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Nucleotide-Based Copying of Nucleic Acid Sequences without Enzymes

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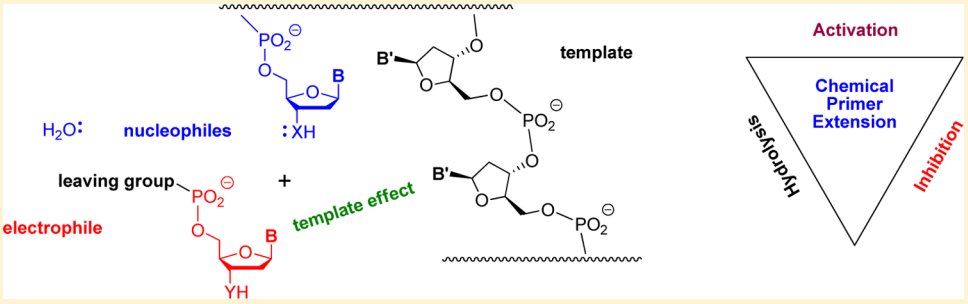

ABSTRACT: Chemical primer extension is the enzyme-free incorporation of nucleotides at the end of an oligonucleotide, directed by a template. The reaction mimics the copying of sequences during replication but relies on recognition and reactivity of nucleic acids alone. Copying is low-yielding, particularly for long RNA. Hydrolysis of active esters and inhibition through hydrolysis products have been identified as factors that prevent high yields, and approaches to overcoming them have culminated in successful template-directed solid-phase syntheses for RNA and phosphoramidate DNA.

Copying the sequence of a DNA or RNA strand into a daughter strand of complementary sequence is the basis of replication and transcription. Copying occurs through extension of a primer hybridized to a template, with individual nucleotides engaging in Watson–Crick base pairing (Scheme 1). Enzyme-free versions are known for amino-terminal DNA primers[1−11] and primers consisting of unmodified RNA.[12−24] The former produce a phosphoramidate linkage upon extension (Scheme 1),[25,26] whereas the latter produce natural, *O3′→P5′*-phosphodiester linkages or their isomeric *O2′→P5′*-phosphodiester counterparts (Figure 1).[12,14,15,24] Unmodified DNA primers are generally too unreactive, but there is hope that highly reactive activating agents[27] or cycles of hydration and dehydration will overcome this problem.[28]

Successful copying is a prerequisite to replication. Because the ability of nucleic acids to store and transfer information is fundamental for life,[29,30] the question whether enzyme-free replication is feasible is of broad interest.[16,29,31] The question is linked to the question of the origin of life on earth,[19,23,31] which some call "one of the most important questions in science".[32]

Mechanistically, chemical primer extension is a reaction of a nucleophile (the primer, shown in blue in Scheme 1) with an electrophile (the activated nucleotide, shown in red). For amino-terminal primers, a detailed kinetic study has shown that there are at least two steps that can be rate-limiting.[7] The first is believed to be the formation of a pentavalent intermediate upon nucleophilic attack of the amine (Scheme 1), the second is probably the subsequent pseudorotation and/or the expulsion of the leaving group. We consider it unlikely that the base pairing event is rate limiting, but the extent to which the nucleotide is bound, as well as the structural details and dynamics of the base pair certainly determine how strong the template effect is, and thus how fast an attack occurs.

Why, then, was enzyme-free copying long considered a low-yielding reaction, so that Orgel[33] wrote: "the prospect of replication in this system is remote, even for RNA"?[18] In principle, the reaction of an alcohol or amine with an active ester or active amide of a phosphate should not pose a fundamental problem. Since there is more than one nucleophilic site in the primer/template/nucleotide mixture, side reactions like undesired transphosphorylations and pyrophosphate formation may occur,[12,34] as well as the formation of regioisomers (*O3′→P5′*[15,35] vs *O2′→P5′* linkages).[36] But, the available data suggested that side reactions are not a major problem.[37−40] Instead, the key complication preventing high-yielding chemical primer extensions appeared to stem from the fact that extensions have to be performed in aqueous solution to ensure that base pairing governs the selection of complementary nucleotides. Water, a competing nucleophile that is similar in reactivity to hydroxy groups of (deoxy)ribose and present at more than 50 M concentration, reacts with activated nucleotides. This has direct and indirect consequences.

