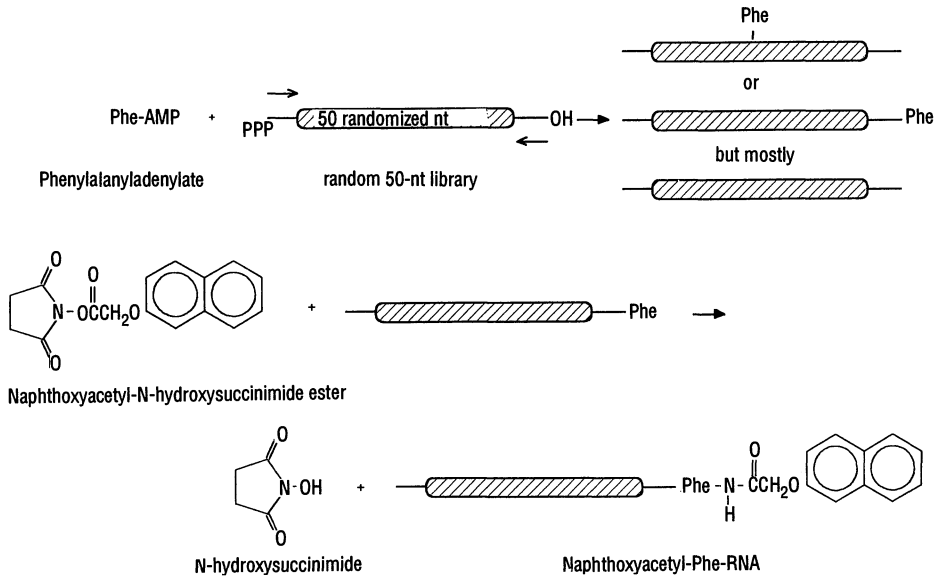

Fig. 2. Catalytic RNA selection by product trapping. A small number of hypothetical catalytic RNAs that can self-acylate is made more hydrophobic with a naphthoxyacetyl label.

