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RNA self-processing: Formation of cyclic species and concatemers from a small engineered RNA

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ABSTRACT

We have engineered a self-processing RNA, derived from the hairpin ribozyme that runs through a cascade of cleavage and ligation reactions thereby changing its topology. The first two cleavage events leave the resulting RNA with a 5′-OH group and a 2′,3′-cyclic phosphate. Thus, upon refolding, intramolecular ligation delivers a cyclic species. In addition, we demonstrate formation of concatemers resulting from multiple intermolecular ligations. Our results demonstrate the potential of RNA for self-supported topology changes and support the suggestion of 2′,3′-cyclic phosphates being suitable activated building blocks for reversible phosphodiester bond formation in the RNA world.

1. Introduction

Over the past two decades, RNA engineering has become a strong field of research. On one hand, synthetic RNAs have enabled sophisticated studies into structure and mechanism of RNAs with various cellular functions. On the other hand, understanding the mechanism of action of those RNAs has helped in the design of new functional entities composed of natural and/or modified ribonucleic acids. Research in the field of chemical biology for example has afforded a variety of ribozymes with useful activities, artificial riboswitches, and diagnostic tools [1–4]. Our contribution to the field have been a number of RNA systems derived from the hairpin ribozyme that allow for controlled cleavage and ligation of suitable RNA substrates [5–11]. In particular, we have developed twin ribozymes for RNA repair and site-specific labeling [5–8], and aptazymes that are controlled by the oxidation state of the allosteric activator flavine mononucleotide [9,10]. In the course of those studies, we also sought to develop a ribozyme that processes itself from a linear transcript to a cyclic RNA species [11]. Apart from the interest in RNA-self-processing, the development of a strategy for the cyclization of RNA to be used as nuclease resistant molecular tools was a strong motivation for this project. The primary RNA resulting from transcription was designed to undergo two cleavage reactions followed by ligation as depicted here in Fig. 1 (103mer transcript → 92mer/94mer after first cleavage → 83mer after second cleavage → cyclic 83mer after intramolecular ligation). It is derived from the hairpin ribozyme [12–14], the cleavage/ligation characteristics of which are particularly suited to this purpose. The hairpin ribozyme cleavage reaction proceeds by nucleophilic attack of the 2′-OH on the neighboring phosphorous leading to a pentavalent intermediate (Fig. 2). From that, the 5′-oxygen of the neighboring nucleoside is released forming the final products, one carrying a 2′,3′-cyclic phosphate, the other a 5′-OH group. Ligation proceeds via the same intermediate, thus requiring fragments with 5′-OH and 2′,3′-cyclic phosphate as substrates (Fig. 2) [12–14]. The hairpin ribozyme is a rather rigid structure undergoing very little conformational change upon cleavage/ligation. Therefore, ligation being the faster reaction compared with cleavage, is favored if cleavage products/ligation substrates are tightly bound to the ribozyme. However, if the ribozyme structure is less stable, such that cleavage products/ligation substrates can easily dissociate, the cleavage reaction becomes dominant [15]. Based on these features, we have previously designed the 103mer transcript [11] to fold in the two conformations shown in Fig. 1a. Both structures should favor cleavage, because upon cleavage the short overhanging 5′-, and 3′-terminal sequences can easily dissociate. According to hairpin ribozyme characteristics, cleavage of the 5′-terminal sequence leaves a 5′-OH group and cleavage of the 3′-terminal sequence leaves a 2′,3′-cyclic phosphate at the remaining 83mer

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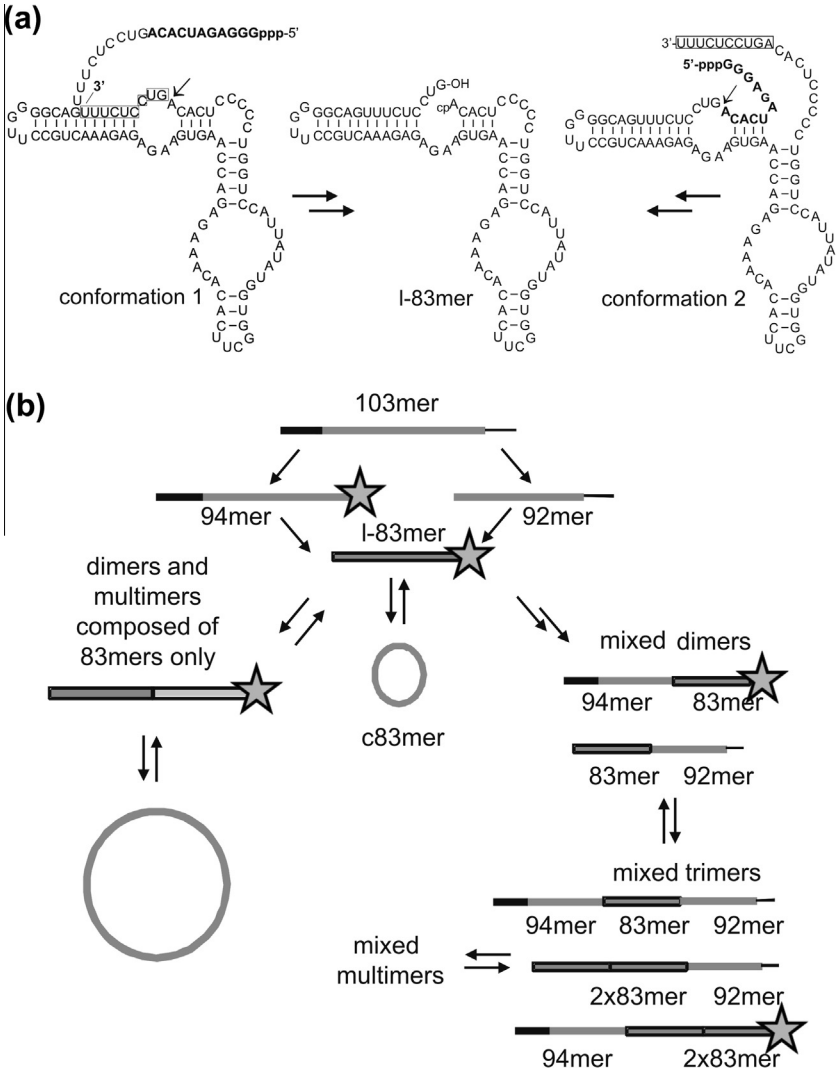

