RNA enzymes with two small-molecule substrates

Faqing Huang*, Zhili Yang and Michael Yarus

Background: The 'RNA world' hypothesis posits ancient organisms employing versatile catalysis by RNAs. In particular, such a metabolism would have required RNA catalysts that join small molecules. Such anabolic reactions now occur very widely, for example in phospholipid, terpene, amino acid and nucleotide synthetic pathways in modern organisms. Present RNA systems, however, do not perform such reactions using substrates that do not base pair. Here we ask whether this lack is a methodological artifact due to the practice of selection–amplification, or a fundamental property of active sites reconstructed within RNA structures.

Results: Three rationally modified RNA enzymes, Iso6-G, Iso6-2G and Iso6-3G, catalyze the formation of (5′→5′) polyphosphate-linked oligonucleotides *in trans*. One of these, Iso6-G RNA, has a specific substrate site for a guanosine triphosphate, GTP, dGTP or ddGTP, and one nonspecific substrate site for a terminal-phosphate-containing small molecule. This ribozyme catalyzes multiple turnovers, proceeding at a constant rate. Guanosine specificity is probably not attributable to Watson–Crick base pairing.

Conclusions: Ribozymes can readily bind multiple small-molecule substrates simultaneously and catalyze reactions that build up larger products, apparently independent of substrate–RNA Watson–Crick base pairing. RNA enzymes therefore parallel proteins, which often overcome the entropic difficulties of positioning multiple small substrates for catalysis of anabolic reactions. These results support the idea of a complex ancestral metabolism based on RNA catalysis.

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Introduction

All organisms rely on catalysts to carry out the impressive variety of reactions required for metabolism. In a hypothetical 'RNA world' such catalysts would have been ribozymes. Because a hallmark of biological metabolism is the production of complex molecules from smaller precursors, many indispensible catalytic steps are those of molecule building or anabolism (e.g. syntheses of amino acids, nucleotides, saccharides and lipids from simpler carbon, nitrogen and phosphate-containing compounds). Thus proteins frequently bind two or more small-molecule substrates and catalyze reactions between them.

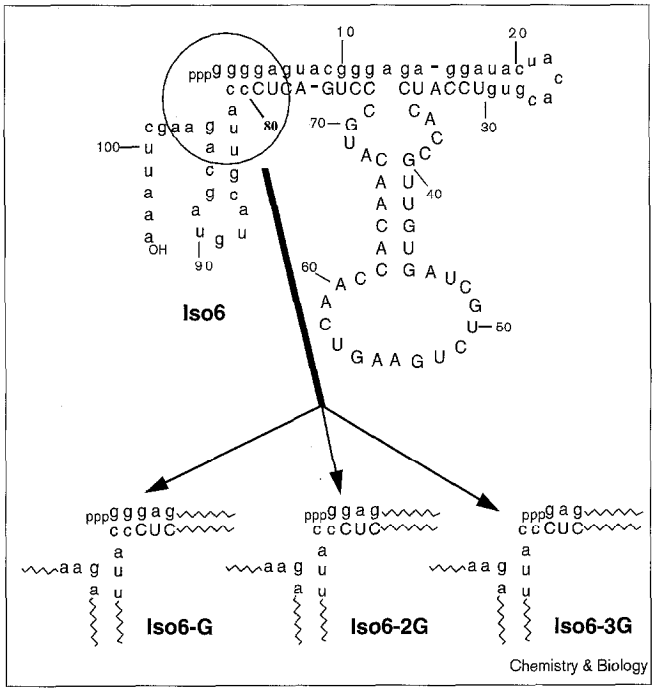
It is notable, therefore, that ribozymes isolated thus far do not perform small-molecule bisubstrate reactions. In fact, even single ribozyme substrates are often bound through attachment to an oligonucleotide with subsequent base-pairing [1–7]. Base-pairing simplifies the need for substrate-binding sites by exploiting a salient property of nucleic acids that automatically supplies strong binding and specific substrate positioning. Because an 'RNA world' [8–10] surely required molecule-building, it is relevant to ask the reasons for the absence of small-molecule-bisubstrate RNA enzymes.

On the one hand, the absence of such RNA enzymes might be an artifact. Present methods for isolating catalytic activities, such as selection–amplification [11–13], require that the RNA catalyst modify itself in order to confer a new molecular property on the catalyst; the new property can then be used to purify it from the majority of inactive molecules. There has been, therefore, an artificial concentration on simplified RNA systems, in which the substrate–catalyst and product–catalyst are the same molecule.

On the other hand, it is also true that bringing three molecular elements together in space is a more difficult feat than the assembly of a simpler complex. When two small-molecule substrates and a set of catalytic groups converge, additional entropic costs must be paid, while preserving relative positions to atomic dimensions in order to permit rapid reaction.

A nontrivial question therefore arises: can an active center composed of RNA act anabolically on small-molecule bisubstrates? We now construct such RNA catalysts by elaborating a unisubstrate ribozyme [14]. We first construct three ribozymes that act on substrates *in trans*. One of these, as predicted from what is known of the parental

Figure 1



Conversion of a self-modifying capping ribozyme (Iso6) into a series of *trans*-acting ribozymes (Iso6-G, Iso6-2G and Iso6-3G). To construct *trans*-active ribozymes, the reaction center (the 5′-terminal α-phosphate) of Iso6 was removed systematically along with different numbers of guanosine nucleotides from the 5′ terminus. Supplementing these 5′-shortened RNAs with the corresponding 5′-triphosphorylated guanosine oligonucleotides reconstitutes the original Iso6 but with an exchangeable reaction center, thereby conferring the capacity for multiple turnovers.

ribozyme, performs multiple turnover reactions on two free nucleotides, most likely without employing Watson–Crick base pairing to fix free substrates at the active site.

The previously selected self-capping RNA, Iso6 [14], has demonstrated varied catalytic activities such as general phosphoryl-coupling, pyrophosphatase, decapping and cap-exchange activities, associated with a single reaction center incorporating its 5′-terminal α-phosphate [15,16]:

$$\text{Nu} + \text{Rpp-}\mathbf{p}\text{RNA} \rightarrow \text{Rpp} + \text{Nu-}\mathbf{p}\text{RNA} \qquad (1)$$

where Nu is water and/or a terminal phosphate-containing molecule, and Rpp- is a pyrophosphate, triphosphate, nucleoside 5′-diphosphate, nucleoside 5′-triphosphate or nucleoside 5′-tetraphosphate. Varying Nu and Rpp-, equation 1 describes all catalytic activities of Iso6 RNA.

