Spontaneous rearrangements in RNA sequences

Helena V. Chetverina, Alexander A. Demidenko, Victor I. Ugarov, Alexander B. Chetverin*

Institute of Protein Research, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia

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Abstract The ability of RNAs to spontaneously rearrange their sequences under physiological conditions is demonstrated using the molecular colony technique, which allows single RNA molecules to be detected provided that they are amplifiable by the replicase of bacteriophage Qβ. The rearrangements are Mg^{2+}-dependent, sequence-non-specific, and occur both *in trans* and *in cis* at a rate of 10^{-9} h^{-1} per site. The results suggest that the mechanism of spontaneous RNA rearrangements differs from the transesterification reactions earlier observed in the presence of Qβ replicase, and have a number of biologically important implications.

Key words: RNA recombination; Transesterification reaction; Qβ phage satellite RNA; Qβ replicase; Molecular colony technique

1. Introduction

Known reactions leading to rearrangements in RNA sequences are either catalyzed by proteins and/or ribozymes often involved in posttranscriptional RNA processing [1–4], or made possible due to activation of phosphate groups [5,6]. Recently we have developed an extremely sensitive assay for reactions producing RNA molecules amplifiable by Qβ replicase [7], the RNA-directed RNA polymerase of bacteriophage Qβ [8]. The assay employs non-replicable supplementing 5′ and 3′ fragments of a Qβ phage satellite RNA. Reaction between the two fragments may result in a replicable RNA which is then amplified by Qβ replicase. The amplification occurs in an agarose-immobilized medium allowing the progeny of single replicable molecules to form separate colonies (the molecular colony technique) [9]. Counting the number of RNA colonies reveals the number of replicable molecules produced in the assayed reaction.

Here we use this assay to demonstrate that RNAs themselves can rearrange their sequences under physiological conditions, without the need for group activation or assistance from proteins or ribozymes.

2. Materials and methods

2.1. RNAs

The 5′ and 3′ fragments of the 133 nt long −-strand of $RQ135^{-1}$ RNA [10], split between positions 52 and 53, were constructed at the DNA level, synthesized with T7 RNA polymerase, and purified [7]. Foreign extensions of the 5′ fragment were derived from the pUC18 polylinker sequence in which the fragment was cloned between the *Hin*dIII and *Pst*I sites: type I fragment was synthesized from the plasmid digested at the *Bam*HI site [7], and type II fragment was synthesized from the *Eco*RI-digested plasmid that had been internally deleted by digesting at the *Pst*I and *Sma*I sites, removing the 3′ overhang with T4 DNA polymerase, and religating with T4 DNA ligase. The 5′ fragments were used either unmodified (3′-OH), or oxidized with periodate (3′-ox) producing a nucleoside dialdehyde at the 3′ end [11], or oxidized and then treated with aniline (3′-P) which eliminates the dialdehyde and produces a 1 nt shorter 3′-phosphoryl RNA [12], as described [7]. RQ mRNAs were prepared by transcription of appropriate plasmids with T7 RNA polymerase [7] and purified by electrophoresis through a 4% polyacrylamide gel [13]. mRNA inserts were placed between positions 53 and 54 of the −-strand of $RQ135^{-1}$ RNA [14]. CAT RQ mRNA and type I DHFR RQ mRNA have been reported [14,15]; the insert in type II DHFR RQ-mRNA had different sequences flanking the mRNA (Fig. 4B).