In this Synopsis, we would like to present a brief overview of our attempts to overcome the "water problem" in our work of the past decade. There were three basic ideas underlying this work: (i) strengthening the template effect, so that the desired nucleophile at the primer terminus is well positioned to react with the nucleotide, whereas bulk water is not, (ii) increasing

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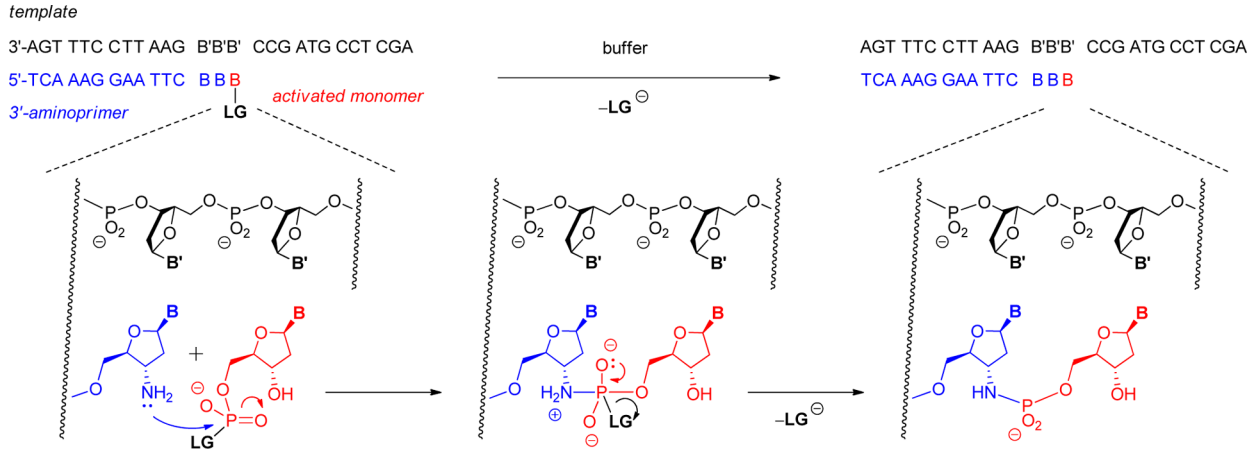
Scheme 1. Extension of a 3′-Aminoterminal Primer by an Activated Deoxynucleotide in a Representative Sequence Context[a]



[a]Stages of the proposed mechanism[7] are shown in the lower part. Bases = B, B′, LG = leaving group.

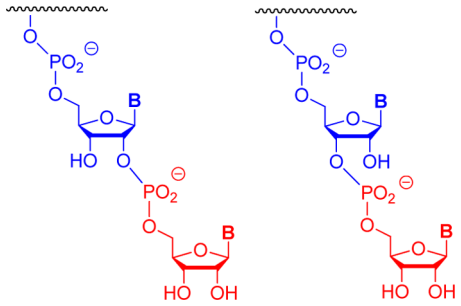


Figure 1. Regioisomeric chemical primer extension products for RNA.

