DNA-Directed Synthesis

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Template-Directed Synthesis in 3′- and 5′-Direction with Reversible Termination**

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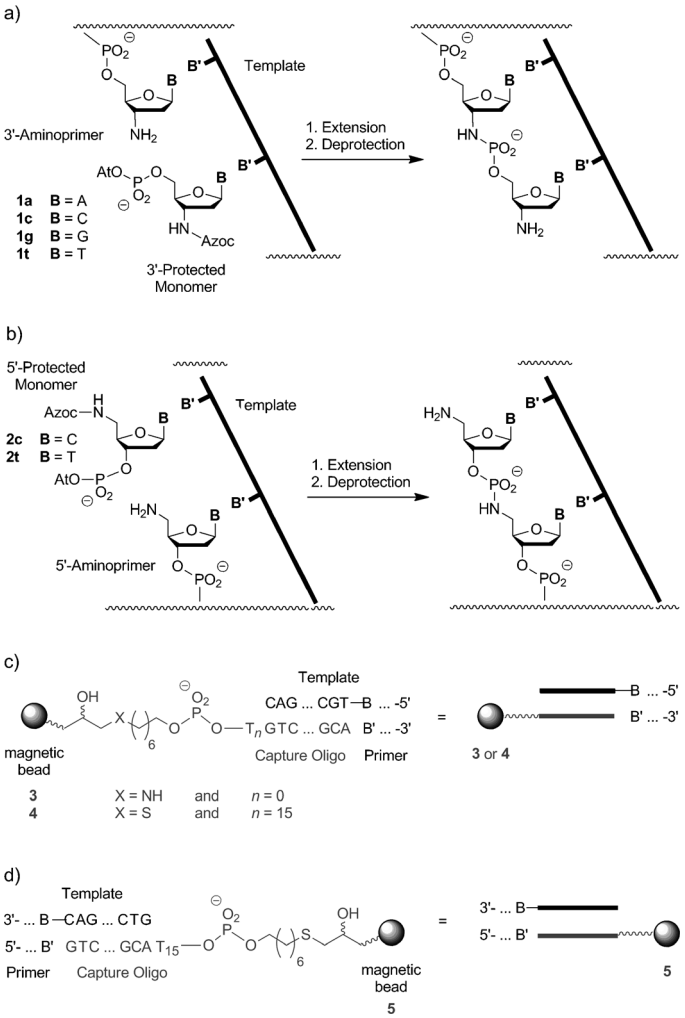
Template-directed synthesis of nucleic acid chains is one of the most important processes of the animate world. In the cell, polymerases catalyze the extension of primers bound to DNA templates, using nucleoside 5′-triphosphates as monomers. Chain growth occurs in 3′-direction.[1] Chemists, using programmable DNA synthesizers, commonly perform DNA and RNA syntheses in template-free fashion through the coupling of 3′-phosphoramidites of protected nucleosides, with chains growing in 5′-direction on a solid support.[2] Control over the sequence and length of the synthetic oligonucleotides is achieved through protecting groups and the programmed flow of solutions.

Chemical primer extension (CPE) is the template-directed elongation of an oligonucleotide primer through the reaction of activated nucleotides with the primer terminus in the absence of enzymes. Chemical primer extension assays have been performed for the elongation of RNA,[3–5] DNA,[6] 3′-amino-terminal DNA,[7–9] 2′-amino-terminal DNA,[10,11] and more-extensively backbone-modified nucleic acids.[12] With CPE, the intrinsic ability of nucleic acids to copy and possibly replicate genetic information can be studied.[13] Most studies, including some on template-free polymerization of nucleotides[14,15] and short oligonucleotides,[16] have focused on prebiotic questions. An increasing number of theoretical studies are appearing.[17–19] But, recent work has also demonstrated the potential of CPE[8a,20] and chemically induced ligation[21] for practical applications. Multiple, stepwise chain extensions, controlled by protecting groups are currently unknown.[22] Also, to the best of our knowledge, enzyme-free primer extension has thus far been limited to chain growth in 3′/2′-direction.

