Reversible Switching of Cooperating Replicators

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(Received 26 January 2016; published 15 February 2017)

How can molecules with short lifetimes preserve their information over millions of years? For evolution to occur, information-carrying molecules have to replicate before they degrade. Our experiments reveal a robust, reversible cooperation mechanism in oligonucleotide replication. Two inherently slow replicating hairpin molecules can transfer their information to fast crossbreed replicators that outgrow the hairpins. The reverse is also possible. When one replication initiation site is missing, single hairpins reemerge from the crossbreed. With this mechanism, interacting replicators can switch between the hairpin and crossbreed mode, revealing a flexible adaptation to different boundary conditions.

DOI: 10.1103/PhysRevLett.118.078102

Introduction.—To study key aspects of Darwinian evolution under controlled conditions, synthetic approaches are widely used. They minimize the complexity of living organisms and allow their full quantitative analysis. Most importantly, evolution can be studied on the molecular level with a focus on central mechanisms such as replication and selection [1–3]. Along this line, nucleic acids have been widely used to study replication mechanisms, ranging from auto- and cross-catalytic processes [4–7] to molecular ecosystems which show Lotka-Volterra oscillations and pattern formations [8,9]. Although auto- and cross-catalytic processes have also been observed in peptide based metabolic networks [10], it remains consensus that the evolution of nucleic acids was a key process in the transition from inanimate matter to life [11,12].

But how can simple replicators prevent the loss of genetic information [13]? The information content of early replicators has to be preserved against processes such as diffusion, dilution, or molecular degradation. Inter- and intramolecular base pairing reduces cleavage and enhances sequence memory due to mutual interaction, favoring oligonucleotides with secondary structures [14,15].

For the evolution of catalytic oligonucleotides (ribozymes), which drive replication [5,6,16–18], intermolecular interactions are especially important to recognize the replication templates. In protein-based, modern biology, as well as for RNA-based replication mechanisms [16–18], typically a short oligonucleotide, called primer, activates an initiation site, recruits the base-by-base replication machinery, and triggers the formation of double stranded oligonucleotides from the single stranded template. Copying an RNA ribozyme produces the complementary sequence. Since it most probably lacks catalytic activity, the complement should serve as a template for subsequent replication [19], which again requires an initiation site. However, the spontaneous emergence of two independent, functional initiation sites in a single ribozyme seems unlikely due to the size of the sequence space. For an oligonucleotide with an initiation site of length l, the probability for a primer of the same length to be complementary is 4^{-l}. When two primers are required, the probability significantly decreases to 4^{-2l}.

As a start, we compare the growth behavior of three replicator types that differ in the number of initiation sites and secondary structures of the oligonucleotides [Fig. 1(a)]:

(i) Strands with one initiation site and without secondary structure lead to a product that cannot replicate further due to the lack of an active initiation site in the replicated strand. Such linear replicators will go extinct by the eventual degradation of the template strand (Supplemental Material [20], S6).

(ii) For hairpin replicators, the replicated complements inherit the initiation site, due to the self-complementary stem sequence. This allows auto-catalytic, exponential replication. However, the secondary structure limits the access to the initiation site, causing a slow replication kinetics.

(iii) A replicator without secondary structure, but with two active initiation sites—one at the initial strand and one at its complement—combines the advantages of linear and hairpin replicators: It replicates exponentially without self-inhibition. But, as described, the spontaneous emergence of such a cross-catalytic replicator is an unlikely event.

The replication behavior of the three replicator types is realized experimentally in a polymerase chain reaction (PCR) [Fig. 1(a)]. We demonstrate experimentally that two auto-catalytic, self-inhibiting hairpin replicators cooperate by a mechanism termed *crossbreeding*. This mechanism provides a transition from one to two initiation sites. It enables cross-catalysis and preserves the hairpin sequence information with increased fitness to sustain even strong degradation conditions. Interestingly, this process can be reversed by *hairpin regrowth*, giving rise to the initial hairpins.

