Freeze-thaw cycles enable a prebiotically plausible and continuous pathway from nucleotide activation to nonenzymatic RNA copying

Stephanie J. Zhang, Daniel Duzdevich, Dian Ding, and Jack W. Szostak

Edited by Joseph Piccirilli, University of Chicago, Chicago, IL; received September 6, 2021; accepted March 22, 2022; published April 21, 2022.

Nonenzymatic template-directed RNA copying using chemically activated nucleotides is thought to have played a key role in the emergence of genetic information on the early Earth. A longstanding question concerns the number and nature of different environments that might have been necessary to enable all of the steps from nucleotide synthesis to RNA copying. Here we explore three sequential steps from this overall pathway: nucleotide activation, synthesis of imidazolium-bridged dinucleotides, and template-directed RNA copying. We find that all three steps can take place in one reaction mixture undergoing multiple freeze-thaw cycles. Recent experiments have demonstrated a potentially prebiotic methyl isocyanide-based nucleotide activation chemistry. However, the original version of this approach is incompatible with nonenzymatic RNA copying because the high required concentration of the imidazole activating group prevents the accumulation of the essential imidazolium-bridged dinucleotide. Here we report that ice eutectic phase conditions facilitate not only the methyl isocyanide-based activation of ribonucleotide 5′-monophosphates with stoichiometric 2-aminimidazole, but also the subsequent conversion of these activated mononucleotides into imidazolium-bridged dinucleotides. Furthermore, this one-pot approach is compatible with template-directed RNA copying in the same reaction mixture. Our results suggest that the simple and common environmental fluctuation of freeze-thaw cycles could have played an important role in prebiotic nucleotide activation and nonenzymatic RNA copying.

Significance

The replication of RNA without the aid of evolved enzymes may have enabled the inheritance of useful molecular functions during the origin of life. Template-directed RNA copying is a postulated step in RNA replication. Key steps on the path to copying of RNA templates have been studied in isolation, including chemical nucleotide activation, generation of a key reactive intermediate, and template-directed polymerization. Here we report a prebiotically plausible scenario under which these reactions can happen together under mutually compatible conditions. Thus, this pathway could potentially have operated in nature without the complicating requirement for exchange of materials between distinct environments.

PNAS 2022 Vol. 119 No. 17 e2116429119
https://doi.org/10.1073/pnas.2116429119

Downloaded from https://www.pnas.org by Dana Farber Cancer Inst on April 25, 2022 from IP address 132.183.13.29.
Under partially frozen conditions, solutes are excluded from growing ice crystals and become concentrated in the interstitial liquid solution. This phenomenon of eutectic phase concentration has been demonstrated to facilitate the synthesis of nucleotide precursors (12), as well as the untempered condensation of activated nucleotides into oligomers (13). Here we report the in situ MeNC-mediated activation of mononucleotides with stoichiometric 2AI by repeatedly subjecting the solution to eutectic phase freezing, which temporarily brings the reactants into close physical proximity and obviates the need for the large excess of 2AI otherwise required to drive activation in relatively dilute liquid phase reactions. We find that all four canonical ribonucleotides (as well as helper oligonucleotides) can be activated with this approach, which also yields high concentrations of bridged species. Consequently, we were also able to show that the reaction is compatible with template-directed nonenzymatic RNA copying. The geochemically plausible conditions that we have identified enable a potentially prebiotic pathway that begins with ribonucleotide 5′-monophosphates, generates 2AI activated nucleotides and then the essential bridged dinucleotides, and finally yields template-directed primer extension products, all in one reaction mixture.