As part of a program on enzyme-free sequence determination by CPE, we have previously shown that azaoxybenzotriazole esters (OAt esters) give faster extensions than methylimidazolides.[8a,c] Near quantitative CPE was observed for 64 different sequence contexts with OAt esters of deoxynucleotides.[8c] Herein, we present a new method that we refer to as chemical primer extension with reversible termination. This method uses protecting groups that are removable under non-denaturing conditions and a solid support on which both template and primer are bound noncovalently. Controlled, stepwise synthesis of oligonucleotides in template-directed fashion is demonstrated both in 3′- and 5′-direction.



Scheme 1. Extension reactions and method for template-directed synthesis with reversible termination. a) Primer extension in 3′-direction, giving *N*3′→*P*5′ phosphoramidate DNA. b) Extension in 5′-direction, giving *P*3′→*N*5′ phosphoramidate DNA. c) Solid supports **3** and **4** for primer extension in 3′-direction, and d) support **5** for primer extension in 5′-direction. Full sequences of templates and capture oligonucleotides are given in the Supporting Information. All strands in this study are oligodeoxynucleotides. Azoc = CO-OCH$_2$N$_3$.

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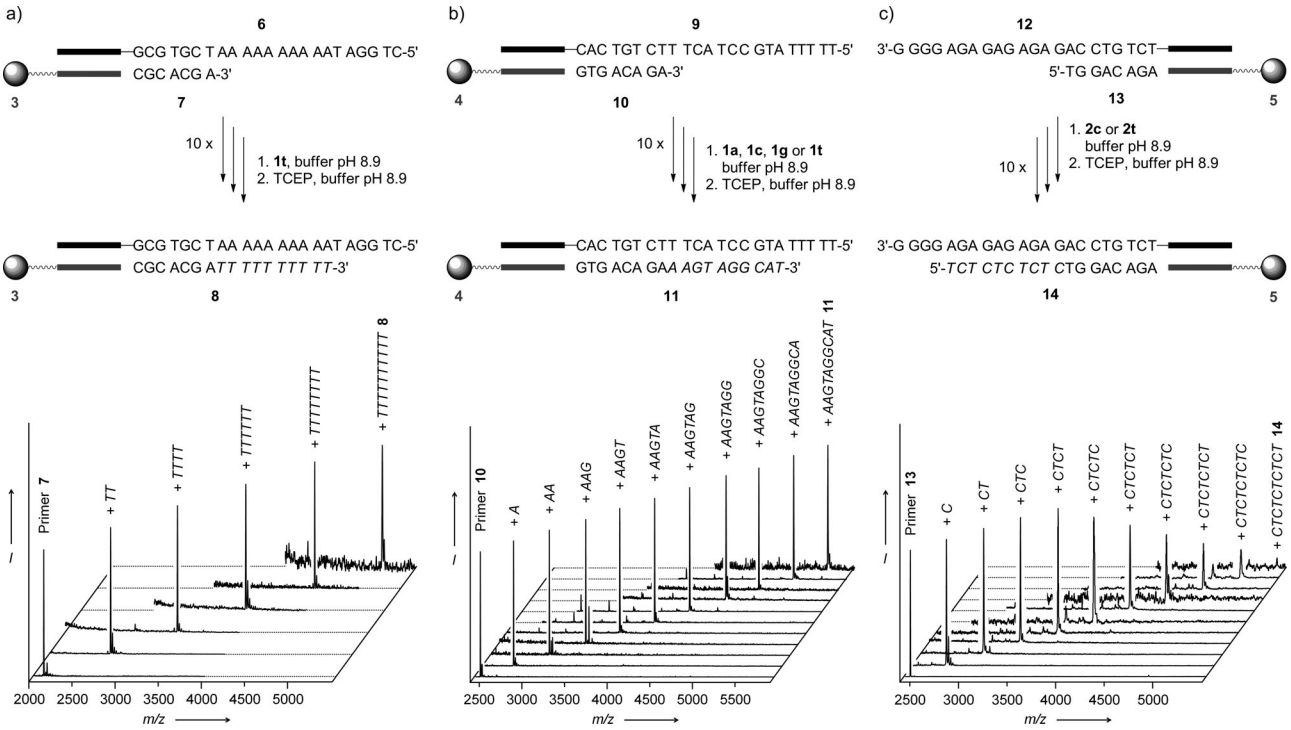
We chose genetic polymers with phosphoramidate linkages that are isoelectronic to natural DNA for our study (Schemes 2a and 2b).[7–11,16,21–23] The azidomethyloxycarbonyl (Azoc) group, first described by Pothukanuri and Winssinger,[24] was selected for the protection of the 3′- and 5′-amino group of the mononucleotides (**1a**–**t**, **2c**, and **2t**; Schemes 1a and 1b) and was introduced using a recently developed carbonate reagent.[25]