In this unusual reaction center, the first, reactive nucleotide (GTP) is not base-paired in the apparent secondary structure (Figure 1). In addition, the first guanine is required for all activities (if the 5′ GTP is replaced by ATP, all activities are lost; F.H. and M.Y., unpublished observations). Finally, guanosine and its nucleotides at millimolar concentrations inhibit Iso6 activities, whereas adenosine and its nucleotides have no observable effects [17]. Based on these observations, we propose a specific guanosine-binding site, usually filled by the 5′ G, and in addition a nonspecific site for an attacking nucleophile [17]. Suppose now that the linkage between the 5′-terminal GTP and the second nucleotide of Iso6 RNA were severed: it is plausible that the remaining RNA would be able to catalyze multiple turnovers using free GTP.

Results

Construction of RNA enzymes

We constructed several 5′-shortened versions of the Iso6 ribozyme designated Iso6-G (Iso6 RNA minus the 5′ GTP), Iso6-2G (lacking two Gs), and Iso6-3G (lacking three Gs), by changing the transcription template (Figure 1). We presumed that these ribozymes would be active with free pppG, pppGpG and pppGpGpG as substrates, respectively.

Preparation of substrates

Some of these prospective substrates are unusual, but were available as products of abortive transcription. If a short dsDNA template is transcribed by T7 RNA polymerase either with all triphosphates or GTP alone, an evident series of triphosphate-terminated oligoriboguanosines is generated (using [α-^{32}P]GTP as the label, Figure 2a), even though only some of the residues are templated. From such a ladder, we eluted a series of oligomers: pppGpG, pppGpGpG, pppGpGpGpG and pppGpGpGpGpG.

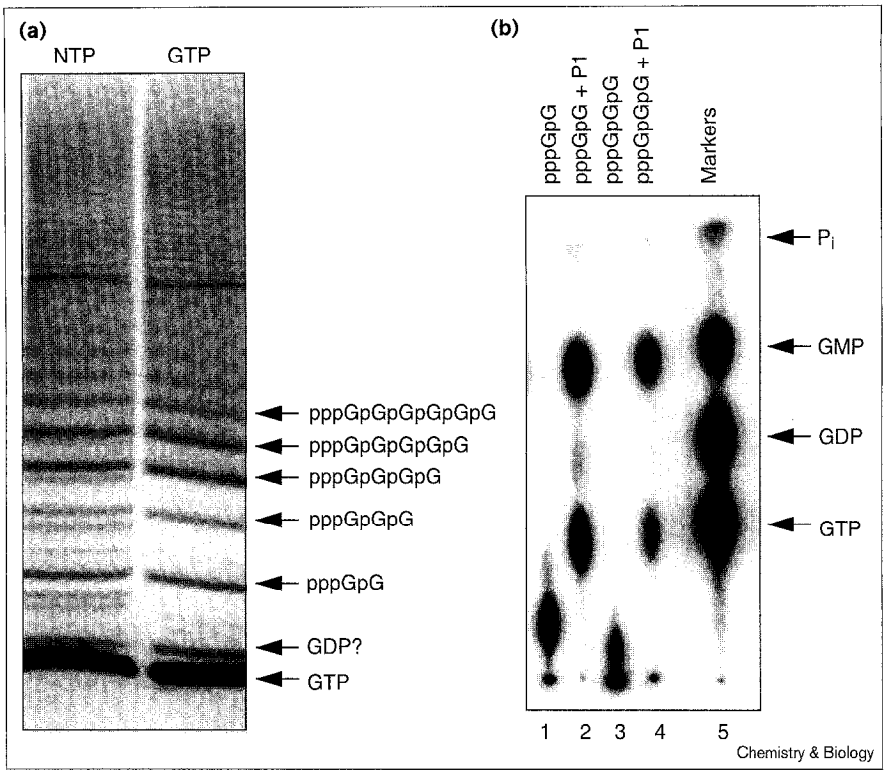
Figure 2b shows how the structures of these compounds were confirmed using nuclease P1, which cleaves every phosphodiester bond between the 3′ sugar of a nucleotide and the 5′ phosphate of the next nucleotide. After purification by polyacrylamide gel electrophoresis (PAGE), P1-digested pppGpG (G2) yields equimolar products migrating with GTP and GMP markers, as expected. Digestion of putative pppGpGpG (G3) yields two moles GMP for each mole of GTP (Figure 2b); a similar trend is observed for other members of the series.

RNA enzyme-catalyzed reactions

Figure 3a pairs substrates of different size with RNAs that have different sized 5′ deletions. In each set of five lanes, GDP alone or GDP plus one of the oligo Gs was incubated with the Iso6-G, Iso6-2G, Iso6-3G or normal Iso6 ribozymes (thereby all 5′ triphosphorylated; see below).

Complementarity between the reactants and the RNA catalysts is striking. For example, when pppGpG is used as a substrate, product (above background levels) only occurs when the Iso6-2G ribozyme is used, which might be expected to provide a complementary substrate site.

Figure 2



Preparation of substrates (5′ triphosphorylated guanosine oligonucleotides) for the RNA enzymes Iso6-2G and Is6-3G. **(a)** T7 transcription of a double-stranded DNA template containing T7 promoter and only CCC as the coding sequence, in the presence of four nucleoside triphosphates (NTP) or GTP alone. The label used was [α-^{32}P] GTP. All transcription products were separated by 20% denaturing PAGE. **(b)** Identification of transcription products. Purified products from (a) were treated with nuclease P1 (lanes 2 and 4), along with untreated samples (lanes 1 and 3) and standard markers (lane 5), and chromatographed on a polyethyleneimine (PEI) thin layer chromatography (TLC) plate, which was developed in 0.5 M potassium phosphate, pH 6.5, 20% MeOH.

Similarly, the G3 product occurs only as a result of Iso6-3G catalysis. G4 and G5 contain extra guanosines and do not match the potential substrate sites of Iso-2G or Iso6-3G, and no products are formed. Iso6 RNA has its own endogenous 5′ guanosines and is unable to react measurably with any pppG(pG)$_n$ substrate *in trans*.

