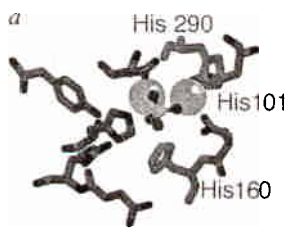
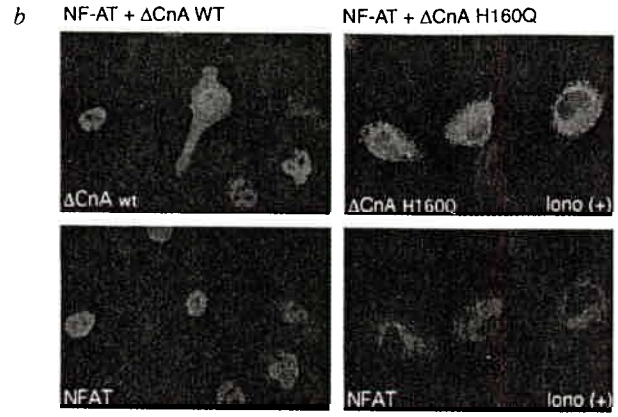


ΔCnA	Staining	Catalytic activity (%)	Nuclear NF-AT4	
WT	N > C	100	95 %	
H101Q	C	0	ND	*
H160Q	C	0	6 %	*
H290Q	C	0	6 %	*
Vector		0	95 %	



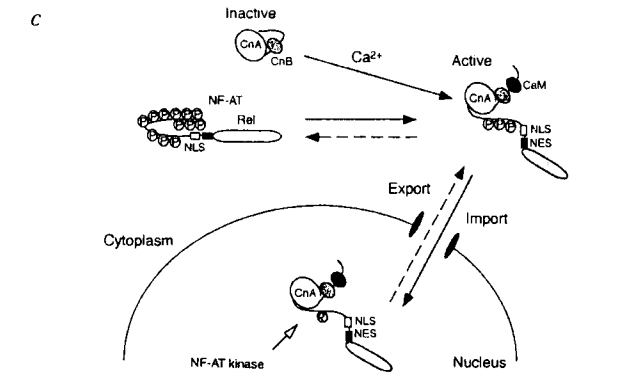


FIG. 4 Catalytically inactive calcineurin mutants are exclusively cytoplasmic and interfere with NF-AT4 translocation. *a*, Model of calcineurin active site based on conserved residues in protein phosphatase (ref. 17). The three histidine residues (at positions 101, 160 and 290) mutated to glutamine are labelled. The two metal ions are shown in blue, adjacent to a phosphate group. These mutants were synthesized *in vitro* and tested for catalytic activity towards the RII substrate[19]. The three inactive ΔCnA mutants are located exclusively in the cytoplasm of transfected cells, as judged by immunofluorescence. When co-transfected with NF-AT4, the H160Q and H290Q mutants suppress the ability of endogenous calcineurin to translocate NF-AT4 when stimulated with ionomycin (asterisk). *b*, Wild-type ΔCnA is primarily nuclear and induces the nuclear translocation of NF-AT4 (left panels). In contrast, ΔCnA-H160Q is exclusively cytoplasmic and fails to translocate NF-AT4 (right panel). *c*, Calcium signalling through calcineurin and NF-AT. Initially, both calcineurin (CnA and CnB), shown in its auto-inhibited form, and NF-AT4, with masked nuclear location signals, reside in the cytoplasm. Calcium activates calcineurin, which ten binds to an dephosphorylates NF-AT4, causing an unmasking of its nuclear location signals. Both NF-AT4, and the associated CnA are translocated to the nucleus, where they encounter a putative NF-AT4 kinase. As calcineurin activity decreases at the end of calcium signalling, the NF-AT4 kinase predominates and promotes the nuclear export of NF-AT4. NLS and NES refers to putative nuclear localization and export signals, respectively.

METHODS. Mutagenesis of ΔCnA and analysis of the catalytic activity of mutants were done essentially as described[19]. For analysis of dominant-negative activity of the calcineurin mutants, BHK cells were transfected with either NF-AT4 alone or with plasmid expressing mutant ΔCnA and CnB. After 16 h expression, cells were treated with ionophore for 30 min and processed for immunofluorescence to assay for NF-AT4 translocation.