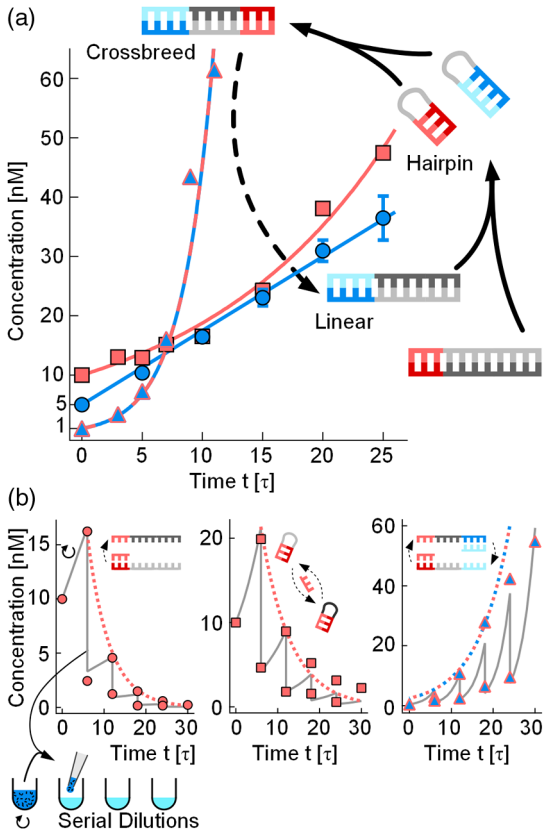

FIG. 1. Three modes of replication: linear, hairpin, and crossbreed. A linear oligonucleotide with one initiation site leads to linear product increase during PCR (blue circles). Hairpins replicate exponentially with a single initiation site. The secondary structure slows replication by self-inhibition (red squares). Linear DNA replicates exponentially if two initiation sites are present (red-blue triangles). Arrows indicate transitions between replication modes. (b) Degradation is implemented by serial dilutions. After n replication rounds, the replicated DNA is transferred to $d-1$ parts of fresh replication medium ($n=6$; $d=5$). Linear replicators (left) vanish. Exponential replicators (middle, right) survive if the replication rate exceeds the dilution rate. Dashed curves are exponential fits, gray curves show the theoretical dynamics between the dilutions [Eqs. (1)–(3)].

The crucial step in both processes is the cross-hybridization of unfinished products to another template, followed by subsequent elongation. Related nucleic acid based mechanisms are known from other minimal replicating systems capable of cooperation [21] and predation [8,22]. In these systems, the unfinished products have a well-defined sequence, whereas in our system they originate from stochastic interruption.

Material and methods.—For the PCR, Phusion High Fidelity DNA Polymerase (20 U/ml) was used in 1 × HF buffer with 0.2 mM NTPs (all from New England Biolabs) and 1× EvaGreen (Biotum Inc.). The reaction volume was between 5 and 15 μl. Each primer was added at a concentration of 250 nM, and template concentrations varied from 0.5 to 30 nM (Supplemental Material [20], S1, Table S3). Pipetting steps were done on ice. Samples were cycled in a C1000 Touch™ Thermal Cycler with a CFX96 detection system (Bio-Rad Laboratories). In each PCR cycle, samples were denaturated for 10 s at 95 °C (after pipetting steps the first denaturation step was extended by 3 min) and annealed for 36 s at 67 °C. Including heating and cooling, the time for one cycle was 72s. The lid temperature was set to 105 °C to prevent condensation and the samples were cooled down to 1 °C after the last cycle. Oligonucleotides were purchased from biomers.net GmbH with HPLC purification (sequences in Supplemental Material [20], S1).

Primers were purchased labeled with Cy3 or Cy5 at their 5′-end in order to measure species concentrations using denaturating PAGE (12.5% PAA gels, 19:1, 50% urea, from Carl Roth). After PCR, samples were mixed with a 2× loading buffer (95% formamide, 5% glycerol and 50 μM EDTA) as preparation for PAGE. The errors of the given fitness and efficiency values were calculated from a pipetting error of 10% and obtained as a standard deviation from multiple experimental runs. Error bars are standard deviations.