Figure 3b further explores the requirements for the successful reaction of matched oligomers and ribozymes. On the left in Figure 3b (lanes 1–11) three reactions are shown, two of these RNA catalyzed. Metal-catalyzed (i.e., nonRNA-catalyzed, using metals in the reaction cocktail) hydrolysis of some pppGpG to ppGpG is evident (compare lanes 1 and 2, and below). This nonenzymatic reaction also doubles the substrate bands in Figure 3a. The RNA-catalyzed reaction of GDP with G2 is shown in lane 7:

$$G(5')pp + pppGpG \xrightarrow{\text{Iso6-2G}} G(5')pppGpG + PP_i \quad (2)$$

Because substantial reaction occurs between Iso6-2G and G2 requires GDP (compare lane 7 with lane 6) it is therefore mostly capping *in trans* (equation 2) with a minority of hydrolysis *in trans*:

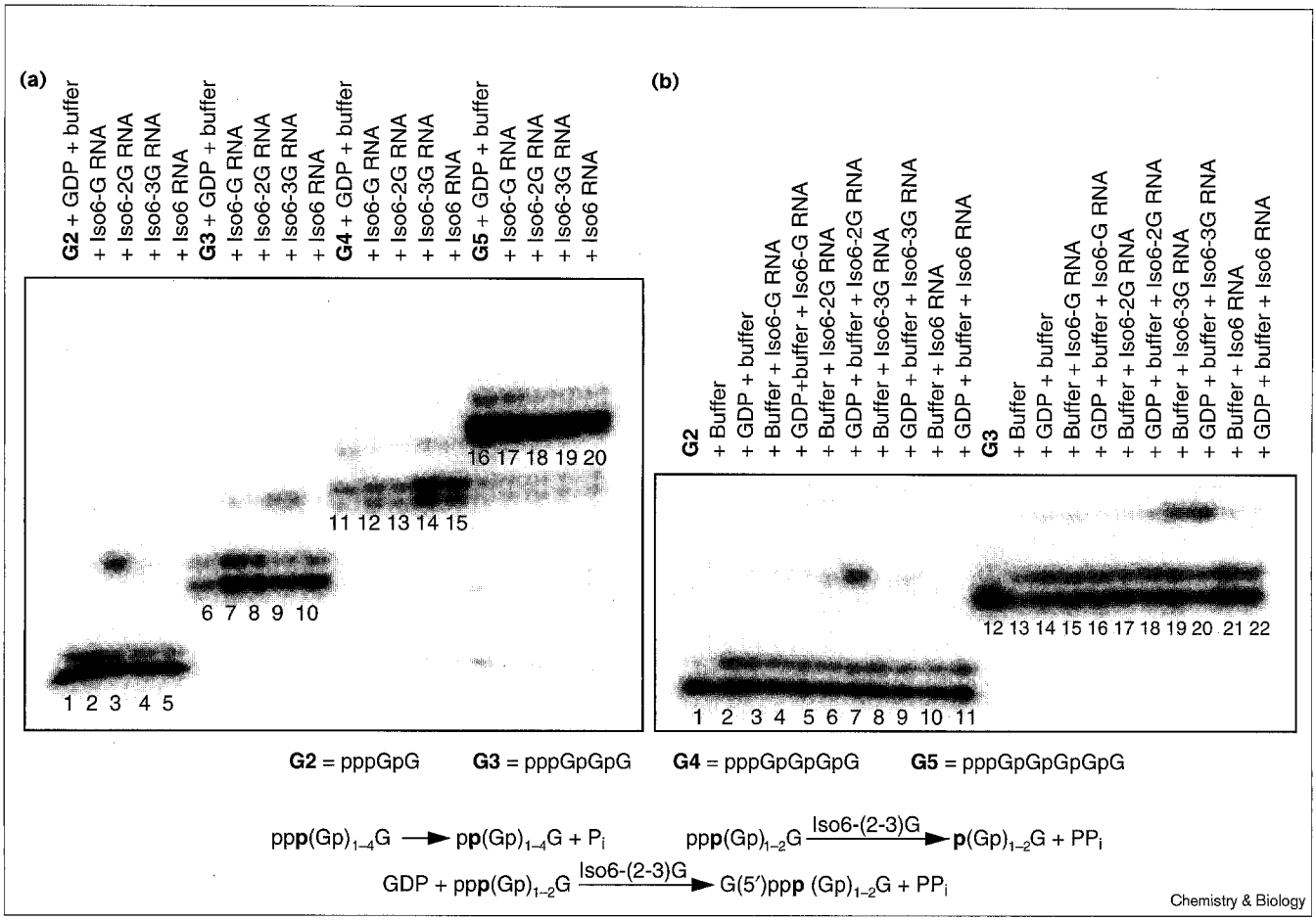
$$pppGpG \xrightarrow{\text{Iso6-2G}} pGpG + PP_i \quad (3)$$

Both these RNA-catalyzed reactions are seen again in the reactions of G3 with Iso6-3G (Figure 3b, lanes 19 and 20). Capping of G3 is of the same order (~30% slower) for G2 and Iso6-2G. In these electrophoretic gel fractionations, pGpG and G(5′)pppGpG comigrate, as do pGpGpG and G(5′)pppGpGpG (see data below).

Figure 3b somewhat overemphasizes the hydrolysis reaction (lane 19) of G3 + Iso6-3G because of elevated input to lane 19 relative to lane 20; in other data, hydrolysis of G2 by Iso6-2G and G3 by Iso6-3G are comparable.

Some of the data supporting these statements about product structures are shown in Figures 4 (G3) and 5 (G2). In Figure 4, nuclease P1 digests fractionated on TLC are shown versus markers. Lanes 1 and 2 (Figure 4a) show that the lowest band in lanes 12–22 of Figure 3b is pppGpGpG, as expected. The product created by incubation without RNA is shown in lanes 3 and 4, where the loss of phosphate to form ppGpGpG is confirmed. The RNA product that does not require GDP (lane 19, Figure 3b) is analyzed in lanes 5 and 6 (Figure 4a), and is the product of catalytic hydrolysis with release of PP_i, pGpGpG. When GDP, a triphosphorylated nucleotide and a reactive RNA are present (lane 20, Figure 3b) their product yields GMP plus cap on P1 nuclease digestion (lanes 7 and 8, Figure 4b) as expected for G(5′)pppGpGpG. The cap marker G(5′)pppG, prepared

