Accelerating chemical replication steps of RNA involving activated ribonucleotides and downstream-binding elements†

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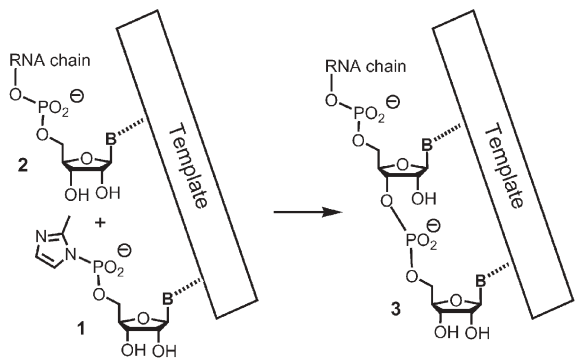
Received (in Cambridge, UK) 28th July 2005, Accepted 25th August 2005
First published as an Advance Article on the web 12th September 2005
DOI: 10.1039/b510775j

Template-directed single nucleotide extension of an RNA primer with oxyazabenzotriazolides of ribonucleotides is shown to be fast and sequence-selective; downstream-binding RNA strands contribute to the acceleration of the reaction.

Among the two classes of nucleic acids (DNA and RNA), RNA shows greater structural diversity, has a larger number of biological functions, and makes up more of the dry weight of cells.[1] Also, RNA lends itself more readily to evolving new functional motifs, starting from randomized sequences.[2] Spontaneous oligomerization of chemically activated nucleotides in the presence of minerals is known for RNA,[3] but not for unmodified DNA.

RNA-directed oligomerization of ribonucleotides that forms or extends a strand complementary to the template is known to occur spontaneously with 2-methylimidazolides (**1**) as activated monomers in aqueous solution in the absence of enzymes (Scheme 1). This replication-like reaction involves the 2′/3′-diol of a ribonucleotide (**2**) as nucleophile and produces extended strands (**3**).[4] Weakly base pairing nucleotides (A and U) do not support these reactions well,[5] and among the strongly base-pairing nucleotides (G and C), only C is free of complications as a template.[6,7] Even for polyC templates, replication assays produce mixtures of chains of different lengths, a problem that can be further exacerbated when enantiomers of nucleotides are present.[8] Known forms of non-enzymatic primer extension are also slow, with typical reaction times of days to weeks, making it desirable to find improved methodologies.



Scheme 1

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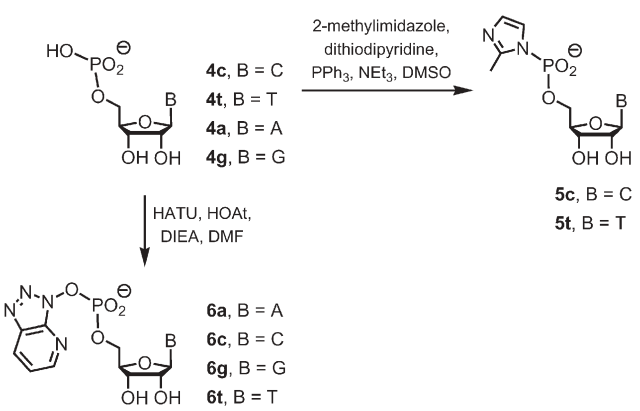
† Electronic supplementary information (ESI) available: syntheses, protocols for assays, representative MALDI spectra, and kinetics. See http://dx.doi.org/10.1039/b510775j

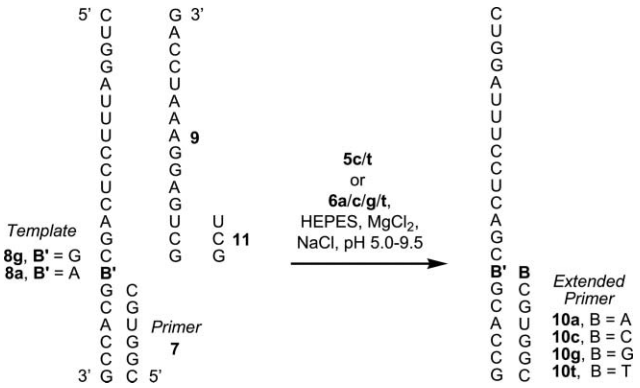
Earlier reports on accelerated primer extension steps have focused on modified DNA.[9,10] To achieve sufficient rates, primers with a 3′-terminal amino group replacing the 3′-hydroxyl group of natural DNA were required. The question of whether rapid reactions can be induced with unmodified nucleic acids as primers has remained open. Here we show that for a system consisting of an unmodified RNA primer and template, rapid template-directed reactions can be achieved with chemically activated ribonucleotides. Downstream-binding RNA elements accelerate these reactions further and restrict them to a defined, single extension step, making them attractive for extending RNA without the constraints of the active sites of polymerases or ligases.