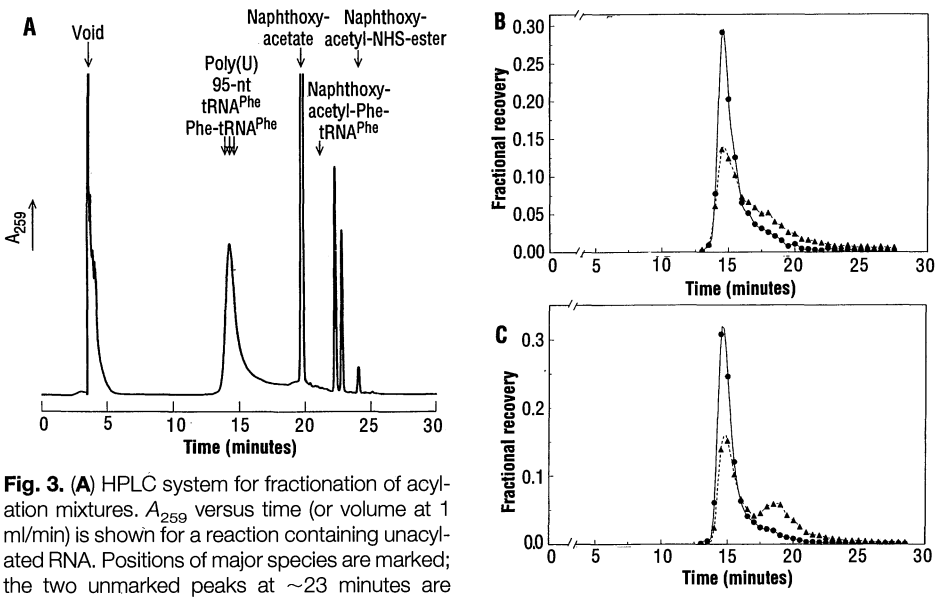

Fig. 3. (**A**) HPLC system for fractionation of acylation mixtures. A_{259} versus time (or volume at 1 ml/min) is shown for a reaction containing unacylated RNA. Positions of major species are marked; the two unmarked peaks at ~23 minutes are products of reaction between tris or its contaminants and naphthoxyacetyl-NHS. (**B**) Chromatography and rechromatography of RNA in randomized initial pool; solid lines and circles, fractionation of RNA reacted with phe-AMP, then naphthoxyacetyl-NHS; dotted line and triangles, refractionation of RNA eluting at 17.5 to 25 minutes. (**C**) Chromatography and rechromatography of RNA at cycle 11 of selection; solid line and circles, fractionation of RNA reacted with Phe-AMP, then naphthoxyacetyl-NHS; dotted line and triangles, refractionation of RNA eluting at 17.5 to 25 minutes.

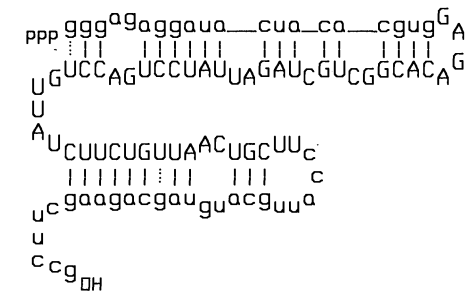

Fig. 4. Isolate 29 RNA, shown in the most stable secondary structure found by Mfold (*19*), and confirmed by chemical probing. Lowercase letters are fixed nucleotides, complementary to the PCR primers, and uppercase letters represent nucleotides originally randomized (*3*).

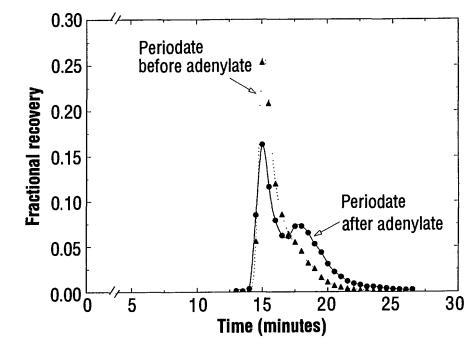

Fig. 5. Isolate 29 oxidized with periodate (*9*) before (triangles) or after (circles) reaction with Phe-AMP, then naphthoxyacetylated and fractionated by HPLC (*6*).

2′(3′) aminoacylation (*7*) (Fig. 1). However, periodate oxidation before exposure to Phe-AMP blocked product formation, whereas the same oxidation had no effect after exposure to Phe-AMP (Fig. 5). Thus the 2′(3′) *cis*-diol of the terminal ribose, which is oxidized very specifically by periodate (9), was required for formation of the chromatographically detected product. Once acylated, the 2′(3′) terminus would be blocked and resistant to subsequent periodate oxidation, as was observed. The 2′(3′) terminal was also essential by other criteria; transcripts extended by one arbitrary nucleotide (*10*) were not acylated, and isolate 29 of normal length, but 2′(3′) terminated by adenosine, was also inactive. Thus isolate 29 aminoacyl-RNA likely bore the aminoacyl at its 3′ terminus, as would a modern aminoacyl-tRNA.

Further confirmation of product structure was sought by synthesis of labeled adenylate, [^{3}H]Phe-AMP (*1*), and characterization of the product after digestion with P1 nuclease. The P1 nuclease releases 5′ mononucleotides from RNA, and would therefore release 2′(3′)-O-Phe–GMP (guanosine monophosphate) from a 3′ terminally acylated isolate 29 RNA (Fig. 4). Controls showed that this product was stable at low pH in P1 nuclease digests. Unlike tRNA, isolate 29 RNA has 3′ terminal G rather than A because, as indicated above, 2′(3′) aminoacyl esters of AMP were spontaneously formed by aminoacyl migration in Phe-AMP (*2*), which was present in reactions at very large molar excess over RNA. Because 2′(3′)-O-Phe-AMP esters were unavoidable contaminants in every reaction, reisolation of this nucleotide might not definitively establish the reaction product.