**Results**

Addressing the incompatibility between template-directed primer extension and the initial in situ activation of mononucleotides requires a plausible pathway to nucleotide activation using a concentration of 2AI comparable to the concentration of nucleotides, rather than the large excess currently required (Fig. 1A). We reasoned that the ice eutectic phase as a medium for activation could temporarily increase the effective local concentration of 2AI, thereby alleviating the requirement for an absolute excess of 2AI relative to nucleotides (Fig. 1B). To begin exploring the effect of ice eutectic phase concentration on nucleotide activation, we treated 10 mM adenosine monophosphate (pA) with 10 mM 2AI, 100 mM 2-methylbutyraldehyde (2MBA), and 200 mM MeNC, at pH 8 under both room temperature and ice eutectic phase conditions (9). With these reactant concentrations, and incubation at room temperature, only 2.0 ± 0.1 mM activated nucleotides (~20% of the 10 mM total available nucleotides) and only 0.11 ± 0.01 mM bridged dinucleotides were formed after 24 h (Fig. 1C). In contrast, using the same concentrations of reactants but subjecting the mixture to ice eutectic phase concentration at −13°C for 24 h yielded 50% total activation, consisting of 3.0 ± 0.1 mM activated nucleotides and 1.0 ± 0.1 mM bridged dinucleotides. The ice eutectic phase not only increases the yield of activated nucleotides but also acts as a storage mechanism, because activated nucleotides are less susceptible to hydrolysis at low temperatures (SI Appendix, Fig. S1 C and D), which further aids in the accumulation of bridged dinucleotides (Fig. 1C).

The possibility that in a prebiotic environment MeNC could have been released periodically in response to day-night cycles of UV irradiation (14) prompted us to test multiple cycles of eutectic phase concentration and MeNC delivery. Instead of adding 200 mM MeNC from the start, 50 mM MeNC was introduced to the solution at the beginning of each of four sequential freeze-thaw cycles. This approach further increased nucleotide activation to 75%, yielding 2.33 ± 0.01 mM activated nucleotides and 2.8 ± 0.3 mM bridged dinucleotides (Fig. 1D). By comparison, periodic addition of MeNC at room temperature without eutectic freezing yielded only 1.30 ± 0.6 mM activated nucleotides and 0.7 ± 0.6 mM bridged dinucleotides, or 27% total activation (Fig. 1E). A range of pHs was examined to rule out the possibility that freezing-induced changes in pH might account for the increase in activation (SI Appendix, Fig. S2).

![Fig. 1](https://www.pnas.org/content/110/32/11746/F1.large.png)

**Fig. 1.** Activation in the ice eutectic phase enhances the yield of activated nucleotides (pN) and promotes formation of bridged dinucleotides (Np*pN). (A) Scheme showing role of ice eutectic concentration. Left: At room temperature, the excess 2AI needed to drive nucleotide activation inhibits bridged dinucleotide accumulation. Right: activation in ice eutectic phase requires only equimolar 2AI and ribonucleotides, allowing bridged dinucleotide accumulation and template copying. (B) Solutes are concentrated in the ice eutectic phase. (C) Nucleotide activation in the ice eutectic phase (left) and at room temperature (right) with one round of MeNC addition and equimolar initial nucleotides and 2AI (SI Appendix, Fig. S3A). (D) Cycles of MeNC addition and eutectic freezing drive continued nucleotide activation. Curve arrows indicate addition of MeNC after each thawing of the reaction mixture. Note that the reaction mixture is frozen as eutectic ice for the entire reaction course except during the brief thawing steps (~30 min at room temperature for the mixture to thaw completely) (SI Appendix, Fig. S3B). (E) Activation is inefficient at room temperature in the absence of ice eutectic freezing. Straight arrows indicate the addition of MeNC at room temperature (SI Appendix, Fig. S3C).
We then varied the frequency of MeNC addition to the system and evaluated the effect on activated nucleotide yield. We found that an interval of roughly 1 day between MeNC additions led to efficient activation (Fig. 2A and B). Further increases in the interval, up to several days, did not significantly change the activation yield (Fig. 2 and SI Appendix, Fig. S2).