2.2. Spontaneous reactions

If not indicated otherwise, samples containing the 5′ and 3′ RNA fragments were annealed at a fragment concentration of 100 nM in 2×incubation buffer (20 mM Tris-HCl, pH 8.0; 200 mM NaCl; 2 mM EDTA) by heating for 2 min at 98°C followed by cooling to 35°C during 1 h, and prior to incubation were diluted 2-fold with water or 20 mM $MgCl_2$ (9 mM final free Mg^{2+} concentration); RQ mRNAs were prepared directly at the 50 nM concentration in 1×incubation buffer with or without 10 mM $MgCl_2$, omitting the annealing. After incubation under specified conditions, a sample containing a 3′-modified 5′ fragment or an RQ mRNA was diluted 30-fold with 1 mM EDTA (pH 8.0) in 10% glycerol (final concentration, 10^{10} molecules per 10 μl) and melted by heating for 4 min at 98°C followed by quick transfer to ice. A sample containing the unmodified 5′ fragment was diluted 20-fold with 1 mM EDTA (pH 8.0), melted, and then oxidized [11] with 35 mM Na-periodate in 100 mM Na-acetate (pH 4.8) during 1 h on ice in the dark; the salts were removed by microdialysis or gel filtration through a Sephadex G-25 superfine (DNA grade, Pharmacia) spun column in 1 mM EDTA (pH 8.0) at room temperature; the concentrations of the fragments and glycerol were adjusted to 10^{10} molecules per 10 μl and 10%, respectively, and the sample was melted again. Formation and melting of interfragment complexes were monitored by gel electrophoresis under non-denaturing conditions followed by silver staining [9] using samples which had been transferred into 1 mM EDTA (pH 8.0) by gel filtration.

2.3. Amplification and analysis of RNA

RNA colonies were grown at 22°C as reported [9,11]. A 10 μl sample was distributed over an agarose slab (18×18×0.37 mm) containing highly purified Qβ replicase [16] and covered with a nylon membrane containing rNTPs. 60 min later or as indicated, the membrane was fixed [9] and hybridized with a ^{32}P-labeled 5′ fragment to reveal the colonies by autoradiography [7]. Then RNAs were extracted from agarose, cloned and sequenced [7]. Secondary structures were predicted online at http://www.ibc.wustl.edu/–zuker/ by program MFOLD 3.0 based on free energy minimization [17].

3. Results

3.1. Experimental design

As previously reported [7], we used derivatives of the 52 nt long 5′ fragment and the 81 nt long 3′ fragment of $RQ135^{-1}$

*Corresponding author. Fax: (7) (095) 924-0493.
E-mail: alexch@vega.protres.ru

Abbreviations: RQ RNA, a Qβ phage satellite RNA replicable by Qβ replicase; 3′-OH, 3′-ox and 3′-P fragments, fragments with the unmodified, oxidized or phosphorylated 3′ end, respectively; DHFR, dihydrofolate reductase; CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein

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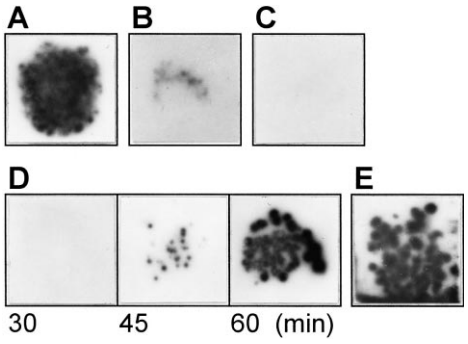
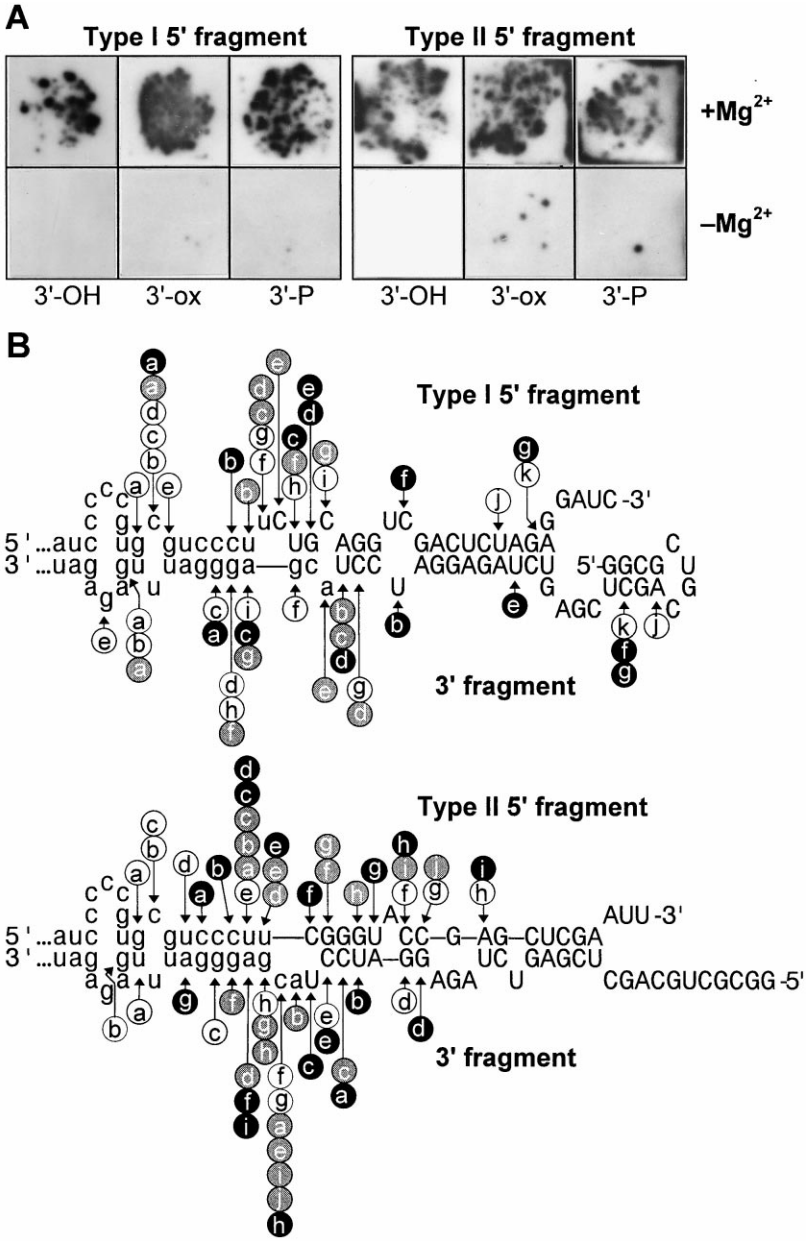


Fig. 1. Suppressing recombination during RNA amplification. A: RNA colonies formed by an annealed mixture of the 3′ fragment and the type I 5′ fragment by 60 min. B: Effect of melting the annealed mixture. C: Effect of oxidizing the melted mixture with sodium periodate. D: Time course of the growth of RNA colonies produced by 10^4 molecules of a replicable RNA (recombinant type A1 [7]) which were introduced into the fragment mixture before that was melted and oxidized. E: RNA colony pattern produced by 60 min by the same amount of the untreated replicable RNA alone. ←

− RNA [10], a Qβ phage satellite [18]. The split ends were extended as shown in Fig. 2B. The 5′ fragments bearing two different extension sequences with unmodified or differently modified (oxidized or phosphorylated) 3′ termini were tested. Replicable RNAs produced in a reaction between the 5′ and 3′ fragments were detected by the molecular colony technique [9].