Fig. 1. Self-processing RNA CRZ-2 derived from the hairpin ribozyme. (a) Sequence of CRZ-two folded in two alternative conformations (left and right structure). Upon two subsequent cleavage events, both RNA structures are converted in the shortened sequence shown in the middle. Cleavage sites are marked with an arrow. (b) Reaction path of self-processing. The original 103mer RNA derived from the hairpin ribozyme cleaves itself to remove the 5′- and 3′-ends. Resulting from a typical hairpin ribozyme reaction, the produced 83mer has a 5′-OH group and a 3′-terminal 2′,3′-cyclic phosphate. Due to the intrinsic hairpin ribozyme activity, it can form cyclic species by intramolecular ligation or concatemers by self-association and intermolecular ligation. Cyclic phosphates are marked with an asterisk.

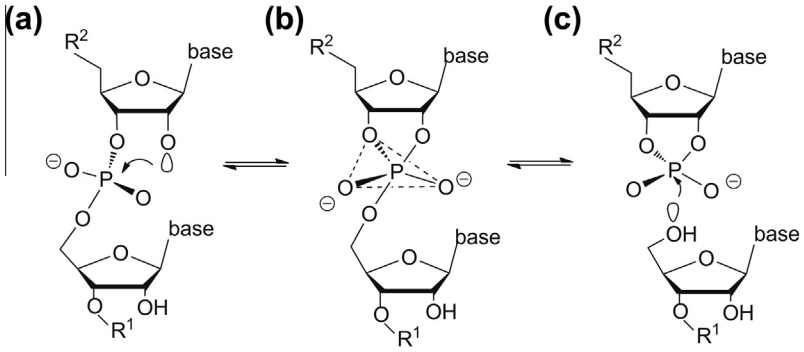

Fig. 2. Mechanism of the hairpin ribozyme catalyzed cleavage and ligation of RNA. Both reactions proceed via the same pentavalent intermediate, generating characteristic fragments with 2′,3′-cyclic phosphate and 5′-OH after cleavage, and requiring these functionalities for ligation. R^1, R^2 = RNA chain.

(Fig. 1). Thus, the produced 83mer still possesses all elements required for hairpin ribozyme activity, and now should favor ligation due to the intramolecular arrangement of the ligation site hampering dissociation. The final ligation would lead to the expected cyclic RNA. To our surprise, upon reaction analysis, we had detected a variety of products, very likely resulting from intermolecular ligation in addition to the predicted intramolecular cyclization [11]. This is a very interesting result, because it shows the potential of

RNA for self-organization and polymerization. Intermolecular ligation apparently is in strong competition with intramolecular reaction, and thus might be considered as a model of self-supported generation of longer RNAs from smaller fragments in the RNA world. We decided to further study the potential of this RNA for concatemerization versus cyclization in order to possibly define conditions that allow controlling the tendency towards the one or the other activity. As a first step towards this goal, we here present an analysis of the formed products and provide support of their cyclic or linear nature.

2. Materials and methods

2.1. General information

All aqueous solutions were made with demineralised water which was further purified with a BarnstedtNanopure system and autoclaved. Deoxynucleotide triphosphates (dNTPs), nucleotide triphosphates (NTPs), Klenow buffer, DNase I, Klenow fragment exo-, *Sma*I, RiboLock™, RiboRuler low range RNA ladder and polynucleotide kinase (PNK) were purchased from Fermentas Company; RNA Ligase (T4 RnL2) and *Vent*® polymerase were obtained from New England Biolabs. Dried phophodiesterase I (PDE I) from *Crotalus atrox* was purchased from Sigma–Aldrich®. Agar, X-Gal and IPTG were purchased from Roth. DNA primers were provided by Biomers. All reagents were of analytical grade and filtered before use.

