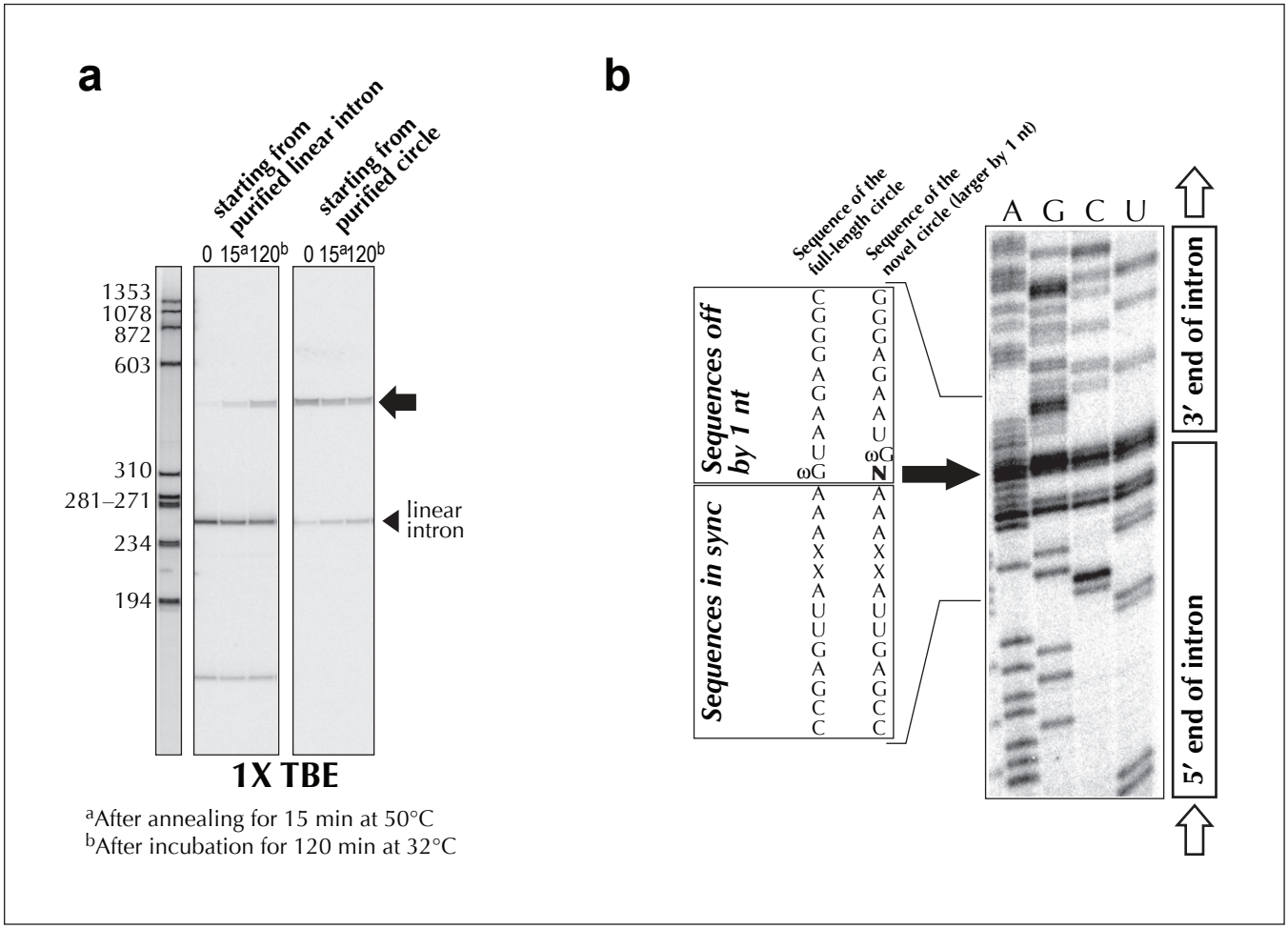
Supplementary Material

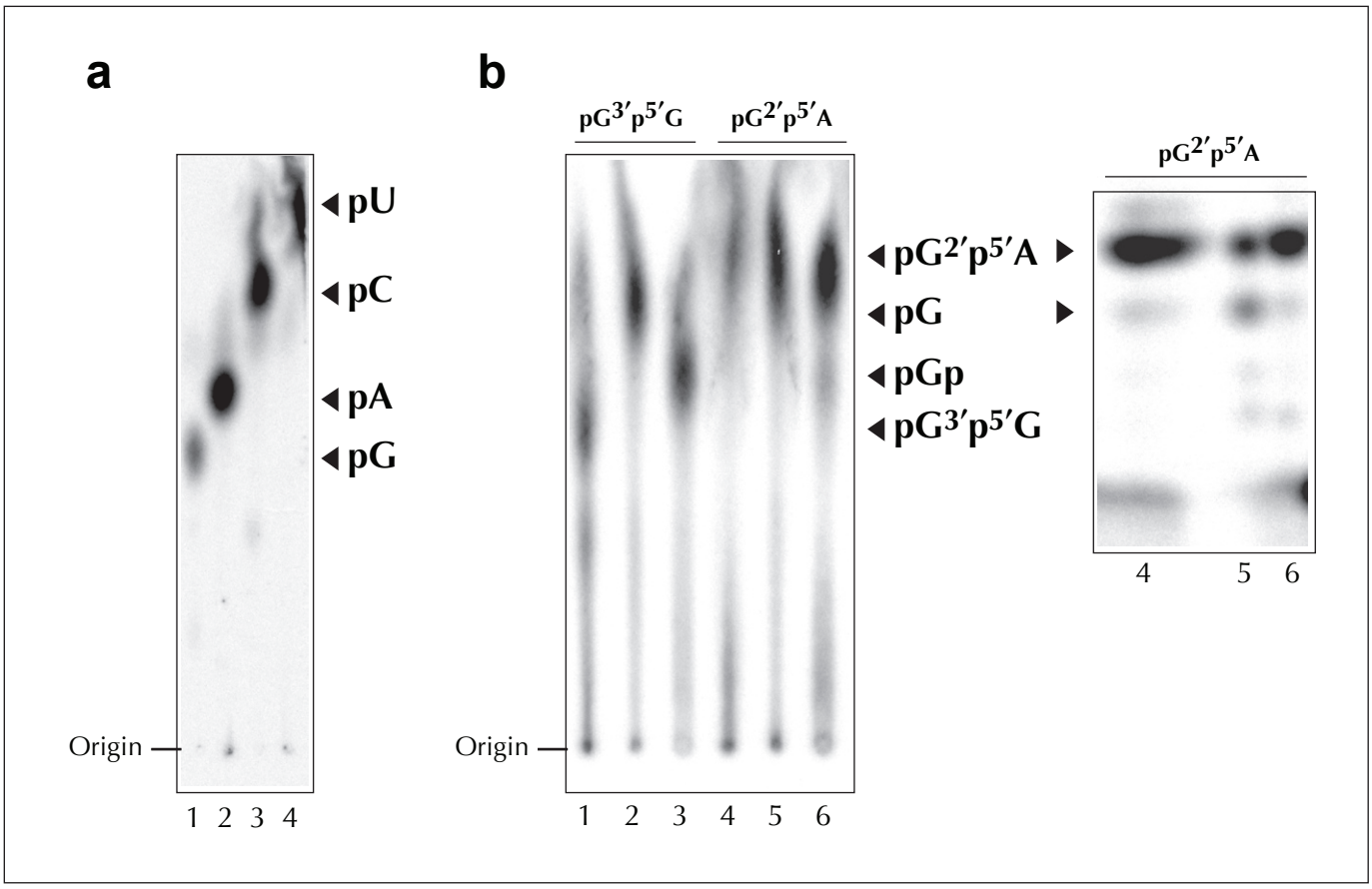
A natural ribozyme with 3′,5′ RNA ligase activity

