Available online at www.sciencedirect.com
ScienceDirect
Gene 389 (2007) 10–18
GENE
SECTION
EVOLUTIONARY GENOMICS
www.elsevier.com/locate/gene

Catalytic activity of hammerhead ribozymes in a clay mineral environment: Implications for the RNA world

Elisa Biondi [a], Sergio Branciamore [a], Luca Fusi [a], Selma Gago [b], Enzo Gallori [a,*]

[a] *Department of Animal Biology and Genetics, University of Florence, Florence, Italy*
[b] *Instituto de Biologia Molecular y Cellular de Plantas, Universidad Politecnica de Valencia, Valencia, Spain*

Received 4 July 2006; received in revised form 11 September 2006; accepted 12 September 2006
Available online 27 September 2006
Received by M. Di Giulio

Abstract

The hypothesized RNA-based world would have required the presence of a protected environment in which RNA, or an RNA-like molecule, could originate and express its biological activity.

Recent studies have indicated that RNA molecules adsorbed/bound on clay minerals are able to persist in the presence of degrading agents, to interact with surrounding molecules, and to transmit the information contained in their nucleotide sequences.

In this study, we assessed the ability of RNA molecules with catalytic activity to perform a specific reaction in a mineral environment. For this purpose, we investigated the self-cleavage reaction of the hammerhead ribozyme of the Avocado Sun Blotch Viroid (ASBVd), both in the monomeric and in dimeric forms. The monomeric transcript was tightly bound on the clay mineral montmorillonite to form a stable complex, while the behaviour of the dimeric transcript was studied in the presence of the clay particles in the reaction mixture.

The results indicated that the hammerhead ribozyme was still active when the monomeric transcript was adsorbed on the clay surface, even though its efficiency was reduced to about 20% of that in solution. Moreover, the self-cleavage of clay-adsorbed molecule was significantly enhanced (~ four times) by the presence of the 5′ reaction product.

The self-cleavage reaction of the dimeric transcript in the presence of montmorillonite indicated that the mineral particles protected the RNA molecules against aspecific degradation and increased the rate of cleavage kinetics by about one order of magnitude.

These findings corroborate the hypothesis that clay-rich environments would have been a good habitat in which RNA or RNA-like molecules could originate, accumulate and undergo Darwinian evolutionary processes, leading to the first living cells on Earth.

Keywords: RNA activities; Mineral surfaces; Prebiotic evolution; RNA–clay interactions

1. Introduction

The hypothesis of an ancestral era of our planet, known as the "RNA World" (Gilbert, 1986), originally proposed by Orgel and Crick (1993) and Woese (1996), has been strengthened by the discovery of catalytic RNA molecules, the ribozymes (Kruger et al., 1982; Guerrier-Takada et al., 1983), and by the fundamental role played by RNA in many biological processes, particularly in ribosome structure and function (Moore and Steitz, 2002; Steitz and Moore, 2003). According to this hypothesis, RNA, or an RNA-like molecule, could have functioned contemporaneously as a carrier of genetic information and as an enzyme, thus resolving the "chicken-and-egg" paradox (Joyce, 2002; Doudna and Cech, 2002).

However, if the RNA world ever existed, it is unlikely to have developed in a free aqueous solution, due to the difficulty of polymerization reactions and the instability of polymers in

Abbreviations: ASBVd, Avocado Sun Bloch Viroid; mASBVd(−), monomeric form of ASBVd in minus polarity; dASBVd(+), dimeric form of ASBVd in plus polarity; CEC, cation exchange capacity; ddH_2O, double distilled water; M, montmorillonite; PARBP33, RNA-binding protein from *Persea americana*; SSA, specific surface area.

* Corresponding author. Via Romana 17, 50125 Florence, Italy. Tel.: +39 055 2288241; fax: +39 055 2288250.
E-mail address: enzo.gallori@unifi.it (E. Gallori).

0378-1119/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2006.09.002

this environment (Pace, 1991; Doudna and Cech, 2002). The presence of the 2′-OH group in ribose, which is the main feature for RNA to be catalytic, also makes the molecule particularly susceptible to hydrolysis.

The occurrence of various steps towards the formation of a very complex molecule, such as RNA, must have required the presence of a protected confined environment, where the primordial genetic molecule could originate and evolve. In recent years, several possible physical settings have been suggested, from boron-bearing minerals (Ricardo et al., 2004) to freezing environments (Vlassov et al., 2004, 2005).

Moreover, numerous observations have reinforced the hypothesis of a surface-mediated origin of life, suggested more than fifty years ago by Bernal (1951). These observations include the synthesis of precursors of nucleotides in the presence of clay minerals (Pitsch et al., 1995; Saladino et al., 2004) and their polymerization into oligonucleotides up to the length of a small ribozyme with or without the need of a primer (Ferris et al., 1996; Huang and Ferris, 2003).

More recently, the work of Hanczyc et al. (2003) and Hanczyc and Szostak (2004) showed that clays can favour the spontaneous conversion of fatty acids into vesicles, with the encapsulation of clay-adsorbed RNA molecules. This suggests a simple solution to the problem of primitive compartmentalization.

In addition to this, laboratory studies on the fate of genetic information in different habitats have demonstrated the role of clay minerals in environmental protection of genetic molecules (DNA, RNA) against biotic (i.e. nucleases) and abiotic (UV, X-ray radiation) degrading agents. Indeed, nucleic acid molecules adsorbed on clays ("clay–nucleic acid complexes") can persist for a long time in hostile environments while still maintaining their biological activities, such as the ability to transform competent bacterial cells, to transmit the genetic information contained in their sequences and to interact with molecules present in the environment (Gallori et al., 1994; Stotzky et al., 1996; Vettori et al., 1996; Scappini et al., 2004; Franchi and Gallori, 2004).

These observations suggest that clay minerals played a central role in the formation and preservation of ancestral genetic material on the early Earth (Franchi et al., 1999; Franchi and Gallori, 2004), promoting their persistence in critical conditions.

At present, it is crucial to understand how nucleic acid-like molecules (primitive RNA) adsorbed on clays might have been in the right conditions to undergo specific chemical reactions, triggering the molecular evolution that led to the first cells.