Measures for glass plates of polyacrylamide gel electrophoresis were $200 \times 150 \times 1.5\ mm^3$ All UV spectra were recorded on a NanoDrop ND 1000 spectrophotometer. Product band intensities were quantified using Gene ImageIR 4.05.

2.2. RNA synthesis

Klenow primers (P1: 5′-AAA GAG GAC TGT GAG GGG GAC CAG GTA ATA TAC CAC CGA AGT *GTG TTT CTC TGG TTC ACT TCT C*-3′ P2: 5′-TAA TAC GAC TCA CTA TAG GGA GAT CAC AGT CCT CTT TGA CGG GGT TCC GTC AAA *GAG AGA AGT GAA CCA GAG AAA CA*-3′) with 21 bp overlap shown in italics were used in the Klenow reaction with Klenow exo$^-$polymerase from Fermentas company following the manufacturer's protocol. The obtained dsDNA was subjected to native agarose gel electrophoresis (1.5%, EtBr stained), product containing bands were cleaved off, and DNA was purified utilizing the QIAquick gel extraction kit (Qiagen).

RNAs were synthesized by in vitro transcription of double stranded DNA templates (1 μM) with T7 RNA polymerase (35–70 ng/μl, self-made) in the presence of the four ribonucleoside triphosphates (2 mM) and 1 U/μl RiboLock™ and 1× HEPES buffer in a total reaction volume of 50 μl for 3 h at 37 °C. The DNA template was digested using DNaseI following the manufacturer's protocol. Final purification was achieved by electrophoresis through 15% denaturing polyacrylamide gels, followed by elution of the product with sodium acetate (0.1–0.3 M, pH 7 at 10 °C) and precipitation with ethanol overnight at −20 °C.

2.3. Synthesis of the inactive linear and cyclic variant of the 83mer

The inactive 83mer (in-l-83) was transcribed from the dsDNA template: 5′-*TAA TAC GAC TCA CTA TA* G**GG AG**A GT**G** CTC CCA AAG AG**G ACT** GTG AAC CAG AGA AA**G** ACA CTT CGG TGG TAT ATT ACC TGG TCC CCC TCA CAG TCC TCT TT-3′ (Sequence of the sense strand is given; T7 promotor sequence in italics, sequence changes in comparison to the linear 83mer resulting from self-processing of the 103mer transcript are indicated in bold). For in vitro transcription, GMP was added to the NTP mix at a 4.8:1 ratio of GMP/GTP. Transcription was carried out following the protocol of Harris and Christian for incorporation of guanosine monophosphorothioate [16].

For cyclization of in-l-83, enzymatic ligation was conducted for 4 h at native conditions using T4 RnL2 in the supplied buffer (composed of 50 mM Tris–HCl, 2 mM $MgCl_2$, 1 mM DTT, 400 μM ATP, pH 7.5) in a total reaction volume of 20 μl following the suppliers protocol.

2.4. Ribozyme self-processing reaction

RNA-transcripts or isolated fragments (50 pmol) were taken up in Tris–HCl buffer (10 mM, pH 7.5). After denaturation for one minute at 90 °C, RNA folding for 15 min at room temperature followed. To initiate the cleavage reaction, $MgCl_2$ hexahydrate to a final concentration of 10 mM was added and cleavage took place within 2 h at 37 °C. For preferential ligation, Mg^{2+} concentration was increased up to 50 mM, and reaction proceeded for 2, 4 or 24 h at 37 °C. The final concentration of RNA in the self-processing reaction was 2 μM. Reaction was stopped by ethanol precipitation over night or 1:1 addition of stop mix composed of urea (7 M) and EDTA (50 mM) for direct PAGE analysis.

2.5. Two-dimensional denaturing gel electrophoresis

RNA (50 pmol) was analyzed using two-dimensional PAGE (first dimension, 12% polyacrylamide; second dimension, 16% polyacrylamide) under denaturing conditions (7 M urea). After the second electrophoresis, the ethidium bromide-stained gel was visualized using Chemi-Smart 2000 WL/LC 26M.