Quentin Vicens & Thomas R. Cech

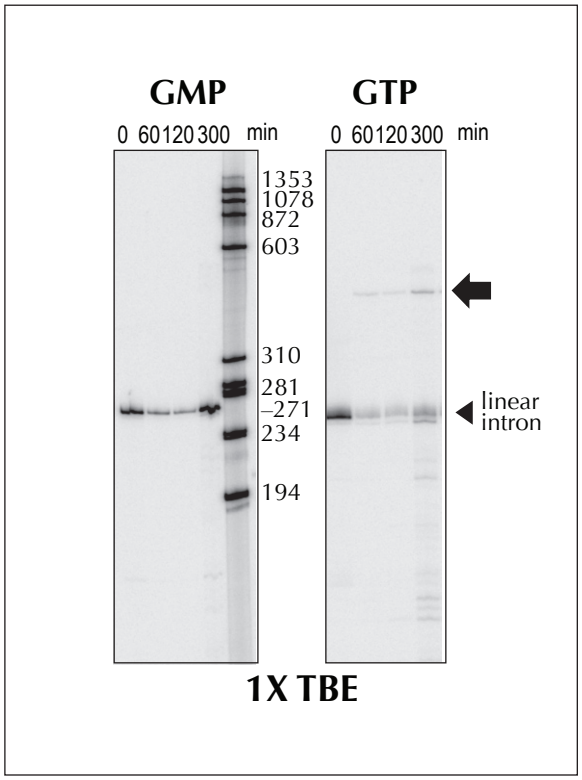
Supplementary Figures



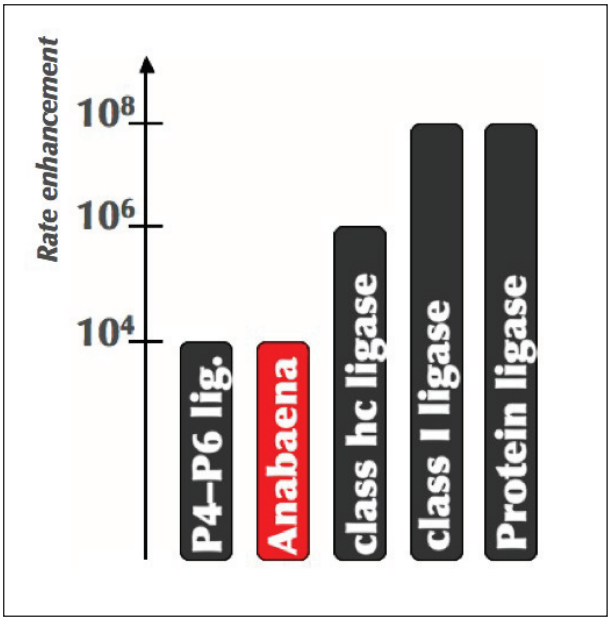
Supplementary Figure 1 | Characterization of the precursor-product relationship between the linear intron and the novel product and determination of the sequence of the novel product. (a) Incubation of the purified linear intron results in circle formation, and vice-versa. Time-course reaction performed under the reaction condition A (see legend to Figure 2) from a purified linear intron (left) and from a purified circle (right). (**b**) Sequencing of the low-mobility product obtained starting from the presursor RNA. The superimposition of the two sequences at the 3′ end of the intron indicates that the full-length circle and the circle presented herein migrate to the same location on the gel.