NF-AT4 kinase involved in the attenuation of calcium signalling in the nucleus. □

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RNA-catalysed RNA polymerization using nucleoside triphosphates

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The hypothesis that certain RNA molecules may be able to catalyse RNA replication is central to current theories of the early evolution of life[1–6]. In support of this idea, we describe here an RNA that synthesizes RNA using the same reaction as that employed by protein enzymes that catalyse RNA polymerization. In the presence of the appropriate template RNA and nucleoside triphosphates, the ribozyme extends an RNA primer by successive addition of up to six mononucleotides. The added nucleotides are joined to the growing RNA chain by 3′,5′-phosphodiester linkages. The ribozyme shows marked template fidelity: extension by nucleotides complementary to the template is up to 1,000 times more efficient than is extension by mismatched nucleotides.

Ribozymes that ligate RNA have been found in nature[7–11] and have been isolated from large pools of random RNA sequences[12,13]. Of particular interest for exploring the possibility of self-replicating RNA are ligase ribozymes that, like RNA polymerases, join RNA substrates using reactions in which pyrophosphate (PP_i) is displaced[11,12]. The class I ligase, a ribozyme derived from random RNA sequences, efficiently catalyses a

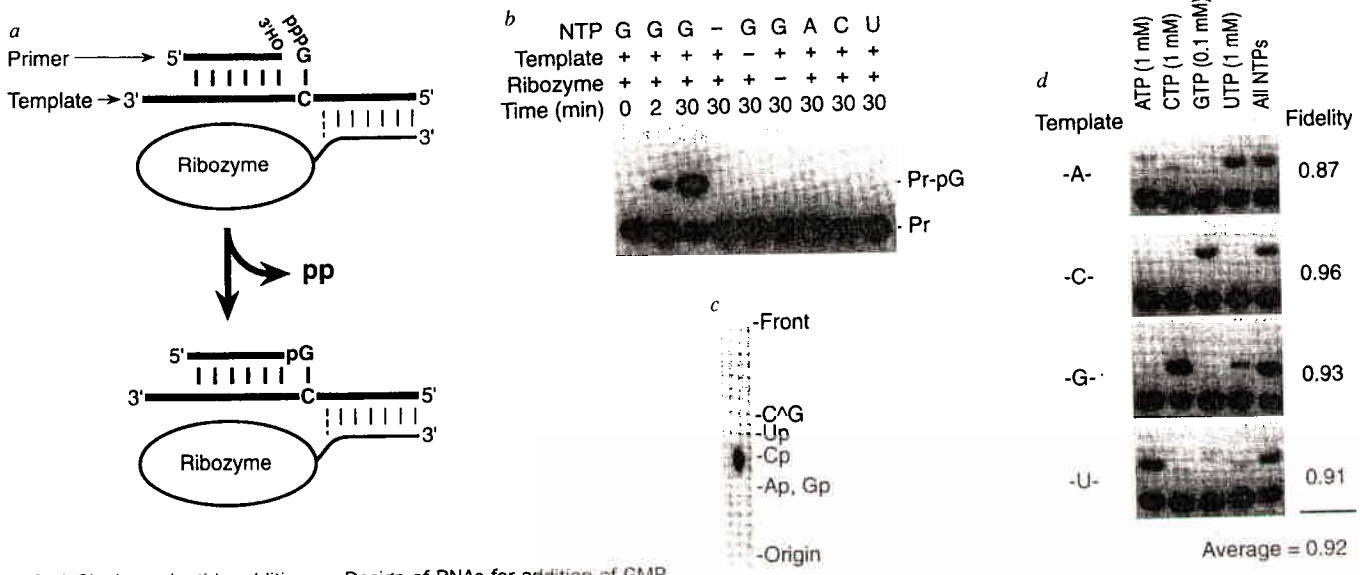


FIG. 1 Single nucleotide addition. *a*, Design of RNAs for addition of GMP using a -C- template. The ribozyme binds the template–primer, in part through base pairing of the 5′ end of the template with the 3′ tail of the ribozyme (thin vertical lines; dashed line denotes a proposed A·A pair[15]). GTP (pppG) binds the primer–template–ribozyme ternary complex such that the primer and the GTP are aligned by contiguous Watson–Crick pairing (thick vertical lines) to the template RNA. The ribozyme promotes attack of the primer 3′ hydroxyl (3′HO) on the GTP α-phosphate, resulting in the extension of the primer by one nucleotide (pG), with concomitant release of inorganic pyophosphate (PP_i). *b*, Time course of GMP addition. ^{32}P-labelled primer (Pr) was incubated with or without the indicated NTP (1 mM), template and ribozyme. Extended primer (Pr-pG) was separated from non-extended primer on a sequencing gel (20% acrylamide/8 M urea). *c*, Nuclease digestion of the extended primer indicates that the added nucleotide is joined to the primer by a 3′,5′-phosphodiester linkage. Unlabelled primer extended using [α-^{32}P]GTP was gel-purified, mixed with carrier RNAs, and digested with nuclease T2, which does not efficiently digest 2′,5′ RNA linkages. Digestion products were separated by thin-layer chromatography on PEI cellulose, developed with 1 M LiCl. Ultraviolet shadowing revealed digestion products of carrier tRNA (nucleoside 3′-monophosphates: Ap, Cp, Gp, Up) as