This not only indicates the robustness of the system in response to changes in the timing of activation but also demonstrates that freezing is a storage mechanism in our system: the accumulation of bridged dinucleotides is consistent with the expectation that activated mononucleotides and bridged dinucleotides should not readily hydrolyze in the ice eutectic state (SI Appendix, Fig. S5). This not only indicates the robustness of the system but also shows that nucleotides activated using this approach behave the same way as synthetically prepared and purified activated nucleotides. We found that nucleotides activated using this approach behave the same way as synthetically prepared and purified activated nucleotides. We activated 5 mM each of CMP and GMP under ice eutectic phase conditions (Fig. 3B) and then added the resulting mixture to a primer-template complex in solution phase. Under these conditions, the primer was extended (Fig. 3C). We were able to rescue high-efficiency activation by treating a mixture of all four nucleotides (10 mM total) with activation chemistry under ice eutectic conditions (Fig. 3B). Dilution experiments showed that the rescue is partially due to the decreased relative concentration of pG (from 10 mM to 2.5 mM, Fig. 3C). Encouraged by these results, we sought to combine ice eutectic phase nucleotide activation and bridged species formation with template-directed primer extension (Fig. 4A). First, we aimed to confirm that nucleotides activated using this approach behave the same way as synthetically prepared and purified activated nucleotides. We activated 5 mM each of CMP and GMP under ice eutectic phase conditions (Fig. 3B) and then added the resulting mixture to a primer-template complex in solution phase. Under these conditions, the primer was extended (Fig. 4C). Next, we asked whether in situ activation and primer extension can occur together in a single reaction mixture during freeze-thaw cycles. In this experiment the primer-template complex was included along with all other reagents from the start and exposed to cycles of ice eutectic phase activation. We found that primer extension was comparable to that observed with pure activated mononucleotides, or with sequential eutectic activation.
of reactants and subsequent solution phase primer extension (compare Fig. 4E to B–D). We then asked whether this in situ pathway could also be combined with our previously developed bridge-forming activation. In this process isocyanide and aldehyde are added to activated mononucleotides to generate enhanced levels of bridged dinucleotides from activated mononucleotides in solution, which thereby promote primer extension. We performed in situ nucleotide activation together with primer extension as in Fig. 4E, but then further treated the mixture with bridge-forming activation at room temperature and incubated the mixture for an additional 24 h. As expected, primer extension was even more efficient, with 52% of the total primer extended to +4 product (Fig. 4 G and H) compared with 39% in the absence of additional bridge-forming activation (Fig. 4 F and H). This experiment demonstrates that ice eutectic phase concentration enables nucleotide activation with bridge-forming activation and primer extension, all in one reaction mixture.

The experiments discussed so far used templates with at most two different template nucleobases. To test the limits of our approach with respect to prebiotic plausibility and heterogeneity, we next asked whether the ice eutectic phase activation of all four nucleotides could be combined with bridge-forming activation and primer extension to copy mixed sequence templates. Mixed-sequence templates containing AU are known to be challenging for nonenzymatic RNA copying (17) but activated downstream helper oligonucleotides greatly facilitate primer extension on templates containing all four bases (3, 5). The experiments discussed so far used templates with at most two different template nucleobases. To test the limits of our approach with respect to prebiotic plausibility and heterogeneity, we next asked whether the ice eutectic phase activation of all four nucleotides could be combined with bridge-forming activation and primer extension to copy mixed sequence templates. Mixed-sequence templates containing AU are known to be challenging for nonenzymatic RNA copying (17) but activated downstream helper oligonucleotides greatly facilitate primer extension on templates containing all four bases (3, 5).