In order to compare the P1 product with a known standard, we prepared synthetic 2′(3′)-O-Phe-GMP by the method of Gottikh *et al.* and further purified it by HPLC (*4*). The ^{3}H of incorporated Phe, after P1 digestion of Phe-RNA, migrated as a single product on thin-layer chromatography (TLC) (Fig. 6), which is consistent with a single terminal site of aminoacylation.

The [^{3}H]Phe-containing P1 nuclease product had a mobility indistinguishable from synthetic 2′(3′)-O-Phe-GMP. The product was resolved from the potential contaminant 2′(3′)-O-Phe-AMP, [isolated by P1 digestion of PheRs-synthesized [^{3}H]Phe-tRNA^{Phe} (yeast)]. Some of the breadth of the product peak (Fig. 6) was likely due to the presence of both 2′ and 3′ phenylalanyl-esters of GMP; different positions of esterification could be resolved under acid conditions like these (*11*). The 2′ and 3′ esters may be formed directly or by rapid 2′(3′) aminoacyl migration at the pH of the aminoacylation reaction (*12*).

The P1 nuclease product from a [^{3}H]Phe-AMP reaction with isolate 29 RNA labeled by [α-^{32}P]GTP (guanosine triphosphate) during transcription is shown in Fig. 7. In this case, fractionation of the P1 nuclease digest demonstrated cochromatography of the ^{32}P of GMP and ^{3}H of phenylalanine, further supporting identification with 2′(3′)-O-Phe-GMP. In addition, the ^{32}P product peak contained 1.9 percent of recovered ^{32}P. A parallel HPLC experiment suggested that 50 percent of the input RNA had been converted to the hydrophobic RNA peak. Isolate 29 contained 24 G's (Fig. 4); therefore we can predict 2.1 percent (1/2 × 1/24) of ^{32}P in product. The P1 digest product therefore represented >0.9 mole per mole of HPLC product; thus the peak recovered here quantitatively accounts for the observed reaction.

Ribonuclease (RNase) A cuts after the 3′ phosphate of pyrimidine nucleotides. RNase A releases [^{3}H]Phe in a form chromatographically indistinguishable from Phe-guanosine (*13*). Thus Phe is unequivically 3′ terminal.

Finally, data in Fig. 8 confirmed that tritium released from isolate 29 [^{3}H]Phe-RNA by mild alkaline hydrolysis was chromatographically indistinguishable from phenylalanine, and, therefore is not (for example) Phe-Phe. Thus the selection (Fig. 1) identified an RNA reacting with adenylate, Phe-AMP, to give a product chemically similar to a biologically derived 2′(3′)-O-aminoacyl-tRNA.

The full complement of divalents in selection reactions (*2*) was not required for aminoacyl transfer. However, both Mg^{2+} and Ca^{2+} was required. The reaction stopped if calcium or magnesium were individually withdrawn. Aminoacylation was not restored if Mg^{2+} was increased from 10 to 15 mM in the absence of Ca^{2+}. However, the addition of 0.5 mM Ca^{2+} to 10 mM Mg^{2+} produced a virtually normal amount of product (in the presence of buffer and the monovalents) (*2*). We do not yet know whether the isolate 29 RNA reaction required the divalents in structural or catalytic roles or both. Nevertheless, the active complex may be termed a metallocatalyst, requiring both Mg^{2+} and Ca^{2+}, but not other divalent metals, for rapid aminoacyl transfer.