well as a 2′,5′-linked carrier dinucleotide (C^G). *d*, Observed Watson–Crick fidelity of primer extension with an optimized ratio of NTP concentrations. Gel slices show the amount of extension after 30 min (-C- and -G- templates) or 180 min (-A- and -U- templates) with individual nucleoside triphosphates and with the combination of four NTPs. Extension rates were determined using these time points, as well as additional time points not shown. The observed fidelity was calculated as the extension rate for the Watson–Crick match divided by the sum of the extension rates for all four individual NTP possibilities. Note that this observed fidelity differs from the standard fidelity (Table 1) in that the observed fidelity is calculated from the extension rates when using a specific ratio of the four NTPs, whereas the standard fidelity is derived from the k_{cat}/K_m^{NTP} values.

METHODS. Reactions were done at 22 °C in 30 mM Tris-HCl, pH 8.0, 200 mM KCl, 60 mM $MgCl_2$ and 0.6 mM EDTA, with 2.5 μM ribozyme, 2.0 μM template, 1 μM primer and the indicated concentration of NTP, using described methods[15]. Ribozyme construct b1-233t was generated as described[15] (b1-233t sequence: 5′-ggaaaaagac aaaucugccc ucagagcuug agaacaucuu cggaugcaga ggaggcagcc uccgguggcu uuaacgccaa cguucucaac aauagcca, where underline represents segment pairing with template RNA). *In vitro* transcription[30] was used to generate template RNAs (5′-ggcuauaNgacuggaacca; single underline, segment pairing with ribozyme; double underline, segment pairing with primer; N, template base designed to pair with the nucleoside triphosphate, that is, for the -A- template, N = A). A synthetic DNA–RNA chimaera served as the primer (5′-AAAccaguc; where upper-case represents DNA, and double underline, segment pairing with template)[15].

reaction in which the 3′-hydroxyl of one substrate RNA attacks the 5′-triphosphate of another substrate molecule, forming a new phosphodiester linkage with concomitant release of pyrophosphate[14,15]. To explore this ribozyme's potential as a starting point for developing an RNA polymerase, we examined whether it could utilize a mononucleoside triphosphate rather than an oligonucleotide triphosphate as one of its substrates (Fig. 1*a*). When incubated with a primer RNA, GTP, and a template RNA designed to align the primer with the GTP by means of Watson–Crick pairing, the ribozyme elongated the primer by addition of GMP (Fig. 1*b*). When assayed over a range of GTP concentrations, the reaction showed saturable kinetics that were consistent with a single GTP-binding site (Michaelis–Menten parameters, $K_m^{GTP} = 5$ mM, $k_{cat} = 0.3$ min^{-1}). In the presence of excess primer–template, catalytic turnover was observed (7.5 turnovers in one hour, using 0.1 μM ribozyme, 5 μM primer, 5 μM template, 3 mM GTP). Analysis of the product confirmed that GMP was joined to the primer by a 3′,5′-phosphodiester linkage (Fig. 1*c*).