We have previously shown that a primer can be extended by up to seven nucleotides by copying the template sequence 3'-UACUCGCCG-5', if six activated trimers (UGA, GAG, AGG, GCC, GCG, CGC) are included in the reaction mix (Fig. 5A). We repeated this experiment as a control, using 1.2 μM primer and 1.5 μM template in the presence of 5 mM of each activated mononucleotide (*pN, n = A, U, C, and G) and 0.5 mM of each of the six activated trimers. After 24 h, 93% of the primer had been extended by one or more nucleotides, with ~61% of the primer extended by seven nucleotides (Fig. 5 B and D), consistent with previous results (3). In order to test monomer and helper oligonucleotide activation, the formation of imidazolium-bridged species, and primer extension all in a one pot reaction, we subjected 1.2 μM primer and 1.5 μM template, as above, along with 5 mM of each NMP and 0.5 mM of each trimer to four cycles of ice eutectic phase nucleotide activation. This procedure resulted in 97% of the primer being extended by one or more nucleotides, and 75% by four or more nucleotides, but only 22% of +7 extended product (Fig. 5 C and E). However, a subsequent 48-h incubation at
the accumulation of the imidazolium-bridged species required for efficient primer extension with respect to changes in the length of freeze-thaw cycles and the frequency of MeNC delivery suggests that the efficiency of nucleotide activation is robust, and therefore any corresponding early Earth environment need not be highly contrived. Ice eutectic-based nucleotide activation may not even have astrobiological implications (23, 24). We observed that the efficiency of nucleotide activation is robust with respect to changes in the length of freeze-thaw cycles and the frequency of MeNC addition. MeNC is thought to have been released from prebiotic feedstocks exposed to UV irradiation from the Sun (7). The relative independence of activation efficiency from the frequency of MeNC delivery suggests that the required temperature cycling conditions are not stringently constrained, and therefore any corresponding early Earth environment need not be highly contrived. Ice eutectic-based nucleotide activation may not even have been required continuously: once the initial pool of activated mononucleotides had been generated, the application of solution-phase bridge-forming activation could potentially prebiotic chemical reactions that at first appeared to be incompatible but were subsequently reconciled. For example, it used to be thought that the oxygenous chemistry leading from formaldehyde to sugars such as ribose, and the nitrogenous chemistry leading from cyanide to the nucleobases were strictly incompatible due to the formation of unreactive glycolonitrile and therefore had to occur in separate environments. However, the cyanosulfidic reaction network studied by Sutherland and colleagues has shown that this is not the case, and that it is actually advantageous for these steps to occur together because the facile reduction of glycolonitrile makes it an excellent starting point for many synthetic reactions (18–20).

Fig. 5. Ice eutectic phase activation enables efficient primer extension with all four nucleotides. (A) Schematic of primer-template complex. (B) Time course of primer extension using pure activated mononucleotides and trinucleotides (5 mM each *pN and 0.5 mM each *pN(pN)2, room temperature). (C) Primer-template duplex was directly treated with pNs (n = A, U, C, and G) and (pN), and isocyanide activation chemistry under ice eutectic phase. The products of the reaction were then incubated for 24 h at room temperature, and finally treated with additional two rounds of bridge-forming activation for another 48 h (see details in Materials and Methods). (D and E) Quantification of primer extension products under conditions (B and C).

Discussion

The plausibility of any scenario for the origin of life on the early Earth is greatly enhanced if different steps or stages in the overall process can occur together under mutually compatible physical and chemical conditions. There are many examples of
slowly reactivate hydrolyzed nucleotides as well as regenerate bridged dinucleotides in solution. Additional experiments will be needed to determine how long this system can replenish hydrolyzed activated nucleotides without the need for additional freezing after the initial activation. It is possible that although the ice eutectic phase concentration favors activation chemistry, the low temperature may inhibit the actual primer extension reaction. Indeed, primer extension during the freeze-thaw phase of our experiments may be occurring primarily during the relatively short but nonnegligible thaw intervals. Nucleotide activation during cycles of partial drying and rehydration may also be relevant (25).

The key ingredient in the chemical nucleotide activation procedure that we used is MeNC, so it is important to consider whether MeNC could be generated in an environment compatible with our multistep pathway. For example, the strong UV absorption of nucleotides might leave insufficient UV light for MeNC generation. However, nucleotides absorb UV light most strongly between 240 nm and 280 nm, whereas the generation of MeNC proceeds with longer wavelength irradiation (26). Specifically, two steps in the plausible synthesis of MeNC exploit the use of UV light: the production of nitroprusside has been achieved with 365 nm or 254 nm light and isocyanide ligand exchange also occurs during exposure to 365 nm light (7), consistent with the broad band irradiation from the young Sun (27). The formation of MeNC using longer wavelength UV irradiation suggests that the generation of MeNC is compatible with the presence of nucleotides. Another consideration is that an important ingredient in MeNC synthesis is methylamine, which others have suggested could have been delivered to the primordial Earth by comets or formed by reduction of HCN with hypophosphite and sponge nickel (7, 28). On the basis of Ugi-type reactions (29), a primary amine such as methylamine will readily react with an aldehyde, forming an iminium ion. However, this may not affect the nucleotide activation chemistry because MeNC will still add reversibly to the iminium ion to produce the nitritium ion required for nucleotide activation (14). In addition, 2-hydroxy-\text{-}N\text{-}methylpropamide, the side product of nucleotide activation, will eventually hydrolyze to regenerate methylamine, which can then take part in another round of MeNC synthesis (7). The prebiotic plausibility of the remaining chemical ingredients has been previously discussed (18, 20, 30), but additional experiments will be needed to reconstitute in situ MeNC generation with simultaneous nucleotide activation.