The aminoacylation of isolate 29 RNA was highly accelerated. Using the double HPLC criterion of Fig. 3, we detected no acylation of the initial pool. That is, controls (for example, without adenylate) were virtually indistinguishable from the full reaction shown in Fig. 3B. Estimating an upper limit for a hypothetical hydrophobic (trailing) peak, we find that after 2 hours at 0°C at an initial concentration of 11 mM Phe-AMP, $\leq 1.3 \times 10^{-3}$ of random RNA has reacted.

$$k_{random} = \frac{\text{fraction-reacted}}{\text{time [Phe} - \text{AMP]}}$$

$$\leq 1.0 \times 10^{-3}\ M^{-1}\ min^{-1}$$

The background rate is estimated as a second-order rate constant because isolate 29's reaction is approximately first order in both RNA and adenylate under our conditions. Lability of the adenylate and the ester product have been neglected. This slow rate is also consistent with previous experiments (*14*), showing that poly(U) and poly(A) are unreactive with phe-AMP.

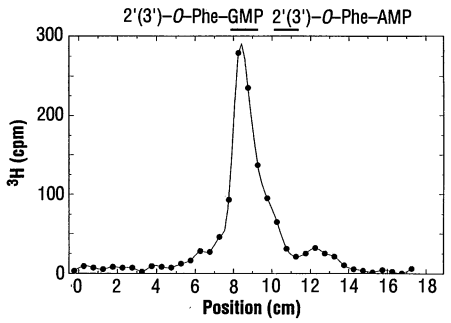

Fig. 6. TLC (*29*) characterization of ^{3}H from a P1 nuclease digest (*31*) of [^{3}H]Phe-RNA from reaction of isolate 29 transcript RNA and Phe-AMP. At the top of the chromatogram, the position of absorbance or radioisotope (of marker nucleotides and derivatives) or ninhydrin color (for marker amino acids and peptides) is shown by labeled bars.

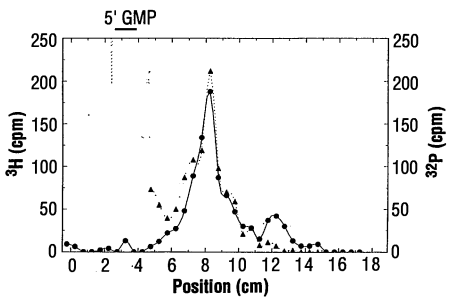

Fig. 7. TLC characterization of ^{3}H (circles) and ^{32}P (triangles) from P1 nuclease digest of [^{3}H]Phe-[α-^{32}P]G-RNA.

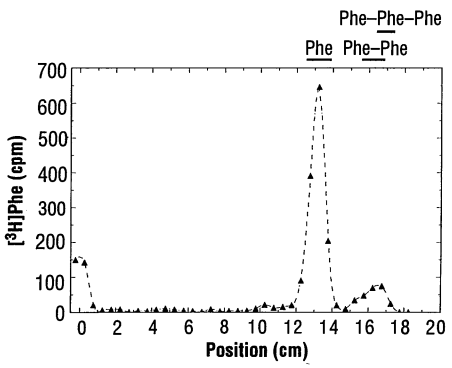

Fig. 8. TLC characterization of ^{3}H released by mild alkaline hydrolysis (*32*) of [^{3}H]Phe-RNA.

We have observed 50 percent reaction of isolate 29 RNA in 2 minutes at 0°C with 2 mM Phe-AMP.

$$k_{\text{isolate 29}} = \frac{1}{(\text{Phe} - \text{AMP})t_{1/2}} = \frac{1}{(0.002)2} = 2.5 \times 10^2\ \text{M}^{-1}\ \text{min}^{-1}$$

By comparison with initial randomized RNA, the isolate 29 RNA reaction was approximately 250,000 times or more accelerated.

Therefore an RNA catalyzes the aminoacyl group transfer reaction which comprises the second step of the protein aaRs reaction. The RNA selected here (Fig. 4) is a good catalyst (*15*), in the sense that it rapidly becomes acylated under "ordinary" conditions and concentrations of components. However, we have not shown whether it is an enzyme (cyclically forms a Michaelis complex).

