# Thermal, Autonomous Replicator Made from Transfer RNA

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Evolving systems rely on the storage and replication of genetic information. Here we present an autonomous, purely thermally driven replication mechanism. A pool of hairpin molecules, derived from transfer RNA replicates the succession of a two-letter code. Energy is first stored thermally in metastable hairpins. Thereafter, energy is released by a highly specific and exponential replication with a duplication time of 30 s, which is much faster than the tendency to produce false positives in the absence of template. Our experiments propose a physical rather than a chemical scenario for the autonomous replication of protein encoding information in a disequilibrium setting.

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*Introduction.*—In modern biology, an RNA-dominated machinery encodes proteins and proteins replicate genetic information. Before this interlinked machinery evolved, early life most probably replicated genes using a minimal pool of rather short RNA sequences [1]. RNA world [2,3] approaches advocate chemical base-by-base replication [4,5] under high salt conditions [6]. However, the translation of proteins relies only on a redundant three-letter code of codons. An intriguing idea is then to replicate a succession of codons rather than single bases. Here we explore a physical, not chemical, replication mechanism for codon successions.

As a consequence of the second law of thermodynamics, replicators must be driven by disequilibrium conditions. The demonstrated replication is solely driven by wasteless thermal energy, which could be provided by thermal convection, for example. The replicator is built from four halves of transfer RNA (tRNA), forming hairpins with a sequence encoding toehold (Fig. 2). tRNA molecules define the genetic code as they translate codons into amino acids in the translation process of proteins. Arguably, it is one of the most ancient molecules of biology [7–9].

In a sense, we create a physically driven version of the chemical ligation chain reaction [10] that replicates a two-letter code cross catalytically [11–13]. Instead of joining the matching strands by chemical backbone ligation, the substrates are joined within seconds by physical base pairing. Our replication is driven by two modes of thermal oscillation. The binding energy is first stored using an initial high temperature step (95 °C) where tRNA molecules are quenched on ice water into monomolecular hairpin states. Subsequent moderate temperature oscillations between 10 °C and 40°C connect the hairpins exponentially in a cross-catalytic reaction (Fig. 1). The approach is inspired by isothermal DNA machines [14–17] and nonautonomous replicators [18]. As discussed below, no highly specialized RNA sequence was required. We randomly chose half of a tRNA sequence to create the replicator. The thermal approach is compatible with hydrothermal molecule traps [19–21], thermal microconvection [22], and might have been selected by asymmetric hydrolysis of the strand backbone [23].

*Materials and methods.*—Alanine-TGC tRNA of Methanobacterium thermoautotrophicum was cut left of the anticodon and was predicted [24] to fold into a hairpin with a toehold consisting of anticodon and upstream bases [Fig. 2(a)]. Inside the hairpin loop, we replaced an adenine base by the UV-fluorescent 2-aminopurine (2-AP) which is quenched by hybridization [25] once the hairpins are opened and bound. From this hairpin $A$, hairpins $B$, $a$, and $b$ were constructed. The stem-loop region of $B$ is complementary to the stem loop of $A$, but has a different sequence at the toehold. The 8-base long toehold regions of $a$ and $b$ are complementary to toehold regions of hairpins $A$ and $B$. Both $a$ and $b$ have again complementary stem-loop regions. To prevent binding crosstalk and keep the reaction symmetric, the backward read sequence of $A$ and $B$ was chosen. The resulting sequences are 5′-UGC AAG GCG GAG GCC CCG GGU UCAP AAU CCC GGU GGG UCC A-3′ for hairpin $A$ with AP = 2-aminopurine, 5′-U GGA

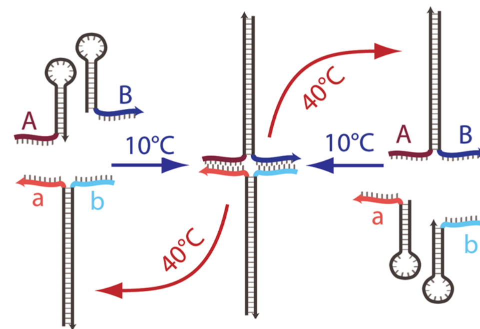


FIG. 1 (color online). Thermal replication using a cross-catalytic replication reaction. A purely thermally driven exponential replication of sequence succession $ab$ is expected from metastable hairpins and microscale convection.