Results.—We use PCR as an experimental proxy for a replication mechanism. Temperature oscillations provide oscillatory dehybridizing conditions, which prevent product inhibition and allow exponential growth. In Fig. 1, the DNA concentration is plotted over time for all three replicators. For the linearly replicating species, 5 nM template produces 1.25 ± 0.11 nM of copies in every PCR cycle. Thus, the concentration c increases linearly with time t, according to $c(t) = 5\ \text{nM} + 1.25\ \text{nM}\, t/\tau$, where $\tau = 72$ s is the length of a cycle. For exponential replicators, we assume that $c(t) = c_{t=0} e^{\varepsilon t/\tau}$, where ε is the replication efficiency. The fitted efficiencies for the hairpins and crossbreeds are $\varepsilon = 0.06 \pm 0.01$ and $\varepsilon = 0.38 \pm 0.02$, respectively. The secondary structure of the hairpin replicator blocks the initiation site, causing slower replication. We also find that efficiencies depend on the details of the initiation sites and the templates (Supplemental Material [20], S1).

However, molecules do not only form, but also degrade. Typically, this happens with an exponential kinetics [23]. Here, degradation is experimentally implemented by serial dilution transfers of PCR products to fresh resources, in particular polymerase, NTPs and primers, which leads to selection for fast replicators [Fig. 1(b)]. After n PCR cycles, we use a dilution factor of d; i.e., one part of the PCR product is added to $d-1$ parts of fresh medium. In order to prevent extinction, the replication rate has to exceed the dilution rate. For exponential replication with the time $t' = n\tau$ between two dilution steps m and $m+1$, species go extinct if the fitness $F = c(t_{m+1})/c(t_m) = (e^{\varepsilon t'/\tau})/d = e^{\varepsilon n - \ln(d)} < 1$. Linear replicators will always go extinct under dilution (Supplemental Material [20], S6).

Figure 1(b) shows how the dynamics of the three replicator types behave under the selection pressure of degradation with $n=6$ and $d=5$: Linear replicators with one active initiation site go extinct as expected. The hairpin species reaches a fitness $F = 0.43^{+0.06}_{-0.07}$, which also leads to

extinction, whereas the fast replicating crossbreeds survive with $F = 2.26^{+0.15}_{-0.16}$. The fitness values are obtained from the exponential fits, indicated as dashed curves in Fig. 1(b). For the crossbreed replication, the fit is done until $t = 16\tau$.

The gray curves show the theoretical dynamics between the dilutions from a discrete time model, in which replication is described by

$$c_L^{j+1} = c_T + c_P^{j+1} = c_L^j + c_T\varepsilon_L, \quad (1)$$

$$c_{\mathrm{HP}}^{j+1} = c_{\mathrm{HP}}^j(1+\varepsilon'), \quad (2)$$

$$c_{\mathrm{CB}}^{j+1} = c_{\mathrm{CB}}^j[1+\varepsilon'(1-c_{\mathrm{CB}}^j/K)], \quad (3)$$

where c_L^j, c_{HP}^j, and c_{CB}^j are the concentrations of the respective species after j cycles. For the linear replicator, the template concentration c_T remains constant during replication, but generates product c_P with the linear efficiency ε_L. For hairpin and crossbreed replication the efficiency is $\varepsilon' = e^{\varepsilon} - 1$ (Supplemental Material [20], S5). For the crossbreed replicators, a logistic approach with the carrying capacity K was chosen. The parameters used in all figures are summarized in Table S5 (Supplemental Material [20], S5).

The hairpin information does not have to be lost under the given dilution conditions. Two hairpin species that differ by their initiation sites can cooperate with matching loop sequences and preserve their information in fast replicating crossbreeds [Fig. 2(a), Supplemental Material [20], S2]. The mechanism is a consequence of nonprocessive replication, triggered, for example, by a premature temperature increase before the hairpin replication process is finished. By interrupting the replication, an incomplete complement of the hairpin is produced. It detaches from its template and cross-hybridizes to another hairpin species at lower temperatures. Subsequent elongation creates a crossbreed species with initiation sites of both original hairpins. The crossbreeding mechanism can also start from interrupted replication of the other hairpins ($*$) and leads to a second variant of the crossbreed species ($**$), which differs by an inverted sequence. The color of the crossbreeds initiation site at the $5'$ end reveals from which hairpin the replication was initiated, while the color of the loop domain shows whether the original hairpin or its complementary strand was used. Thus, depending on the hairpin on which the replication is initiated, four different crossbreed molecules emerge [Fig. 2(a), right side]. They form two complementary pairs, differing by a sequence inversion in the central regions.