There seem to be four salient implications. First, this makes accessible an unexplored type of RNA catalyst. Plausibly, the RNA facilitates attack of a terminal RNA ribose alkoxide on the activated carbonyl of Phe-AMP. Thus these RNAs potentially provide a forum for study of RNA-activated nucleophilic attack at a carbonyl carbon. This might reveal new RNA catalytic facilities, hinted at by the work of Piccirilli *et al.* (*16*) on hydrolysis of an aminoacyl oligonucleotide, and Noller *et al.* (*17*) on peptidyl transfer, but not previously available in an efficient, simple form.

A second point concerns the requirement for Ca^{2+}. It has been argued that great catalytic variety is accessible to RNAs via the varied chemistry of associated divalents (*18*). An uncharacterized, but essential function for Ca^{2+}, not fulfilled by Mg^{2+} (or by low concentrations of Cu^{2+}, Mn^{2+}, or Zn^{2+}) supports this idea. A testable speculation on the mechanism may be based on the known ability of Ca^{2+} to use ribose hydroxyl oxygens as direct ligands (*19*). Such Ca^{2+} binding could activate the ribose hydroxyl groups by helping to dissociate a hydrogen ion. Whatever the specific role of Ca^{2+}, these results also argue for use of a variety of ions in RNA selections.

The third implication is evolutionary. Aminoacyl adenylates and aminoacyl-RNAs are universal translational intermediates in modern organisms. Therefore they are ancient, probably dating to the last common ancestor of all life on earth, or before. On the hypothesis that modern biology derives by succession from an earlier RNA world (*20*), in which RNA was the predominant catalyst, it is necessarily predicted that RNA be able to catalyze some form of translation, so as to give rise to coded peptides. The rise of specific peptides as catalysts and structural elements is, in fact, the event which marks the logical gateway from the RNA world to the current nucleoprotein world. The current finding, that an RNA can efficiently catalyze a universal step in modern translation, is therefore a step toward an experimental demonstration that the gateway exists.

Amino acid activation (step 1 of the aaR's reaction) is now an immediate goal. In this connection, the group 1 active center has a discriminating site for the splicing cosubstrate guanosine (*21*), and much smaller RNAs have been selected that selectively bind ATP (*22*) and guanosine diphosphate (*23*). RNAs also bind and distinguish the side chains and enantiomorphs of both polar amino acids like arginine (*24*) and nonpolar ones like valine (*25*). RNAs therefore bind both substrates for amino acid activation. Protein aaR's apparently catalyze activation (aminoacyl-AMP synthesis) by entropic means, binding and apposing ATP and the amino acid so as to favor their reaction, rather than by more sophisticated catalytic devices (*26*). It seems likely that RNA, for which binding of both nucleotides and amino acids is already established, will be able to activate amino acids by this same strategy. Therefore a complete RNA aaR's now appears conceivable.

Finally, peptidyl transferase and isolate 29 RNA probably have similar transition states, because peptidyl transferase is inhibited by a transition state analog based on a tetrahedral, polar carbonyl (*27*). Because this work suggests that such a transition state can be hosted by an RNA, it supports the idea (*17*) that RNA can also catalyze peptide synthesis. The evolution of translation, from the origin of the simplest genetic code (*28*) through the advent of aminoacyl-RNAs and peptidyl transferase may therefore require only currently demonstrable RNA activities.