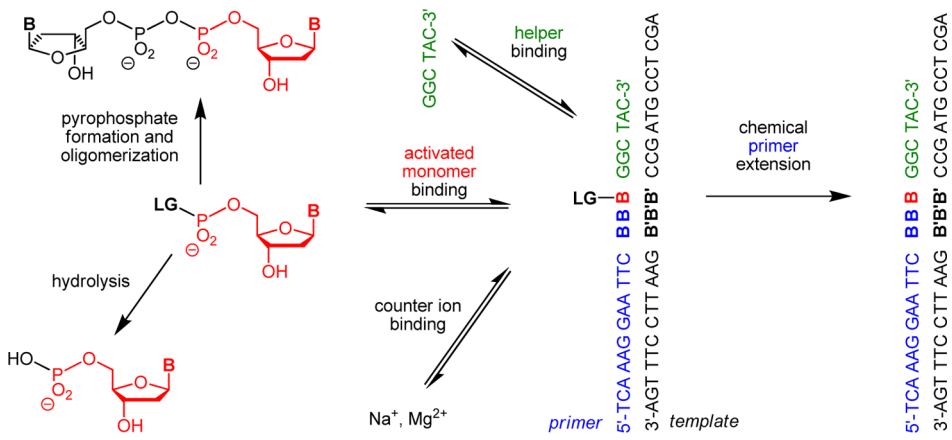
the absolute rate of primer extension through proper activation of the nucleotide, and (iii) actively countering the direct (hydrolysis) and indirect effect of hydrolysis of activated nucleotides (inhibition). We focus on two systems most frequently studied in our laboratory – RNA (Figure 1) and DNA with a 3′-terminal 3′-amino-2′,3′-dideoxynucleoside primer (Scheme 1). We will largely ignore the effect of sequence on rates, as well as the issue of sequence fidelity, focusing instead on yields. This Synopsis is not a review, and we apologize for being unable to do the work of others in the field justice.

STRENGTHENING THE TEMPLATE EFFECT

In an aqueous buffer, the binding equilibrium between an activated nucleotide at millimolar concentration and a primer/template duplex at micromolar concentration will lie predominantly on the unbound side. Strongly pairing nucleotides that form three hydrogen bonds with the template base (G and C) bind more strongly than those that form only two hydrogen bonds upon base pairing (A and T/U). Purines (A and G) stack more strongly than pyrimidines (C and T/U). So, G is incorporated most readily, and much of the early work on enzyme-free copying has focused on C-rich templates.[15,19,37−42] Unfortunately, semiconservative replication is not feasible when more than 50% of one specific base is required in a genetic sequence.[19,43] Other means must be used instead of limiting oneself to a C-rich template to achieve replication.

Effect of the Helix Conformation. One way to influence the template effect is to switch between A-type and B-type conformation.[44] Helix geometry affects the position of nucleophile and electrophile. Szostak et al. showed that extensions of aminoterminal primers occur faster on RNA, LNA,[45] and *O2′→P5′*-linked DNA templates than on DNA,[6,46] confirming results by Orgel and Göbel that suggest that A-type helices better support copying.[17,18,41,42,47] An NMR study

Scheme 2. Reaction Pathways and Binding Equilibria for an Activated Nucleotide in Chemical Primer Extension with a "Helper" Strand[3,7,20−22]



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indicated that nucleotides can change from C2′-endo to C3′-endo conformation upon binding to RNA.[48]

Modified Nucleobases. Another way to improve yields is to substitute weakly pairing nucleobases with more strongly pairing analogues, and base analogues were used by Orgel,[49] Göbel,[50] Switzer,[51] and Szostak.[6] For example, replacing adenine with diaminopurine leads to base pairs with three hydrogen bonds.[6,49−51] Replacing thymine or uracil with 5-propynyluracil provides a larger stacking surface.[6,51]

Effect of Salts. An increase in salt concentration stabilizes duplexes and thus the template effect, and typical chemical extensions are performed at high salt concentrations. Polymerases use magnesium ions, and most buffers for chemical primer extension also contain Mg^{2+}. Softer divalent cations have also been tested.[14,35,36,52] Göbel and co-workers showed that low yields with guanosine-rich templates are caused by quadruplex formation that can be avoided by using Li^+ cations.[17,47]