The primer extension reaction was highly specific for elongation using GTP, the Watson–Crick match to the template. The other three nucleotides added 600 to 1,000 times less efficiently (Fig. 1*b*). When the template RNA was substituted with templates designed to code for other nucleotides, addition by the Watson–Crick match was always favoured over addition by mismatch possibilities (Table 1). The k_{cat}/K_m^{NTP} values for all 16 template–NTP (where NTP is any nucleoside triphosphate) combinations can be used to calculate a standard Watson–Crick fidelity of 0.85 (Table 1). In other words, if given all four NTPs at equimolar concentrations, extension by the matched nucleotide typically would comprise 85% of the total extension. Certain asymmetric NTP ratios produced observed fidelities significantly greater than 0.85; the observed fidelity averaged 0.92 when the GTP concentration was uniformly lowered to one tenth of the concentration of the other three NTPs (Fig. 1*d*). At an observed fidelity of 0.92, extension by the Watson–Crick combination is, on average, 34 times faster than that by each of the three non-Watson–Crick combinations (0.92, compared to 0.027).

To examine the ribozyme's ability to extend a primer by more than one nucleotide, two additional template bases were inserted in the region between the primer–template helix and the template–ribozyme helix (Fig. 2*a*). This insertion makes three template residues available to specify the successive addition of up to three nucleotides. The template was also joined to the 5′ terminus of the ribozyme; tethering of the template to the ribozyme typically enhances extension efficiency by 2–6-fold. When incubated with all four NTPs and primer, such ribozyme–template

fusions catalysed primer extension by three nucleotides (Fig. 2). As polymerization proceeds, the primer–template helix shifts relative to the site of chemistry, changing the identity of the Watson–Crick pairs at most positions in the helix. With most templates shown in Fig. 2, none of the primer–template base pairs (counting back from the 3′ terminus of the growing strand) is the same during all three elongation steps. This shows that, as with polymerases found in nature, there is no absolute requirement for any particular Watson–Crick pair at any position of the primer–template helix.

The accuracy of extension was reassessed using the ribozyme–template format that permits multiple nucleotide addition. Although extension slowed considerably when switching from the single-nucleotide-addition format, the average fidelity of polymerization across from the four homopolymeric template segments (0.88; Fig. 2*e*) was similar to that observed for single nucleotide addition (0.92; Fig. 1*d*). Another similarity between the two formats was that correct extension directed by G or C template residues was 10 to 40 times more efficient than that directed by A or U residues (Table 1 and Fig. 2).

Although much lower than the ≥0.996 fidelity seen with viral polymerases that synthesize RNA using RNA templates[16,17], the observed fidelity is significantly greater than would be expected from the intrinsic stability of Watson–Crick matches compared with the stability of mismatches. Watson–Crick pairs at the ends of RNA helices are sometimes no more stable than are mismatches[18]. Using the relative stabilities of terminal base pairs and mismatches[18] to predict the fidelity of polymerization yields a maximal (using optimal NTP ratios) fidelity averaging about 0.40–a mere twofold advantage of Watson–Crick matches over non-Watson–Crick combinations (0.40, compared to an average of 0.20 for each of the three mismatches).

Use of nucleoside triphosphates to extend an RNA or DNA molecule is a fundamental reaction in all living organisms. The reaction shown in Fig. 2 is similar to that of a telomerase, a specialized DNA polymerase that uses a short segment of a built-in RNA template to direct the extension of chromosome ends. It has been proposed that before the advent of protein catalysis, a ribozyme with activity similar to that of the transfer RNA nucleotidyltransferase served as a telomerase[19]. Although tRNA nucleotidyltransferase is primarily responsible for adding and maintaining the CCA-3′ tail of the tRNA acceptor stem, this cellular enzyme can function as a simple telomerase for viral genomic RNAs that assume tRNA-like structures at their 3′ ends[20,21]. To the extent that our ribozyme can add a CCA-3′ tail to an RNA primer (Fig. 2*c*), we confirm that RNA can catalyse this reaction which is thought to have been important during the RNA world.

More central to the RNA-world hypothesis than an RNA telomerase is an RNA replicase, an RNA polymerase ribozyme which, with the aid of an RNA template encoding a second polymerase molecule, would be capable of autocatalytic replication[1–6]. Previous efforts to design ribozymes with polymerase properties have focused on derivatives of self-splicing introns that promote RNA oligonucleotide disproportionation reactions, where one RNA molecule becomes longer at the expense of a second RNA. Although progress has been made by this approach[22–27], reactions using self-splicing intron derivatives still suffer from low Watson–Crick fidelity; the highest fidelity achieved thus far has been 0.65 (ref. 28). More important, polymerization by oligonucleotide disproportionation is intrinsically prone to undesirable side reactions, which has led to the assertion that the substrates of a replicase ribozyme must have been more analogous to those of contemporary polymerases; that is, they must have been activated by a moiety like pyrophosphate rather than by RNA[6].