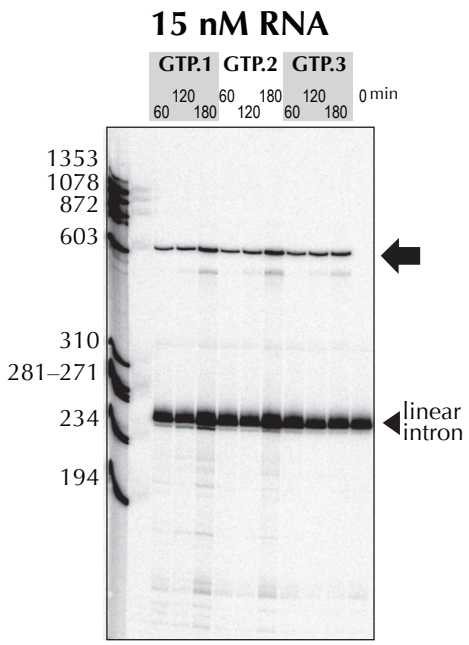
Supplementary Figure 2 | Determination of the cleavage specificities of the nucleases used in this study. (**a**) Nuclease P1 cleaves after any nucleotide. The P4–P6 RNA was body-labeled using either α^{32}P-GTP (lane 1), α^{32}P-ATP (lane 2), α^{32}P-CTP (lane 3), or α^{32}P-UTP (lane 4) and digested using nuclease P1. (**b**) RNase T1 is 3′,5′-specific but nuclease P1 is not. 5′-end labeled guanylyl-3′,5′-guanosine (pGpG, **9**, containing a 3′,5′ linkage; lane 1) and guanylyl-2′,5′-adenosine (pGpA, **10**, containing a 2′,5′ linkage; lane 4) visualized by TLC (left). Complete nuclease P1 digestions are in lanes 2 and 5. Complete RNase T1 digestions are in lanes 3 and 6. Because the digestion products of pGpA on TLC were not completely clear, samples 4–6 were also visualized by 20% polyacrylamide gel electrophoresis (right). Here it is clearly seen that RNase T1 treatment (lane 6) did not significantly digest the starting material (compare with lane 4), whereas nuclease P1 treatment (lane 5) resulted in partial conversion of the pGpA dinucleotide to pG.

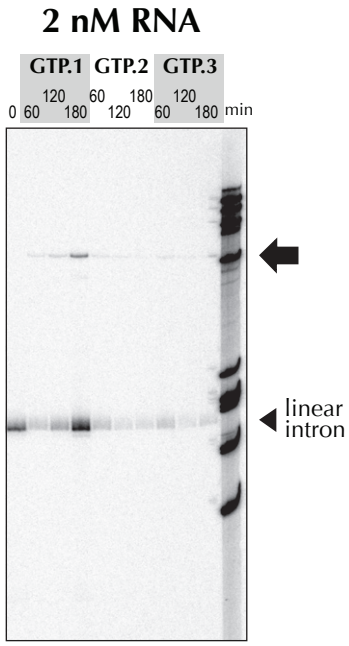


Supplementary Figure 3 | Incubation of the purified linear intron excised using GMP does not result in circle formation. Time-course reaction performed under the reaction condition A (see legend to Figure 2) from a purified linear intron excised using GMP (left) or GTP (right). The RNA is at 5.0 nM in both panels.



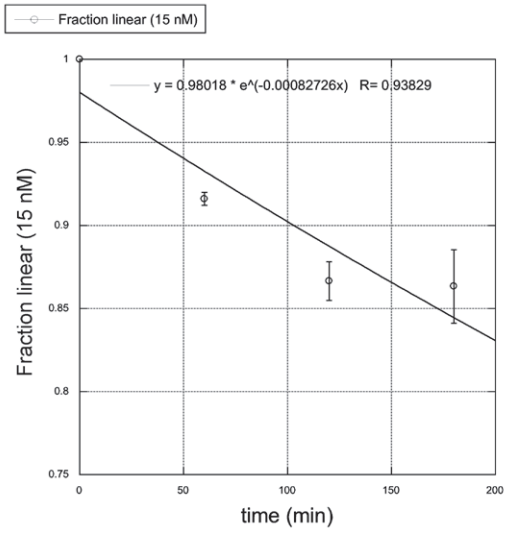
Supplementary Figure 4 | Rate enhancements of various ligases. Rate enhancement are defined as Rate (catalyzed) / Rate (uncatalyzed). The rates in the black bars are from main-text ref.[9].