The syntheses of Azoc-protected monomers **1a**, **1c**, **1g**, **1t**, **2c**, and **2t** are shown in the Supporting Information. Amino-terminal oligodeoxynucleotide primers were synthesized using literature protocols.[8c,26] The Azoc protection chosen avoids uncontrolled polymerization and intramolecular cyclization,[11a] and deprotection of the 3′-, or 5′-amino group after the elongation can be induced with a water-soluble phosphine. High yields are required for both the extension and the deprotection reaction for efficient, multiple primer extension. Azidomethyl groups have been employed in second generation sequencing to protect 3′-hydroxy groups reversibly.[27,28] These are not suitable for amines. The Azoc group is smaller than common protecting groups for amines,[29] thus avoiding steric hindrance during monomer binding. The Azoc group performed better than larger, photolabile protecting groups, which were tested during the early stages of our study.[8b,22]

To establish a successful protocol for primer extension in a controlled, stepwise fashion, with cleavage of the protecting groups under non-denaturing conditions, we tested several solid supports. First, streptavidin-coated magnetic beads and biotin-labeled capture oligonucleotides were tested, a combination described earlier for RNA-templated reactions and twofold primer extension with 3′-aminoprimers.[5c,8b] The protein-coated surface proved reactive towards 3′-aminoprimers, as indicated by signal loss in MALDI-TOF mass spectra and the results of control experiments. Switching to epoxy-modified magnetic beads, combined with amino- or thiol-modified capture oligonucleotides gave magnetic-bead-based supports **3**, **4**, and **5**, which do not suffer from this limitation. Supports **3** and **4** were used for CPE in 3′-direction (Scheme 1c) and **5** was used for CPE in 5′-direction (Scheme 1d). The Supporting Information provides details and characterization data for both types of DNA-loaded magnetic beads. The active form of the supports was generated by hybridizing template and primer to the capture oligonucleotide on their surface.

With monomers and supports in hand, we performed template-directed syntheses (Scheme 2). First, we tested Azoc-protected **1t** as the monomer, and tris(2-carboxyethyl)-phosphine (TCEP) in aqueous buffer as the cleavage reagent in the deprotection step that involves a Staudinger reduction. High yields in extension and deprotection were observed for ten cycles, as demonstrated by MALDI-TOF MS readout after every other elongation cycle (Scheme 2a).



Scheme 2. Tenfold template-directed synthesis with reversible termination. a) Extension of aminoprimer **7** on poly(dA) template **6** in 3′-direction, using thymidine **1t** (100 mM) and pyridine (50 mM)[8b] at 4°C for 6 h. Deprotection: TCEP (250 mM) at 20°C for 10 min; b) Extension of aminoprimer **10** on mixed template **9** in 3′-direction, using monomers **1a**, **1c**, **1g**, or **1t** (40 mM of the complementary monomer) at 20°C for 4–12 h; Deprotection: TCEP (100 mM) at 0°C for 30 min; c) Extension of aminoprimer **13** on GA-template **12** in 5′-direction, using monomers **2c** or **2t** under conditions otherwise identical to those given for b). The upper part of each graphic shows the reaction scheme and the lower part shows overlays of MALDI-TOF MS spectra acquired during the assay. All extension steps were performed in buffer containing HEPBS (200 mM), NaCl (400 mM), and $MgCl_2$ (80 mM) at pH 8.9. All deprotection steps used the same buffer additionally containing TCEP (100 or 250 mM, as detailed above). Primer sections with phosphoramidate linkages are shown in italics. HEPBS = *N*-(2-hydroxyethyl)piperazine-*N*′-(4-butanesulfonic acid).