To this aim, we investigated the ability of clay-bound RNA molecules to perform specific reactions in a mineral environment. In particular, we studied the self-cleavage reaction carried out by the so-called "hammerhead" ribozyme. These ribozymes are small RNA motifs which, in the presence of a divalent metal ion, self-cleave at a specific phosphodiester bond (via a transesterification reaction) to produce 2′,3′ cyclic phosphodiester and 5′ hydroxyl termini (Hutchins et al., 1986; Forster and Symons, 1987). These ribozymes are present in nature in small circular naked RNA molecules, named viroids, which are the smallest plant pathogens known (Diener, 2001; Flores et al., 2004). Since viroids are the only RNA molecules able to undergo replication without DNA intermediates and without coding for any protein, they are considered possible relics of the RNA world, and their ribozymes can be viewed as "molecular fossils" (Diener, 2001).

In the present paper, we report the results of studies on the influence of the clay mineral montmorillonite on the self-cleavage activity of the hammerhead ribozyme in the Avocado Sun Bloch Viroid (ASBVd) (Symons, 1981; Flores et al., 2000). In particular, we investigated: i) the self-cleavage mechanism of the hammerhead ribozyme present in the monomeric form of the viroid (mASBVd); ii) the activity of mAS BVd when the molecule was tightly bound on the montmorillonite surface; iii) the self-cleavage activity of the dimeric form of the viroid (dASBVd) when the reaction was performed in the presence of montmorillonite.

2. Materials and methods

2.1. Plasmid templates

Plasmids containing the cDNA of the monomeric and dimeric forms of ASBVd were kindly provided by Dr. Flores, Universidad Politécnica de Valencia-CSIC, Spain.

p18A plasmid is derived from pSPT18, with the insertion of monomeric ASBVd cDNA (Marcos and Flores, 1992); pBdAS BVd (A28) plasmid is a pBluescriptII vector containing the cDNA of the viroid in dimeric form.

2.2. Sequence analysis

Viroid cDNAs inserted in p18A and A28 plasmids were checked for predicted sequences with primer SEQ-ASBVdM-Fwd (5′-CGTTAGAACGCGGCTACAAT-3′) and primer M1320-Fwd (5′-CCTTTGTCGATACTGGTACT-3′), respectively. Sequencings were performed by the C.I.B.I.A.C.I. Centre (University of Florence) with an ABI PRISM 310 genetic analyzer (Applied Biosystems) and the relative sequencing kit.

2.3. Preparation of RNA

Monomeric ASBVd transcript in minus polarity (307 nt) was prepared from plasmid p18A linearized with *Eco*RI (New England Biolabs) and transcribed with T7 polymerase (ROCHE). Dimeric ASBVd in plus polarity (587 nt) was transcribed with T3 polymerase from plasmid A28 linearized with *Xba*I.

Full-length RNA transcripts were then purified by extraction from denaturing polyacrylamide gel and were ethanol precipitated. To ensure the removal of residual acrylamide, samples were further purified by passing the RNAs through a sephadex G-25 column (ROCHE).

The 5′fragment for analysis of mASBVd(−) was obtained by equal gel-extraction after a 2 h self-cleavage reaction of the monomer.

2.4. Structural analysis

Analysis of the theoretical structures of dASBVd and mAS BVd transcripts and calculation of the relative energies were performed with the softwares RNADraw (Matzura and Wennborg, 1996) and *mfold* 3.2 (Zuker, 2003).

2.5. Preparation of homoionic montmorillonite

The clay mineral used in the experiments was montmorillonite (M) Volclay SPV-200 (American Colloids), which is a 2:1 (Si/Al) smectite clay with a cation exchange capacity (CEC) of 76.4 cmolc kg^{-1} and a specific surface area (SSA) of 78 m^2 g^{-1}. The <2 μm fraction of montmorillonite was made homoionic to Na^+ following the procedure reported by Banin et al. (1985).

2.6. Preparation of stable mASBVd(−)/M complexes

Samples (5 μg) of mASBVd(−) transcripts were mixed with 100 μl of a suspension (20 mg/ml) of montmorillonite in the presence of [Ca^{2+}] 4.0 mM, in a final reaction volume of 200 μl. After 2 h of incubation, the samples were centrifuged (15,000 ×*g*, 20 min, RT). Pellets were washed for 1 h with 200 μl of ddH_2O containing [Ca^{2+}] 4.0 mM, as previously described (Franchi et al., 2003) and were centrifuged (15,000 ×*g*, 20 min, RT) to remove unbound RNA; this procedure was repeated until the absorbance of supernatant at λ=260 nm was zero (Kanna and Stotzky, 1992; Gallori et al., 1994).

2.7. Removal of tightly bound RNA from RNA–clay complexes

RNA was desorbed from montmorillonite by adding 100 μl of stop/elution solution (formamide 50%, urea 8 M, EDTA 50 mM pH 8.0, Blu Bromophenol 0.025%, Xylene cyanol 0.025%). After 30 min of incubation at RT, 3.4 μg of RNA (about 70% of the amount initially added) were desorbed from the clay. Samples were denatured at 95 °C for 2 min and directly loaded in denaturing polyacrylamide gel for subsequent analysis.

2.8. In vitro self-cleavage of mASBVd(−)

2.8.1. RNA free in solution

In vitro mASBVd(−) transcript self-cleavage reactions were performed at 55 °C by mixing equal volumes of RNA and self-cleavage buffer 2× (Tris–HCl 100 mM, pH 7.5, Mg^{2+} 200 mM). The reactions were quenched after 30 min with stop/elution solution and analyzed by denaturing PAGE (5%, 8 M urea) with the software QuantityOne (BioRad) after SybrGold staining (Invitrogen).

For the kinetic assays used to study the dependence of cleavage on the mASBVd(−) concentration, we prepared three samples of mASBVd(−), i.e. 100, 10, 1 ng/μl, and added an equal volume of self-cleavage buffer 2× to each of them, giving a monomer final concentration of 50, 5, 0.5 ng/μl. For each kinetic assay, samples were taken at 15′, 30′, 45′, 60′, 120′ and quenched with equal volumes of stop/elution solution.

To investigate the time-course of the self-cleavage reaction in the presence of the 5′fragment, we prepared three samples of mASBVd(−) transcripts (10 ng/μl) containing the 5′fragment at a concentration of 0, 6 or 30 ng/μl. Self-cleavage buffer 2× was added to each sample. The samples were incubated at 55 °C and aliquots were collected at 0′, 15′, 30′, 45′, 60′, 120′; the reaction was stopped with equal volumes of stop/elution solution.