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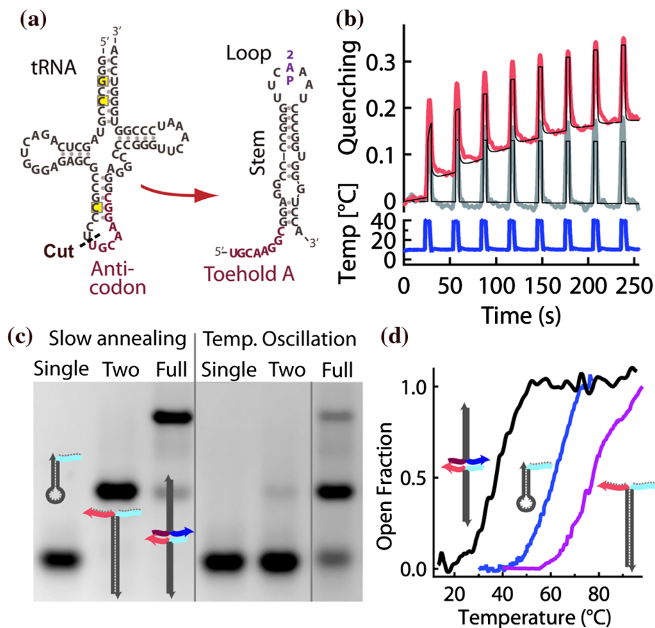


FIG. 2 (color online). Replication results. (a) As fuel, a typical tRNA is cut next to the anticodon, forming a hairpin with a toehold sequence $A$. (b) Quenching of 2-aminopurine rises as duplexes are formed in the full reaction with 500 nM of four hairpins $a, b, A, B$, and 35 nM template duplex $ab$ (red). The non-cross-catalytic mixture of two hairpins $A, B$ with template $ab$ shows no signal (gray). An explicit numerical model fits the fluorescence quenching accurately in both cases (black). (c) Gel electrophoresis of the resulting products. Slow annealing of single, two, or four hairpins leads to equilibrium expectation of hairpins, duplexes, and quadruples. Under thermal oscillation, only the full reaction transforms four hairpins into duplexes and quadruples. (d) Melting curves of quadruple ($37 \pm 3\,°C$, black), hairpin ($62 \pm 2\,°C$, blue), and duplex ($> 75\,°C$, purple).

CCC ACC GGG AUU UGA ACC CGG GGC CUC CAC GUU CCG-3′ for hairpin $B$, 5′-G GGC CAC CCA GGU AAG UUU ACC UCC GGG GCC CGC CUU GCA-3′ for hairpin $a$, 5′-FAM-CGG AAC GUG GGC CCC GGA GGU AAA CUU ACC UGG GUG GCC C-3′ for hairpin $b$ with a fluorescent FAM modification at the 5′ end for gel electrophoresis and 5′-G GGC CAC CCA GGU AAG UUU ACC UCC GGG GCC CGC GCA CUU-3′ for a hairpin with mutated toehold sequence. RNA hairpins (IBA Göttingen) at 2.5 $\mu$M in buffer (150 m$M$ NaCl, 20 m$M$ TRIS $p$H 7, 500 n$M$ $MgCl_2$, Carl Roth) were heated to 95 °C and quenched by ice water (0 °C) to form the hairpin conformation. Electrophoresis was performed in a 3.5% high-resolution agarose gel (2.5 g agarose high-resolution no. K297.2, Carl Roth, 70 ml TB buffer) by the FAM-labeled hairpin $b$. The TB buffer was 10-fold diluted from a stock of 54 g TRIS, 27.5 g Boric acid, 500 $\mu$M $MgCl_2$ at $p$H 8 in one liter of pure water.

*Setup.*—Nucleotides, pipette tips, and reaction tubes were cooled on ice. Hairpins and the annealed template duplex were mixed on ice within 35 s and put on a $2 \times 2$ array of Peltier-Elements (MPC-D701, Micropelt GmbH, Freiburg) covered by silicon and prepared with a 500 $\mu$m thin silicone rubber sheet (KU-TCS50, Kunze, Oberhaching) with a hole of 4 mm in diameter. The 10 $\mu$l reaction chamber was covered with a sapphire slide for UV transparency. Fluorescence imaging was provided by a microscope (AxioTech Vario, Zeiss) through a $20\times$, numerical aperature = 0.65 quartz objective (Partec, Germany) using a standard tryptophan filter set (F36-300, AHF Tübingen) and illuminated with a UV-LED (LED285W, Thorlabs). Emission was detected with a photomultiplier tube (MP 973, Perkin-Elmer Optoelectronics). The temperature was increased from 10 °C to 40 °C every 27 s for 3 s. The temperature was measured inside the chamber using the temperature dependent fluorescence of 5 $\mu$M BCECF (mixed isomers, B-1151, Invitrogen) in 10 mM TRIS before the reaction.