To explore the possibility of a spontaneous reaction between the fragments, it was important to exclude the participation of replicase, i.e. to separate the step of replicable RNA formation from its amplification. To achieve this goal, we took advantage of our previous observations that recombination between the fragments in the presence of Qβ



replicase depended on their pre-annealing and required the 3′ hydroxyl group at the 5′ fragment [7]. Fig. 1A shows that a large number of RNA colonies appeared within 60 min if an annealed mixture of the 5′ and 3′ fragments was applied to the replicase-containing agarose slab; the number was greatly reduced upon melting the mixture (Fig. 1B), and no RNA colonies were seen if the melted mixture was also treated with periodate (Fig. 1C) which specifically oxidizes ribose hydroxyls at the RNA 3′ termini [11]. However, such a treatment did not prevent colony growth if the mixture contained a replicable RNA (Fig. 1D). Only $\leq 1\%$ of the added replicable molecules produced colonies in this experiment and also in the experiment of Fig. 1E, in which the RNA was not treated and not mixed with the fragments. The latter indicated that the deficit in the number of colonies was not due to interference from the fragments present in large excess or to a destruction of the replicable RNA during melting or oxidation. A likely reason is that a large proportion of the $RQ135^{-1}$ RNA molecules acquire a non-replicable conformation [10].

Accordingly, the following experimental scheme was employed to ascertain whether RNAs could react spontaneously. The fragment mixture was pre-annealed, incubated in the absence of a protein or nucleotides and, prior to growing RNA colonies, diluted, melted and oxidized (in experiments with the 3′-terminally modified 5′ fragments the oxidation step was omitted, see below). If RNA colonies appeared upon melting and oxidizing the fragment mixture, this would indicate that replicable RNAs had formed in the sample prior to this treatment.

3.2. Intermolecular reactions

Fig. 2A shows that replicable RNAs do form spontaneously. The reaction yield strongly depends on the presence of Mg^{2+} and is independent of variations in the 5′ fragment structure. The 3′-terminal modifications have no appreciable effect; hence, in contrast to recombination in the presence of Qβ replicase [7], the 3′ hydroxyl of the 5′ fragment is not essential. Sequences of the RNAs produced show that the fragments react by their internal parts and demonstrate that reactive sites are not confined to specific sequences or structures. Rather, they are distributed more or less randomly over ≈ 30 nt long segments of the fragments, with a bias toward unpaired or loosely paired nucleotides in at least one of the fragments (Fig. 2B). The absence of reactive sites beyond these segments is a likely result of the selective amplification by Qβ replicase of those molecules whose structure is most similar to that of the original RQ RNA and which possess a stable secondary structure [19]. The results further suggest that the reaction occurs within complexes formed by base-paired fragments: all counterparts of a given nucleotide are restricted to the nearby 10 nucleotides of the other fragment (Fig. 2B). The results indicate that the observed reaction is not due to a cryptic ribozyme structure that might be produced by the RNA fragments, but is an intrinsic chemical property of RNA.