Figure 3



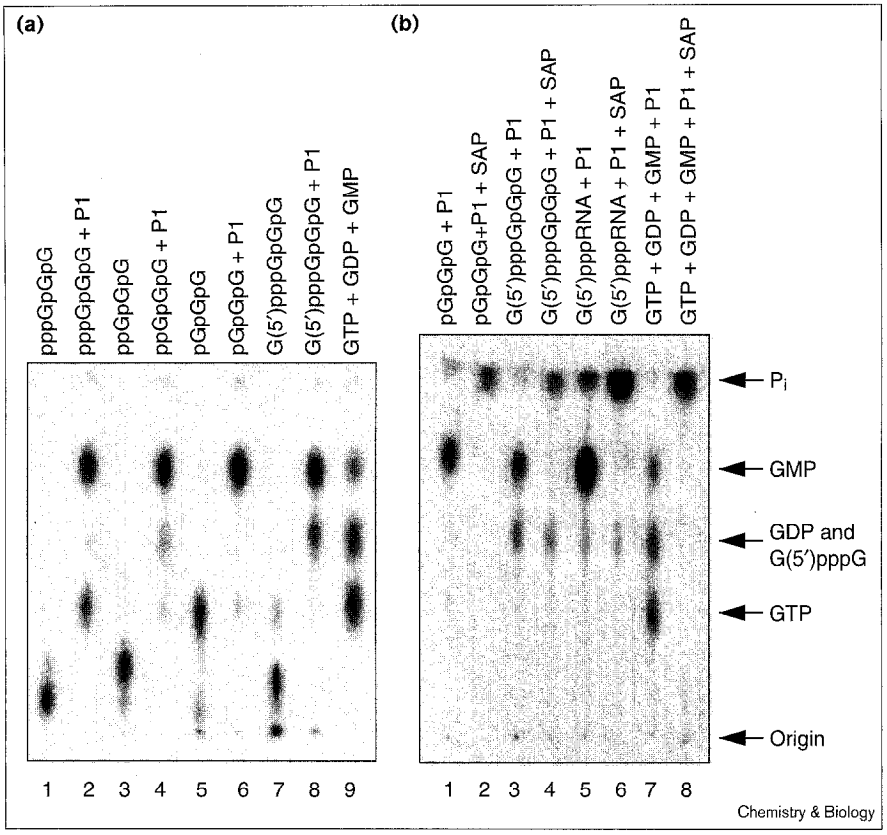
Substrate specificity of shortened RNA enzymes. **(a)** RNA enzyme-catalyzed reactions of the 5′-triphosphosphorylated guanosine oligonucleotides (designated as G2–G4, [α-^{32}P]GTP-labeled) with GDP. The first lane in each set (lanes 1, 6, 11 and 16) was a control under the same reaction conditions. With substrate–enzyme pairs of G2–Iso6-2G and G3–Iso6-3G, three bands were separated by 20% denaturing PAGE. The bottom bands in each set represent starting substrates. The middle bands represent uncatalyzed hydrolysis products, 5′-diphosphorylated guanosine oligonucleotides (see Figure 4). The top bands represent catalyzed reaction products (lanes 3 and 9; see Figure 4). With G3 and G4 as substrates, no catalyzed products were observed using any of the RNA enzymes. **(b)** Catalyzed reactions of substrates G2 and G3 by RNA enzymes Iso6-2G and Iso6-3G. Lanes 1 and 12 are standard RNA substrates, pppGpG and pppGpGpG, without incubation. As shown by 20% denaturing PAGE, G2 reactions are catalyzed only by Iso6-2G (lanes 6 and 7), and the same relationship exists for G3 and Iso6-3G (lanes 19 and 20). Without GDP, pyrophosphate release is catalyzed to produce 5′-monophosphate of guanosine oligonucleotides (lanes 6 and 19), which migrate at the same rates as capped products under the PAGE conditions (see Figure 4).

by nuclease P1 digestion after reaction of GDP with Iso6 RNA [14], is shown in lanes 5 and 6 of Figure 4b. The legend of Figure 4 describes the origin of each of the samples whose structure is characterized.

Caps have internal phosphates, uniquely resistant to phosphatase. Reaction with shrimp alkaline phosphatase (SAP) allows further structural confirmation of capped products (Figure 4b). For example, controls such as the catalytic hydrolysis product pGpGpG digested with P1 nuclease yields all GMPs, which are then converted completely to free phosphate by SAP (lanes 1 and 2, Figure 4b). The same is true of the control mixtures consisting of GMP, GDP and GTP (lanes 7 and 8; Figure 4b). In contrast, *bona fide* caps from full-sized Iso6 capped with GDP (lanes 5 and 6; Figure 4b), or the product of capping *in trans* (lanes 3 and 4; Figure 4b) give SAP-resistant phosphate that migrates near GDP, which can be identified with G(5′)pppG by comparison with the known capped product [14] from the complete RNA (lane 6; Figure 4b).

The conclusions just drawn for the reaction of G3 with Iso6-3G also follow for the reaction of G2 with Iso6-2G *in trans*. Figure 5 summarizes some data which parallels that described just above: Iso6-2G in the presence of

Figure 4



Identification of RNA enzyme-catalyzed reaction products from Figure 3 using TLC under the same conditions as in Figure 2. **(a)** Digestion of G3 and its reaction products (isolated from 20% denaturing gels in Figure 3b) by nuclease P1. pppGpGpG, starting substrate (bottom band in Figure 3b, lane 12); ppGpGpG, uncatalyzed hydrolysis product (middle band in Figure 3b, lanes 13–22); pGpGpG, catalyzed hydrolysis product in the absence of GDP (top band in Figure 3b, lane 19); G(5′)pppGpGpG, catalyzed capping product in the presence of GDP (top band in Figure 3b, lane 20). Markers (lane 9) were prepared by hydrolysis of [α-^{32}P]GTP for 2 h at 95°C. **(b)** Differentiation of RNA-catalyzed hydrolysis products versus capping products (isolated from 20% denaturing gels in Figure 3b, lanes 19 and 20). Although migrating at the same rate on 20% denaturing gels (Figure 3b, lanes 19 and 20), digestion by nuclease P1 and shrimp alkaline phosphatase (SAP) gave different products, from which the hydrolysis and capping products can be distinguished. G(5′)pppRNA (lane 5) was prepared from reaction of GMP with Iso6 RNA [14].

GDP simultaneously releases PP_i by hydrolyzing pppGpG (leaving pGpG) and caps pppGpG to give G(5′)pppGpG. Although pGpG and G(5′)pppGpG migrate at the same rate under PAGE conditions, they can be differentiated by SAP digestion (Figure 5). These reactions with free small molecules parallel the reactions of the 5′ triphosphate in the intact Iso6 RNA [14], and therefore preserve the original reactions with HOH and phosphate nucleophiles, although now the reactions occur *in trans*.

Two small-molecule substrates

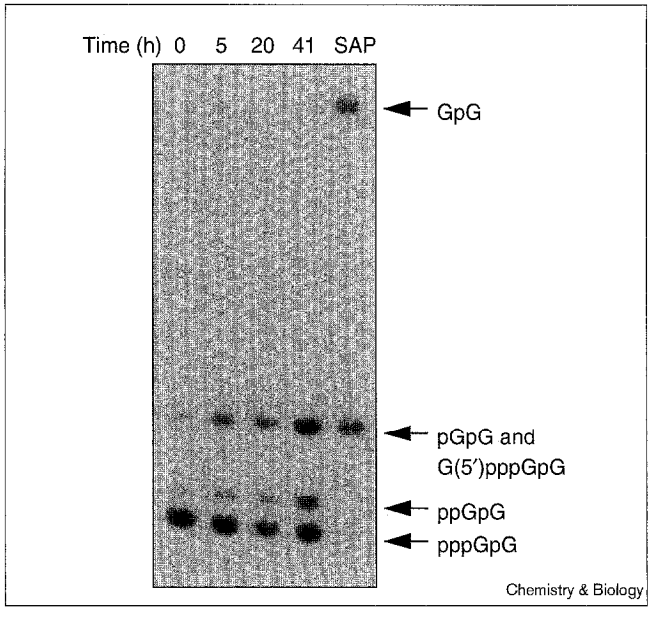
Finally, we turn to reactions that probably do not depend Watson–Crick base pairing: GTP as substrate with Iso6-G as catalyst. For example, using [α-^{32}P]-GTP and GDP as substrates, Iso6-G might catalyze reactions between GTP and GTP or GDP (Figure 1b), simultaneously releasing pyrophosphate (PP_i):