*Theory and calibration.*—Concentrations of all constituents were predicted over time based on published kinetic data [18,26,27] (see the Supplemental Material [28]) and transformed to fluorescence $F(t)$ by linear superposition of calibrated AP-fluorescence contributions. Dimer $ab$ and quadruple $ABab$ were created by a slow annealing of 1 $\mu$M of the corresponding hairpins, cooling them from 95 °C to 5 °C within 2 h. By comparing gel electrophoresis [Fig. 2(c)] with 2-AP fluorescence in the buffer at 10 °C and 40 °C, the relative 2-AP-fluorescence contributions $\{\text{hairpin, duplex, quadruple}\} = \{1, 0.3, 0.5\}$ were inferred (see the Supplemental Material [28]). The melting temperatures of annealed species were measured with microscale thermophoresis [29].

*Results.*—Let us assume an RNA molecule can either form high-energy hairpins with itself or complexes at lower energies with other RNA molecules. When a heated molecule is cooled rapidly, the kinetics to form a hairpin is typically faster than finding a partner RNA with more paired bases and lower free energy. As a consequence, RNA hairpins can store metastable thermal energy, which we use to drive a replication reaction.

The following replication is cross catalytic under a moderate temperature oscillation (Fig. 1). Four partially complementary hairpins $a$, $b$, $A$, and $B$ were derived from half a tRNA molecule (Fig. 1) and prepared to be in a metastable state. The toehold succession $ab$, both memorized in the duplex $ab$ or $AB$, is replicated. At 10 °C, the duplexes $ab$ or $AB$ bring the hairpins with complementary toeholds in mutual proximity and enhance the kinetics to form the quadruple $ABab$, releasing the energy from the hairpin loop. Subsequent moderate heating (40 °C) separates $ABab$ into duplexes $AB$ and $ab$, which both replicate again with hairpins $a, b$ and $A, B$, respectively. The duplex amount doubles in each temperature cycle and a purely thermally fuelled and driven exponential replication of sequence succession $ab$ is expected. Temperature oscillations could be provided by convection in millimeter-sized rock pores or clefts [19,22].

The replication was monitored by time-resolved fluorescence [Fig. 2(b)]. At time = 0 s, all four hairpin species $A$, $B$, $a$, and $b$ were present at 500 n$M$ concentration, together with 35 n$M$ of annealed duplexes $ab$. Hybridization of the hairpins to duplexes and quadruples reduces the time dependent fluorescence $F_{(t)}$ of the UV-fluorescent base analog 2-aminopurine [25] in the loop of hairpin $A$. As a result, the quenching $Q = 1 - F_{(t)}/F_{(t=0)}$ rose as the replication progresses and saturated in less than 10 min, indicating a fast progression of the reaction [Fig. 2(b), red]. In contrast, a reaction of hairpins $A$ and $B$ with template $ab$ resulted in no quenching increase [Fig. 2(b), gray] and hence no replication.

The thermal cycling times were chosen to exceed the on rates of toehold hybridization [18,26,27] $k_{\text{on}} = 1.3\ (\text{s}\,\mu M)^{-1}$ at the initial hairpin concentration of 500 n$M$. The opening-closing kinetics of the hairpins were faster (0.1–1 ms) [26] than the average toehold binding time constant (1 s) [18,27], enabling the catalysis of the hairpin opening. In each cycle, the temperature was set to 10 °C for 27 s, followed by 40 °C for 3 s [Fig. 2(b)]. The periodic peaks in fluorescence report the separation of quadruple $ABab$ into duplexes $AB$ and $ab$ at 40 °C.

The choice of temperatures is confirmed by melting curves [Fig. 2(d)], revealing clearly separated melting transitions of hairpins, duplexes, and quadruples. Running the reactions between 10 °C and 40 °C ensures a robust replication without an opening of hairpins or separation of duplexes.

