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Communication

Potentially Prebiotic Activation Chemistry Compatible with Nonenzymatic RNA Copying

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ABSTRACT: The nonenzymatic replication of ribonucleic acid (RNA) may have enabled the propagation of genetic information during the origin of life. RNA copying can be initiated in the laboratory with chemically activated nucleotides, but continued copying requires a source of chemical energy for *in situ* nucleotide activation. Recent work has illuminated a potentially prebiotic cyanosulfidic chemistry that activates nucleotides, but its application to nonenzymatic RNA copying had not been demonstrated. Here, we report a novel pathway that activates RNA nucleotides in a manner compatible with template-directed nonenzymatic copying. We show that this pathway, which we refer to as bridge-forming activation, selectively yields the reactive imidazolium-bridged dinucleotide intermediate required for copying. Our results will enable more realistic simulations of RNA propagation based on continuous *in situ* nucleotide activation.

RNA is a leading candidate for the primordial genetic polymer because of its capacity to function as both a hereditary and enzymatic biomolecule.[1−3] The emergence of life in the RNA World would have required nonenzymatic RNA replication prior to the emergence of ribozyme-catalyzed replication.[4−6] Primer extension is a model of RNA copying in which nucleotides **1** are added to a primer when guided by a template sequence (Figure 1).[7−9] Nonenzymatic primer extension relies on activation of the mononucleotide phosphate groups.[10−14]

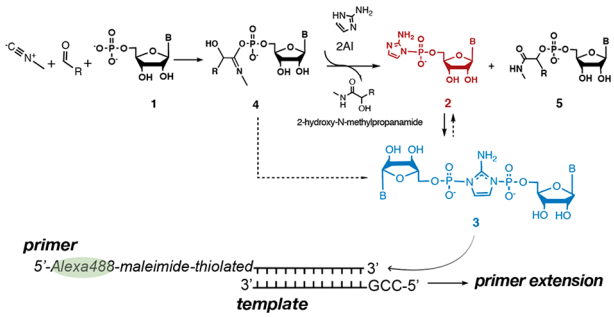

Figure 1. Components of nonenzymatic RNA primer extension. Nonenzymatic template-directed RNA polymerization at the 3′-end of a primer proceeds via **3**, which forms spontaneously in a pool of chemically activated nucleotides **2**. Isocyanide nucleotide activation chemistry is incompatible with primer extension due to the required excess 2AI, which inhibits accumulation of **3**.

While alternative phosphate activation pathways for primer extension exist,[15−17] our laboratory has demonstrated efficient copying of various short RNA templates using 2-aminoimidazole (2AI) activated ribonucleotides (2AImpN **2**),[14] and shown that polymerization proceeds predominantly through spontaneously generated 5′-5′-imidazolium-bridged dinucleotides **3**[18−20] (Figure 1). The superiority of 2AI as a phosphate activating group over other imidazole derivatives is due at least in part to the higher accumulation and greater stability of the corresponding bridged dinucleotide.[21] Activated mononucleotides hydrolyze to generate free 2AI, which in turn attacks the bridged dinucleotide to yield two 2AImpNs **2**.[19,21] Bridged dinucleotides also decay through hydrolysis, yielding one 2AImpN **2** and one nucleoside monophosphate (NMP **1**).

A prebiotically relevant model requires *in situ* activation that is also compatible with primer extension.[11,14,22] Recent advances in prebiotic cyanosulfidic chemistry suggest a robust chemical pathway that may have generated the major building blocks of life.[23−27] Sutherland and co-workers have also recently reported a route to selective phosphate activation with methyl isocyanide, aldehyde, and imidazole[28] in a pH regime that is potentially compatible with primer extension and without modifications to the nucleobases.[21,28] This prompted us to seek conditions under which this nucleotide activation chemistry could be applied to template-directed nonenzymatic RNA copying (Figure 1).