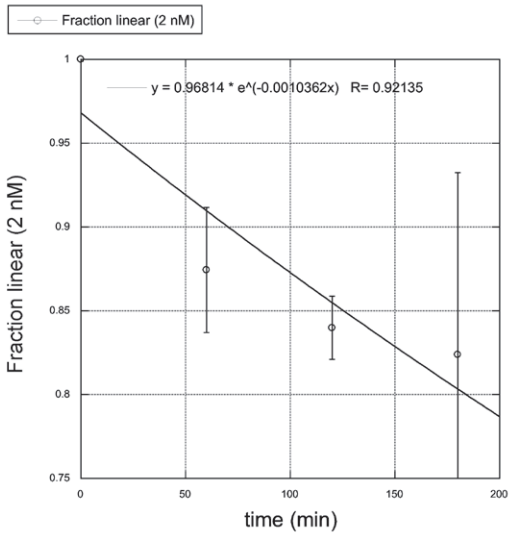




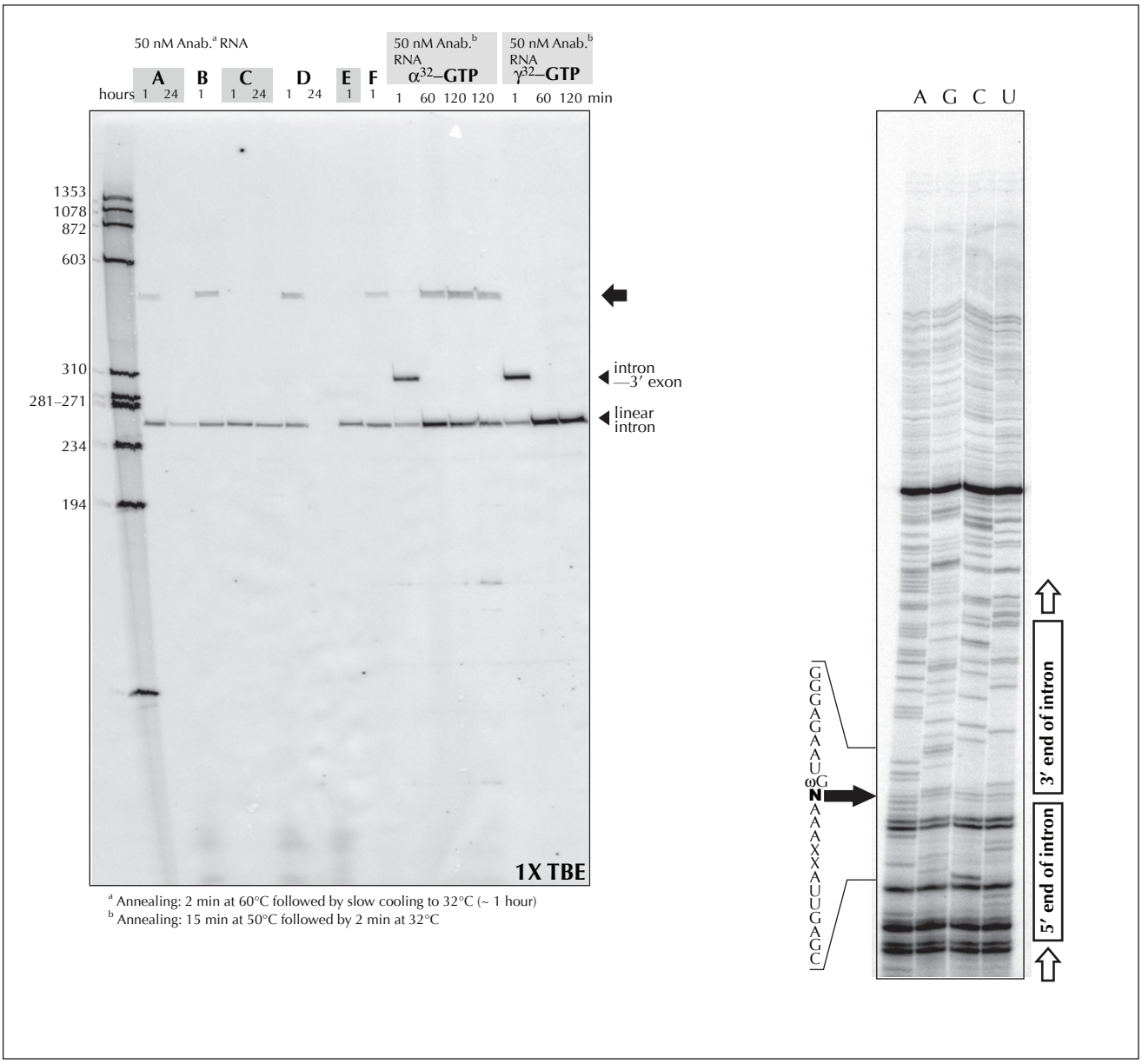
Time (min)	GTP.1	GTP.2	GTP.3	AVER.	STDEV.
0	1.000	1.000	1.000	**1.000**	**0.001**
60	0.915	0.920	0.913	**0.916**	**0.004**
120	0.867	0.878	0.855	**0.867**	**0.012**
180	0.868	0.883	0.839	**0.863**	**0.022**

Time (min)	GTP.1	GTP.2	GTP.3	AVER.	STDEV.
0	1.000	1.000	1.000	**1.000**	**0.001**
60	0.843	0.883	0.896	**0.874**	**0.037**
120	0.864	0.818	0.838	**0.840**	**0.019**
180	0.869	0.887	0.716	**0.824**	**0.109**