Conversely, when both of these crossbreed variants are present, they give rise to the original hairpin species by a hairpin regrowth mechanism triggered by a missing second active replication initiation site [Fig. 2(a), lower pathway]. The key process is again an interrupted elongation followed by cross-hybridizing and subsequent elongation. Depending on the crossbreed molecule from

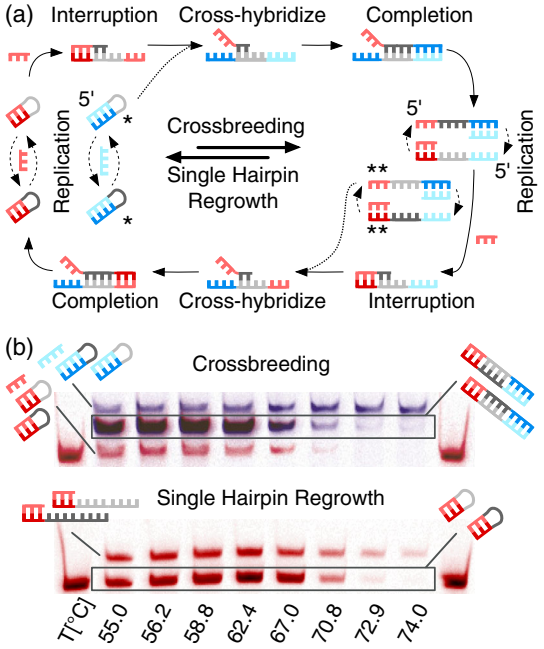

FIG. 2. Crossbreeding and hairpin regrowth. (a) Two hairpins with same loop but different initiation sites cooperate to form crossbreeds. Replication is interrupted and an incomplete strand emerges. It cross-hybridizes to another hairpin and replication is completed (upper pathway). Two crossbreed variants are possible, differing by a sequence inversion in their center. The inversion allows hairpins to regrow: The replication is again interrupted and completed after cross-hybridization (lower pathway). The processes can equally well start with the species marked by $*$ or $**$. (b) Different lengths and fluorophores (Cy3, Cy5) allow us to distinguish the species in gel electrophoresis. The replication of cooperating hairpins leads to crossbreeds (top). Starting from crossbreeds with one active initiation site the hairpins regrow (bottom). Both processes work for a wide range of annealing temperatures. First and last lanes are Cy5-labeled 84-mers as length reference.

which the elongation is initiated, all four hairpins can regrow (Supplemental Material [20], S8). Interestingly, the hairpin regrowth process is suppressed when both initiation sites are active (Supplemental Material [20], S7).

The gels in Fig. 2(b) show crossbreeding (top) and hairpin regrowth (bottom). Replication products of long and short hairpins are distinguished by their length (124 vs 84 bases) and fluorophores. Apart from the primers, no specially designed sequences were used in the experiment. In the crossbreeding experiment, the two hairpin species are mixed and the products are analyzed on a gel after 18 rounds of replication. A third band with intermediate length, which corresponds to the crossbreed species (104 bases), is visible in both color channels. In addition, an intercalating dye was used to compare the products to a DNA ladder (Supplemental Material [20], S7).