$$G(5')*ppp + pp*pG \xrightarrow{\text{Iso6-G}} G(5')*ppp*pG + PP_i \quad (4)$$

$$G(5')pp + pp*pG \xrightarrow{\text{Iso6-G}} G(5')pp*pG + PP_i \quad (5)$$

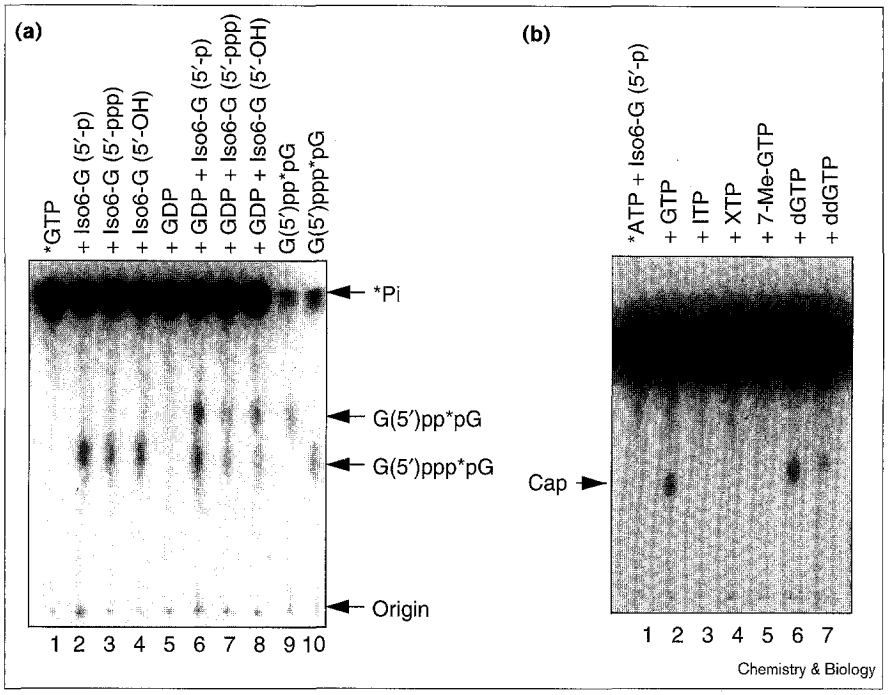
where the asterisk indicates the location of ^{32}P. The two free nucleotides on the left-hand side of the equations

Figure 5



Time course for the Iso6-2G ribozyme-catalyzed reaction with G2 ([α-^{32}P]GTP-labeled) analyzed using 20% denaturing PAGE. The sample for the last lane was digested with SAP. Reaction conditions: 1 μM RNA, 10 μM G2, 0.5 mM GDP, 20 mM Ca^{2+} and MES, pH 5.5, 37°C.

Figure 6



(a) Iso6-G RNA catalyzed reactions of GTP with other terminal phosphate-containing nucleophiles. Reaction conditions: 0.2 mM [α-^{32}P]-GTP (*GTP, lanes 1–8), 0.2 mM GDP (lanes 5–8), 5 μM RNA (lanes 2–4 and 6–8), 20 mM MES (pH 5.5), 20 mM Ca^{2+}, 25 h at room temperature. After incubation, 3 μl samples were digested with 1 unit of SAP in supplied buffer for 1 h at 37°C, to convert nonreacted *GTP into *P_i (capped products are unaffected). The samples (1 μl) were then spotted on a cellulose PEI TLC plate and developed in 0.5 M potassium phosphate (pH 6.5) containing 20% methanol. ^{32}P on the TLC plate was analyzed by phosphorimaging (BioRad GS525). The two capped guanosine standards, G(5′)pp*pG and G(5′)ppp*pG, were prepared by first reacting GDP and GTP, respectively, with uniformly [α-^{32}P]-GTP labeled Iso6 pp*pRNA to generate G(5′)pp*pRNA and G(5′)ppp*pRNA [14], digestion with nuclease P1 (1 unit, 1 h at 37°C in 10 mM NaOAc, pH 5.0), and then further digestion with SAP. **(b)** Substrate specificity of Iso6-G. [α-^{32}P]-ATP (*ATP, 1 mM, all lanes) and 5 μM Iso6-G (5′-p, all lanes) were incubated with 1 mM different nucleoside 5′-triphosphates in 20 mM MES (pH 5.5), 20 mM Ca^{2+}, for 30 min at 37°C. The samples were then digested with SAP and analyzed as above.

occupy the nonspecific nucleophile-binding site (GTP and GDP) and the former 5′-terminal guanine site is occupied specifically by GTP. In some of these experiments we incorporated three different 5′ catalyst termini: triphosphate (ppp-), monophosphate (p-), and hydroxyl (HO-) (Figure 1), to determine whether the ribozyme terminus, which is adjacent to the active site (Figure 1), affects reactions.

Figure 6a shows TLC product analysis after incubation with GTP alone (lanes 1–4), or with GTP and GDP (lanes 5–8). In all cases where the ribozyme was present, capped products were observed co-migrating with capped markers: G(5′)ppppG from GTP (lanes 2–4), and a mixture of comparable amounts of G(5′)ppppG and G(5′)pppG when both GTP and GDP were present (lanes 6–8). The 5′ terminus of the Iso6-G catalyst does affect activity slightly, and 5′ pIso6-G (monophosphorylated RNA), is the most active ribozyme.