2.6. Sequencing analysis

RNA was reverse transcribed by SuperScript™ III Reverse Transcriptase (Invitrogen) with RT-primer, 5′-ATA GGA TCC AAA GAG GAC TGT GAG GGG G-3′ according to the manufacturer's protocol. Non-purified single stranded cDNA was amplified with Taq (0.025 U/μl, self-made) and Vent® (0.01 U/μl) DNA polymerase with the RT-primer, reverse primer, 5′-ATA TCT AGA GAC GGG GTT CCG TCA AAG-3′, dNTPs (1 μM) and 1× Thermo Pol PCR buffer 4S in a total reaction volume of 100 μl. Amplified double stranded DNA was phosphorylated using 0.5 μg DNA, supplied reaction buffer A (with final concentrations: 50 mM Tris–HCl pH 7.6 10 mM $MgCl_2$, 5 mM DTT and 0.1 mM spermidine) 1 mM ATP and 0.5 U/μl PNK) in a total volume of 20 μl at 37 °C for 30 min, followed by heat inactivation of the PNK applying 70 °C for 10 min. Phosphorylated DNA was purified using QIAgen PCR purification kit and ligated into the *SmaI* restriction site of pUC18 (Stratagene). Ligation was conducted using a relation of 1:3 for vector: insert. 5 ng/μl plasmid and 15 ng/μl insert were added to 20 U of T4 DNA ligase in the supplied reaction buffer (composed of 50 mM Tris–HCl 10 mM $MgCl_2$, 1 mM ATP 10 mM DTT) in a total volume of 20 μl. Ligation proceeded for 16 h, starting at 18 °C for 3 h. followed by decreasing the temperature by 2 grad every 3 h. After 9 h, temperature was decreased to 10 °C and hold for 2 h and finally to 4 °C and hold for another 2 h. Then, the mixture was treated at 70 °C for 10 min for heat inactivation. The resulting plasmid was transformed into *E. coli* TG1 competent cells (cc) using a heat shock transformation protocol. Two hundred and fifty microliter of ccTG1 were carefully mixed with 20 μl of ligation mix and left on ice for 30 min, followed by immediate incubation at 42 °C for 45 s, and then putting the cells back on ice for additional 5 min. Growing was reinitiated at 37 °C in a shaking incubator at 200 rpm for 1 h. Agar plates including Ampicillin with a final concentration of 100 μg/ml were treated with X-Gal (40 mg/ml – 40 μl per plate) and IPTG (0.25 M –– 30 μl per plate). Colonies of interest were

identified via blue-white screening, and the DNA was purified and sequenced by GATC Biotech AG.

2.7. Digestion with phosphodiesterase

The isolated linear 83mer (2.25 μM) in 150 μl ligation buffer (10 mM Tris–HCl pH 7.5, 50 mM $MgCl_2$) was incubated at 37 °C for two hours. Lyophilized PDE I was solved in water and 2 μl were added to the RNA mixture to a final concentration of 0.7 μg/μl. The same amount of water was added to a control reaction mixture. The first aliquot (25 μl) was taken immediately after mixing the reagents. It was added to an equal volume of stop mix (7 M urea and 50 mM EDTA) and frozen in liquid nitrogen (time zero). Further aliquots were taken every two minutes and treated as described above. Samples were analyzed by PAGE (denaturing, 15%) after heat denaturation at 90 °C for two minutes.

2.8. Gene bank indices: EMBL Nucleotide Sequence Database

Trimer: Accession#: HF565114
Exemplary dimer: Accession#: HF565115
Exemplary dimer: Accession#: HF565116

3. Results and discussion

We have prepared the 103mer transcript (now called CRZ-2) and followed its processing reaction by polyacrylamide gel electrophoresis (Fig. 3). Processing is observed already during in vitro transcription as the characteristic product bands occur (Fig. 3a, lane 1). This is not surprising, since transcription proceeds at 37 °C, at pH 7.5, and in the presence of Mg^{2+} and other cations, conditions that also support hairpin ribozyme activity [14]. From the in vitro transcription mixture, the full lengths 103mer transcript as well as the presumed linear 94-, 92- and 83mer fragments were isolated and incubated at typical hairpin ribozyme cleavage conditions (Fig. 3b). As expected, the 103mer was cleaved again to the 92/94mer and 83mer, and the 92/94mer was further cleaved to the 83mer, as shown by the corresponding bands in lane 1 and 2. However, in addition to the incubated fragments, a number of bands with strongly retarded migration were observed (Fig. 3b, bands ***a***–***e***). As already mentioned above, we hypothesized that these bands represent oligomeric products of the linear 83mer,

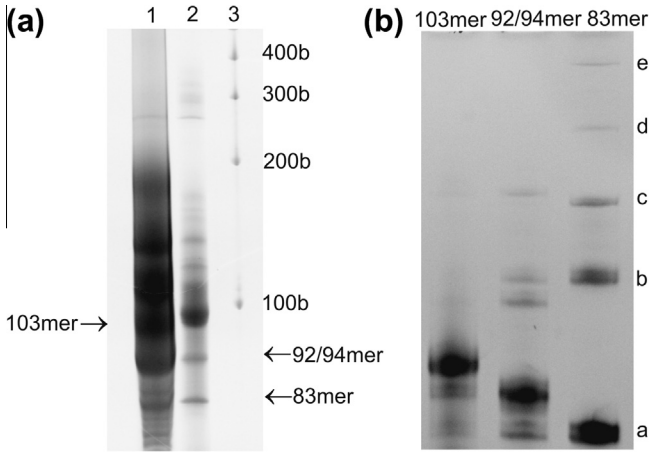

Fig. 3. In vitro transcription and self-processing of CRZ-2. (a) Fifteen percentage denaturing polyacrylamide gel, lane 1: in vitro transcription, lane 2: incubation of the isolated full length transcript in reaction buffer (10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 37 °C, 2 h(cleavage) and 50 mM $MgCl_2$, 37 °C 2 h (ligation), lane 3: RNA ladder; (b) Re-incubation of isolated 103mer, 92/94mer and 83mer in cleavage buffer at 37 °C for 2 h. Individual species resulting from reaction of the 83mer were labeled ***a***–***e***.