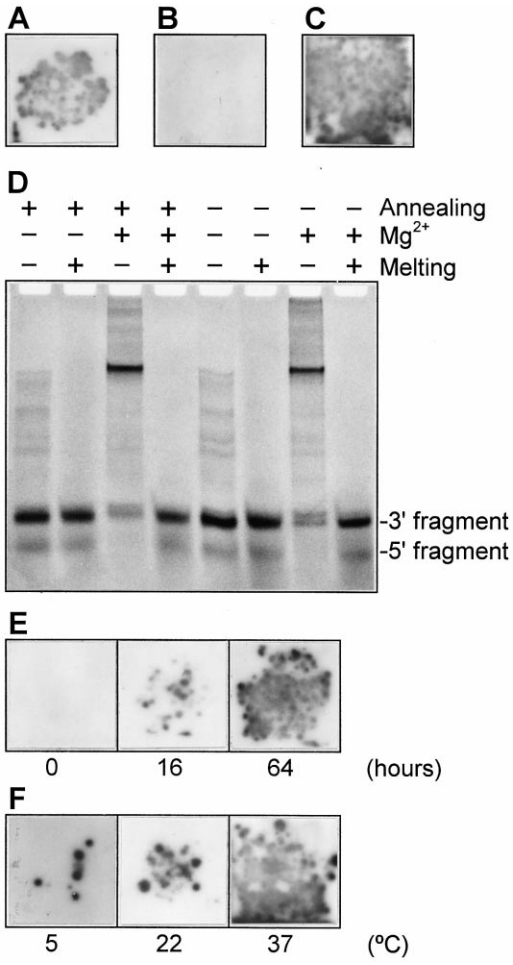


Fig. 3. Properties of the intermolecular reaction studied with the 3′-oxidized type I 5′ fragment. Unless otherwise indicated, the incubations were for 64 h at 37°C in the presence of 9 mM Mg^{2+}. A: The fragment mixture was oxidized after incubation. B: The 3′ and 5′ fragments were incubated separately at a 100 nM concentration, then melted and mixed just before colony growth. C: Fragment pre-annealing was omitted. D: Effect of annealing and incubating the samples in the presence of Mg^{2+} on the formation of interfragment complexes. E: Time course of replicable RNA formation at 37°C. F: replicable RNAs formed within 64 h at different temperatures.

The reaction was studied in more detail by using a 3′-oxidized 5′ fragment. In this case, oxidation of the fragment mixture prior to amplification did not decrease the number of RNA colonies (cf. Fig. 3A and sample type I, 3′-ox of Fig. 2A) and therefore may be omitted. This experiment also showed that the prolonged incubation in the presence of Mg^{2+} did not expose the 3′ hydroxyls that could provide for the replicase-assisted recombination. One might suggest

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Fig. 2. Spontaneous reactions between RNA molecules. A: RNA colonies produced by a mixture of the 3′ fragment and the indicated version of the 5′ fragment incubated with or without 9 mM Mg^{2+} during 64 h at 37°C. B: Distribution of crossover sites. The nucleotides that become linked are marked with identical encircled symbols; the circle filling distinguishes the RNAs produced by 3′-OH (white), 3′-ox (gray), and 3′-P (black) 5′ fragments. If there is a homology between the fragments around a crossover site, then the site's most upstream location in the 5′ fragment is indicated. The original $RQ135^{-1}$ RNA sequences are (partially) shown in lower case, and their extensions are shown in upper case.