2.8.2. RNA adsorbed on clay

Pellet obtained after adsorption of mASBVd(−) transcripts on montmorillonite was resuspended in 15 μl of self-cleavage buffer 1× (Tris–HCl 50 mM, pH 7.5, Mg^{2+} 100 mM).

Cleavage with the 5′fragment was performed by resuspending the mASBVd(−)/M complex in 15 μl of self-cleavage buffer 5′ (Tris–HCl 50 mM, pH 7.5, Mg^{2+} 100 mM, [5′F] 50 ng/μl).

After 30 min of incubation at 55 °C, the reaction was stopped and RNA was desorbed with 100 μl of stop/elution solution.

2.9. In vitro self-cleavage of dASBVd(+)

In vitro dASBVd(+) self-cleavage reactions were carried out in the presence and absence of clay. Aliquots of purified transcripts (400 nt) were heated for 2 min at 95 °C in ddH_2O and snap-cooled on ice. Self-cleavage buffer was then added to a final concentration of Tris–HCl 50 mM, pH 8.0, $MgCl_2$ 10 mM, plus 1 μg/μl montmorillonite, or the same volume of ddH_2O, and the reaction mixtures (20 μl) were incubated at 25 °C. Samples of 2 μl (40 ng) were taken at different times; the reaction was stopped with 10 volumes of stop/elution solution, and the sample was heated for 2 min at 95 °C, snap-cooled on ice, and directly loaded on denaturing polyacrylamide gel.

2.10. Northern-blot hybridization

RNAs from dASBVd(+) transcript self-cleavage reactions were separated by 8 M urea denaturing 5% PAGE in TBE. After ethidium bromide staining, RNAs were electroblotted to nylon membranes (Hybond-N, Amersham Pharmacia Biotech) and fixed by UV irradiation. Membranes were hybridized at 70 °C in the presence of 50% formamide with a ^{32}P-labelled RNA probe specific for monomeric ASBVd in minus polarity (Daros et al., 1994).

2.11. Self-cleavage analysis

mASBVd(−) transcript self-cleavage reactions were analyzed by ethidium bromide and SybrGold staining (Invitrogen) with the GelDoc2000 gel documentation system (BioRad) and the relevant software QuantityOne. Self-cleavage efficiency (*E*), or the relative percentage, was evaluated with the relation $E=I_2/(I_1+I_2)$, where I_1 and I_2 are the intensities of the gel bands corresponding to the monomeric entire transcript and 3′ fragment, respectively.

The extent of the dASBVd(+) self-cleavage reactions was monitored by autoradiography using a phosphorimager (Fuji) and the relative software ImageGauge. Fractions (*F*) of self-cleavage products (or the relative percentages) were evaluated

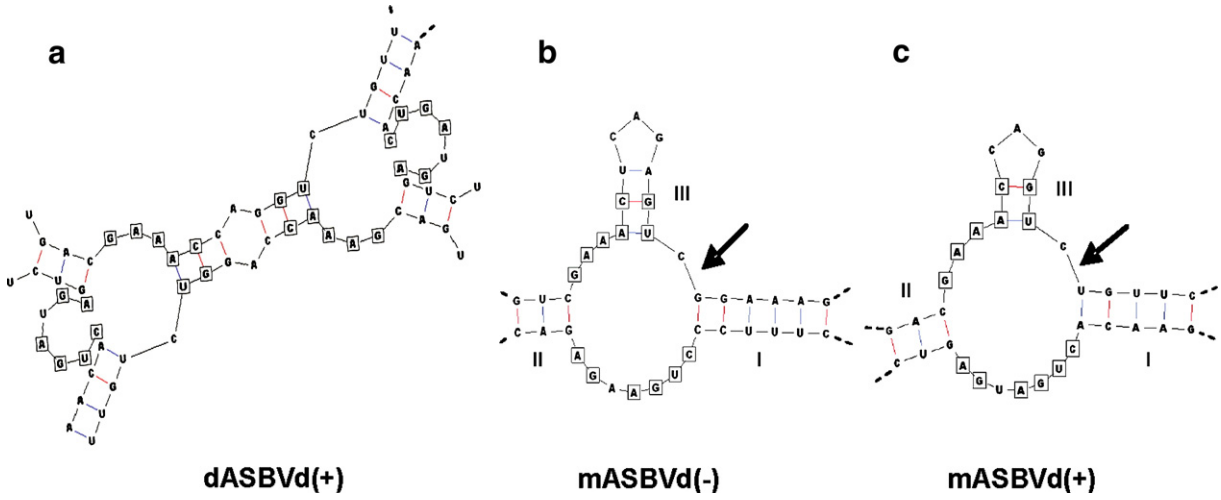

Fig. 1. Enlargement of the secondary structures of viroid ASBVd containing the self-cleaving hammerhead. (a): Double hammerhead of plus polarity dASBVd. (b): Single hammerhead of minus polarity mASBVd. (c) Single hammerhead of plus polarity mASBVd. Stems are numbered I to III (Forster and Symons, 1987), sites of cleavage are indicated by the arrows, and conserved nucleotides are boxed. Structures were generated with the software RNADraw.

with the relation $F=(I_2+I_3+I_4+I_5)/(I_1+I_2+I_3+I_4+I_5)$, where I_1 is the intensity of the entire dimeric transcript, while I_2 to I_5 are the intensities of the bands corresponding to the 4 longest cleavage fragments.

Observed kinetics were fitted with the equation $F_t=F_\infty(1-e^{-k_{obs}t})$, where F_t is the fraction of cleaved transcript at time t, F_∞ is the fraction of cleaved transcript at the end point, and k_{obs} is the observed kinetic rate constant.

2.12. Statistics

All experiments were performed at least in triplicate. Data are expressed as mean±standard error of the mean (S.E.M.).

3. Results

3.1. Dimeric and monomeric forms of ASBVd

The secondary structure of the dimeric and monomeric transcripts of ASBVd were initially checked using RNADraw (Matzura and Wennborg, 1996).

The secondary structure generated for the dimeric transcript in plus polarity (dASBVd(+)) was supposed to originate a double hammerhead structure (Fig. 1, a), which could produce a high yield of five cleavage fragments already during *in vitro* transcription (Fig. 2, a and b).

The sequences of the two polarities (mASBVd(+) and (−)) of the monomeric transcript (Fig. 1, b and c) revealed two possible low-stability hammerhead secondary structures, as previously described by Hutchins et al. (1986).