A major hurdle to the compatibility of activation chemistry and RNA copying is that excess 2AI is required to drive nucleotide activation, but excess 2AI specifically inhibits primer extension by attacking the imidazolium-bridged dinucleotide intermediate **3** (Figure 1). We report a new pathway that both circumvents this issue and yields significantly higher concentrations of bridged dinucleotides than the spontaneous self-reaction of activated mononucleotides **2**.[21]

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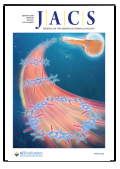

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As a first step to combining isocyanide activation with primer extension, we sought reaction conditions compatible with both. Optimal primer extension requires Mg^{2+} and mildly basic buffer (pH ≈ 8).[21,29] We examined the effects of Mg^{2+} concentration and pH on the activation of NMPs **1** to 2AImpN **2** using isocyanide (Figure S1) and acetaldehyde. All four canonical ribonucleotides were activated under primer extension conditions (Figures S2, S3a). However, an undesirable Passerini reaction product **5**,[30] which depletes the starting NMP **1** pool (supplementary text, Scheme S1), also formed (Figure S2, Tables S1, S2). In a screen of longer chain aldehydes and ketones in place of acetaldehyde, 2-methylbutyraldehyde (2MBA) decreased the formation of **5** from 12% to 3%, while increasing the yield of 2AImpN **2** from 31% to 81% (Figure S4, Table S3 with 30 mM Mg^{2+}). The higher yield of **2** may stem from reduced hydrolysis of the imidoyl intermediate **4** without affecting the 2AI attack on the phosphate group.

Although the above optimizations define reaction conditions compatible with primer extension, there remained a significant obstacle. The high concentration of 2AI required for NMP **1** activation prohibits accumulation of the imidazolium bridged dinucleotides **3** necessary for RNA copying by driving the equilibrium toward 2AImpN **2** (Figure 1, Table 1).[21]

Table 1. Yields of 2AImpA 2 and Bridged Dinucleotide 3 at Different 2AI Concentrations Measured by NMR Spectroscopy[a]

[2AI] (mM)	Activation (**2**) yield (%)	Bridged dinucleotide (**3**) yield (%)
10	6 ± 2	1.8 ± 0.5
20	13.4 ± 0.6	2.8 ± 0.9
50	28.1 ± 0.2	2.1 ± 0.2
100	33.0 ± 0.5	0.8 ± 0.4
200	48 ± 2	0
400	34 ± 1	0
800	19 ± 1	0

[a]All reactions were carried out using AMP **1** (10 mM), acetaldehyde (200 mM), methyl isocyanide (200 mM), 2AI (varied), Mg^{2+} ($MgCl_2$, 10 mM), and HEPES (200 mM) at pH 8.0 and t = 6 h. Errors are standard deviations of the mean, n = 3 replicates.

Confirming this effect on primer extension required an assay compatible with isocyanide activation chemistry. Because the isocyanide chemistry modified the fluorophores used for primer labeling (Figure S5), we developed a postlabeling strategy for measuring primer extension (supplementary text, Figures S6, S7). Using a standard primer extension reaction in which the template sequence is 5′-CCG-3′, we found that the excess 2AI (200 mM) required for efficient activation severely inhibits primer extension in the presence or absence of activation chemistry (Figure S8). Thus, the requirement for excess 2AI appears to be a fundamental incompatibility between primer extension and *in situ* activation with isocyanide.

Reflecting on the overall primer extension pathway—from nucleotides **1**, via activated nucleotides **2**, to the bridged dinucleotides **3** that actually promote the elongation of the primer—we asked whether the isocyanide activation chemistry might be relevant to the formation of the bridged dinucleotide. We therefore introduced 2AImpN **2** to a mixture of isocyanide, aldehyde, and AMP **1** without any free 2AI. We found that not only did the bridged dinucleotide species **3** form, but it accumulated to a significantly higher level than through the self-reaction of activated monomers in the absence of activation chemistry. For an equimolar mix of AMP **1** and 2AImpA **2** in the presence of isocyanide activation, ^{31}P NMR spectra show 16% bridged dinucleotide **3** at t = 229 min (the time point at which the concentration of bridged dinucleotide peaks), compared with only 2% in its absence (Figure 2). To