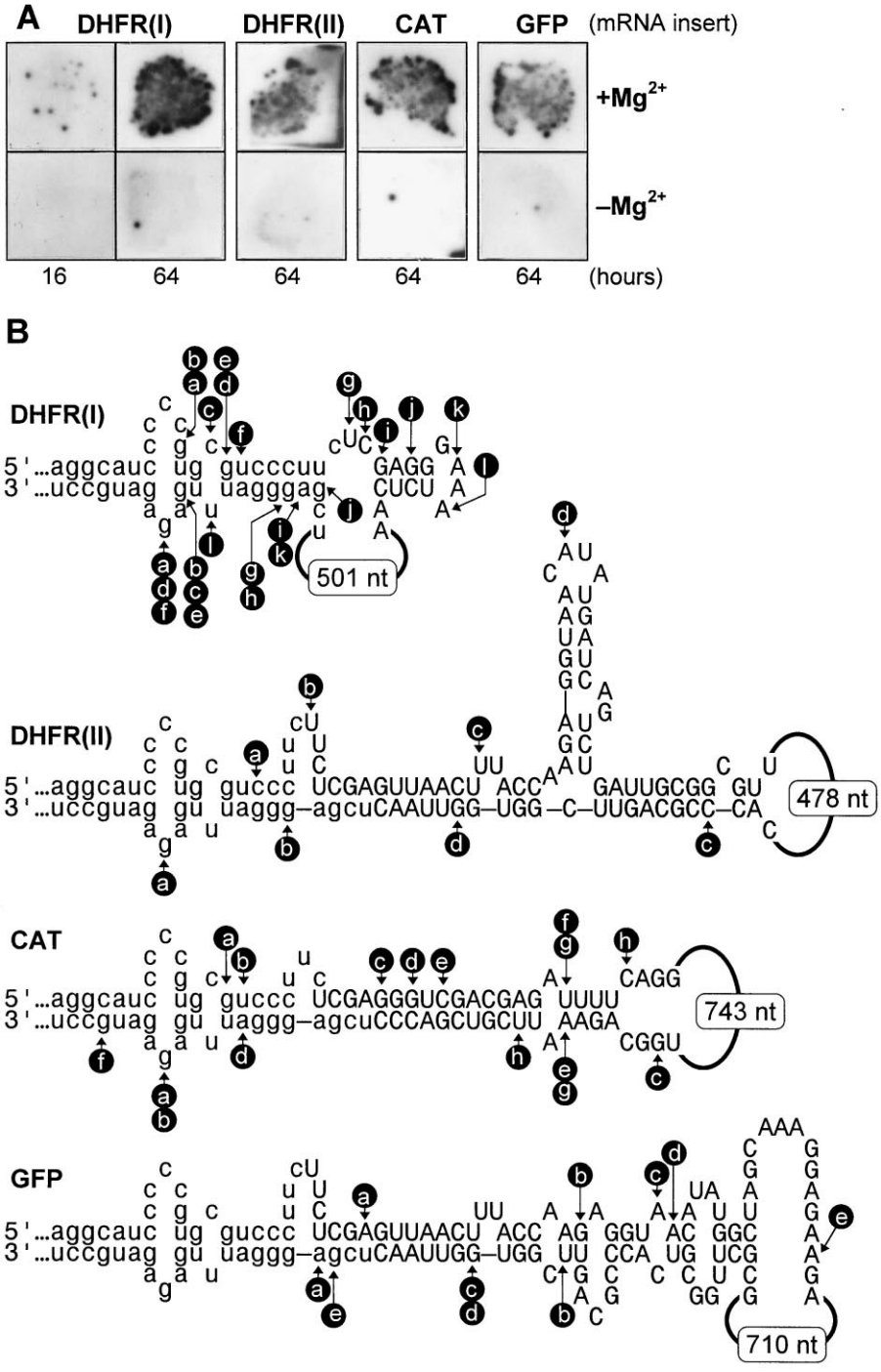

Fig. 4. Generation of replicable RNAs by spontaneous deletions of mRNA inserts from RQ mRNAs. A: RNA colonies produced by RQ mRNAs carrying the indicated mRNA inserts upon incubation with or without 9 mM Mg^{2+} at 37°C. B: Distribution of crossover sites.

that during the prolonged incubations Mg^{2+} simply exposed other reactive groups by cleaving the fragments, whereas the covalent bond between the fragments was formed by Qβ replicase. This possibility was rejected because RNA colonies did not appear if the 5′ and 3′ fragments were separately incubated in the presence of Mg^{2+} and mixed before applying to the agarose (Fig. 3B). The reaction yield did not change if preannealing was omitted (Fig. 3C) or if the fragment concentration varied from 5 to 500 nM (not shown), indicating a pseudo-first order reaction in which a chemical step is rate-limiting. This is because the reaction proceeds within stable intermolecular complexes which readily form in the presence of Mg^{2+} irrespective of pre-annealing (Fig. 3D). Fig. 3E reveals the roughly linear reaction kinetics. In a series of independent experiments it was determined that the number of replicable RNAs generated by 64 h was 4.0 ± 1.8 times greater than by 16 h (mean ± S.D., $n = 10$). The apparent reaction rate was $\approx 3 \times 10^{-10}$ h^{-1} per RNA molecule. Since only about 1% of replicable molecules produced colonies (Fig. 1D,E), the mean reaction rate can be estimated as 10^{-9} h^{-1} per site from the ≈30 nt long reactive segment of a fragment. Fig. 3F shows that in the temperature range studied the rate in-

creases approximately 3-fold per 10° increment, which is similar to a ribozyme-catalyzed RNA cleavage [20].