92mer and 94mer. Oligomerization would require association of the linear fragments to form active complexes, which is well possible due to sequence complementarities. Furthermore, dimerization of the hairpin ribozyme was described previously [17], and thus it is well possible that self-association followed by intermolecular reactions plays also a role in the system described here. This in turn would explain the production of concatemers by intermolecular ligation. First support for this suggestion, in our system was obtained from sequence analysis. The slowly migrating products in the range of 200–400 bp (Fig. 3a) were isolated and pooled, reverse transcribed and amplified for cloning and sequencing (in Supplementary Fig. S1). Sequence analysis showed the presence of dimers and trimers (for sequence codes see Section 2). However, no concatemers longer than trimers were found. A most likely explanation for this observation is that due to the presence of Mg^{2+} in the isolation and reverse transcription buffers, longer concatemers were re-cleaved to trimers, dimers and monomers as shown by the pattern of cDNA obtained from reverse transcription of isolated species (Supplementary Fig. S1). Furthermore, RT-PCR amplification of a concatemer is quite challenging, because the PCR primers will not only bind to the 3′-end of the template but also to internal sites at each repeating sequence unit of the concatemer. This in turn will produce a majority of monomer units instead of concatemers after amplification. From the mixture of the obtained cDNAs, after native polyacrylamide gel electrophoresis only the upper bands corresponding to the designated longer concatemers were isolated (in Supplementary Fig. S1), cloned and sequenced. In addition to full length double stranded concatemers, these upper bands in a native gel may also represent nicked duplexes containing several monomer strands bound to the corresponding concatemeric strand, such that for cloning and sequence analysis monomer units are over-represented. Indeed, out of 34 sequenced samples, 20 monomers, 13 dimers and only one trimer were found. Thus, there is no direct evidence for the existence of long concatemers. However, due to the clear detection of di- and trimers, the system's general capability of self-processing involving concatemerization has been shown.

All following analyzes were done exclusively with the isolated linear 83mer. It cannot undergo further cleavage; however, ligation can take place leading to a cyclic monomer and/or linear and cyclic oligomers, due to the presence of both, the 5′-OH group and the 3′-terminal cyclic phosphate as described above (Fig. 1). First, we analyzed the products being represented by bands ***b***–***e*** (Fig. 3) resulting from incubation of the isolated 83mer (band ***a***). As a first attempt to differentiate between the linear and cyclic 83mer, we prepared an inactive variant of the linear 83mer by introduction of a number of sequence changes (see Section 2), and part of that was enzymatically circularized. In order to generate the 5′-phosphate required for circularization by enzymatic ligation, in vitro transcription was carried out in the presence of guanosine monophosphate as transcription initiator, thus delivering RNAs with 5′-monophosphate instead of the 5′-triphosphate (see Section 2). Circularization was performed with T4 RNA ligase II. The linear species (in-l-83) runs faster through a 15% denaturing polyacrylamide gel than the circular species (in-c-83) (Fig. 4). Comparison of this pattern with the bands resulting from the ribozyme reaction confirms the identity of band ***a*** being the linear 83mer. However, it has to be noted that the cyclic inactive 83mer (lane 4) has no corresponding band in lane 3. Apparently, there is no cyclic 83mer formed upon the ribozyme reaction, or it shows a different migration behavior in the gel compared with the inactive cyclic 83mer. The latter assumption may apply, because the sequence changes made for inhibition of ribozyme activity are likely to influence the structure of the cyclic species, and this in turn would result in distinct retention of the two cyclic species, in spite of both having the same size.

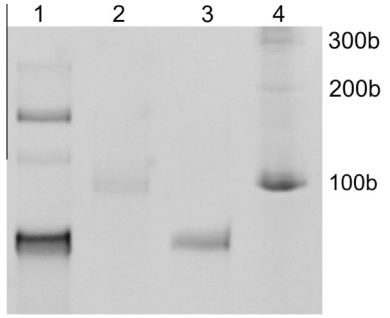

Fig. 4. Fifteen percentage polyacrylamide gel analysis of a separately synthesized inactive cyclic (lane 2) and linear (lane 3) 83mer in comparison to the ribozyme-derived active 83mer (lane 1). Lane 4: RNA ladder.