To substantiate the replication mechanism, the concentrations of all reaction species have been modeled (see the Supplemental Material [28]). The simulated concentrations were converted into quenching and showed good agreement for all reactions [Fig. 2(b), black]. In Fig. 2(c), the products of the reaction were analyzed with high-resolution agarose gel electrophoresis. Slow annealing from 95 °C to 5 °C within 2 h of single hairpins, two hairpins, and all hairpins resulted in clearly distinguishable bands, revealing the equilibriums of the reactions, which correspond to single hairpins, duplexes, and quadruples, respectively. After 15 temperature cycles the full reaction yielded up to 80 ± 5% of information carrying constituents, while in the reactions with one or two hairpins the high-energy hairpin conformation was maintained [Fig. 2(c)]. These results confirmed the finding presented in Fig. 2(b).

Specificity was tested by replacing the toehold of one of the four hairpins with a random sequence [Fig. 3(a)]. Although duplexes could still be formed in equilibrium, no replication was observed, reproducing results from only two hairpins [Fig. 2(b), gray]. In the absence of matching toehold sequences, the hairpins cannot enter a cross-catalytic cycle, demonstrating the high specificity of the replication.

Without thermal cycling, quadruples do not open actively and cannot serve as templates for cross catalysis. As expected, isothermal reactions were dominated by the background rate and independent of the initial template concentration [Fig. 3(b), shades of red]. A further removal of one or more hairpins from the reaction, again similar with an introduction of a wrong toehold, further reduced the background rate [Fig. 3(b), shades of blue and yellow]. No significant levels of duplexes were found without thermal activation of cross catalysis.

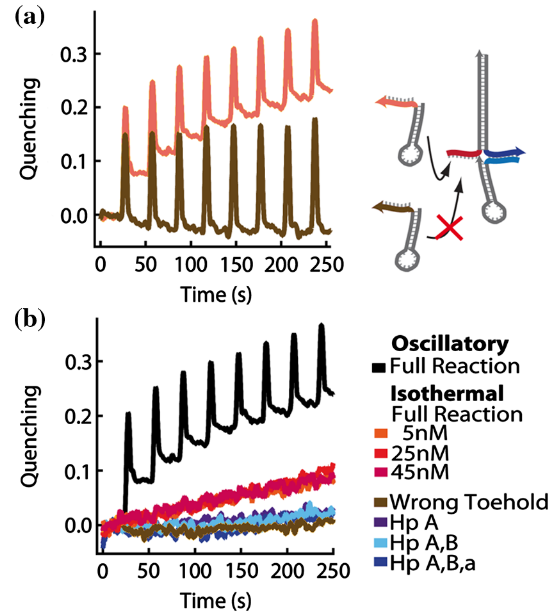


FIG. 3 (color online). Specificity. (a) No replication was found for a mutated toehold sequence (brown). (b) With isothermal driving, the complete reaction replicated slowly and showed no dependence on the template concentration (template duplex = {5, 25, and 45 n$M$}, hairpin = 500 nM). Isothermal reactions using a nonmatching toehold sequence (yellow) or without hairpins (blue) showed no signature of replication.

An exponential replicator with doubling time $\tau$ and false positive background rate $k_0$ enhances the concentration of replica $C$ according to

$$dC/dt = C\ \ln(2)/\tau + k_0. \tag{1}$$

We tested the concentration dependence of $dC/dt$. In Fig. 4(a), replications were performed with increasing template concentration $ab$ between 5 and 50 nM. The quenching was modeled [Fig. 4(a), dashed lines] and the time derivative $dC/dt$ of the information carrying molecules $C = [AB]_{\text{calc}} + [ab]_{\text{calc}} + 2[ABab]_{\text{calc}}$ was averaged within the first three replication cycles. In the beginning, the hairpin concentration was not yet significantly depleted and replication did not yet saturate. The fit with Eq. (1) revealed a duplication time $\tau = 28 \pm 5$ s together with a background rate $k_0 = 3.0 \pm 0.3$ n$M$/s. Error bars were determined from three independent experimental runs. The duplication time matched the total cycling time $\tau_{\text{cycle}} = 30$ s, indicating that the sequence information was doubled with each temperature oscillation.