Methylimidazolides **5c** and **5t** were prepared as reference compounds, using slight modifications of literature protocols (Scheme 2).[11–13] Hydroxyazabenzotriazole (OAt) esters of ribonucleotides (**4a–t**) were then prepared in one-step syntheses using HATU[14] (see electronic supplementary information). Thymine was used as nucleobase in **5t** and **6t**, as, unlike uracil, its mass is sufficiently different from that of cytosine to allow for unambiguous identification of extension products *via* MALDI-TOF mass spectrometry. Even though the potential for oligomerization is higher in the case of ribonucleotides than in the case of deoxyribonucleotides,[10] activation conditions were found for all four monomers that give **6a–t** in satisfactory yields after precipitation. Exploratory studies on the hydrolytic stability of **6a–t** in D_2O by NMR gave half-life times >16 h.

Scheme 3 shows the primer (**7**) and templates (**8a/g**) employed. Either template constitutes a 'difficult' case, where the templating base is a purine. Purines as templating bases lead to poorer incorporations than pyrimidines, as the incoming nucleotides have small nucleobases that offer little stacking surface, making it



Scheme 2



Scheme 3

difficult to retain the monomer long enough at the template. Adenine as templating base is the 'worst case', as it also forms a weak base pair.

Primer extension reactions were monitored using MALDI-TOF mass spectrometry under conditions that allow for quantitative detection.[15] Mass spectrometry does not provide information on whether 2′–5′ or 3′–5′-linked products are formed, so the distribution of regioisomers is currently unknown. To determine second order rate constants, the duplex between template and primer was treated as one and the monomers as the other reactant. This facilitates kinetic analyses, but is an approximation valid only for assay temperatures below the melting point of the duplex and at a given monomer concentration, as preassociation equilibria between monomers and templates influence rates, and hydrolysis competes with extension.[16]

First, a concentration range of monomers was established that provides sufficient primer conversion for detection of extension products using methylimidazolides **5c** and **5t** and buffer components well established for their use.[17] While conversion at 3.6 mM monomer concentration was minimal, even for G-template **8g**, reactions at 20 mM monomer concentration provided detectable extension products for either template, **8g** and **8a** (Table 1). Downstream-binding pentadecamer **9** not only limited the reactions to a single extension step, it also increased yields detectably. The 5′-terminal nucleotide of **9** offers additional stacking surface to incoming monomers, and can thus be expected to help monomers bind at the terminus of the primer. Monomers alone can only induce such an effect at concentrations high enough to favor the formation of non-covalent helices on templates.[17]

Reactions of OAt esters **6c** and **6t** with **7/8/9** are faster than those with methylimidazolides under the established buffer conditions. But unlike the latter, the pH optimum for their reaction with the primer is at elevated pH (Fig. 1), possibly due to the basicity of the aza nitrogen at position 7 of HOAt.[18] Interestingly, rates level off at pH 8.9, while primer conversion continues to rise when increasing the pH to 9.5. At pH 8.9, the rate acceleration for the OAt ester-driven reaction on template **8a** over that with **5t** at pH 7.7 is more than 42-fold, accompanied by higher conversion. For G-template **8g**, the rate constant reaches 36 $h^{-1}M^{-1}$ at pH 8.9 and near-quantitative conversion after 20 h is achieved with **6c** in the presence of **9** at pH 9.5 (Fig. 2). Exploratory experiments show that primer conversion for reactions of **6t** with **7** on template **8a** at pH 8.9 can be significantly enhanced by addition of a fresh aliquot of **6t** after the first reaction phase has gone to completion. Further, lowering the concentration of NaCl to 0.2 M reduces the conversion of **7** in the presence of **8g** and **9** at pH 8.9 by less than 10%.

We performed exploratory reactions with mixtures of OAt esters to study the sequence selectivity of the reactions at elevated pH, a feature critical for practical applications. Assays with monomers **6c** and **6t** at 10 mM concentration showed preferential incorporation of the complementary nucleotide for either template, even at pH 9.5 (Table 2). Assays with all four competing monomers (**6a/c/g/t**) and template **8g** at pH 8.9 or pH 9.5 again showed the desired selectivity, even at 5 mM monomer concentration. The product distributions in Table 2 are conservative values, as salt-adducts (M + Na and M + Mg) were not corrected for, increasing peak intensities for the side products.

Table 1 Effects of downstream-binding strand and monomer concentration on primer extension (268 μM strands, pH 7.7)

oligomers	monomer	$c_{monomer}$ [mM]	conversion[a] [%]	k_2 $[h^{-1}M^{-1}]^b$	$t_{½}$ [h]
7/8g	**5c**	3.6	⩽ 1	—[c]	—[c]
7/8g	**5c**	20	13	1.3 ± 0.1	27
7/8g/9	**5c**	20	25	1.3 ± 0.1	27
7/8a	**5t**	20	3	—[c]	—[c]
7/8a/9	**5t**	20	4	0.2 ± 0.05	153

[a] After 20 h reaction time. [b] Second order rate constant; oligonucleotide complexes treated as one reactant, activated nucleotide as the other. [c] Conversion too low for useful fits.