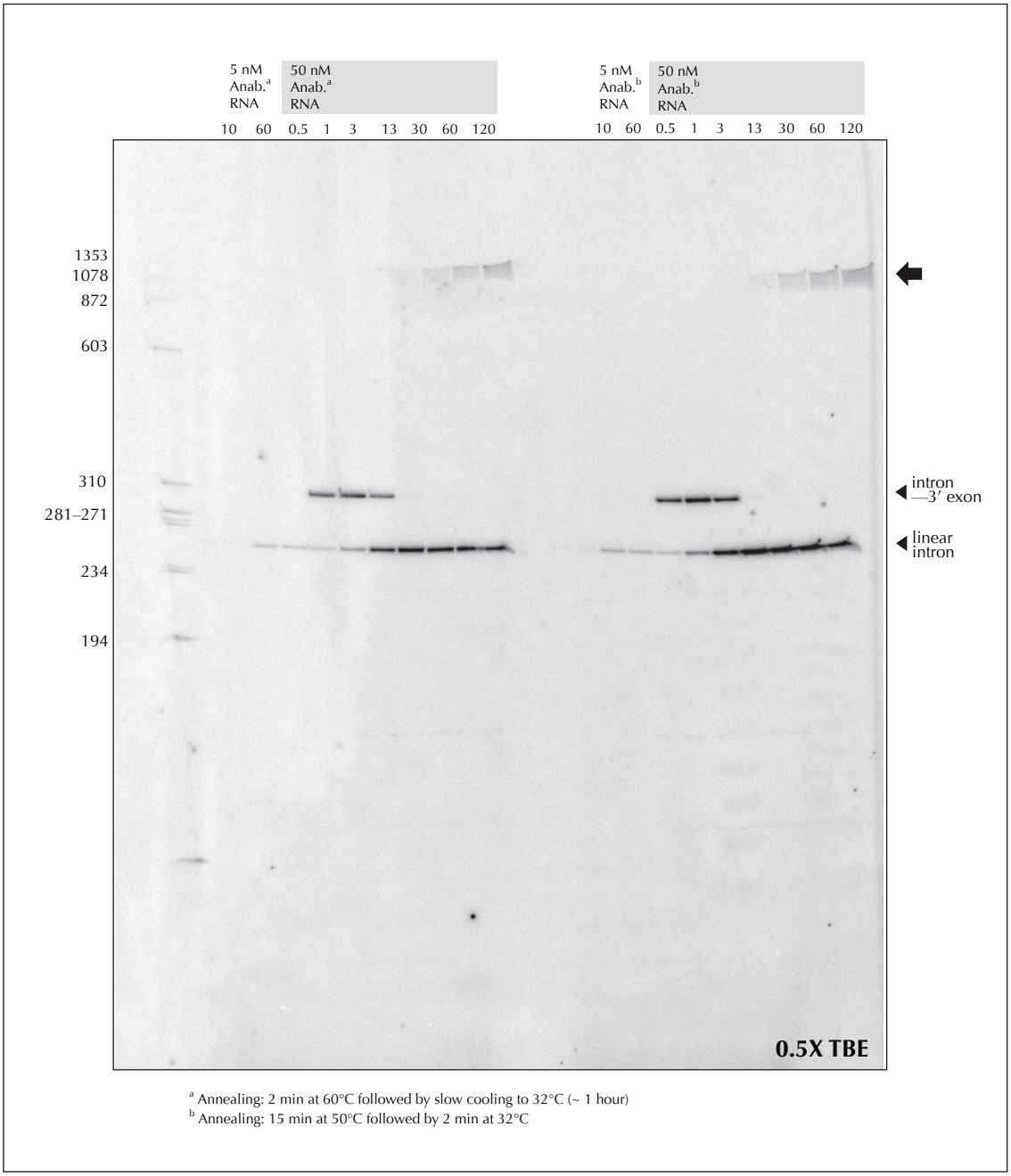


Supplementary Figure 5 | As the RNA concentration decreases, the rate of circle formation remains unchanged. Time-course experiment over 3 hours run at 0.75X TBE (reaction condition: A) from a purified linear intron at 15 nM (top left) and at 2 nM (top right). The experiments were performed in triplicate (referred to as GTP.1, GTP.2, and GTP.3 for both concentrations). Determination of the fraction of linear intron for each experiment (middle). The average fraction linear was plotted as a function of time (bottom). The error bars denote the standard deviation from the three experiments. The equations used to calculate a first-order catalytic rate constant are displayed on each graph.

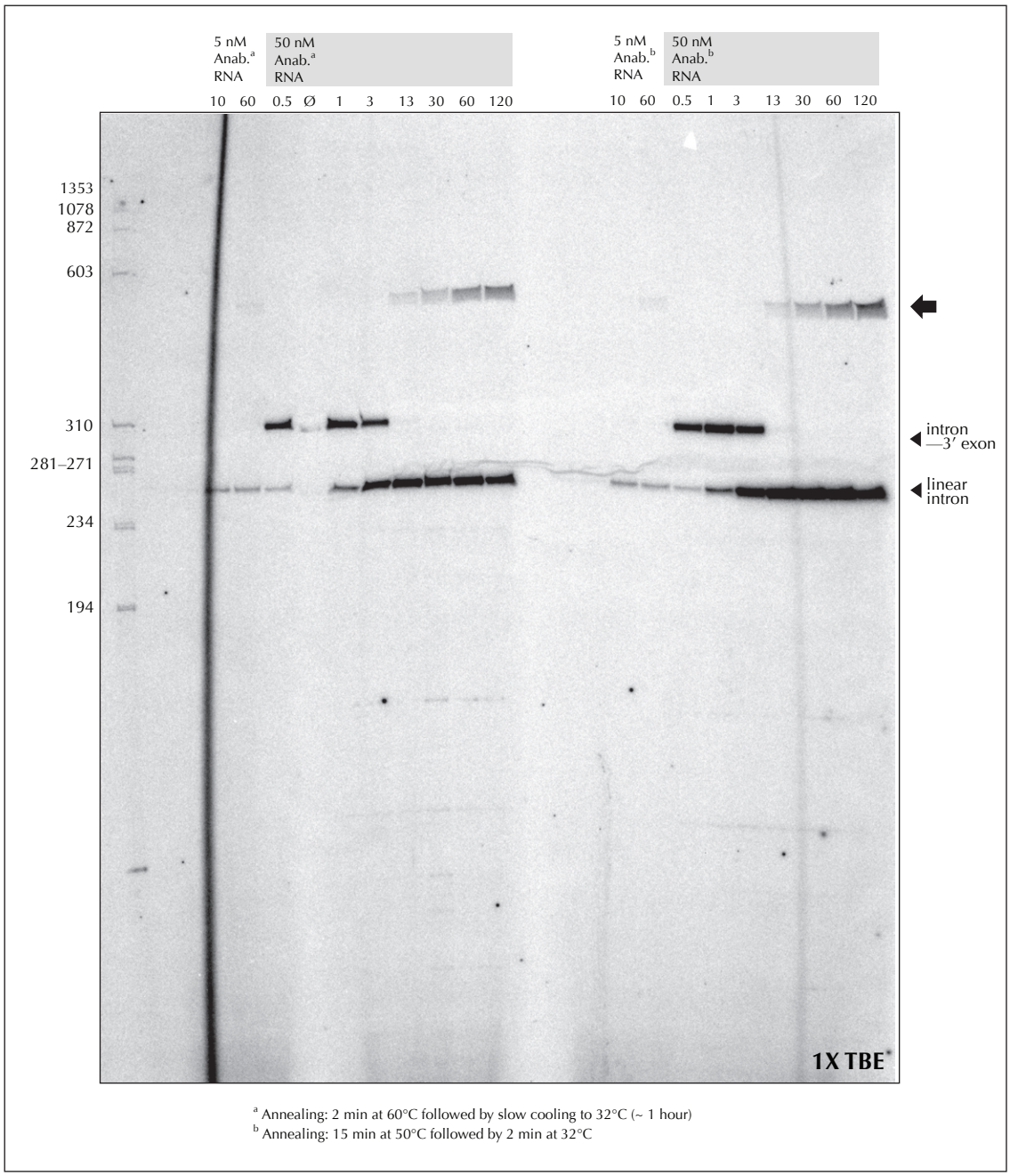


[a] Annealing: 2 min at 60°C followed by slow cooling to 32°C (~ 1 hour)
[b] Annealing: 15 min at 50°C followed by 2 min at 32°C