REFERENCES AND NOTES

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3. For the RNA pool, selected RNAs were T7 RNA polymerase transcripts (*10*) of: 5′-CGG AAG CTT CTG CTA CAT GCA ATG G - N_{50} - CAC GTG TAG TAT CCT CTC CC ≪ T ATA GTG AGT CGT ATT AGA ATT CGC-3′. N implies equimolar nucleotides and ≪ the transcript start. For renaturation, 4.7 × 10^{14} molecules of gel-purified 95-nucleotide (nt) transcript RNA (from 1.7 × 10^{14} independently synthesized DNA templates) having a central tract of 50 randomized positions were heated at 65°C in 20 μl of 1 mM Mg^{2+} for 3 minutes, then quenched in ice. For the reaction with adenylate during selection, a salt-and-buffer mixture was added containing 100 mM Hepes (final pH about 7), 20 mM potassium acetate, 200 mM NaCl, 5 mM $CaCl_2$, 10 μM $CuSO_4$, 2 μM $FeCl_3$, 10 mM $MgCl_2$, 100 μM $MnCl_2$, and 5 μM $ZnCl_2$ in a final volume of 50 μl, with 6 to 8 mM Phe-AMP (*1*) present initially. During acylation Cu^{2+}, provided for possible utilization in an RNA structure (*19*), is far below that used to hydrolyze aminoacyl-RNA (*8*). For selections, incubation was continued on ice for 30 minutes, then at room temperature for 15 minutes. The reaction was stopped by the addition of sodium acetate (pH 5.2) to 300 mM in 150 μl, then two volumes of ethanol, then centrifugation. The reaction during characterization was as described, except for 10 mM $MgCl_2$ and 5 mM $CaCl_2$ (no other divalent metals), 2 mM initial Phe-AMP, 100 μM EDTA, and 0.5 to 2 μg of gel-purified isolate 29 RNA at 0°C. Products were characterized when about 50 percent of the RNA appeared acylated by HPLC.
4. B. P. Gottikh, A. A. Krayevsky, N. B. Tarussova, P. P. Purygin, T. L. Tsilevich, *Tetrahedron* **26**, 4419 (1970). For the synthesis of Phe-2′(3′)-*O*-GMP, a modification of the above was used; 375 μmol of carbonyldiimidazole (Sigma) and tBOC-L-phenylalanine (Sigma) were dissolved in dry tetrahydrofuran. After 10 minutes at 23°C, this mixture was added to 20 μmol of disodium 5′ GMP in 500 μl of acetonitrile and H_2O (1:4 by vol). After 20 minutes, this was poured into 15 ml of acetone at 20°C and centrifuged; the sedimented solids were dried under vacuum. The phenylalanine was deprotected by dissolving in 500 μl of trifluoroacetic acid (Fluka), and rapidly removing the trifluoroacetic acid at reduced pressure. The deprotected product was first purified by preparative TLC (*30*). An apparently uniform band in about 16 percent yield (R_F = 0.49) was eluted in water. However, the water-eluted TLC band was resolved into two materials by HPLC on C_{18} glass (Rainin 80-225-C5) in a water-acetonitrile gradient. One (70 percent by A_{260}) was the desired product Phe-2′(3′)-*O*-GMP, which had a spectrum like that of 5′-GMP and was resistant to periodate until treated with mild alkali. The unexpected product (30 percent) was sensitive to periodate, and resistant to mild alkali. On the basis of these properties and a new absorbtion peak at 275 nm, the contaminant may have been aminoacylated at the G base.
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6. For HPLC, centrifuged RNA products were redissolved in 200 μl of 100 mM ammonium acetate, pH 4.5, containing 50 percent (v/v) DMSO. This material was filtered and injected into a two-pump Waters chromatograph with a 481 absorbance monitor, and an electronic gradient controller. A C_4 porous glass column (Rainin Dynamax-300A; 4.6 × 250 mm) was eluted for 2 minutes with 100 mM ammonium acetate, pH 4.5, then with a 1 hour exponential gradient (Waters profile 3) at 1 ml/min consisting of 100 per-

cent 100 mM ammonium acetate, pH 4.5, to 52 percent ammonium acetate and 48 percent acetonitrile (Mallinckrodt HPLC grade). Underivatized RNA elutes in about 31 percent acetonitrile at 23°C.
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31. P1 nuclease digests were conducted with about 1 to 2 μg of RNA and 4 units of P1 nuclease (Bethesda Research Labs) in 10 μl of 30 mM sodium acetate, pH 5.2 at 23°C for 15 minutes
32. Hydrolysis utilized 50 mM Na_2CO_3 buffered at pH 10 for 15 minutes at 37°C.
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