Materials and Methods

General Information.

Materials. Reagents and solvents were obtained at the highest available purity from Acros Organics, Alfa Aesar, Fisher Scientific, Sigma-Aldrich, ThermoFisher Scientific, or Tokyo Chemical Industry Co., and were used without further purification unless noted below. Nucleoside-5’-monophosphates, free acid, were purchased from Santa Cruz Biotechnology. 2-aminoimidazole hydrochloride and 2,2’-dipyridyl disulfide were purchased from Combi Blocks. The 5’-phosphorylated trimers, 5’-arabinoseimidazole activated trimers, and the 5’-arabinoseimidazole activated mononucleotides were prepared as previously described (3). RNA oligonucleotides for standard primer extension experiments were purchased from Integrated DNA Technologies. The synthesis and storage of methyl isocyanide was as previously described (9). All reactions were carried out in DNase/RNase-free distilled water.

NMR spectroscopy. \textsuperscript{1}H and \textsuperscript{31}P-NMR spectra were obtained using a Varian INOVA NMR spectrometer operating at 400 MHz and 161 MHz, respectively. Samples in H_2O/D_2O mixtures were analyzed using WettD suppression to collect \textsuperscript{1}H-NMR data. Chemical shifts (d) are shown in parts per million. Coupling constants (J) are given in hertz (Hz) and the notations s, d, t, and m represent singlet, doublet, triplet, and multiplet multiplicities, respectively. pH measurements. pH values were determined by a micro pH probe (Orion 9863B) equipped with a needle tip and a SevenCompact meter (Metter Toledo S220).

Data analysis. All NMR spectra were analyzed using MestReNova (version 12.0.3). The yields of conversion were determined by the relative integration of the signals in the \textsuperscript{1}H or \textsuperscript{31}P-NMR spectra.

Preparation, Storage, and Concentration Determination of Stock Solutions. Stock solution of nucleoside-5’-monophosphosphate disodium salt was prepared in DNase and RNase-free distilled water. After adjusting the pH to the reported values with NaOH/HCl, the stock solution was filtered with 0.22-\textmu m syringe filters (Millipore Sigma). Each stock solution was then aliquoted and kept at −20 °C until further use. Stock solution of 2-aminoimidazole hydrochloride was prepared in DNase and RNase-free distilled water, filtered with a 0.22-\textmu m syringe filter (Millipore Sigma), and kept at −20 °C until further use. The pH was adjusted to the reported values with NaOH/HCl right before the reaction to prevent possible polymerization at basic pH.

The concentrations of the nucleoside-5’-monophosphate solutions were determined by analysis of serial dilutions on a UV spectrophotometer. The absolute concentrations of other stock solutions were determined by comparing the integrals of \textsuperscript{1}H-NMR peaks of interest to the calibrant, trimethyl phosphate, by NMR spectroscopy.

Sample Preparation for Ice Eutectic Phase Experiments. Reaction mixtures were first flash-frozen by immersion in liquid nitrogen, followed by incubation at −13 °C in a standard laboratory freezer unit for the indicated time (11, 15, 31).