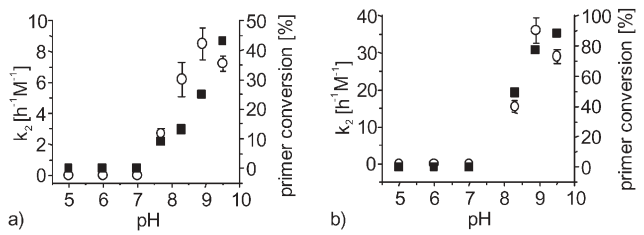


Fig. 1 pH-dependence of primer extension (268 μM strand concentration, 20 mM monomers) in the presence of **9**; a) with template **8a** and monomer **6t**, b) with template **8g** and monomer **6c**. Open circles (left axes) are second order rate constants, filled squares (right axes) are primer conversion values after 20 h.

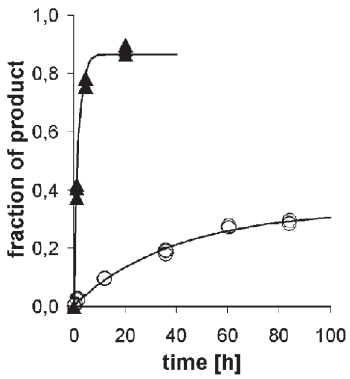


Fig. 2 Kinetics for extension of primer **7** on template **8g**, using **5c** at pH 7.7 (open circles) or using **6c** at pH 9.5 in the presence of **9** (filled triangles).

Table 2 Sequence selectivity of accelerated primer extension[a]

oligomers	monomers	$c_{monomers}$ [mM]	pH	k_2[b] [$h^{-1}M^{-1}$]	$t_{½}$[b] [h]	product ratio (**10a/c/g/t**)
7/8a/9	**6c/6t**	10	8.9	12.1 ± 2.6	5.8	—/36/—/**64**
7/8a/9	**6c/6t**	10	9.5	14.8 ± 4.1	4.1	—/18/—/**82**
7/8g/9	**6c/6t**	10	8.9	26.9 ± 9.7	2.6	—/**82**/—/18
7/8g/9	**6c/6t**	10	9.5	22.3 ± 8.9	3.1	—/**75**/—/25
7/8g/9	**6a/6c/6g/6t**	5	8.9	36.2 ± 8.2	3.8	14/**56**/10/20
7/8g/9	**6a/6c/6g/6t**	5	9.5	38.5 ± 11.7	3.6	17/**66**/11/6

[a] Conditions: 268 μM oligomers, 200 mM HEPES, 400 mM NaCl, 80 mM $MgCl_2$; primer conversion 14–64% after 20 h. [b] For desired product.

Table 3 Temperature-dependent extensions promoted by **11**[a]

oligomers	monomer	T [°C]	conversion [%]	k_2 [$h^{-1}M^{-1}$]	$t_{½}$ [h]
7/8g	**6c**	24	11	10.3 ± 1.1	3.4
7/8g/11	**6c**	24	37	14.5 ± 0.5	2.4
7/8g	**6c**	4	8	1.7 ± 0.3	20.7
7/8g/11	**6c**	4	67	3.1 ± 0.1	11.2

[a] Same conditions as for data in Table 2.

Our methodology may be evolved further by using short downstream-binding elements. For example, tetramer GCUG, composed entirely of 2′-*O*-methyl ribonucleotides, has a very similar effect on the rate of OAt ester-driven primer extensions to that of RNA pentadecamer **9** and leads to greater selectivity (see Figure S9, electronic supplementary information). This suggests that such base-stable and easy-to-synthesize analogs of natural RNA can be used as downstream-binding elements to accelerate primer extension reactions at specific sites. Further, exploratory reactions with trimer GCU (**11**) as downstream-binding element showed that non-enzymatic primer extension with OAt esters can be more efficient at low temperatures (Table 3). At room temperature, reactions in the presence of **11** reached only 37% conversion, probably because the trimer is largely unbound, but when the temperature is lowered to 4 °C, **11** increases primer conversion to almost twice that value. The more efficient conversion at lower temperature is reminiscent of a feature of the polymerase chain reaction (PCR), where primer extension occurs at lower temperature and higher temperatures favor dissociation of strands, stopping the reaction.

Our data show that primer extensions in all-RNA systems can be accelerated by employing azabenzotriazole esters at elevated pH and that the rate can be modulated by a downstream-binding oligoribonucleotide. This demonstrates a propensity of RNA to undergo spontaneous replication steps beyond levels previously known. Further, it shows the ability of RNA to regulate these steps with 'microRNA', such as trimer **11**. Our method may also become useful for practical applications. The conversion observed in our exploratory assays on sequence selectivity is sufficient to determine single nucleotide polymorphisms[10] based on RNA, using either mass spectrometry[19] or possibly optical methods after incorporating fluorophore-labeled monomers.[20] Compared to DNA, RNA generated by transcription has the advantage of being single stranded and inexpensive to produce. Also, since our methodology does not require any chemical modification of the template, primer, or downstream-binding element, either may be the product of transcription. Finally, our method may become attractive for labeling biologically relevant RNAs without the constraints of enzymes, or for studies on possible scenarios for the origin of life.[21]

This work was supported by DFG (grant No. 1063/1-3) and Fonds der Chemischen Industrie (project 164431). S. V. was a recipient of a predoctoral fellowship from the State of Baden-Württemberg.

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