3.3. Intramolecular reactions

Since the above reactions took place between internal parts of the associated fragments, one could expect that similar reactions would occur within an RNA molecule, resulting in deletions of its internal segments. Fig. 4 shows that this is the case. In these experiments we used artificially prepared RQ mRNAs comprised of mRNA sequences embedded within the $RQ135^{-1}$ sequence. Earlier we found that long mRNA inserts inactivated the RQ RNA vector as a Qβ replicase template [14]; hence, RNA colonies would only appear if the inserts were deleted. The dependence on Mg^{2+}, the reaction kinetics (Fig. 4A), and the random distribution of reaction sites (Fig. 4B) all resembled the intermolecular reaction, suggesting that the same mechanism operated in each case.

4. Discussion

4.1. Two different mechanisms of non-replicative RNA recombination

Recently, we described a transesterification reaction resulting in recombination of RNA molecules [7]. There are two basic differences between that reaction and the reaction reported here. (i) The two reactions are quite dissimilar chemically. In the reaction reported earlier, the transesterification is a result of an attack of a free 3′ hydroxyl on an internucleotide phosphodiester bond, whereas in the newly described reaction the 3′ hydroxyls do not participate. (ii) While the latter reaction requires nothing but RNA and Mg^{2+}, the former reaction is only observed in the presence of Qβ replicase and is at least two orders of magnitude faster than the spontaneous reaction. Since RNA colonies became visible by 45 min of growth at 22°C (Fig. 1D), the recombinants seen by 60 min in Fig. 1A must had been formed within the first 15 min of incubation in the presence of Qβ replicase at this temperature, whereas the spontaneous reaction between the same fragments produced a smaller number of recombinants in 64 h at 37°C (Fig. 2A, sample type I, 3′-OH).

The mechanism by which Qβ replicase promotes the attack of 3′ hydroxyls at phosphodiester bonds is unknown. Since a chemically related reaction is accelerated by hybridizing the reacting RNA segments to a complementary sequence [6], one could suggest that replicase plays a structural role by juxtaposing the reacting groups. Alternatively, replicase might catalyze the transesterification reaction like it catalyzes the addition of nucleotides to the growing end of the nascent RNA strand [8].

The facts that 3′ hydroxyls do not participate in the spontaneous reaction and that the RNA fragments react by their internal sites might suggest that this reaction includes two Mg^{2+}-catalyzed RNA cleavages generating the 2′,3′-cyclic phosphate and 5′ hydroxyl termini [21] followed by cross-ligation of the termini. However, we cannot exclude other chemical mechanisms, e.g. those involving the formation of a branched lariat-like structure or a pyrophosphate bond.

4.2. Biological implications of the spontaneous reactions

The results show that RNAs are able, on a biologically meaningful time scale, to spontaneously and randomly rearrange their sequences under physiological conditions. The reactions can occur both *in trans* and *in cis*, and this may generate a virtually unlimited diversity of new RNA sequences. Inasmuch as the rearrangements require nothing but RNA itself and Mg^{2+}, they must be ubiquitous in nature. This finding extends our knowledge of the chemical flexibility of RNA and its versatility as a carrier of genetic information, and has a number of important implications.

RNA rearrangements are intensively studied by virologists who often observe recombinations between viral RNA genomes [22], as well as the generation of defective interfering particles via deletions of significant portions of the viral genome [23]. Some of these rearrangements may be due to the spontaneous reactions reported here. Spontaneous reactions between RNA molecules might even provide for the evolution of DNA genomes. With the observed reaction rate of 10^{-9} h^{-1} one rearranged RNA molecule can arise every minute in a typical mammalian cell with a total RNA length of 5×10^{10} nt [24], amounting to up to 10^{20} of such molecules generated during the life span of the human body, some of which may be reverse transcribed and integrated into the genome [25]. Other possible implications include a mechanism for the genetic exchange between RNA viruses and their hosts [22] and a new possibility for gene evolution in the prebiotic RNA world [26,27].

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