Helper Oligonucleotides. In our work, we asked whether the template effect can be strengthened through a third strand that binds downstream of the templating base (Scheme 2). The downstream-binding oligonucleotide or "helper oligonucleotide" offers additional stacking surface to the incoming nucleotide and should help to shield the electrophilic center from water. Helpers were found to increase rates (approximately 3-fold) as well as the fidelity of chemical extensions.[3,7,20] Well-pairing monomers can bind cooperatively to templates,[48,53−55] accelerating primer extensions,[46] and probably providing a helper-like effect. We extended the concept to "micro helpers", i.e., short sequences that can be replaced or washed away, so that several extension steps can benefit from a helper effect.[21,22]

Temperature. Lowering the temperature shifts the binding equilibrium between nucleotides and primer:template duplex to the bound side. There are suggestions that arctic conditions could have favored the origin of life.[56] Unfortunately, lower temperatures also reduce the absolute rate of reactions, which is particularly undesirable for RNA-based extension assays that typically run on the time scale of weeks. Therefore, more reactive nucleotide monomers were called for.[20]

■ ACTIVATING NUCLEOTIDES

Nature uses triphosphates as building blocks for polymerase-catalyzed extensions. They are kinetically stable but liberate pyrophosphate readily in the active sites of enzymes.[57] Triphosphates are unreactive in chemical primer extension reactions, even in the presence of divalent cations. Organic leaving groups are being employed instead. Early studies used monomers activated in situ through carbodiimides.[12] In 1966, water-soluble carbodiimides were shown to induce the ligation of hexanucleotides (T_6) on a polydeoxyadenosine template in up to 5% yield.[58] Sulston and Orgel similarly activated adenosine-5′-monophosphate (AMP) in the presence of polyuridylic acid and observed an AMP dimer in 10% yield.[12] Whether an initial carbodiimide adduct or a species formed from it was the kinetically relevant electrophile is not known. Later work showed that side reactions include formation of dinucleosidic pyrophosphates,[12,34] hydrolyzed monomers,[53,59] and accelerated hydrolysis of the condensing agents in the presence of phosphates.[60,61] Oró achieved the nontemplated formation of pentanucleotides using cyanamide and montmorillonite,[62] but preactivated nucleotides gave considerably higher yields and quickly gained the upper hand, both for template-directed[13,19] and nontemplate-directed oligomerizations.[19,63,64]

Preactivated Nucleotides. A brief account of how activated nucleotides can be prepared can be found in ref 72. The most frequently used reaction to produce active amides of the nucleotides is a Mukaiyama redox condensation[65] with PPh_3 and dipyridyl disulfide as condensing agents, followed by precipitation from acetone saturated with sodium perchlorate.[66] Other methods for the formation of active amides include the reaction of a nucleotide with 1,1′-carbonyldiimidazole[67] and reactions of nucleosides with phosphoryl chloride in the presence of appropriate heterocycles.[6,46,68]

Imidazole (**Im**, Figure 2) as leaving group has dominated the field of activated nucleotides for template-directed enzyme-free

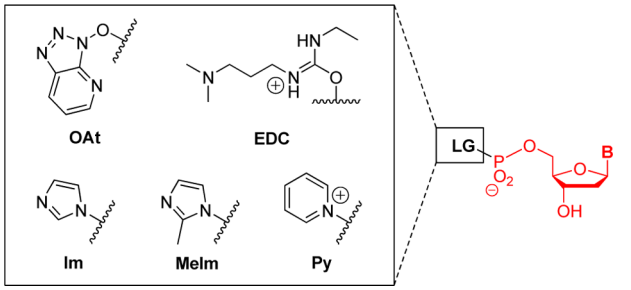

Figure 2. Structures of activated 2′-deoxynucleotides. Possible leaving groups are shown, including a postulated 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (**EDC**) adduct and pyridinium (**Py**) intermediate.