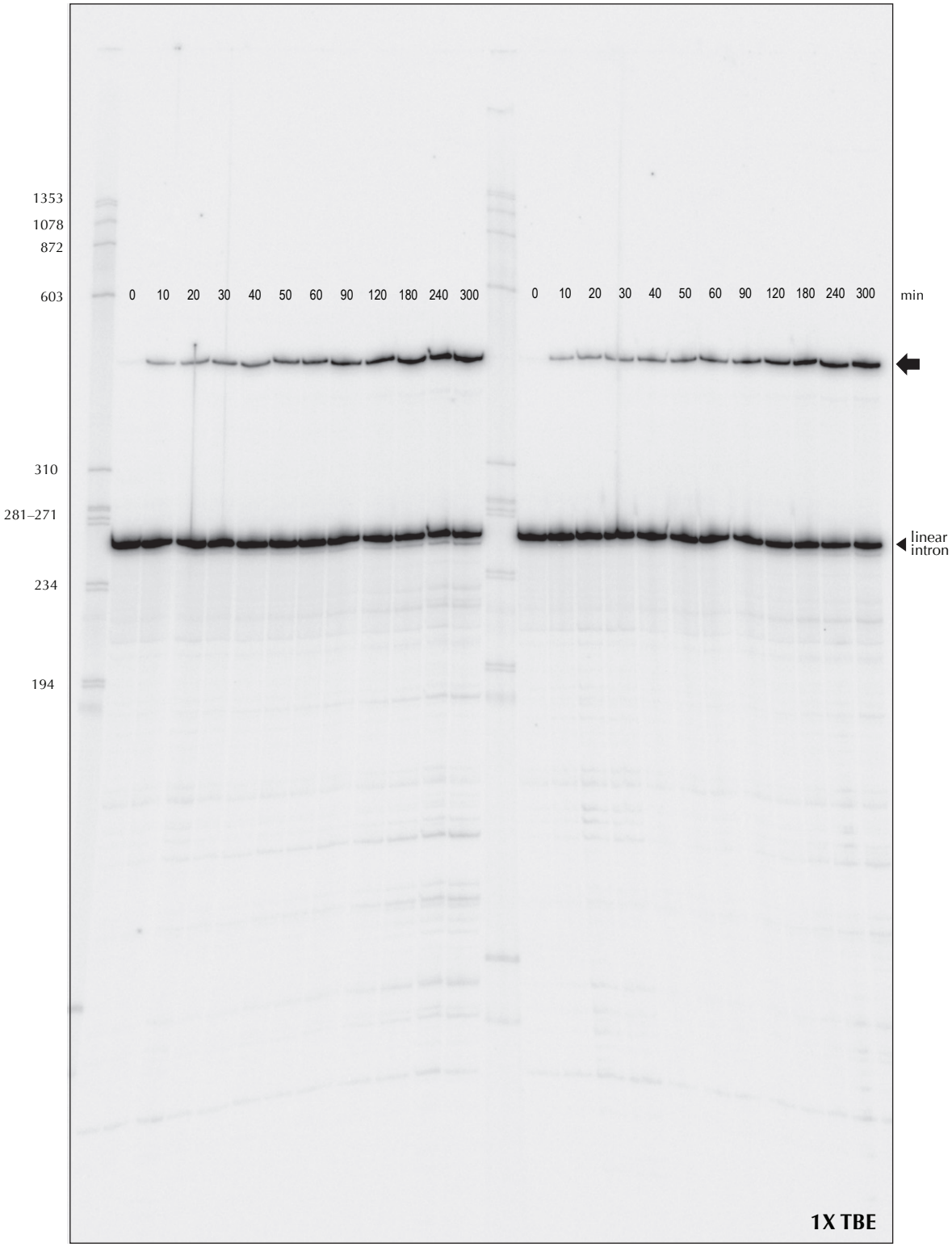
Supplementary Figure 6 | Full-length versions of the gels shown in Fig. 2a, 2f and 2d.



Supplementary Figure 7 | Full-length gel of the time-course experiment shown in Fig. 2b. Two independent experiments were run side-by-side. The RNA was annealed according to two different protocols (see footnote). A 1-hour long timecourse was also performed at 5.0 nM RNA. The part of the gel depicted in Fig. 2b is the left part of this gel.



Supplementary Figure 8 | Full-length gel of the time-course experiment shown in Fig. 2c. The two independent experiments run at 0.5X TBE were run side-by-side on this gel at 1X TBE. The RNA was annealed according to two different protocols (see footnote). A 1-hour long timecourse was also performed at 5.0 nM RNA. The part of the gel depicted in Fig. 2c is the left part of this gel.



Supplementary Figure 9 | Full-length gel of the time-course experiment shown in Fig. 2h. Two independent experiments were run side-by-side. The part of the gel used in Fig. 2h corresponds to the experiment shown on the right.

Supplementary Methods

In vitro transcription of the unlabeled precursor RNA

The *pAtRNA-1* plasmid[1] containing a 40-nucleotide 5' exon (beginning at base 1 of the mature $tRNA^{Leu}$), a 249-nt intron, and a 45-nt 3' exon (ending immediately prior to the CCA sequence), was linearized using the restriction enzyme Ear I (New England Biolabs (NEB) #R0528S), purified by phenol/chloroform extraction followed by ethanol precipitation, and transcribed for 1 h at 37°C in a 25 μL-reaction mix containing: ~3.0 μg/μL linearized plasmid; 5.0 mM each rNTP; 8.0 mM $MgCl_2$ (at such concentrations, all the Mg^{2+} ions are chelated by the rNTP, which inhibits cotranscriptional self-splicing [1]); 2.0 mM spermidine.HCl; 10 mM DTT; 0.01% Triton X-100; 4.0 ng/μL inorganic pyrophosphatase (Sigma #I1891-100UN); 40 U RNasin Plus RNase inhibitor (Promega #N261B); 2 μL T7 RNA polymerase (prepared in house by Anne Gooding); 50 mM Tris.HCl pH 7.5.