The mASBVd(−) form was expected to present a higher *in vitro* self-cleavage efficiency than the plus polarity, as the latter has only two pairs of nucleotides in stem III instead of three. mASBVd(+) did not show any activity during *in vitro* self-cleavage in the presence of Mg^{2+} ions (data not shown), while mASBVd(−) performed a low-efficiency self-cleavage reaction in the same experimental conditions (Fig. 2, c and d).

3.2. In vitro mASBVd(−) self-cleavage

The catalytic activity of mASBVd(−) was tested by varying several experimental parameters, i.e. pH, T and Mg^{2+}

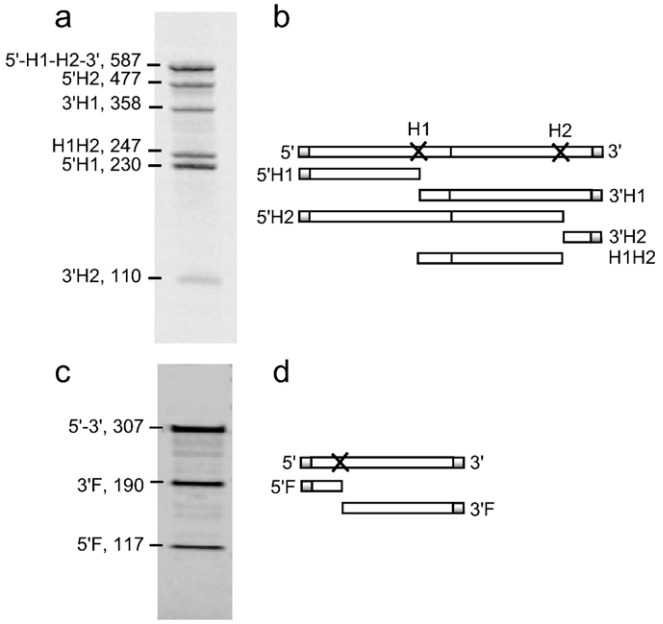

Fig. 2. Cleavage of dimeric (+) and monomeric (−) ASBVd RNAs. (a) and (c): Denaturing PAGE showing RNA transcripts of plus dASBVd (a) and minus mASBVd (c) after self-cleavage reaction. Labels on the left indicate lengths in nucleotides and name of fragments according to the diagrams in (b) and (d). (b): Schematic representation of the self-cleavage as in (a). 5′-H1-H2-3′: Full-length transcript of dimeric plus ASBVd; 5′H1: 5′fragment resulting from the self-cleavage in site H1; 3′H1: 3′fragment resulting from the same site; 5′H2, 3′H2: fragments resulting from the cleavage in site H2; H1H2: exact monomeric ASBVd resulting from the reaction in both cleavage sites. (d): Schematic representation of the self-cleavage as in (c). 5′–3′: Full-length transcript of monomeric minus ASBVd; 5′F and 3′F: 5′ and 3′fragments of cleavage, respectively. Crosses indicate sites of cleavage (named H1 and H2 for the dimeric form); vector and viroid sequences are represented by grey and white bars, respectively.

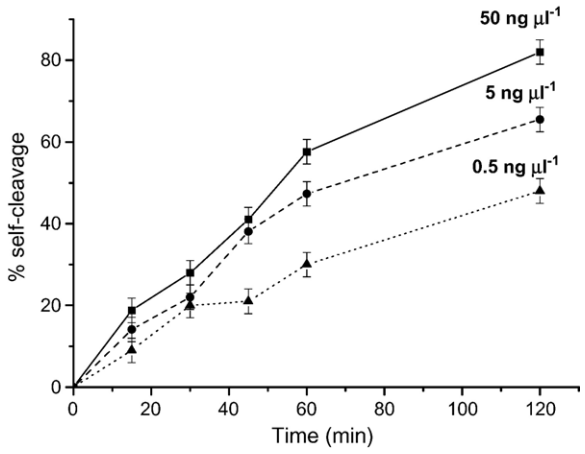

Fig. 3. Effect of mASBVd(−) concentration on percent of self-cleavage. Time-course of three self-cleavage reactions at [mASBVd(−)] = 50 ng/µl (■), 5 ng/µl (●) and 0.5 ng/µl (▲).

concentration. The results (data not shown) indicated that pH 7.5, 55 °C and Mg^{2+} 100 mM allowed the highest percentage (~80%) of self-cleavage of the monomer in free solution after 2 h of incubation. Hence, these values were chosen for the following analyses.

We also found that the mASBVd(−) cleavage efficiency depended on the monomer concentration (Fig. 3). The curves representing the time-course of the self-cleavage reaction at 0.5, 5.0 and 50.0 ng/µl of mASBVd(−) are shown. The results indicated that a higher monomer concentration produced more efficient self-cleavage. In particular, 80% self-cleavage was reached with a monomer concentration of 50 ng/µl, while the formation of products decreased to 65% and 48% at 5 ng/µl and 0.5 ng/µl, respectively. These observations suggest that the reaction proceeded in both a monomolecular and bimolecular manner, with the formation of double hammerhead structures.

On the basis of previous observations by Sheldon and Symons (1989), who found that the low-stability hammerhead

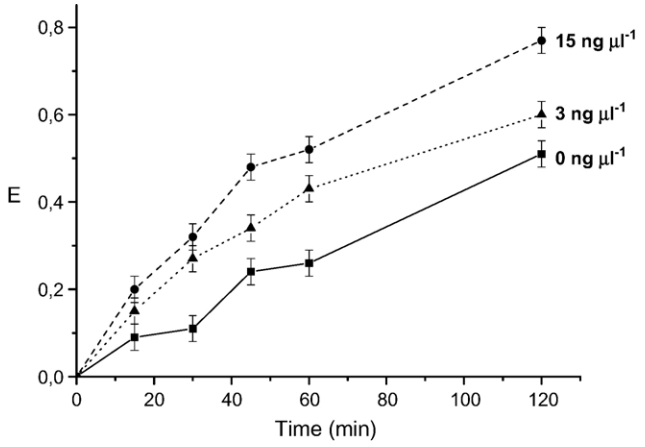

Fig. 4. Effect of 5′fragment on self-cleavage efficiency (*E*). Time-course of three self-cleavage reactions at [mASBVd(−)] = 5 ng/µl in presence of 5′fragment at 0 ng/µl (■), 3 ng/µl (●) and 15 ng/µl (▲).