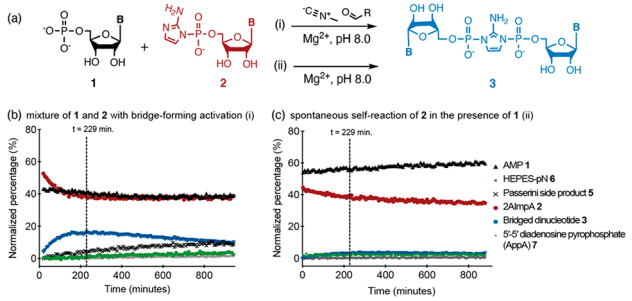

Figure 2. Bridge-forming activation. (a) Prebiotically plausible chemistry drives bridged dinucleotide **3** formation. Analyses of the reaction over the course of 15 h by ^{31}P NMR (b) with and (c) without bridge-forming activation. The relative percentage of each species is calculated based on the corresponding peak integration normalized to the number of phosphorus atoms. Reaction conditions: AMP **1** (5 mM), 2AImpA **2** (5 mM), Mg^{2+} (30 mM, $MgCl_2$), HEPES (200 mM) at pH 8.0 with or without methyl isocyanide (200 mM) and 2MBA (200 mM).

differentiate this scenario from the one in which excess 2AI drives NMP **1** activation, we call it bridge-forming activation. In bridge-forming activation, species **3**, required for primer extension, is efficiently generated in the presence of 2AImpN **2**, isocyanide, and aldehyde (supplementary text).

In any prebiotically plausible scenario for RNA copying, the ratio of activated to unactivated nucleotides would vary with time. We find that bridge-forming activation functions across a broad range of ratios, with bridged dinucleotide **3** detected in every case in which activated mononucleotide **2** is present (Figures S9, S10a–c) but not its absence (Figure S10d).

Interestingly, the treatment of 100% activated mononucleotides **2** with the bridge-forming activation reagents also efficiently yielded bridged dinucleotide **3** with little accompanying hydrolysis (Figure S11): from t = 12 min to t = 135 min, only 1% of the mononucleotides hydrolyzed whereas the bridged dinucleotide **3** yield was 40%. These observations suggest a significant contribution from a novel pathway in which the activation chemistry directly mediates the bridging of two already-activated nucleotides **2** (rather than only the bridging of pairs of activated **2** and unactivated nucleotides **1**) (Scheme S2c).

We next considered whether bridge-forming activation shows any preference for 2AI over 2-methylimidazole (2MI), the historically most common activating imidazole.[10,21] Treatment of an equimolar mixture of AMP and 2MImpA **8** with bridge-forming activation did yield 2MI-bridged dinucleotide **9**, though markedly less than with 2AImpN **2**. Without bridge-forming activation, no detectable 2MI-bridged dinucleotide **9** formed (Figure S12). The significant difference in bridged dinucleotide accumulation between 2AI- **2** and 2MI-activated mononucleotides **8** led us to consider how they would behave together. Remarkably, the reaction yielded only the 2-aminoimidazolium bridged dinucleotide **3** (Figure S13). Thus bridge-forming activation is highly selective toward 2AI

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over 2MI in a nucleotide concentration regime that is functional in primer extension.

Encouraged by these results, we sought to apply bridge-forming activation to primer extension. To copy a 5′-GCC-3′ template, various concentrations of 2-AI activated C and G mononucleotides were mixed with unactivated C and G and treated or not treated with bridge-forming activation (Figure 3). As a control we performed primer extension with 5 mM