To demonstrate the robustness of the mechanism, the experiments are performed over a wide range of annealing temperatures. For higher temperatures, the process seems to become weaker, but so does the replication of the short

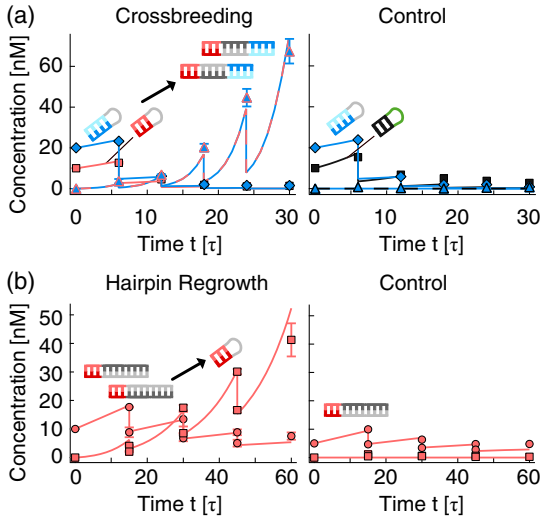

FIG. 3. Crossbreeding and hairpin regrowth under serial dilution. (a) Two hairpins with the same loop sequence go extinct under dilution conditions with $n = 6$ and $d = 5$ (left). However, the hairpins form crossbreeds, which slowly emerge [$\beta = 4.5 \times 10^{-5}$ nM^{-1}, see Eqs. (4) and (5)] and take over the population. Hairpins with nonmatching orthogonal loop sequences vanish after a few cycles and no crossbreeds emerge (right, $\beta = 0$ nM^{-1}). (b) Linearly replicating crossbreeds go extinct under milder dilutions ($n = 15$; $d = 2$). When both crossbreed variants are present, the sequence inversion allows hairpin regrowth (left, $\alpha = 3.2 \times 10^{-4}$ nM^{-1}). Otherwise, all information is lost (right, $\alpha = 0$ nM^{-1}).

hairpin that has a two bases shorter primer (18 bases) compared to the long hairpin. Thus, the weak binding of the shorter primer limits the reaction. Furthermore, crossbreeding occurs independently of the polymerase (Supplemental Material [20], S3).

Can this mechanism help the hairpin species to survive the strong dilution used previously ($n = 6$; $d = 5$)? The answer is *no*. When a pair of cooperating hairpins replicate under these boundary conditions, they still vanish with fitnesses $F = 0.27 \pm 0.05$ and $F = 0.24 \pm 0.04$ [Fig. 3(a), left]. But their sequence information is transferred to the emerging crossbreeds that survive the dilutions with $F = 2.37 \pm 0.02$. As a control, a short orthogonal hairpin was used that cannot cooperate with the long hairpin [Fig. 3(a), right]. In this case, the long hairpin vanishes with a fitness of $F = 0.24^{+0.05}_{-0.04}$ and the short orthogonal hairpin with $F = 0.35^{+0.07}_{-0.06}$. No species with a fitness $F > 1$ emerges and all the sequence information is lost.

To demonstrate that the mechanism preserves the sequence information of the hairpins, the reaction has to be reversed. Such a hairpin regrowth process is started from the two crossbreed variants with one active initiation site, allowing only linear replication [Fig. 2(b), bottom]. After 35 rounds of replication the short hairpin species is visible on the gel. The long hairpin can be regrown in the same way, but using the other primer (Supplemental Material [20], S8).

Thus, even though the cooperating hairpins in Fig. 3(a) vanish, their sequence information does not. When the conditions change, hairpins can regrow as described in Fig. 2(a) (lower pathway) and survive a serial dilution transfer. To show this experimentally, both crossbreed variants are replicated in the absence of any hairpins, but with only one active initiation site [Fig. 3(b), left]. The now linear replicating crossbreeds go extinct even under milder dilution conditions ($n = 15$; $d = 2$). However, the sequence inversion of the two crossbreed variants allows the regrowth of an exponentially growing hairpin replicator, which survives with $F = 2.24 \pm 0.01$. Without this mechanism, the crossbreeds vanish and no hairpins form [Fig. 3(b), right].