In Fig. 1, reaction conditions are as follows: 10 mM pA, 10 mM 2AI, 100 mM 2MBA, 30 mM MgCl_2, 50 mM Na\textsuperscript{+}-Hepes pH 8, plus (Fig. 1C) one addition of 200 mM MeNC at ice eutectic phase or room temperature, or (Fig. 1D) periodic addition of the MeNC beyond the initial 50 mM in three aliquots of 50 mM each under ice eutectic phase at −13 °C or (Fig. 1E) at room temperature.

Primer Extension Reactions Using Only Monomers. Control primer extension reactions. The primer-template duplex was first annealed in a 40 μL solution containing 2 μM thiol-modified primer (5’-OmC6-DAGUGAGUAACUC, IDT; see Sample preparation and food analysis for use of the thiol to dye-label products), 3.1 μM template (CCGGAGUAACUGA, IDT), 125 mM Na\textsuperscript{+}-Hepes (pH 8.0), and 62.5 mM MgCl\textsubscript{2} by heating at 95 °C for 3 min followed by cooling to 23 °C at a rate of 0.1 °C/s. The reaction was initiated by the addition of 10 μL 100 mM activated mononucleotides.

Eutectic phase primer extension reactions. The primer-template duplex was first annealed in a 100 μL solution containing 2.4 μM thiol-modified primer (5’-OmC6-DAGUGAGUAACUC, IDT), 3.0 μM template (CCGGAGUAACUGA, IDT), 125 mM Na\textsuperscript{+}-Hepes (pH 8.0) by heating at 95 °C for 3 min followed by cooling to 23 °C at a rate of 0.1 °C/s. The reaction was initiated by the addition of 100 μL solution containing 10 mM pH, 10 mM 2AI, 100 mM 2MBA, 30 mM MgCl\textsubscript{2}, 50 mM Na\textsuperscript{+}-Hepes pH 8, after four cycles of ice eutectic phase activation (50 mM MeNC each addition). For bridge-forming activation, the reaction mixture was incubated at room temperature with an additional 100 mM 2MBA and 200 mM MeNC for 24 h.

Trimer-Assisted Primer Extension Reactions. Control primer extension reactions. The primer-template duplex was first annealed in a 40 μL solution containing 3.6 μM thiol-modified primer (5’-OmC6-DCCGCGAGACAC, IDT), 4.5 μM template (5’-CCGGAGUAACUGA, IDT), 125 mM Na\textsuperscript{+}-Hepes (pH 8.0), and 62.5 mM MgCl\textsubscript{2} by heating at 95 °C for 3 min followed by cooling to 23 °C at a rate of 0.1 °C/s. The final reaction mixture contained 0.5 mM each activated trimer and 5 mM each activated monomer.

Eutectic phase primer extension reactions. The primer-template duplex was first annealed in a 100 μL solution containing 2.4 μM thiol-modified primer (5’-OmC6-DAGUGAGUAACUC, IDT), 3.0 μM template (CCGGAGUAACUGA, IDT), 125 mM Na\textsuperscript{+}-Hepes (pH 8.0) by heating at 95 °C for 3 min followed by cooling to 23 °C at a rate of 0.1 °C/s. The reaction was initiated by the addition of 100 μL solution containing 10 mM pH, 10 mM 2AI, 100 mM 2MBA, 30 mM MgCl\textsubscript{2}, 50 mM Na\textsuperscript{+}-Hepes pH 8, and periodic addition of MeNC in four aliquots of 50 mM each under ice eutectic phase. The reaction mixture...
was then further incubated with an additional 100 mM 2MBA and 100 mM MeNC on day 5 and day 6.