The RNA polymerization in Fig. 2 demonstrates features essential for polymerization by an RNA replicase, such as template dependence, use of appropriately activated mononucleotides, regioselectivity for the formation of 3′,5′-phosphodiester

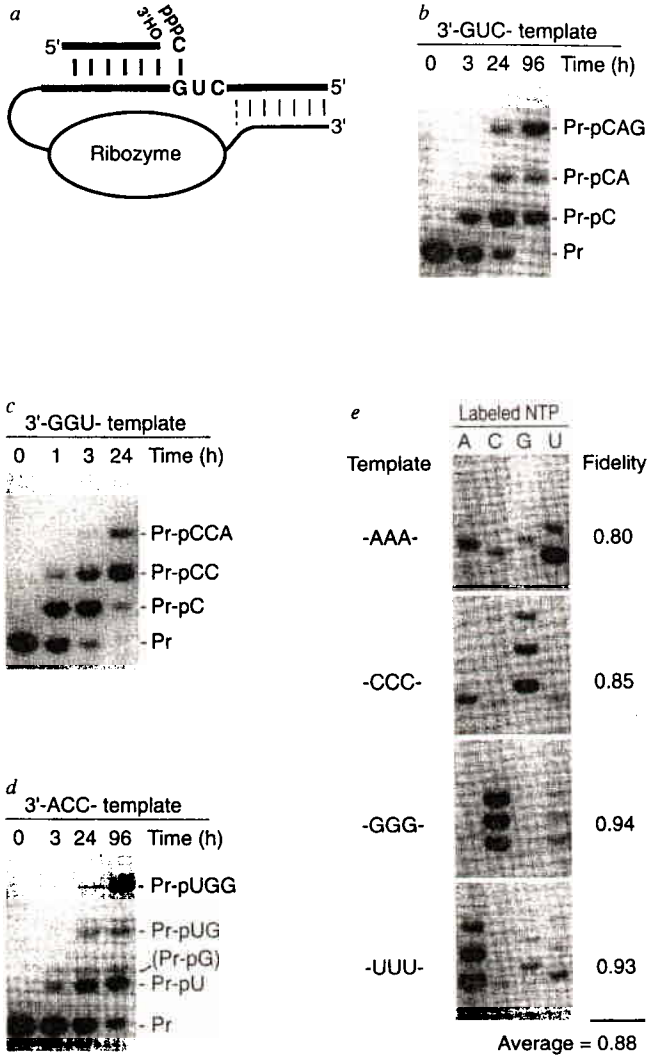


FIG. 2 RNA polymerization. *a*, Design of a ribozyme–template fusion for sequential addition of three nucleotides encoded by a 3′-GUC- template segment. *b–d*, Time course of polymerization using ^{32}P-labelled primer, a mixture of all four NTPs, and the indicated template segment. The identities of the indicated extension products were confirmed by comparison to reactions in which individual nucleotides were omitted (data not shown). In some cases, minor bands can be detected that represent incorporation by a non-Watson–Crick alternative (for example, band Pr-pG in *d*). *e*, Observed Watson–Crick fidelity of polymerization when using the four homopolymeric template segments. Reactions were as in *b–d*, except that the primer was not labelled and the NTP mixture was spiked with the indicated [α-^{32}P]NTP (final specific activity, 1.6 Ci mmol^{-1}). Gel slices show the amount of extension after 3 h (-CCC- and -GGG- templates) and 24 h (-AAA- and -UUU- templates).

METHODS. Reactions were done as for Fig. 1*b*, except that they contained all four NTPs (1 mM GTP; 10 mM ATP, CTP, UTP) and an additional 30 mM $MgCl_2$ to compensate for the Mg^{2+} expected to be complexed by the added NTPs. Ribozyme constructs were the same as construct b1-233t (Fig. 1), except the 5′ terminal residues (G1 and G2) were replaced with a template segment (5′-ggcuauaNNNgacugguacaa, where the single underline represents segment pairing with the ribozyme 3′ terminus, and double underline, segment pairing with primer; NNN are template bases designed to specify the nucleoside triphosphates that extend the primer). After stopping the reactions, samples were incubated (at 80 °C for 3 min) with a competitor DNA (5′-atttgtctttttttgtaccagtcNNNtatagcca) designed to hybridize with the template RNA. Incubation with competitor DNA led to better gel resolution because it prevented reassociation of the extended primer with the template. For each template in *e*, the observed fidelity was calculated as the incorporation by the nucleotide matching the template, divided by the incorporation by all four nucleotides.