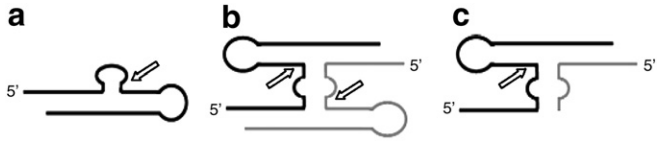

Fig. 5. Schematic representation of the three possible structures of self-cleavage for mASBVd. (a): Single hammerhead; (b): double hammerhead; (c) partial-double hammerhead. Arrows indicate the sites of cleavage.

ribozyme of newt can form a partial-double hammerhead structure with its 5′ cleavage fragment, we evaluated the effect of the 5′fragment of cleavage (5′F) of mASBVd(−) on self-cleavage efficiency. The reaction kinetic was studied at two 5′F concentrations: 3 and 15 ng/µl. The results (Fig. 4) showed that the 5′fragment catalyzes *in trans* the mASBVd(−) self-cleavage reaction. This is an indication of the formation of a partial-double hammerhead structure.

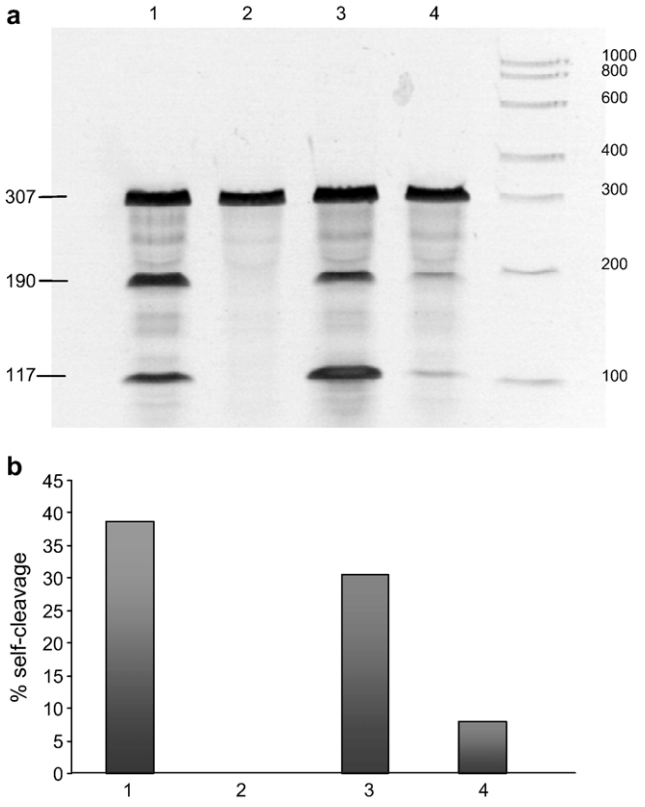

Fig. 6. Self-cleavage reactions of mASBVd(−) on montmorillonite surface. (a): Denaturing PAGE of different samples. Stain of the gel was performed with SybrGold (Invitrogen). Lane 1: self-cleavage of mASBVd(−) transcript in free solution; lane 2: same transcript adsorbed on montmorillonite surface, submitted to all the self-cleavage conditions but without the addition of Mg^{2+}, and desorbed from the clay; lane 3: mASBVd(−) transcript adsorbed on the clay surface, submitted to the self-cleavage conditions in presence of 50 ng/µl of 5′ fragment, and then desorbed; lane 4: same as in lane 3, but in absence of 5′ fragment in the reaction. Right side: RNA ladder with the corresponding sizes in nucleotides. The positions and number of nucleotides of the uncleaved transcripts and of the resulting self-cleavage fragments thereof are indicated on the left. (b): Histogram showing the different percentage of self-cleavage products for every correspondent lane in (a). Note that the lesser catalytic activity of the adsorbed monomer in respect to the reaction in free solution, is almost all restored by the presence of the 5′fragment.

Therefore, the mASBVd(−) self-cleavage reaction could be interpreted as the result of the concurrent action of three structures that mASBVd(−) can assume during the self-cleavage reaction: simple, double and partial-double hammerhead (Fig. 5).

3.3. Self-cleavage of mASBVd(−) adsorbed on the montmorillonite surface

The ability of the ribozyme adsorbed on the clay surface to carry out the catalytic reaction was investigated under the same conditions used for the free solution experiments.

The mASBVd(−)/M complex was resuspended in the cleavage buffer and incubated at 55 °C for 30 min. The reaction was then quenched and RNA was desorbed from the clay surface, as reported in Materials and methods.

The reaction products were revealed on PAGE electrophoresis by SybrGold staining. The results (Fig. 6) showed that mASBVd(−) tightly adsorbed on clay could perform the self-cleavage reaction, although its efficiency was reduced from 38% in free solution to 8% when the ribozyme was complexed.

To ensure that RNA did not come off from clay surface during the self-cleavage, the clay–ribozyme complex was centrifuged after 30′ of incubation in the cleavage buffer, and the supernatant loaded on denaturing PAGE. No RNA could be detected in the cleavage buffer (data not shown).

These results indicate that the hammerhead ribozyme was still active when tightly bound on the clay surface.

3.4. Effect of the 5′fragment on self-cleavage of clay-adsorbed mASBVd(−)

We studied the ability of clay-adsorbed catalytic RNA molecules to interact with complementary strands and the possibility that the interaction could enhance their reactivity by the formation of a specific molecular structure. To this aim, we assessed the formation on the mineral surface of partial-double hammerhead structures between clay-adsorbed monomer transcripts and 5′F.

The stable mASBVd(−)/M complex, obtained as previously described, was resuspended in the cleavage conditions in the presence of 50 ng/μl of free 5′F. The results of PAGE electrophoresis (Fig. 6) indicated that 5′F increases the self-cleavage efficiency of mASBVd(−)/M from 8%, in the absence of the fragment, to 30.5%. This demonstrates not only the possibility of interactions between the two molecules on the mineral surface but also that the ribozyme can assume the more active catalytic structure even when complexed with clay particles.

3.5. Self-cleavage of dASBVd(+) in the presence of montmorillonite

Recent studies on the catalysis of hammerhead ribozymes *in vitro* have indicated that some RNA-binding proteins, of viral and cellular origin, can stimulate the reaction by acting as chaperones (Herschlag et al., 1994; Huang and Wu, 1998). In particular, Daros and Flores (2002) showed that the self-cleavage reaction of the dimeric form of ASBVd is enhanced by a chloroplast RNA binding protein, PARBP33.