Sample Preparation and Product Analysis. Sample purification and preparation. Ten-microliter aliquots were removed at given time points and desalted using a ZYMO Oligo Clean & Concentrator spin column (ZYMO Research) (adapted from ref. 9). The isolated material was resuspended in 30 μl 100 mM Na+-Hepes (pH 7.50) and disulfide bonds were reduced using a 10-fold molar excess of Tris-(2-carboxyethyl) phosphate hydrochloride. Alexa 488 C5 maleimide dissolved in anhydrous dimethyl sulfoxide at a concentration of 1 mM was added to the primer-template duplex dropwise, and the coupling reaction was allowed to proceed in the dark at room temperature for 2 h. The labeled primer-template duplex was separated from free dye using ZYMO DNA Clean & Concentrator spin columns, resuspended in 5 μl of 100 mM Na+-Hepes buffer, and mixed with 30 μl of quenching buffer containing 8.3 M urea, 1.3 × Tris-borate-EDTA buffer (pH 8.0), 0.005% bromophenol blue, 0.04% Orange G, and 75 μM RNA complementary to the template. To denature the labeled product from the template prior to polyacrylamide gel analysis, and sequencher the template with its complement (added in excess as a competitor), the sample was heated to 95°C for 3 min. and cooled to 23°C at 0.1°Cs.

Analysis of the polymerization products. Twenty percent polyacrylamide gels were prepared using the SequaGel-UreaGel system (National Diagnostics, Atlanta, GA). The gels were allowed to prerun at a constant power of 20 W for 40–45 mins. Fifteen-microliter aliquots of samples were separated by 20% (19:1) denaturing polyacrylamide gel electrophoresis at a constant power of 5 W for 10 min and then 25 W for 1.5 h. Gels were imaged on a Typhoon 9410 scanner (GE Healthcare, Little Chalfont, Buckinghamshire, UK) and quantified using the accompanying ImageQuant TL software. All reactions were performed in duplicate or greater as indicated.

Data Availability. Raw gel and NMR data are available at the Open Science Framework (OSF): https://osf.io/sjtwu/?view_only=c38e3a8bcd14c280bf53234912891.

ACKNOWLEDGMENTS. J.W.S. is an Investigator of the Howard Hughes Medical Institute. This work was supported in part by grants from the Simons Foundation (290363) and the NSF (CHE-1607034) to J.W.S. The authors thank Dr. Li Li for his expert advice on preparation of 5′-aminoimidazolium activated trimers; members of the Szostak laboratory for helpful discussions and comments on the manuscript.

1. B. J. Weimann, R. Luhrmann, L. E. Orgel, H. Schneider-Bernloehr, J. E. Sulston, Template-directed synthesis with adenosine-5′-phosphorimidazolide. Science 161, 387 (1968).

2. S. R. Vogel, C. Deck, C. Richert, Accelerating chemical replication steps of RNA involving activated ribonucleotides and downstream-binding elements. Chem. Commun. (Camb.) 39, 4922–4924 (2005).

3. L. Li et al., Enhanced nonenzymatic RNA copying with 2-aminomidazolide activated nucleotides. J. Am. Chem. Soc. 139, 1810–1813 (2017).

4. T. Walton, J. W. Szostak, A highly reactive imidazolium-bridged dinucleotide intermediate in nonenzymatic RNA-primer extension. J. Am. Chem. Soc. 138, 11996–12002 (2016).

5. N. Prywes, J. C. Blain, F. Del Fato, J. W. Szostak, Nonenzymatic copying of RNA templates containing all four letters is catalyzed by activated oligonucleotides, eLife 5, e17756 (2016).

6. D. Ding, L. Zhou, C. Giugiuc, J. W. Szostak, Kinetic explanations for the sequence biases observed in the nonenzymatic copying of RNA templates. Nucleic Acids Res. 50, 35–45 (2022).

7. A. Marani, D. A. Russell, T. Javelle, J. D. Sutherland, A light-releasable potentially prebiotic nucleotide activating agent. J. Am. Chem. Soc. 140, 8657–8661 (2018).

8. T. Walton, J. W. Szostak, A kinetic model of nonenzymatic RNA polymerization by cytidine-5′-phosphoro-2′-aminimidazolide. Biochemistry 56, 5739–5747 (2017).

9. S. S. Zhang, D. Ihodiev, J. W. Szostak, Potentially prebiotic activation chemistry compatible with nonenzymatic RNA-copying. J. Am. Chem. Soc. 142, 14810–14813 (2020).

10. C. Menor-Salvà, M. R. Marin-Vaysia, Prebiotic chemistry in eutectic solutions at the water-ice microenvironment conducive to nonenzymatic biopolymer formation. Esa Sp Pubs 545, 145–148 (2004).