TABLE 1 Template dependence of single nucleotide addition

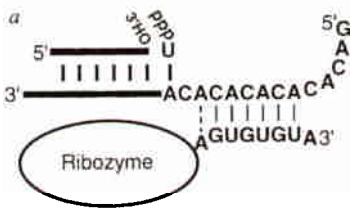
Template	Relative efficiencies of extension*				Standard fidelity
	ATP	CTP	GTP	UTP	
-A-	0.062	0.054	0.12	1.0 (1.80)	0.81
-C-	0.0010	0.0014	1.0 (68)	0.0013	0.996
-G-	0.0017	1.0 (28)	0.0017	0.071	0.93
-U-	1.0 (2.5)	0.015	0.34	0.048	0.71
				Average	0.85

Reactions were performed as for Fig. 1. For each template–NTP combination, the apparent second-order rate constant, k_{cat}/K_m^{NTP}, was determined from the slope of the line plotting observed rate constants as a function of NTP concentration (using NTP concentrations at least 10 times lower than the K_m^{NTP}). For each template, the four k_{cat}/K_m^{NTP} values were normalized to the k_{cat}/K_m^{NTP} value of the Watson–Crick match, yielding the relative efficiencies of addition. (The relative efficiency of addition for a mismatch is the same as its error frequency[29].) Standard fidelity was calculated as the k_{cat}/K_m^{NTP} value of the Watson–Crick match, divided by the sum of the k_{cat}/K_m^{NTP} values for all four NTP possibilities. The average fidelity is the geometric average of the fidelities. (Because the overall accuracy of RNA polymerization is represented by the product of the fidelities for each added nucleotide, geometric averages are reported throughout this study.)

* The k_{cat}/K_m^{NTP} values ($M^{-1}\,min^{-1}$) for Watson–Crick matches are shown in parentheses.

bonds, and absence of sequence requirements within the primer–template helix. However, it is deficient in two key respects: the template is covalently linked to the catalyst, and polymerization beyond three nucleotides is blocked, presumably by specific base pairing of the template to the ribozyme. The single-nucleotide-addition experiments demonstrate that the ribozyme can function with an external rather than a tethered template (Fig. 1). The block to polymerization by the ribozyme–template helix is more difficult to overcome, but it was partially circumvented when template and ribozyme were designed to base-pair in multiple registers (Fig. 3*a*). When using this strategy with an external template, polymerization of up to six nucleotides was detected after a long (6-day) incubation (Fig. 3*b*).

The progress made in deriving an RNA polymerase from random RNA sequences bodes well for the eventual demonstration of autocatalytic RNA replication. It will be of interest to examine the extent to which *in vitro* evolution and engineering efforts can improve the properties of our ribozyme. Extended copying of any RNA sequence may be possible if the 5′ portion of the template was recognized primarily by sugar–phosphate contacts rather than by pairing to template bases. Other critical improvements would include more avid NTP binding and increased template fidelity. □



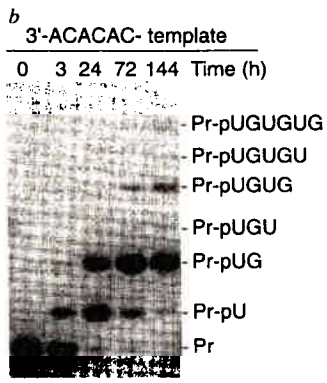


FIG. 3 RNA polymerization of up to 6 nucleotides. *a*, Design of template and ribozyme sequences such that the external template can pair with the ribozyme in multiple registers. *b*, Time course of polymerization. The reaction was done as for Fig. 1*b*, but contained UTP (20 mM), GTP (2 mM) and an additional 60 mM $MgCl_2$. The ribozyme was the same as construct b1-233t (Fig. 1), except that the 3′ terminal segment (5′-aauagcca) was replaced by (5′-agugugua). The template RNA was 5′-gacacacacacacaga-cuggaacca. After stopping the reactions, samples were heated in the presence of the appropriate competitor DNA, as for Fig. 2.

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