The most obvious way to differentiate between linear and cyclic nucleic acids is exonucleolytic digestion. However, analysis of isolated species is not possible, since the cyclic RNA upon isolation from the reaction mixture would undergo immediate re-cleavage to its linear counterpart. The linear RNA would be removed by exonuclease digestion, thus evoking further re-cleavage until the cyclic species is consumed and all RNA is digested. Therefore, we added phosphodiesterase I (PDE I) directly to the reaction mixture. This procedure cannot prevent re-cleavage of cyclic species as soon as linear species in the mixture are digested; however, the decay reaction can be better temporarily followed. To this end, after addition of the exonuclease, at time intervals indicated in Fig. 5, aliquots were taken out of the reaction mixture, and reaction was stopped and analyzed by gel electrophoresis. Bands ***b***, ***d*** and ***e*** quickly disappeared, whereas band ***c*** was detectable over 4 min, and only then slowly disappeared from the gel. This implies that band ***c*** represents a cyclic species that first has to undergo re-cleavage before it can be digested, whereas bands ***b***, ***d*** and ***e*** being digested immediately, correspond to linear RNAs. Band ***a***, representing the linear 83mer, as was confirmed by the previous experiment (Fig. 4), is also still detectable after 4 min of PDE treatment, very likely because of its high excess in the reaction mixture.

In order to further confirm the result of this analysis, a two-dimensional PAGE experiment [18–20] was conducted. It was shown previously that migration of linear and circular nucleic acid species is distinctly dependent on the gel pore size [21]. Therefore, electrophoresis was run in the first dimension using 12% polyacrylamide, followed by 16% polyacrylamide in the second dimension. As a result, all linear species should form a diagonal, whereas circular RNAs are expected to be located beyond. In order to better fit the diagonal to linear species, an RNA size standard, being exclusively composed of linear RNA sequences, was added to the reaction mixture (double mix). As shown in Fig. 6, apart from species c, which clearly stands out, all other spots form a diagonal with the fragments of the RNA size standard, thus further supporting the cyclic nature of c.

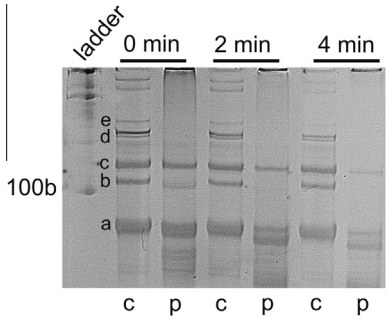

Fig. 5. Analysis of the exonucleolytic digestion of the RNA mixture obtained from incubation of the linear 83mer in ligation buffer (50 mM $MgCl_2$, 37 °C, 2 h). Aliquots were taken at the indicated time intervals and subjected onto a 15% denaturing polyacrylamide gel. Left lane: RNA ladder, lanes marked with c display control reactions including water instead of PDE, lanes marked with p display RNA reaction mixtures including PDE.

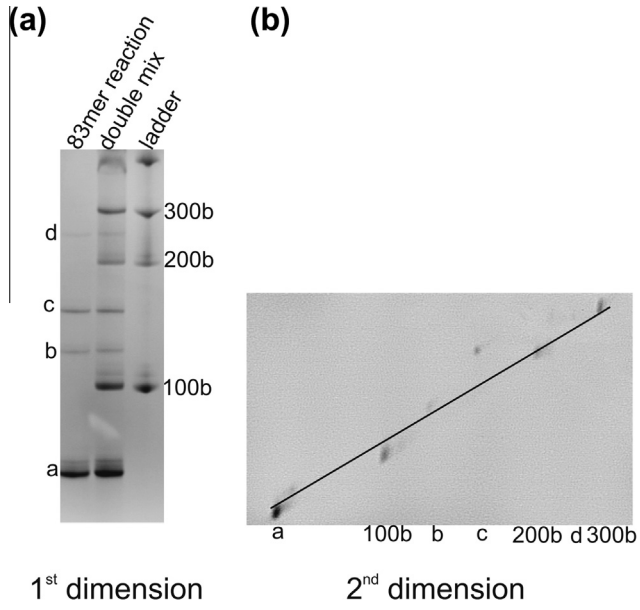

Fig. 6. 2D polyacrylamide gel electrophoretic analysis of self-processing of the linear 83mer using 12% (first dimension a) and 16% (second dimension, b) denaturing polyacrylamide gels. The self-processing reaction was left to proceed for 4 h in ligation buffer at 37 °C. For better visualization, the reaction mixture of the 83mer self-processing reaction was mixed with the RNA ladder prior to subjecting onto the gel. Individual species were labeled as ***a***–***e*** according to Fig. 3.