Substrate specificity at the GTP site is easily demonstrated. We reacted [α-^{32}P]-ATP (which cannot enter the G site, and so must act as the nonspecific phosphate nucleophile; see below) with varying nucleoside 5′-triphosphates (NTPs) in the presence of Iso6-G (Figure 6b). The experiment thereby determines which NTP can specifically substitute for GTP at the GTP site:

$$A(5')^*ppp + pppN \xrightarrow{\text{Iso6-G}} A(5')^*ppppN + PP_i \quad (6)$$

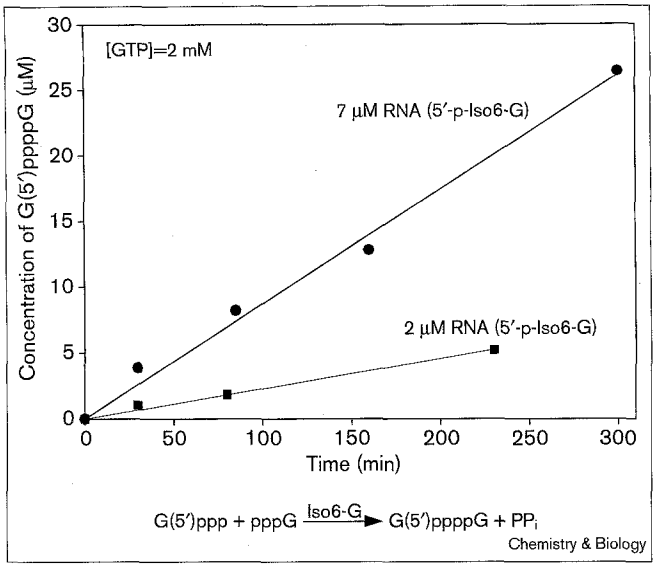
Only 5′-triphosphate derivatives of guanosine can act as the first GTP of the original Iso6 RNA (Figure 6b); neither ATP, ITP, XTP nor N7-methylated GTP is accepted by Iso6-G at this specific site. The 2′- or 3′-OH of the sugar does not appear to be involved in the reactivity; modifications to the guanine base completely abolish observable capped product formation, however. The Iso6-G ribozyme therefore binds guanosine triphosphate by recognizing guanine, confirming the specific nature of this site. Specificity probably involves Iso6-G contacts with the 2-amino and N7 positions of guanine on opposite sides of the base. Thus the site created in Iso6-G is embracing, surrounding the base, and probably is not a Watson–Crick base pair.

Multiple turnovers occur in this system. Figure 7 shows G(5′)ppppG formation kinetics; the capped product G(5′)ppppG increases linearly with time for at least 4–5 h. Product formation is first order in RNA; that is the rate is proportional to the RNA concentration. One RNA molecule catalyzes the formation of ~4 molecules of G(5′)ppppG under these conditions. Iso6-G RNA is therefore able to catalyze multiple-turnover reactions with a rate that is first order with respect to the RNA concentration and invariant with respect to time, as expected.

Kinetics of the Iso6-G ribozyme

The picture developed to this point, of an RNA enzyme containing two distinct binding sites for two small molecules, can account quantitatively, as well as

Figure 7



Turnover and RNA enzyme (Iso6-G) concentration. Reaction conditions: 2 or 7 μM 5′-p-Iso6-G RNA with 2 mM [α-^{32}P]-GTP, 20 mM MES (pH 5.5), 20 mM Ca^{2+}, for varying times at 37°C. The samples were analyzed as above and quantitated by phosphorimaging.

qualitatively, for the activity of Iso6-G. To show this, consider the rate of the GTP–GTP and GTP–ADP reactions as a function of free nucleotide concentration (Figure 8). With a fixed RNA concentration (5 μM), the rate of the GTP–GTP reaction (Figure 8a,b) increases with GTP concentration. The specific activity of *GTP decreases with increasing GTP concentration, which reduces product intensity in the right hand lanes of Figure 8a, although product concentration increases. Fitting the data to a kinetic equation (equation 7, see the Materials and methods section) appropriate to Iso6-G RNA and the GTP-only bisubstrate system (Figure 8b), we obtained the saturating rate constant k_{cat} and two Michaelis constants K_1 and K_2 at the two distinct substrate sites (Table 1). Comparing these parameters with those of the original Iso6 RNA [17], it becomes clear that K_1 (1.6 mM) represents specific GTP binding to Iso6-G at a site equivalent to the 5′-terminal GTP (Figure 1), because, remarkably, K_2 (15 μM) is the same as that of nonspecific GTP binding to intact Iso6 RNA [17]. The reaction seems unimpaired; the maximal rate of Iso6-G is reduced only about fourfold relative to Iso6, despite the absence of a covalent connection to the substrate in Iso6-G.

The roles of the two sites are better separated in the kinetics of a GTP–ADP system. Using ADP instead of GDP avoids possible interference by GDP at the specific GTP site [17]. At fixed concentrations of RNA (5 μM) and ADP (0.5 mM) with varying GTP concentration, two major reactions compete (Figure 8c,d). At low concentrations of GTP, both reaction rates increase with increasing GTP concentrations. The rate of A(5′)pppG formation decreases at high GTP concentrations because of competition at the nonspecific site, however. Cap-exchange reactions are also possible (A(5′)pppG reacting with GTP to give G(5′)ppppG), but are much slower [16] and would not affect these quantitative conclusions.

By fitting the experimental data to appropriate kinetic equations 8 and 9 (see the Materials and methods section) for the GTP–ADP system (Figure 8d), we define a set of kinetic parameters k_{cat}, K_1, K_2 and K_3 (Table 1). These parameters agree with those obtained from the GTP-only system (Figure 8a,b). K_1 for GTP is again 1.6 mM, and K_3 for ADP is 11 μM, consistent with the Michaelis constant of GDP for the nonspecific nucleophile-binding site of the original Iso6 RNA [17]. These data, therefore, show quantitatively that the invocation of two distinct substrate sites on Iso6-G, represented by K_1 and K_2 (K_3) for the specific GTP site and the nonspecific nucleophile site, respectively, can account for the rate of reaction with a variety of substrate configurations and concentrations.