Template-directed synthesis in the opposite (5′) direction was also first tested with a thymidine monomer (**2t**). Again, mass spectra were obtained from small samples of the support that were denatured by heating in deionized water (see the Experimental Section for details). After eight extension cycles, the extended primer was still detectable by MALDI-TOF MS, before the signal disappeared in the noise. Our study demonstrates, for the first time, that CPE in 5′-direction is of a similar efficiency as the established primer extension in 3′-direction.

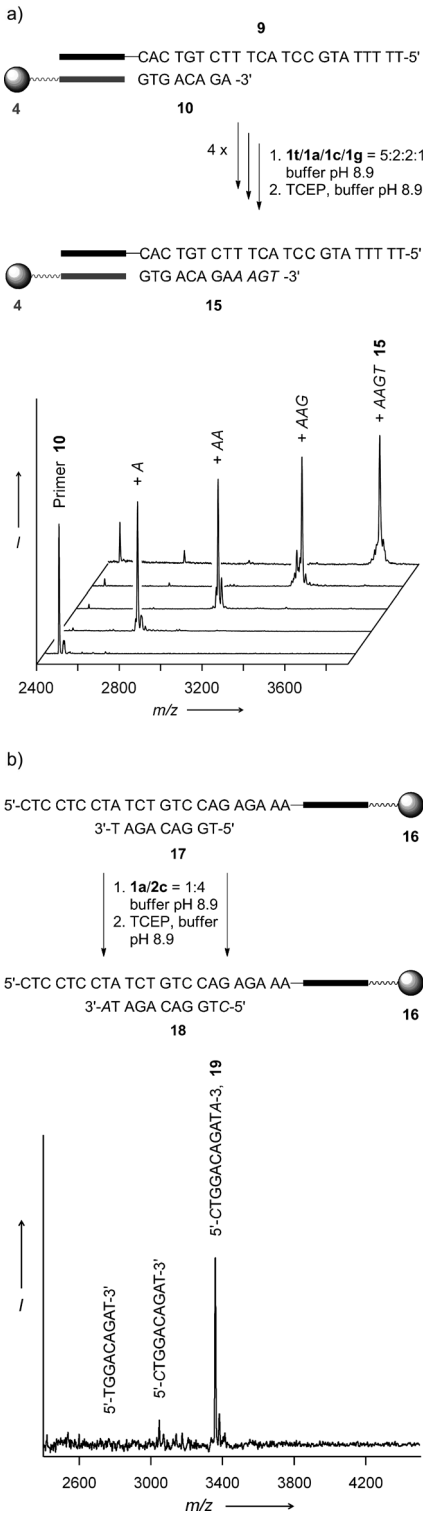
We then studied extension on mixed-sequence templates. The tenfold primer extension in 3′-direction was observed on two different template sequences containing all four different nucleobases (Scheme 2b and Supporting Information). Furthermore, tenfold extension of the 5′-amino-terminal primer was induced by performing extension cycles with **2c** or **2t**, using a template with A and G as templating bases (Scheme 2c).

Since the MALDI-TOF MS signal becomes increasingly weak as the primer grows, the completeness of a CPE reaction with reversible termination was checked by performing an assay with a Cy3-labeled 3′-aminoprimer and analyzing the product by polyacrylamide gel electrophoreses after tenfold extension (see the Supporting Information, Scheme S11).

Finally, exploratory experiments were performed with mixtures of nucleotide monomers to test the sequence fidelity of the CPE. Determining fidelity is important for a better understanding of the ability of genetic polymers to undergo spontaneous replication under potentially prebiotic conditions.[13b] It is also important for possible applications, such as enzyme-free sequencing. The ratio of monomers chosen was 5:2:2:1 (**1t**/**1a**/**1c**/**1g**), a mixture that roughly reflects the relative incorporation propensity of the nucleotides to undergo incorporation.[9b] We performed fourfold CPE with reversible termination using three different templates and found that in all cases the most prominent peak corresponded to the mass of the correctly extended primer (Scheme 3 and the Supporting Information). A more detailed study is under way and will be reported separately.

A first experiment using a Janus-headed primer, with amino groups at both the 3′- and the 5′-terminus was performed, using a system where the template strand was immobilized directly on the solid support. When this doubly reactive primer was exposed to a mixture of the required 3′-reactive and 5′-reactive monomers, the correctly extended primer was observed, with little singly extended primer remaining when the assay was stopped (Scheme 3b).