11. J. L. Bada, C. Bigham, S. L. Miller, Impact melting of frozen oceans on the early Earth. Implications for the origin of life. Proc. Natl. Acad. Sci. U.S.A. 91, 1248–1250 (1994).

12. P. A. Monnard, D. W. Deamer, J. W. Szostak, The eutectic phase in water ice: A microenvironment conducive to nonenzymatic biopolymer formation. Esa Sp Pubs 545, 145–148 (2004).

13. D. Walton, D. W. Deamer, J. W. Szostak, Eutectic phase polymerization of activated ribonucleotide mixtures yields quasi-equimolar incorporation of purine and pyrimidine nucleobases. J. Am. Chem. Soc. 125, 13734–13740 (2003).

14. J. D. Sutherland, L. B. Muller, F. F. Buchet, Potentially prebiotic Passerini-type reactions of phosphates. Synlett 14, 2161–2163 (2008).

15. A. Kanavarioti, P. A. Monnard, D. W. Deamer, Eutectic phases in ice facilitate nonenzymatic nucleic acid synthesis. Astrobiology 1, 271–281 (2001).

16. J. W. Szostak, An optimal degree of physical and chemical heterogeneity for the origin of life? Philos. Trans. R. Soc. Lond. B Biol. Sci. 366, 2894–2901 (2011).

17. X. Li, L. E. Orgel, Nonenzymatic template-directed synthesis on hairpin oligonucleotides. J. Am. Chem. Soc. 114, 7963–7969 (1992).

18. D. J. Ritson, J. D. Sutherland, Synthesis of aldehydic ribonucleotide and amino acid precursors by photoredox chemistry. Angew. Chem. Int. Ed. Engl. 52, 5848–5847 (2013).

19. J. Xu et al., Photocatalytic reductive homologation of hydrogen cyanide using sulfite and ferricyanide. Chem. Commun. (Camb.) 54, 5566–5569 (2018).

20. B. H. Patel, C. Perivivale, D. J. Ritson, C. D. Duffy, J. D. Sutherland, Common origins of RNA, protein and lipid precursors in a cyanosulfidic protometabolism. Nat. Chem. 7, 301–307 (2015).

21. E. L. Gibbs, D. C. B. Whittet, A. C. A. Boogert, A. G. M. G. Tielens, Intersetellar ice: The Infrared Space Observatory legacy. Astrophys. J. Suppl. Ser. 171, 25–73 (2004).

22. J. I. Lunine, Physical conditions on the early Earth. Philos. Trans. R. Soc. Lond. B Biol. Sci. 361, 1721–1731 (2006).

23. R. J. Worth, S. Sigurdsson, C. H. House, Seeding life on the moons of the outer planets via lithopanspermia. Astrobiology 13, 1155–1165 (2013).

24. R. Wordworth et al., A coupled model of episodic warming, oxidation and geochemical transitions on early Mars. Nat. Geosci. 14, 127 (2021).

25. B. Damer, D. Deamer, The hot spring hypothesis for an origin of life. Astrobiology 20, 429–452 (2020).

26. M. J. Cavaluzzi, P. N. Borer, Revised UV extinction coefficients for nucleoside-5′-monophosphates and unpaired DNA and RNA. Nucleic Acids Res. 32, e13 (2004).

27. J. F. Kasting, Early Earth: Faint young sun redox. Nature 464, 687–689 (2010).

28. J. C. Aponte et al., Pathways to metacenicetic glycoline and methylamine. ACS Earth Space Chem. 1, 3–13 (2017).

29. A. Dömling, Recent advances in isocyanide-based multicomponent chemistry. Curr. Opin. Chem. Biol. 6, 306–313 (2002).

30. D. Ritson, J. D. Sutherland, Prebiotic synthesis of simple sugars by photoredox systems chemistry. Nat. Chem. 4, 895–899 (2012).

31. J. Attwater, A. Wochner, P. Holliger, In-ice evolution of RNA polymerase ribozyme activity. Nat. Chem. 5, 1011–1018 (2013).