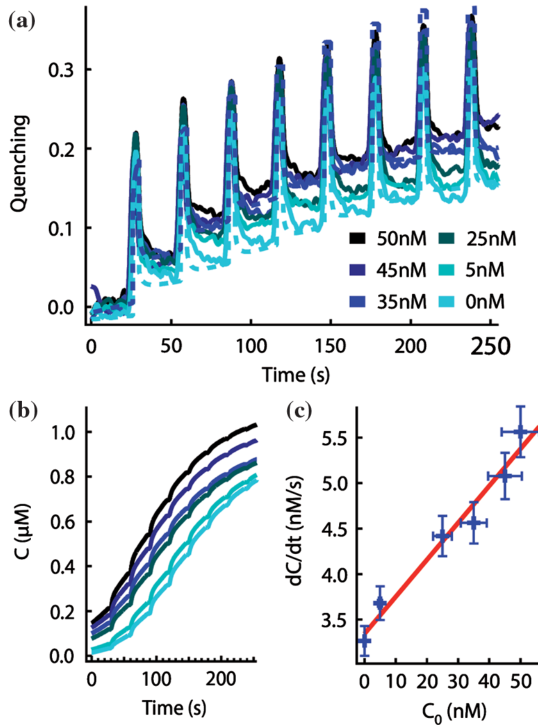


FIG. 4 (color online). Exponential replication. (a) Measured (color) and simulated (dashed lines) quenching of complete reactions with different initial template concentration are in agreement. (b) The increase of the replicated product raised with increasing template concentration. (c) Time derivative of the modeled template concentration $dC/dt$ within the first three temperature cycles plotted versus the initial template concentration. A fit by Eq. (1) revealed a doubling time $\tau = 28 \pm 5$ s, matching the thermal cycling time of 30 s.

*Discussion.*—To maintain information against chemical degradation and physical removal, fast exponential replication is desirable if not necessary [30,31]. The replicator shows exponential growth rates on the time scale of seconds to minutes due to the fast kinetics of RNA hybridization and the efficient template separation by thermal cycling. In comparison with chemical, protein-free replicators [11–13], the hairpin replicator is more than 2 orders of magnitude faster.

Does the hybridization of toehold and hairpin loops suffice to gain free energy in the reaction $A + B + a + b \leftrightarrow ABab$? Transforming two hairpins into a duplex yields [24] $\Delta G_{\text{duplex}} = -45\,\text{kcal/mol}$ and the hybridization of one toehold $\Delta G_{\text{codon}} = -13\,\text{kcal/mol}$, resulting in a total gain from base pairing of $\Delta G_{\text{rep}} = 2\Delta G_{\text{duplex}} + 2\Delta G_{\text{codon}} = -116\,\text{kcal/mol}$. In contrast, the entropic penalty [16] $\Delta G_{\text{entropic}} = RT\ln(Q)$, estimated from $Q = ([A][B][a][b]/c_0^4)/([ABab]/c_0)$ at an intermediate reaction point with $[A][B][a][b] = [ABab] = c = 250\,\text{nM}$ is more than fourfold smaller (26 kcal/mol), allowing the reaction to proceed.

The replicator requires moderate thermal cycling. As for the case of protein-catalyzed DNA replication [22,32], suitable temperature oscillation can be provided by laminar microconvection. Together with thermophoresis in elongated clefts, molecules are trapped by a thermogravitational column [19]. Both effects were implemented simultaneously in the same chamber [21]. Notably, such settings can accumulate prebiotic lipids to form cell-like vesicles [20]. One can envision that hairpins are quenched as they are accumulated in an elongated convection trap [19], fueling the replication reaction with concentrated, quenched hairpins. Cooling within seconds is readily achieved in microconvection [22]. Replication then becomes fully reversible and does not create waste products. The experiments were performed under physiological salt concentrations, with 0.5 m$M$ $MgCl_2$ to stabilize the binding of hairpin toeholds. As the replication is based on specific interactions with more than 9 bases, we expect the performance to prevail under significant levels of RNA with competing sequences.

The starting pool of RNA oligomers could be produced by nontemplated RNA polymerization from surfaces [33] or other precursors [34] and accumulated by thermophoretic trapping [19]. Recent models indicate that the combined trapping and base-pair selective degradation leads to a prereplication stabilization of complementary sequence motifs where matching duplexes and hairpin conformations dominate [23]. Over time, hairpins with the shown dynamics could be selected since replication settles with fast kinetics in double-stranded structures [e.g., Fig. 3(a)], saving the replicating molecules from replication. Thermophoretic trapping of the larger complex could further select participating hairpins [19,21].

*Conclusion.*—We have shown that a two-letter code can be selectively and exponentially replicated by physical base pairing from a pool of thermally quenched RNA hairpins. Replication is driven by moderate temperature oscillation and—besides a background rate—doubles in each thermal oscillation. It has not escaped our attention that the replication demonstrated here with half a tRNA suggests a possible physico-chemical replication-translation mechanism [35] from the concatenation of multiletter codes using complete tRNA molecules.

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