Prior to being used in catalytic assays, the transcription product (precursor RNA) was purified using either of the following two methods:

1. Gel-filtration on a G-25 Sephadex quick spin column (Roche #1 273 990) according to the manufacturer's protocol (with the addition of 20.0 μL H_2O during the wash step to maximize recovery);
2. Denaturing gel electrophoresis (6% acrylamide/bisacrylamide [29:1]; 8.0 M urea; 1X TBE). The gel slice corresponding to the precursor RNA was crushed and soaked for 12 h at 4°C in 1.0 mL of an elution buffer containing: 1.0 mM EDTA; 250 mM NaCl; 10 mM Tris.HCl pH 7.5. The solution was filtered through a 0.2 μm filter, precipitated for 16 h at -20°C in 70% EtOH, 100 mM Na acetate pH 5.3, centrifugated for 35 min at 13,500 g, and dried for 10 min under vacuum (SpeedVac).

The precursor RNA was stored at -20°C in water.

Self-splicing and circle formation assays

The *Anabaena* precursor RNA was diluted to 500 nM in a buffer (buffer A) containing: 15 mM $MgCl_2$; 25 mM NaCl; 25 mM HEPES pH 7.5. The RNA was subsequently incubated either for 2 min at 60°C in a heating block and slow cooled for ~1 h to 32°C, or for 15 min at 50°C and then for 2 min at 32°C. The catalytic activity was independent of the chosen folding protocol (Supplementary Figs. 7 and 8).

Reactions in the initial self-splicing assays proceeded for 1 h or 24 h at various temperatures in 10 μL-reaction mixes containing: 50 nM RNA; 0.2 mM GTP; 10 μCi α^{32}P-GTP (PerkinElmer); salts and buffer as described[2]. Reactions were stopped by the addition of a solution containing: 500 mM EDTA; 7.0 M urea; 0.02% bromophenol blue; 0.02% xylene cyanol; 1X TBE (100 mM Tris-base; 83 mM boric acid; 1.0 mM EDTA).

Subsequent reactions (circle formation) were performed in a similar manner, exclusively in buffer A. Incubations proceeded for the various times specified in the figures at 32°C (equivalent to condition #A in[2]). 10 μCi γ^{32}P-GTP (PerkinElmer) were employed in place of α^{32}P-GTP for the experiment shown in Figure 1G.

Denaturing polyacrylamide gel electrophoresis (PAGE) and data analysis

Products were separated on denaturing sequencing gels (6% acrylamide/bisacrylamide [29:1]; 7.0 M urea; 1X TBE). The gels were dried under vacuum and placed for 18–30 h in phosphorimager screens (GE Healthcare). The screens were scanned using a phosphorimager (Amersham Bioscience/GE). The gel images from the time-course experiments used to determine the catalytic rate constant were analyzed using ImageQuant TL v. 2005 (Amersham Bioscience/GE). A first-order catalytic rate constant (k) was calculated in Excel (Microsoft) using plots of fraction of linear intron (F_{linear}) versus time (t) by fitting to the equation, $F_{linear} = F_o\, e^{-kt}$, where F_o is the initial fraction of linear intron.

Gel-purification of the linear intron and of the circle

The linear intron was obtained by performing five 100 μL-reactions in buffer A, each containing: 50 nM RNA; 0.2 mM GTP; 10 μCi α^{32}P-GTP (PerkinElmer). Reactions were incubated for 13 min at 32°C, and stopped by the addition of 4.0 μL of 0.5 M EDTA pH 8.0 per tube. The reactions were pooled, concentrated on a 1.5-mL 10,000 MWCO (molecular weight cut-off) centrifugal filter device (Millipore #42421) for 25 min at 4°C and 13,800 g, and purified by denaturing PAGE as described above, with the following modifications: the band was crushed in a 2.0 mL sterile syringe, then placed for 1 h at -80°C; the linear intron was eluted for 4 h in 1.3 mL of the elution buffer and concentrated on a 4.0 mL 10,000 MWCO centrifugal filter device (Millipore #UFC8 01008) for 15 min at 4°C and 4,000 g. The RNA was precipitated as described above.

The purified linear intron was resuspended in 25 μL of buffer A, incubated for 2 h at 32°C (without addition of any GTP), and purified as described above for the linear intron. The purified circle was resuspended in 1.0 mM EDTA. The final radioactivity of the purified circle was measured by the number of counts per minute (cpm) for 1.0 μL of the RNA solution diluted into 3.0 mL ScintiSafe Econo 1 solution (Fisher Scientific #SX20-5), obtained using a scintillation counter (Beckman #LS3801). The purified circle was stored at -20°C and 2,000 cpm/μL.

Sequencing across the circularization site

A DNA primer complementary to the P4–P5 region of the *Anabaena* intron was ordered from Sigma-Proligo (sequence: 5'-GCCTTGTCTATAGCTAGATTTAGGTTTCCCTG-3'). 60 pmoles of primer were 5' end labeled for 30 min at 37°C in a 10-μL mix containing the following reagents: 10 U T4 polynucleotide kinase (New England Biolabs #M0201S); 1X T4 polynucleotide kinase reaction buffer (NEB; 10 mM $MgCl_2$, 5 mM DTT, 70 mM Tris-HCl pH 7.6); 20 μCi (~ 50 pmoles) γ-^{32}P ATP (PerkinElmer). The end-labeled primer was purified by gel-filtration (see above). The primer was resuspended in 100 μL 1.0 mM HEPES pH 8.0 to a final concentration > 500,000 cpm/μL.

Sequencing reactions for each nucleotide were performed each in a 18-μL reaction mix containing the following components: ~ 2 pmoles of purified *Anabaena* circle (pre-incubated in the presence of 3 μL of labeled DNA primer for 2 min at 65°C, then for 10 min at 35°C), 200 U Superscript III reverse transcriptase (Invitrogen #18080-093), 3.0 mM MgCl2, 75 mM KCl, 5 mM DTT, 50 mM Tris-HCl pH 8.3, 0.5 mM (each) dNTP, 0.5 mM (each) one of the four ddNTP. The reaction proceeded for 1 min at 50°C prior to the addition of the reverse transcriptase, then for 15 min at 50°C, before being stopped by the addition of 5.0 μmoles NaOH followed by incubation for 5 min at 95°C. 29 μL of an acid stop mix (4:25 (v/v) mixture of 1.0 M unbuffered Tris-HCl and stop dye containing: 85% formamide, 0.5X TBE, 50 mM EDTA, pH 8.0, 0.02% bromophenol blue; 0.02% xylene cyanol) were added to each of the four 22 μL-sequencing reaction mixes. These four mixes were incubated for 5 min at 95°C and separated for 5 h on a denaturing sequencing PAGE (15% acrylamide/bisacrylamide [29:1]; 7.0 M urea; 1X TBE). The gels were dried and visualized as described above.