Lastly, we calculated the relative amount of species occurring at different concentrations of the startingmaterial (in Supplementary Fig. S2). Intermolecular reactions, hence the formation of oligomers, would profit from higher concentrations of the linear 83mer, whereas intramolecular cyclization is independent of concentration. Reaction was carried out at 0.4, 2 and 10 μM concentration of the linear 83mer (Supplementary Fig. S2). The intensity of individual bands was measured and the relative percentage amount within each lane was calculated (Table 1). In general, reaction is enhanced at higher concentration being mirrored in the higher consumption of the linear 83mer (species a), and thus indicating a considerable part of intermolecular reaction. In addition, it is seen that the relative amount of species b increases from the 0.4 μM reaction to the 10 μM reaction. On the opposite, the relative amount of species c is kept constant within error. Even though, this quantification has a rather high error rate, it provides further support to the interpretation made above that species ***b*** is a linear oligomer of the starting 83mer (presumably the dimer), while species

Table 1
Topology analysis. Calculation of the relative percentage of individual species in the reaction mixtures at 0.4, 2 and 10 μM starting concentration of the linear 83mer.

Band[a]	0.4 μM[b]	2 μM[b]	10 μM[b]	Suggested topology
e	1.2	2.0	4.5	–
d	8.2	7.3	7.0	–
c	8.1	6.7	9.5	Circular
b	4.2	13.5	16.2	Linear
a	78.3	70.5	62.8	Linear (83mer educt)

[a] Bands ***a***–***e*** according to Fig. 4.
[b] Concentration of the linear 83mer as starting material. Individual numbers represent the relative percentage of species ***a***–***e*** in the respective reaction mixture.

c is a circularized product (presumably the circularized monomer). We cannot reliably determine the nature of species ***d*** and ***e***. There is no information from the 2D experiment on the nature of these two species, since the concentration of ***d*** and ***e*** was too low to be reliably detected after electrophoresis in the second dimension. According to the data in Table 1, ***d*** could be a cyclic species, whereas ***e*** is rather linear. From the PDE digestion experiment discussed above, one would however suggest that both ***d*** and ***e*** are linear RNAs, since both disappeared immediately after PDE treatment.

In conclusion, we have demonstrated the high potential of a small engineered RNA derived from the hairpin ribozyme for self-processing forming circles and concatemers of different size. This once more underlines the power of RNA for self-supported topology changes and sequence oligomerization. Furthermore, our results support the previous suggestion of 2′,3′-cyclic phosphates being suitable activated building blocks for reversible phosphodiester bond formation [22], even more so, if catalytic RNA structures support the reaction. Our results are in agreement with previous reports emphasizing the importance of cyclic phosphates in the RNA world by demonstrating the formation of ribonucleotides with 2′,3′-cyclic phosphate from a small collection of pre-biotic stock molecules [23,24].

Furthermore, the designed system demonstrates a potential strategy for easy preparation of cyclic RNA molecules. Due to their higher nuclease stability compared with linear RNAs, cyclic RNA molecules are advantageous for intracellular application of aptamers, ribozymes or other functional RNAs in molecular medicine and diagnostics [25–27]. Although, the reaction cascade described here does not deliver a stable cyclic RNA. Due to the intrinsic activity of the hairpin ribozyme, upon isolation the formed cycle is re-cleaved to its linear counterpart until equilibrium is reached. Preventing re-cleavage of the cyclic RNA requires switching hairpin ribozyme activity in a strictly controlled way. This is technically challenging, but might be manageable by engineering a self-processing RNA variant that is controlled by an external effector. We have shown previously [29] that activity of an inactive hairpin ribozyme variant can be switched on by an external oligonucleotide cofactor compensating for missing sequence elements required for activity. Implementing this principle in the system described here would have the advantage that upon removal of the external cofactor, activity is switched off, thus rendering the cyclized RNA stable. Furthermore, even though the hairpin ribozyme requires a number of conserved nucleotides for activity, large parts of the sequence can be varied as desired [28]. Thus, stable cyclic versions of specific aptamers or other RNAs of interest may be prepared by the strategy shown here combined with external activity control. In addition, the competitive oligomerization may be repressed by computer aided sequence optimization. Work along this line is in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.06.013.