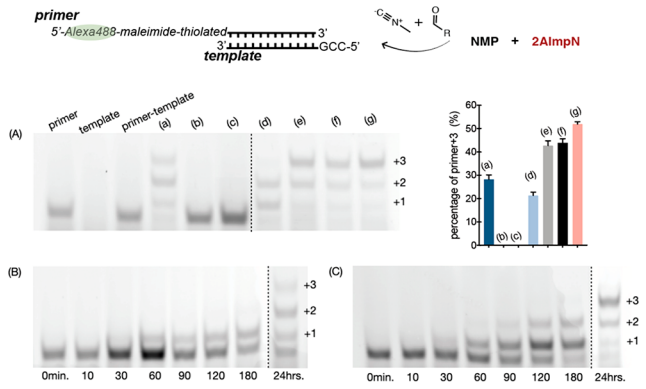

Figure 3. Primer extension using bridge-forming activation. (A) Products from primer extension reactions using (a) 10 mM 2AImpN **2** (*N* = C and G), (b) 10 mM NMPs **1** plus 200 mM 2AI, (c) 10 mM NMPs **1** with isocyanide chemistry, (d) an equimolar mixture of 10 mM 2AImpNs **2** and 10 mM NMPs **1** without bridge-forming activation and (e) with bridge-forming activation, (f) 20 mM 2AImpNs **2** without bridge-forming activation and (g) with bridge-forming activation. Bar graph: quantification of primer +3 from lanes (a) through (g). Reaction conditions: 2AImpNs **2** and NMPs **1** were added to primer (1 μM), template (1.5 μM), HEPES (200 mM) pH 8.0, and Mg^{2+} (30 mM, $MgCl_2$) with isocyanide chemistry (gray and coral pink) or without (dark blue, light blue, black). Extension products were assayed by PAGE (Figure S14) at 24 h. Error bars indicate standard deviations of the mean, n = 3 replicates. (B, C) Time course of primer extension reactions using equimolar mixture of **1** and **2** with (B) and without (C) bridge-forming activation. Positions of primer and +1 to +3 products are indicated. Reaction conditions as in (d) and (e).

each 2AImpG and C and observed a baseline distribution of +1, + 2, and +3 products (Figure 3a). The addition of 10 mM NMPs **1** inhibited the reaction because unactivated mononucleotides **1** compete for the binding sites of bridged dinucleotides **3** (compare Figure 3a to Figure 3d). In contrast bridge-forming activation increased product yield, with 43% +3 products compared to 21% without the bridge-forming activation (Figure 3d, e). This distribution of products from an equimolar ratio of activated **2** and unactivated mononucleotides **1** plus bridge-forming activation is comparable to that found with the use of 20 mM pure activated mononucleotides **2** (Figure 3f). Finally, applying bridge-forming activation to 10 mM pure activated mononucleotides **2**, with no initial unactivated nucleotides **1**, resulted in even more +3 product (53%) (Figure 3g). Note that in these experiments the product length is template-limited, because there are no template bases beyond the +3 position. We confirmed the identities of the primer extension products using bridge-forming activation by liquid chromatography–mass spectrometry (LC-MS). The major component of the peaks corresponding to the primer and the +1 to +3 products in the UV trace all have the correct mass, consistent with being the expected products of primer extension (Figure S15, Table S5). Additionally, no mismatches were observed. These experiments demonstrate the compatibility of isocyanide-based nucleotide activation with nonenzymatic RNA copying.

Bridge-forming activation provides several advantages for primer extension. It requires lower mononucleotide concentrations (1.5–5 mM) to generate appreciable proportions of bridged dinucleotide than required by spontaneous bridging (10–100s mM range) or direct activation with free 2AI (50 mM-400 mM NMP). The higher proportion of bridged dinucleotides raises the possibility that the Mg^{2+} concentration can be further reduced. High Mg^{2+} concentrations are notoriously problematic for primer extension, causing bridged dinucleotide hydrolysis,[31] monomer cyclization,[29,32,33] and template degradation.[29,34]

Although bridge-forming activation is compatible with primer extension and promotes the formation of the required intermediate, it depends on a source of previously activated mononucleotides. One possibility is that initial activation occurs under partial dry-down conditions where all reactants including 2AI are at very high concentrations, followed by dilution to nucleotide concentrations sufficient for bridge-forming activation and primer extension, but with low enough free 2AI to minimize loss of the bridged dinucleotides. Additional processes that might sequester or degrade 2AI should also be investigated. For example, UV radiation, the presumptive energy source for producing isocyanide, photodegrades 2AI on the order of days[35] although the photodegradation rates of **2** and **3** remain unknown (supplementary text).

A highly desirable feature of bridge-forming activation is the potential reactivation of spent nucleotides for further rounds of polymerization. Previously identified activation chemistries lead to damaging side reactions that destroy both templates and substrates,[29,36–40] whereas bridge-forming activation relies on RNA-compatible and specific reagents. Further work is needed to demonstrate nucleotide reactivation in the context of continuous rounds of primer extension.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.0c05300.

Methods and supplemental figures and scheme (PDF)

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Notes

The authors declare no competing financial interest.

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