To check whether montmorillonite could influence the self-cleavage of dASBVd RNA *in vitro*, acting as a "natural prebiotic chaperone" without the need of stable complexes, we tested the ability of dimeric transcripts of ASBVd in plus polarity (dASBVd(+)) to perform the reaction in the presence of montmorillonite.

The catalytic activity of the ribozyme was tested by varying several experimental parameters, i.e. pH, *T* and Mg^{2+} concentration. The results (data not shown) indicated that pH 8.0, 10 mM Mg^{2+} and 25 °C, after 2 h of incubation, were the optimal parameters for our purposes. Hence, these values were used for the following analyses.

To test the influence of clay, we performed the reaction in the presence of 1 mg/ml of montmorillonite, homoionic for sodium. Two parallel kinetic reactions were arranged, in the absence and

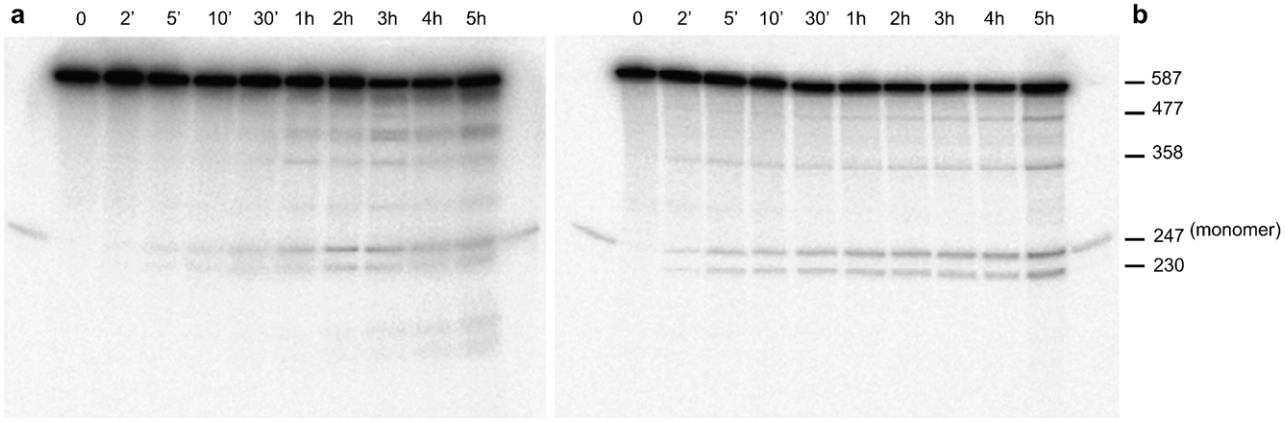

Fig. 7. Effect of the presence of montmorillonite on self-cleavage reaction of dASBVd(+) transcript. Northern-blot of self-cleavage kinetics with transcripts incubated under cleavage conditions in the absence (a), or presence (b) of 1 mg/ml Na-montmorillonite. Hybridization was performed with a mASBVd(−) radioactively labelled probe. Lateral lanes of the two gels: mASBVd(−) transcripts as controls of blotting and hybridization. The positions and nucleotides of the uncleaved transcript and of the resulting self-cleavage fragments thereof are indicated on the right (115 nt fragment run out of the gel; 247 nt fragment corresponded to the exact monomer resulting from the cleavage on the two sites).

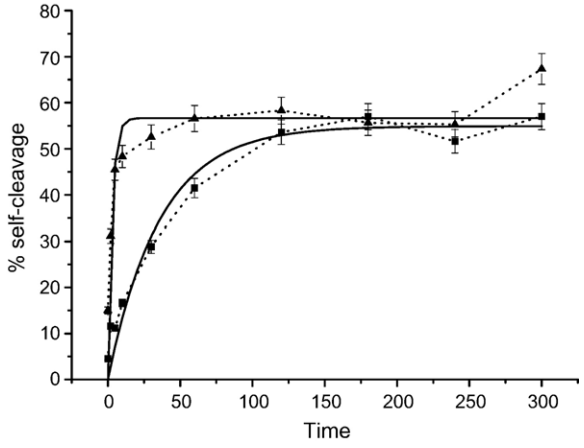

Fig. 8. Time-course of the self-cleavage reactions as in Fig. 7. Solid lines represent the fittings of the two kinetics in presence (▲) or absence (■) of 1 mg/ml Na-montmorillonite, according to the equation $F_t=F_\infty(1-e^{-k_{obs}t})$, where F_t is the fraction of cleaved transcript at time t, F_∞ is the fraction of cleaved transcript at the end point, and k_{obs} is the observed kinetic rate constant.

the presence of M. Samples were taken at different times (0′, 2′, 5′, 10′, 30′, 1 h, 2 h, 3 h, 4 h, 5 h), run on urea PAGE and analyzed by Northern-blot hybridization (see Materials and methods).

Comparative analysis of the reaction products (Fig. 7) showed different effects of the presence absence or presence of montmorillonite. Degradation of RNA molecules was clearly reduced in the presence of the clay, and the ribozyme was able to undergo the self-cleavage reaction even after long periods of incubation (5 h). Instead, the corresponding aliquots in free solution showed higher levels of degradation. During the reaction, degradation bands appeared, which were probably due to the non-enzymatic degradation of RNA. Moreover, the self-cleavage kinetic was enhanced by the presence of clay particles, giving rise to 28% of self-cleavage products already at 2 min, versus only 10% in the corresponding aliquot in free solution.

Data from the two kinetic experiments were plotted and fitted with the equation $F_t=F_\infty(1-e^{-k_{obs}t})$, where F_t is the fraction of cleaved products at time t, F_∞ is the fraction of cleaved products at the plateau, and k_{obs} is the observed kinetic constant of the reaction.

The fitting parameters for the reaction in free solution were $k_{obs}=0.027\ min^{-1}$ and $F_\infty=0.55$, while the reaction in the presence of montmorillonite showed $k_{obs}=0.343\ min^{-1}$ and $F_\infty=0.58$ (Fig. 8). These data indicate that the self-cleavage kinetic of dASBVd(+) in the presence of montmorillonite was enhanced by about one order of magnitude with respect to the same reaction in free solution, even though the two F_∞ values were comparable.

4. Discussion

In this paper, we report the results of the studies on the catalytic activity of ribozymes in the presence of the clay mineral montmorillonite. These studies extend and strengthen previous investigations on the biogenic properties of clay-adsorbed RNA molecules, aimed at assessing the possibility of development of the RNA world on a mineral substrate. Such an environment could have favoured not only the formation and persistence of RNA oligomers (Ertem and Ferris, 1996; Franchi and Gallori, 2004) but also their evolution towards increasingly complex molecular organizations.