References

[1] Wieland, M., Ausländer, D. and Fussenegger, M. (2012) Engineering of ribozyme-based riboswitches for mammalian cells. Methods 56, 351–357.
[2] Liang, J.C., Bloom, R.J. and Smolke, C.D. (2011) Engineering biological systems with synthetic RNA molecules. Mol. Cell 43, 915–926.
[3] Silvermann, S.K. (2008) Catalytic DNA (deoxyribozymes) for synthetic applications-current abilities and future prospects. Chem. Commun. 14, 3467–3485.
[4] Wieland, M. and Hartig, J.S. (2008) Artificial riboswitches: synthetic mRNA-based regulators of gene expression. ChemBioChem 9, 1873–1878.
[5] Welz, R., Bossmann, K., Klug, C., Schmidt, C., Fritz, H.-J. and Müller, S. (2003) Site-directed alteration of RNA sequence mediated by an engineered twin ribozyme. Angew. Chem., Int. Ed. 42, 2424–2427.
[6] Ivanov, S.A., Vauléon, S. and Müller, S. (2005) Efficient RNA ligation by reverse-joined hairpin ribozymes and engineering of twin ribozymes consisting of conventional and reverse-joined hairpin ribozyme units. FEBS J. 272, 4464–4474.
[7] Vauléon, S., Ivanov, S.A., Gwiazda, S. and Müller, S. (2005) Site-specific fluorescent and affinity labelling of RNA by using a small engineered twin ribozyme. ChemBioChem 6, 2158–2160.
[8] Drude, I., Vauleon, S. and Müller, S. (2007) Twin ribozyme mediated removal of nucleotides from an internal RNA site. Biochem. Biophys. Res. Commun. 363, 24–29.
[9] Strohbach, D., Nowack, N. and Müller, S. (2006) Redox-active riboswitching: allosteric regulation of ribozyme activity by ligand-shape control. Angew. Chem., Int. Ed. 45, 2127–2129.
[10] Strohbach, D., Turcu, F., Schuhmann, W. and Müller, S. (2008) Electrochemically induced modulation of the catalytic activity of a reversible redoxsensitive riboswitch. Electroanalysis 20, 935–940.
[11] Pieper, S., Vauléon, S. and Müller, S. (2007) RNA self-processing towards changed topology and sequence oligomerization. Biol. Chem. 388, 743–746.
[12] Feldstein, P.A., Buzayan, J.M. and Bruening, G. (1989) Two sequences participating in the autolytic processing of satellite tobacco ringspot virus complementary RNA. Gene 82, 53–61.
[13] Hampel, A. and Tritz, R. (1989) RNA catalytic properties of the minimum (-)sTRSV sequence. Biochemistry 28, 4929–4933.
[14] Lilley, D.M. (2011) Catalysis by the nucleolytic ribozymes. Biochem. Soc. Trans. 39, 641–646.
[15] Zhuang, X., Kim, H., Pereira, M.J., Babcock, H.P., Walter, N.G. and Chu, S. (2002) Correlating structural dynamics and function in single ribozyme molecules. Science 296, 1473–1476.
[16] Harris, M. and Christian, E. (1999) Use of circular permutation and end modification to position photoaffinity probes for analysis of RNA structure. Methods 18, 51–59.
[17] Butcher, S.E. and Burke, J.M. (1994) A photo-cross-linkable tertiary structure motif found in functionally distinct RNA molecules is essential for catalytic function of the hairpin ribozyme. Biochemistry 33, 992–999.
[18] Gunnarsson, G.H., Thormar, H.G., Gudmundsson, B., Akesson, L. and Johnsson, J.J. (2004) Two-dimensional conformation-dependent electrophoresis (2D-CDE) to separate DNA fragments containing unmatched bulge from complex DNA samples. Nucleic Acids Res. 32, e23.
[19] Gunnarsson, G.H., Gudmundsson, B., Thormar, H.G., Alfredsson, A. and Johnsson, J.J. (2006) Two-dimensional strandness-dependent electrophoresis: a method to characterize single-stranded DNA, double-stranded DNA, and RNA-DNA hybrids in complex samples. Anal. Biochem. 350, 120–127.
[20] Gunnarsson, G.H., Gudmundsson, B., Thormar, H.G., Alfredsson, A. and Johnsson, J.J. (2006) Two-dimensional strandness-dependent electrophoresis. Nat. Protoc. 1, 3011–3018.
[21] Umekage, S. and Kikuchi, Y. (2009) In vitro and in vivo production and purification of circular RNA aptamer. J. Biotechnol. 139, 265–272.
[22] Lutay, A.V., Zenkova, M.A. and Vlassov, V.V. (2007) Nonenzymatic recombination of RNA: possible mechanism for the formation of novel sequences. Chem. Biodiversity 4, 762–767.
[23] Powner, M.W., Gestland, B. and Sutherland, J.D. (2009) Synthesis of activated pyrimidine ribonucleotides in prebiotically plausible conditions. Nature 459, 239–242.
[24] Powner, M.W., Sutherland, J.D. and Szostak, J.W. (2010) Chemoselective multicomponent one-pot assembly of purine precursors in water. J. Am. Chem. Soc. 132, 16677–16688.
[25] Sundaram, P., Kurniawan, H., Byrne, M.E. and Wower, J. (2012) Therapeutic RNA aptamers in clinical trials. Eur. J. Pharm. Sci. 48, 259–271.
[26] Zhou, J., Bobbin, M.L., Burnett, J.C. and Rossi, J.J. (2012) Current progress of RNA aptamer-based therapeutics. Front. Genet. 3, 234.
[27] Thiviyanathan, V. and Gorenstein, D.G. () Aptamers and the next generation of diagnostic reagents. Proteomics Clin. Appl., http://dx.doi.org/10.1002/prca.201200042.
[28] Drude, I., Strahl, A., Galla, D., Müller, O. and Müller, S. (2011) Design of hairpin ribozyme variants with improved activity for poorly processed substrates. FEBS J. 278, 622–633.
[29] Vauléon, S. and Müller, S. (2003) External regulation of hairpin ribozyme activity by an oligonucleotide effector. ChemBioChem 4, 220–224.