Nuclease enzymatic digestions

Control P4–P6 ΔC209 RNAs[3] were transcribed as described above for the *Anabaena* intron, except for the following differences: 40 mM $MgCl_2$, 0.5 μg DNA template. Four transcription reactions were performed, each containing 10 μCi of one of the four α^{32}P-NTP (PerkinElmer). Trancriptions proceeded overnight at 25°C and RNAs were purified as described above.

The G$^{3'}$p$^{5'}$G dinucleotide containing a regular 3',5' linkage was chemically synthesized by Dharmacon Inc. at Thermo Fisher Scientific (Lafayette, CO), and deprotected according to the manufacturer's protocol. The G$^{2'}$p$^{5'}$A dinucleotide containing a 2',5' linkage was purchased from Sigma (#G9011). Both dinucleotides were 5' end labeled for 45 min at 37°C in a 20-μL reaction mix containing the following: 30 nmoles dinucleotide, 5.0 μCi γ^{32}P-GTP (PerkinElmer), 25 μM ATP, 20 U T4 polynucleotide kinase (NEB #M0201S); 1X T4 polynucleotide kinase reaction buffer (see composition above). The labeled

dinucleotides were diluted to 2,000 cpm/μL in 0.5X TE buffer (5.0 mM Tris-HCl pH 7.5, 0.5 mM EDTA).

To confirm ribonuclease specificity[4-6] under the conditions employed herein, 2,000 cpm of either the control P4–P6 RNAs, the dinucleotides or the *Anabaena* circle were digested to completion using either nuclease P1 (USBiological #N7000) using conditions derived from[7] (2 U nuclease P1, 20 mM Na acetate pH 5.3; incubation for 1 h at 50°C) or a combination of RNase A (gift of David Zappulla) and RNase T1 (Ambion #AM2283) (or RNase T1 only for the dinucleotides) (5 mg/mL RNase A, 2 U RNase T1, 50 mM Tris-HCl pH 7.5, 1 mM EDTA; incubation for 1 h at 37°C).

The products of enzymatic digestion were fractionated for ~ 45 min by ascending thin-layer chromatography (TLC) on 20 x 20 cm PEI-cellulose plates containing a fluorescent indicator (Sigma #Z122882-25EA) using 1.0 M LiCl, 50 mM HEPES pH 7.0 as the solvent[7]. TLC plates were dried at room temperature and either UV shadowed (254 nm) or exposed for 16 h in a phosphorimager screen (GE Healthcare) and analyzed similarly to sequencing gels.

Circle formation from body-labeled RNA

A 25 μL-transcription reaction of the precursor RNA was performed as described above, with the addition of 50 μCi α^{32}P-ATP (PerkinElmer). The precursor RNA was purified using denaturing gel electrophoresis as described above, and resuspended in 25 μL 0.1 mM EDTA. This preparation of the body-labeled precursor was split in two. One half self-spliced in a 100-μL reaction mix as described above but in the presence of 0.2 mM GTP only (no labeled GTP added), while the other half self-spliced in the presence of 0.2 mM GMP. The resulting products were purified, eluted and precipitated as described above, before being resuspended to a concentration of ~ 100 nM (~ 5,000 cpm/μL) into a solution containing 0.5 mM EDTA and 50 mM HEPES pH 7.0. The concentration of the RNA was calculated from the specific activity (3,000 Ci/mmole; 10 μCi/μL) and the corresponding number of cpm for 1.0 μL of the α^{32}P-ATP stock solution, as well as from the ratio of labeled over unlabeled ATP

during transcription (0.000133) and the number of adenosine residues in the self-spliced intron (93).

Subsequent reactions (circle formation from the intron self-spliced using GTP) were performed in triplicate, exclusively in buffer A as described above, but at RNA concentrations of 2.0 nM, 5.0 nM and 15 nM. Incubations proceeded for 60, 120, 180 and 300 min at 32°C (equivalent to condition #A in[2]). Incubation of the intron self-spliced using GMP was only performed at 5.0 nM RNA, for 60, 120 and 300 min at 32°C. The products were separated by denaturing PAGE and the data were analyzed as described above. A first-order catalytic rate constant was calculated as described above at concentrations of 2.0 nM and 15 nM for the intron self-spliced using GTP.

Figure preparation

The figures presented in this manuscript were prepared using Adobe Illustrator and Adobe Photoshop (Adobe).

References

1. Zaug, A.J., McEvoy, M.M. & Cech, T.R. *Biochemistry* **32**, 7946-7953 (1993).
2. Vicens, Q., Paukstelis, P.J., Westhof, E., Lambowitz, A.M. & Cech, T.R. *RNA* **14**, 2013-2029 (2008).
3. Juneau, K., Podell, E., Harrington, D.J. & Cech, T.R. *Structure* **9**, 221-231. (2001).
4. Hoadley, K.A., Purtha, W.E., Wolf, A.C., Flynn-Charlebois, A. & Silverman, S.K. *Biochemistry* **44**, 9217-9231 (2005).
5. Koepke, J., Maslowska, M., Heinemann, U. & Saenger, W. *J. Mol. Biol.* **206**, 475-488 (1989).
6. Wodak, S.Y. *J. Mol. Biol.* **116**, 855-875 (1977).
7. Zaug, A.J. & Cech, T.R. *Nucleic Acids Res.* **10**, 2823-2838 (1982).