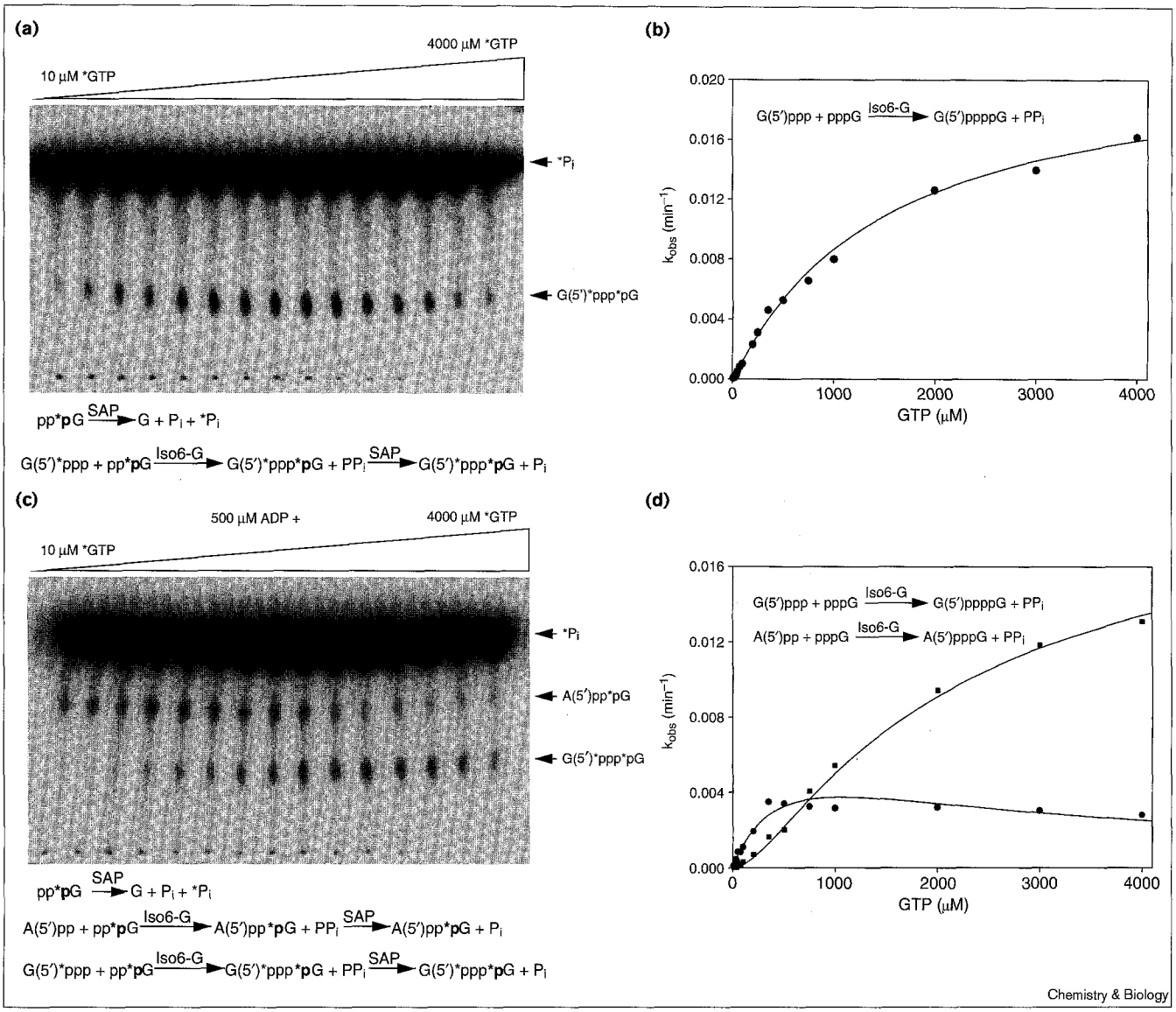
Discussion

These studies exhibit considerable substrate specificity by the RNA enzymes. The active sites of the RNA enzymes Iso6-G, Iso6-2G and Iso6-3G display notable complementarity to their substrates. For example, although G2, G3, G4 and G5 might be thought of as more or less complementary to the active site produced in Iso6-2G, only the precisely fitted substrate, G2, is reactive. This complementarity goes beyond hindrance for substrates that are too large: for example, G2 almost surely can be fitted within the matched, but larger active site of Iso6-3G, yet is unreactive.

Complementarity is most remarkable with Iso6-G, which has the smallest substrate and therefore the fewest functional groups for specific interaction. Severing the covalent bond to the 5′-terminal G of the original RNA apparently leaves intact a site that embraced the guanine of the terminal nucleotide. In truncated Iso6-G RNAs this site acts as a specific guanosine triphosphate (GTP, dGTP or ddGTP)-binding site. This specific site cannot be occupied by N7-methylated guanine, suggesting that the N7 position is closely hindered. ITP also gives no observable reaction, despite base-pairing that would be similar, and only slightly weaker, than GTP. These observations suggest that, as implied by the secondary-structure model (Figure 1), the specific guanine site is not created using Watson–Crick base-pairing principles.

The same Iso6-G truncation also leaves intact a nonspecific binding site for phosphate-containing nucleophiles, believed to have originated as the site for the PP_i leaving

Figure 8



Quantitation of two distinct substrate sites in Iso6-G. Reactions were carried out under the following conditions: 5 μM 5′-p-Iso6-G incubated with varying concentrations of [α-^{32}P]-GTP in 20 mM MES (pH 5.5), 20 mM Ca^{2+}, for 30 min at 37°C. The total counts per minute (cpm) of all samples stayed the same, whereas the specific activity (mCi/mmol) of [α-^{32}P]-GTP decreased with increasing total GTP concentrations. After incubation, the samples were SAP-digested and analyzed on cellulose PEI TLC plates (developed in 0.4 M potassium phosphate, pH 6.5 containing 20% methanol). Quantitation was performed by profile integration after baseline correction using Molecular Analyst (BioRad). **(a)** TLC analysis of the GTP-only bisubstrate system (GTP–GTP reaction) with increasing GTP concentrations. **(b)** Dependence of the reaction rate on the concentration of GTP. The experimental data (dots) were fitted (curve) to a bisubstrate kinetic equation (equation 7). **(c)** Iso6-G catalyzed reactions of varying GTP concentration at a fixed ADP concentration (0.5 mM). **(d)** Kinetics of the two-substrate system. Equations 8 and 9 (see the Materials and methods section) for the GTP–ADP system were used to fit the two sets of product data.

group of the initially selected pyrophosphatase [14–17]. This site is believed to rely on coordination of phosphates to a required calcium ion [17].

The current findings demonstrate that RNA enzymes can be constructed that bind multiple small molecules using varied and appropriate forces and catalyze multiple turnover reactions between these substrates, as do proteins. More impressively, even the rate-limiting first-order steps in such reactions are slowed only fourfold, suggesting that the critical details of the transition state are only slightly affected by truncation. It would now be informative to produce the same type of catalyst using non-nucleotide substrates.

Table 1

Enzymatic characteristics of Iso6-G RNA.

	Site 1 (specific)	Site 2 (nonspecific)
Substrate	GTP, dGTP or ddGTP	R-p
K_M (mM)	1.6 ± 0.3	0.011 ± 0.006 for ADP 0.015 ± 0.007 for GTP
k_{cat} (min^{-1})	0.022 ± 0.002	

Kinetic parameters are for 5′-monophosphate-terminated Iso6-G in 20 mM MES and calcium, pH 5.5, at 37°C.

Significance

The discovery of RNA catalysis suggested an 'RNA world' ancestral to the present one. This hypothetical ancient biochemistry is nevertheless subject to empirical inquiry. RNA reactions demonstrable today can be added to the repertoire of the hypothetical organisms that might have populated an RNA world. Here we have shown that this RNA repertoire can include catalysis of anabolic reactions between small molecules.