To this aim, we investigated the catalytic activity of the hammerhead ribozyme present in the viroid ASBVd.

The self-cleavage activity of mASBVd(−) in solution indicated that, in addition to the simple monomeric structure, hammerhead ribozymes could assume a double and partial-double hammerhead structure, allowing the formation of specific bimolecular structures.

The results of the self-cleavage reaction of clay-bound mASBVd indicated that the ribozyme was still active when adsorbed on the mineral, even though its efficiency was reduced. This reduction was probably due to a decrease in mobility of the molecule after its adsorption, which translated into a lesser flexibility to assume the catalytically active conformation of the hammerhead ribozyme.

Moreover, the self-cleavage of the monomeric transcript tightly bound on montmorillonite was clearly enhanced (~4-fold) by the presence of the 5′ reaction product. This demonstrates that adsorption on the clay surface did not prevent the molecular interactions required to produce the partial-double hammerhead structure, and thus the formation of catalytically active bimolecular structures. This finding is a step forward with respect to our previous results concerning the possibility of clay-adsorbed homopolymers to interact with complementary molecules in the surroundings (Franchi and Gallori, 2005).

The experiments on self-cleavage of dASBVd(+) in the presence of montmorillonite demonstrated an evident effect of protection of the molecules against aspecific degradation. This suggests that in a clay-rich environment not bound RNA molecules are still protected against degradation. This could be due: i) to the ability of the charged clay surface to interact with surrounding ions and water molecules (Sposito et al., 1999), “sequestering” them in a way that they are not able to react with RNA; and ii) to the direct interactions between the groups of the clay and RNA molecules, which could originate less susceptible structures.

The dynamic interactions between RNA molecules and clay minerals could explain the observed enhancement of the reaction kinetic parameters. In fact, the kinetic rate constant of the self-cleavage reaction in the presence of montmorillonite was enhanced by about one order of magnitude ($k_{obs}=0.343\ min^{-1}$) with respect to the same kinetic in the absence of clay ($k_{obs}=0.027\ min^{-1}$). This could be due to a lowering of the activation energy by the clay. In this view, montmorillonite can be considered a real catalyst of the self-cleavage reaction of hammerhead ribozymes, in agreement with the well known catalytic activity of clays (Anderson and Banin, 1975; Rao et al., 1980; Ferris et al., 1989). Otherwise, the observed effect on ribozyme activity could be due to a novel feature of the clay mineral, which could act as a natural prebiotic chaperone to help the hammerhead ribozymes reach the most catalytically active conformation.

The results obtained in this study corroborate our previous observations (Franchi and Gallori, 2005) that clay-rich environments would have been a good habitats on the primordial Earth in which the first genetic molecules could originate and evolve.

We can suppose that an RNA-like world could have arisen in microstructures present inside of clay minerals, in an environment rich in phosphate and in contact with aqueous solutions containing the simple organic molecules precursors of the constituents of primordial genetic molecules. This setting would have allowed not only the concentration of precursors on the surfaces of mineral particles, catalyzing their polymerization into macromolecules, but also the protection of resulting polymers against environmental degradation (e.g. strong UV and X-ray radiation). So, the genetic polymers have had the time necessary to evolve towards an increasing complex organization, triggering the molecular evolution that led to the first living cells.

Acknowledgement

E. B. wishes to thank Dr. Ricardo Flores, Instituto de Biologia Molecular y Cellular de Plantas, Universidad Politecnica de Valencia, for the helpful training in viroid manipulations and for the kind hospitality in his laboratory.

References

Anderson, D.M., Banin, A., 1975. Soil and water and its relationship to the origin of life. Orig. Life 6 (1–2), 23–36.

Banin, A., Lawless, J.C., Mazzucco, J., Church, F.M., Margulis, L., Orenberg, J.B., 1985. pH profile of the adsorption of nucleotides on montmorillonite. Orig. Life Evol. Biosph. 15, 89–101.

Bernal, J.D., 1951. The Physical Basis of Life. Routledge, London.

Daros, J.A., Flores, R., 2002. A chloroplast protein binds a viroid RNA in vivo and facilitates its hammerhead-mediated self-cleavage. EMBO J. 21 (4), 749–759.

Daros, J.A., Marcos, J.F., Hernandez, C., Flores, R., 1994. Replication of avocado sunblotch viroid: evidence for a symmetric pathway with two rolling circles and hammerhead ribozyme processing. Proc. Natl. Acad. Sci. U. S. A. 20;91 (26), 12813–12817.

Diener, T.O., 2001. The viroid: biological oddity or evolutionary fossil? Adv. Virus Res. 57, 137–184.

Doudna, J.A., Cech, T.R., 2002. The chemical repertoire of natural ribozymes. Nature 418, 222–228.

Ertem, G., Ferris, J.P., 1996. Synthesis of RNA oligomers on heterogeneous templates. Nature 379 (6562), 238–240.

Ferris, J.P., Ertem, G., Agarwal, V.K., 1989. The adsorption of nucleotides and polynucleotides on montmorillonite clay. Orig. Life Evol. Biosph. 19, 153–164.

Ferris, J.P., Hill Jr., A.R., Liu, R., Orgel, L.E., 1996. Synthesis of long prebiotic oligomers on mineral surfaces. Nature 2;381 (6577), 59–61.

Flores, R., Daros, J.A., Hernandez, C., 2000. Avsunviroidae family: viroids containing hammerhead ribozymes. Adv. Virus Res. 55, 271–323.

Flores, R., et al., 2004. Viroids: the minimal non-coding RNAs with autonomous replication. FEBS Lett. 567, 42–48.

Forster, A.C., Symons, R.H., 1987. Self-cleavage of plus and minus RNAs of a virusoid and a structural model for the active site. Cell 49, 211–220.

Franchi, M., Gallori, E., 2004. Origin, persistence and biological activity of genetic material in prebiotic habitats. Orig. Life Evol. Biosph. 34, 133–141.

Franchi, M., Gallori, E., 2005. A surface-mediated origin of the RNA world: biogenic activities of clay-adsorbed RNA molecules. Gene 346, 205–214.

Franchi, M., Bramanti, E., Morassi Bonzi, L., Orioli, P.L., Vettori, C., Gallori, E., 1999. Clay–nucleic acid complexes: characteristics and implications for the preservation of genetic material in primeval habitats. Orig. Life Evol. Biosph. 29, 297–315.