To explain both mechanisms quantitatively (Fig. 3, solid lines), the previous equations, Eqs. (2) and (3), are completed by two terms:

$$c_{\mathrm{HP}}^{j+1} = c_{\mathrm{HP}}^{j}(1 + \varepsilon') + \alpha (c_P^j)^2, \tag{4}$$

$$c_{\mathrm{CB}}^{j+1} = c_{\mathrm{CB}}^{j}[1 + \varepsilon'(1 - c_{\mathrm{CB}}^{j}/K)] + \beta c_{\mathrm{HP1}}^{j} c_{\mathrm{HP2}}^{j}, \tag{5}$$

where α and β describe the hairpin regrowth and crossbreeding reactions (Supplemental Material [20], S5).

This model allows us to simulate what happens when boundary conditions are time dependent. In the *in silico* experiment in Fig. 4 we assume that the activities of initiation sites oscillate with a phase shift. When both sites are active, hairpins and crossbreeds replicate exponentially and crossbreeding takes place. In the case of a single active initiation site, the corresponding hairpin replicates exponentially, whereas the crossbreeds replicate linearly and allow hairpin regrowth. The dilutions are performed after every cycle ($n = 1$; $d = 1.18$). For the two hairpin species we assumed the same efficiency of $\varepsilon' = 0.03$, corresponding to the lowest hairpin efficiency measured during an

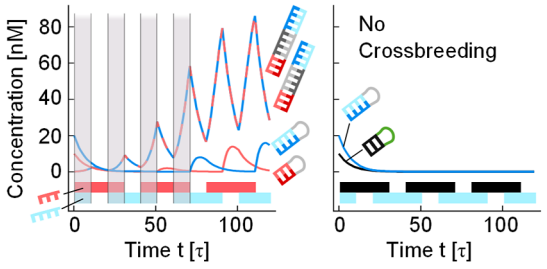

FIG. 4. Surviving an alternating number of initiation sites (blue and red-black). A simulation of hairpin replicators under changing environmental conditions shows how hairpins benefit from crossbreeding. Here, the dilution is implemented after every cycle ($n = 1$; $d = 1.18$) and the initiation sites are switched on and off periodically with a phase shift. Left: When both initiation sites are active (gray bars), the hairpins replicate and form exponentially replicating crossbreeds ($\beta = 4.5 \times 10^{-5}$ nM^{-1}). With a missing initiation site, only one hairpin replicates, crossbreeds replicate linearly and regrow the hairpins ($\alpha = 3.2 \times 10^{-4}$ nM^{-1}). Crossbreeding and hairpin regrowth allow the hairpins to maintain their sequence information. Right: Without crossbreeding the hairpins vanish ($\alpha = \beta = 0$ nM^{-1}).

experiment. Thus, the fitness of the hairpins is $F = 0.88$ when their initiation site is active and $F = 0$ otherwise. This should lead to extinction. But when the hairpins cooperate and form crossbreeds, the hairpins survive due to the hairpin regrowth process, in which new exponentially replicating hairpins are generated for only one active initiation site (Fig. 4, left). Without the crossbreeding process ($\beta = 0$), the hairpins vanish (Fig. 4, right).

Discussion.—Evolution is based on passing on the information of individual molecules despite molecular degradation or dilution. Our results show that this can be achieved by a peculiar cooperation mechanism in which the benefit is not an increase of the replication rate, but an information transfer from slower to faster replicators. Even under strong degradation conditions, the information of the hairpin species can be stored cooperatively despite their extinction.

Because of the reversibility of the process, hairpins can regenerate when one initiation site is missing (Fig. 4). This offers a basic risk-spreading mechanism [24–26] that preserves sequence information under changing boundary conditions: Two active initiation sites favor fast growing crossbreeds, while the presence of only one site under the chosen lower dilution rate favors regrowth of the corresponding slow growing hairpin. Switching between the two modes of replication, cooperating hairpins can prevent extinction.

We thank Erwin Frey, Ulrich Gerland, Simon Lanzmich, and Christof Mast for discussions and Friederike Möller, Matthias Morasch, and Jonathan Liu for corrections to the manuscript. Financial support from the Deutsche Forschungsgemeinschaft SFB1032 Project A4 and the Simons Collaboration on the Origins of Life (SCOL 327125) is gratefully acknowledged. G. U. and D. B. conceived the experiments and wrote the paper. G. U. and T. R. performed the experiments.

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