The present reactions are also interesting in themselves. RNA Iso-G, RNA Iso6-2G and RNA Iso-3G catalyze reactions *in trans* to form oligonucleoside 5′→5′ polyphosphates. These polyphosphates are similar to molecules with signaling and calcium mobilization activities [18–22]. In addition to these biological alarmones, closely similar RNA catalysts might be able to produce unmodified eukaryotic message cap G(5′)pppG. Finally, relatives of Iso-G could make analogs of the many protein enzyme cofactors that contain 5′→5′ oligophosphate linkages, such as NAD. Thus, given only that RNA was then made from that 5′ nucleoside oligophosphate precursors, each of these types of compounds could have occurred and played roles in an organism that depended entirely on RNA enzymes.

Materials and methods

Preparation of RNA enzymes

RNA enzymes, Iso6-G, Iso6-2G, and Iso6-3G, were prepared by T7 transcription (3 h at 37°C) of dsDNA templates, assembled through PCR amplification (16 cycles) of 5000-fold diluted Iso6 dsDNA template [14] using two primers: 5′-TTTAAGCTTCTGCTACATGCAATGG and either 5′-GCTAATACGACTCACTATA***G***GGAGTAC GGGAGAGGATACTACACGTG (for Iso6-G, the bold and italicized G indicates the initiating nucleotide for the RNA), 5′-GCTAATACGACTCACTATA***G***GAGTACGGGAGAGGATACTACACGTG (for Iso6-2G) or 5′-GCTAATACGACTCACTATA***G***AGTACGGGAGAGGATACTACACGTG (for Iso6-3G). All DNA oligos were manufactured by Operon Technologies. 5′-ppp-, -p-, or -HO-Iso6-G RNA was prepared by T7 transcription of its DNA template in the presence of GTP, 1:20 GTP:GMP or 1:20 GTP:G, respectively. All RNA enzymes were purified using 8% denaturing PAGE to single nucleotide resolution as described previously [14].

Preparation of RNA substrates

RNA substrates, pppGpG (G2), pppGpGpG (G3), pppGpGpGpG (G4), and pppGpGpGpGpG (G5), were prepared by abortive T7 transcription (3 h at 37°C) of a dsDNA template assembled from DNA oligos, 5′-GCTAATACGACTCACTATA***GGG***AGTACGGGAGAGGATACTACACGTG and 5′-***CCC***TATAGTGAGTCGTATTAGC (bold and italicized letters serve as the template for transcription). [α-^{32}P]-GTP was used as the label during the transcription. Transcription was tested using either four nucleotides or GTP only (Figure 2), but substrate preparation was carried out with GTP only. These guanosine oligonucleotides were purified using 20% denaturing PAGE, eluted, EtOH-precipitated (in the presence of 20 μg/ml) and redissolved in water. Oligonucleotide concentrations were calculated based on UV absorbance at 260 nm using extinction coefficients of 21.6, 31.7, 41.8 and 51.9 mM^{-1} cm^{-1} [23] for G2, G3, G4 and G5, respectively.

Preparation of markers

^{32}P-labeled markers, *P_i, *pG (GMP) and p*pG (GDP), were prepared by hydrolysis of [α-^{32}P]-GTP in water for 2 h at 95°C. Caps, G(5′)pp*pG and G(5′)ppp*pG, were prepared by first reacting GDP and GTP, respectively, with [α-^{32}P]-GTP-labeled Iso6 RNA [14] followed by nuclease P1 digestion (for conditions, see following section with nuclease P1 digestion).

Substrate and product analysis

All nuclease P1 digestion was performed under the following conditions: 3 μl of sample and 1 unit of nuclease P1 (Pharmacia) for 1 h at 37°C in 10 mM NaOAc, pH 5.0. Phosphatase digestion was carried out under the following conditions: 3 μl of sample and 1 unit of SAP for 1 h at 37°C in 1 × supplied buffer (United States Biochemical). TLC analysis was performed under the following conditions: PEI TLC plates (JT Baker), developing in 0.5 M potassium phosphate, pH 6.5, and 20% MeOH. ^{32}P on the TLC plate was analyzed by phosphorimaging (BioRad GS525).

RNA enzyme-catalyzed reactions

All Iso6-2G and Iso6-3G catalyzed reactions, unless specified differently, were performed under following conditions: 1 μM RNA, 5 μM oligo guanosine (α-^{32}P-GTP-labeled) , 0.5 mM GDP, 20 mM Ca^{2+} and Mes, pH 5.5 for 20 h at 37°C. Iso6-G catalyzed reactions (Figures 6–8) were performed under different conditions, which are specified in the figure legends of different experiments.

Kinetics of Iso6-G-catalyzed reactions

Kinetics of Iso6-G-catalyzed reactions (Figure 8) were performed for 30 min, based on the observation of a constant rate of product formation for several hours (Figure 7). The concentration of product with different initiating substrates was used to calculate the kinetic parameters. According to the original Iso6 RNA kinetics [17], Iso6 has a nonspecific binding site for a terminal phosphate-containing nucleophile. In addition, Figure 6b shows that Iso6-G has a specific guanosine binding site. Therefore, in this GTP-only system (Figure 8a,b), assuming fast equilibrium between substrates and the enzyme and that the two binding sites are independent, the rate-relationship as a function of GTP concentration can be expressed as [24]:

$$k_{ob}^{G(5')ppppG} = k_{cat}\frac{[GTP]}{K_1+[GTP]}\frac{[GTP]}{K_2+[GTP]} \qquad (7)$$

where k_{cat} is the saturating rate constant, and K_1 and K_2 are Michaelis constants for GTP binding at two different sites. The first term

$$\frac{[GTP]}{K_1+[GTP]}$$

describes GTP saturation at the specific GTP site, and the second term represents GTP saturation at the nonspecific nucleophile site:

$$\frac{[GTP]}{K_2+[GTP]}$$

For the GTP–ADP system (Figures 8c,d), supposing that GTP and ADP compete at the nonspecific nucleophile site and ADP does not interfere at the specific GTP site, the rates of two reactions can be

expressed by modifying the equation for the GTP-only system (above). For G(5′)ppppG formation:

$$k_{ob}^{G(5')ppppG} = k_{cat}\frac{[GTP]}{K_1+[GTP]}\frac{[GTP]}{K_2\left(1+\frac{[ADP]}{K_3}\right)+[GTP]} \quad (8)$$

For A(5′)pppG formation:

$$k_{ob}^{A(5')pppG} = k_{cat}\frac{[GTP]}{K_1+[GTP]}\frac{[ADP]}{K_3\left(1+\frac{[GTP]}{K_2}\right)+[ADP]} \quad (9)$$

where k_{cat} is the saturating rate constant, K_1 the Michaelis constant for GTP at the GTP specific site and K_2 and K_3 the Michaelis constants for GTP and ADP, respectively, at the nonspecific nucleophile site.

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