Franchi, M., Ferris, J.P., Gallori, E., 2003. Cations as mediators of the adsorption of nucleic acids on clay surfaces in prebiotic environments. Orig. Life Evol. Biosph. 33, 1–16.

Gallori, E., et al., 1994. Transformation of *Bacillus subtilis* by DNA bound on clay in non-sterile soil. FEMS Microbiol. Ecol. 15, 119–126.

Gilbert, W., 1986. The RNA world. Nature 319, 618.

Guerrier-Takada, C., Gardiner, K., Marsh, T., Pace, N., Altman, S., 1983. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell 35 (3 Pt 2), 849–857.

Hanczyc, M.M., Fujikawa, S.M., Szostak, J.W., 2003. Experimental models of primitive cellular compartments: encapsulation, growth, and division. Science 302, 618–622.

Hanczyc, M.M., Szostak, J.W., 2004. Replicating vesicles as models of primitive cell growth and division. Curr. Opin. Chem. Biol. 8 (6), 660–664.

Herschlag, D., Khosla, M., Tsuchihashi, Z., Karpel, R.L., 1994. An RNA chaperone activity of non-specific RNA binding proteins in hammerhead ribozymes catalysis. EMBO J. 13 (12), 2913–2924.

Huang, W., Ferris, J.P., 2003. Synthesis of 35–40 mers of RNA oligomers from unblocked monomers. A simple approach to the RNA world. Chem. Commun. (Camb) 21 (12), 1458–1459.

Huang, Z.S., Wu, H.N., 1998. Identification and characterization of the RNA chaperon activity of hepatitis delta antigen peptides. J. Biol. Chem. 273 (41), 26455–26461.

Hutchins, C.J., Rathjen, P.D., Forster, A.C., Symons, R.H., 1986. Self-cleavage of plus and minus RNA transcripts of avocado sun-blotch viroid. Nucleic Acids Res. 14, 3627–3640.

Joyce, G.F., 2002. The antiquity of RNA-based evolution. Nature 418 (6894), 214–221.

Kanna, M., Stotzky, G., 1992. Transformation of *Bacillus subtilis* by DNA bound on montmorillonite and effect of DNase on the transforming ability of bound DNA. Appl. Environ. Microbiol. 58, 1930–1939.

Kruger, K., Grabowski, P.J., Zaug, A.J., Sands, J., Gottschling, D.E., Cech, T.R., 1982. Self-splicing RNA: autoexcision and autocyclization of the ribosomal RNA intervening sequence of Tetrahymena. Cell 31 (1), 147–157.

Marcos, J.F., Flores, R., 1992. Characterization of RNAs specific of avocado sunblotch viroid synthesized in vitro by a cell-free system from infected avocado leaves. Virology 186, 481–482.

Matzura, O., Wennborg, A., 1996. RNAdraw: an integrated program for RNA secondary structure calculation and analysis under 32-bit Microsoft Windows. CABIOS 12 (3), 247–249.

Moore, P.B., Steitz, T.A., 2002. The involvement of RNA in ribosome function. Nature 418, 229–235.

Orgel, L.E., Crick, F.H., 1993. Anticipating an RNA world. Some past speculations on the origin of life: where are they today? FASEB J. 7 (1), 238–239.

Pace, N.R., 1991. Origin of life-facing up the physical setting. Cell 65, 531–533.

Pitsch, S., Eschenmoser, A., Gedulin, B., Hui, S., Arrhenius, G., 1995. Mineral induced formation of sugar phosphates. Orig. Life Evol. Biosph. 25 (4), 297–334.

Rao, M., Odom, D.G., Orò, J., 1980. Clays in prebiological chemistry. J. Mol. Evol. 15 (4), 317–331.

Ricardo, A., Cardigan, M.A., Olcott, A.N., Benner, S.A., 2004. Borate minerals stabilize ribose. Science 303, 196.

Saladino, R., Crestini, C., Ciambecchini, U., Ciciriello, F., Costanzo, G., Di Mauro, E., 2004. Synthesis and degradation of nucleobases and nucleic acids by formamide in the presence of montmorillonites. Chembiochem 5 (11), 1558–1566.

Scappini, F., Casadei, F., Zamboni, R., Franchi, M., Gallori, E., Monti, S., 2004. Protective effect of clay minerals on adsorbed nucleic acids against UV radiations: possible role in the origin of life. Int. J. Astrobiol. 3, 17–19.

Sheldon, C.C., Symons, R.H., 1989. RNA stem stability in the formation of a self-cleaving hammerhead structure. Nucleic Acids Res. 17, 5665–5677.

Sposito, G., Skipper, N.T., Sutton, R., Park, S., Soper, A.K., Greathouse, J.A., 1999. Surface geochemistry of the clay minerals. Proc. Natl. Acad. Sci. U. S. A. 96, 3358–3364.

Steitz, T.A., Moore, P.B., 2003. RNA, the first macromolecular catalyst: the ribosome is a ribozyme. Trends Biochem. Sci. 28 (8), 411–418.

Stotzky, G., Gallori, E., Khanna, M., 1996. In: Akkermans, A.D.L., van Elsas, J.D., de Brujin, F.J. (Eds.), Molecular Microbial Ecology Manual, vol 5.1.2. Kluwer Academic Publishers, Dordrecht, The Netherlands, pp. 1–28.

Symons, R.H., 1981. Avocado sunblotch viroid: primary sequence and proposed secondary structure. Nucleic Acids Res. 9 (23), 6527–6537.

Vettori, C., Paffetti, D., Pieramellara, G., Stotzky, G., Gallori, E., 1996. Amplification of bacterial DNA bound on clay minerals by random amplified polymorphic DNA (RAPD) technique. FEMS Microbiol. Ecol. 20, 251–260.

Vlassov, A.V., Johnston, B.H., Landweber, L.F., Kazakov, S.A., 2004. Ligation activity of fragmented ribozymes in frozen solution: implications for the RNA world. Nucleic Acids Res. 32, 2966–2974.

Vlassov, A.V., Kazakov, S.A., Johnston, B.H., Landweber, L.F., 2005. The RNA world on ice: a new scenario for the emergence of RNA information. J. Mol. Evol. 61 (2), 264–273.

Woese, C.R., 1996. The world of ribosomal RNA. Ribosomal RNA 23–48.

Zuker, M., 2003. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415.