In conclusion, we have developed protected monomers and solid supports for chemical primer extension in 3′- and 5′-direction with reversible termination. To the best of our knowledge, our results include the first instance that CPE was shown to be an efficient process for extension both in 3′- and



Scheme 3. a) Fourfold template-directed synthesis in 3′-direction with reversible termination. Extension of aminoprimer **10** on mixed template **9** using a mixture of monomers **1t** (40 mM), **1a** (16 mM) **1c** (16 mM), and **1g** (8 mM) at 20°C for 12 h. b) Simultaneous template-directed synthesis in 3′- and 5′-direction. Extension of aminoprimer **17** on immobilized template **16** using a mixture of monomers **2c** (40 mM), and **1a** (10 mM) at 4°C for 12 h. Immobilization of the template to give solid support **16** was performed using the same protocol as for solid support **5**. All extension steps were performed in buffer containing HEPBS (200 mM), NaCl (400 mM), and $MgCl_2$ (80 mM) at pH 8.9. All deprotection steps were performed at 0°C for 30 min and used the same buffer additionally containing TCEP (100 mM). Primer sections with phosphoramidate linkages are shown in italics.

5′-direction. The exquisite control over individual steps through the Azoc protecting group allows for the determination of sequence composition after each step. Our results also establish CPE as a third method for the synthesis of genetic polymers. The method complements nontemplated chemical synthesis and template-directed polymerase-catalyzed synthesis. It has not escaped our attention that CPE with reversible termination may be extended to nonenzymatic replication systems that reveal the intrinsic sequence preference of enzyme-free and ribozyme-free, nucleotide-based replication systems and possibly leads to new functional entities through selections that do not require enzymes. We are actively pursuing the experimental realization of such systems.

Experimental Section

General protocol for CPE assays. A slurry of magnetic beads with immobilized capture oligonucleotide (25 μL, 10 mg mL^{-1}, capacity approx. 200 pmol mg^{-1}; 50 pmol oligonucleotide) was added to a polypropylene vessel. The storage buffer (PBS buffer containing 0.02% NaN_3) was removed using magnetic separation. The beads were washed with washing buffer (2 × 5 μL; HEPBS (200 mM), NaCl (400 mM), $MgCl_2$ (80 mM); pH 8.9). A stock solution of HEPBS buffer (2 μL; HEPBS (500 mM), NaCl (1M), $MgCl_2$ (200 mM); pH 8.9), solutions of template (0.75 μL, 75 pmol), 3′-aminoprimer (0.75 μL, 75 pmol), and water (1.5 μL) were added, to give a final volume of 5 μL. Annealing was induced by cooling from 80 °C (5 min) to 40 °C (1 h) and then to 20 °C, with a cooling rate of 0.1 °C s^{-1} between the plateaus. After magnetic separation, beads were washed with washing buffer (2 × 2 μL), Then, stock solution of HEPBS buffer (2 μL) and a solution of the monomer (**1a**, **1c**, **1g**, or **1t**; 3 μL, 66.6 mM) were added, leading to the desired concentrations of oligonucleotides (10 μM), HEPBS (200 mM), NaCl (400 mM), $MgCl_2$ (80 mM), and **1a**, **1c**, **1g**, or **1t** (40 mM). After vortexing, CPE was allowed to proceed at 20 °C for 4 h (**1a**, **1c**, and **1g**) or 12 h (**1t**). The beads were washed with stock solution of the HEPBS buffer (2 × 2 μL). Then, the stock solution of HEPBS buffer (2 μL), water (2 μL), and a solution of TCEP (0.5M, 1 μL) was added, and deprotection was allowed to proceed at 0 °C for 30 min. For monitoring, a sample was drawn (0.15 μL) and washed with ammonium acetate solution (3 × 1 μL, 1M; pH 7). Water (5 μL) was added, and the resulting suspension was heated to 80 °C. The hot supernatant was immediately aspirated, using a magnet to retain the beads, and added to an aqueous suspension of Dowex 50 WX8-200 cation exchange beads (1 μL, ammonium form) for 25 min. An aliquot was used for MALDI-TOF MS. The remaining beads were washed with stock solution of HEPBS buffer (2 × 2 μL) and subjected to the next elongation cycle.

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