reactions. While not a good leaving group itself, protonation of N3 of imidazole produces a sufficiently reactive "active amide". Early work employed phosphorimidazolides of nucleosides with an unsubstituted heterocyclic ring.[13] Yields of 44% of AMP dimer[13] and up to 40mers of GMP were detected in the presence of poly(U) and poly(C), respectively.[14] The 2-methylimidazolides (**MeIm**, Figure 2) became most popular, though, because they produce long RNA oligomers with the desired *O3′→PS′* connectivity in the presence of Mg^{2+} as divalent cation.[15] It was with 2-methylimidazole as leaving groups that the first mixed sequences were copied, so that a nonhomopolymeric sequence was formed as daughter strand through selection from a mixture of nucleotides.[16] The results led to proposal that templates need to contain at least 60% C residues to support successful copying.[19,43]

When our group entered the field, we initially used 2-methylimidazolides of 2′-deoxynucleotides (**MeIm**),[69] together with primers terminating in a 3′-amino-2′,3′-dideoxynucleoside (Scheme 1), so that 2′/3′-regioselectivity was a moot point. We screened a range of possible methods for preactivating deoxynucleotides, including EDC-activation,[64] redox condensation,[65,66] and activation with HATU [*O*-(7-azabenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate].[70] We focused on the rate of primer extension because we were interested in employing chemical primer extension for genotyping and eventually for nonenzymatic sequencing, where rapid conversion is critical. Surprisingly, "uronium salts" that had been developed for activating amino acid building blocks in peptide synthesis gave the most promising results. With HATU as activating agent, 1-hydroxy-7-azabenzotriazole esters[71] of nucleotides (**OAt**, Figure 2) were obtained in high yield and were found to react approximately 5-fold faster than 2-methylimidazolides (**MeIm**).[3,72] When combined

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with helper oligonucleotides at pH 8.9, any of the four nucleotides (dAMP, dCMP, dGMP, and TMP) gave quantitative and sequence-selective incorporation with a half-life time of the aminoterminal primer <2.5 h.[3] The ability of the OAt leaving group to tolerate a basic pH probably helped, keeping more of the 3′-amino group of the primer in the unprotonated form.

After an unexpected acceleration upon addition of organic solvents to an assay solution, we then studied a range of different heterocycles as possible covalent catalysts for OAt ester-based primer extensions. Pyridine showed the strongest effect, most probably because it forms a pyridinium phosphate (**Py**, Figure 2) that is more reactive than the OAt ester but stable enough not to be hydrolyzed instantaneously. At 300 mM pyridine, near-quantitative conversion of primers was observed within seconds or minutes.[4] The Szostak group also employed what we believe to be a covalent catalyst, namely 1-hydroxyethylimidazole in their subsequent work.[5,6,46]

Using OAt esters, we were able to show that all-RNA copying systems do overcome the barrier[19] that weakly pairing template sequences, such as AA or AAA, pose.[21] Assays involving elongation by weakly pairing UMP/*r*TMP were successfully performed at −20 °C, a temperature leading to prohibitively slow reactions with 2-methyimidazolides at the modest concentrations favored by us. Again, high-yielding reactions were most easily induced when "helper oligonucleotides" were employed.

In Situ Activation. We then revisited in situ activation, both because it gives a simpler experimental protocol (a plus for diagnostic applications) and because it had the potential to give higher yields because hydrolyzed monomers could be reactivated after suffering hydrolysis. Again, we focused on individual elongation steps of deoxynucleotides with an aminoterminal primer. We were able to find a combination of EDC and 1-methylimidazole that gave full conversion of the primer at just 0.5 M monomer for any of the four bases (N = A, C, G, and T).[8] Similar reaction conditions were recently employed by us to label microRNAs on microarrays with high sequence selectivity.[73]

Labeling. Both enzyme-free extension and ligation have proven useful for detecting or reading out nucleic acid sequences.[73−76] Nucleotides suitable for labeling with fluorophores or biotin are shown in Figure 3.

OVERCOMING INHIBITION

With proper activation chemistry, high-yielding chemical primer extensions can be induced both for aminoterminal DNA primers and RNA primers. In a detailed study involving tens of thousands of mass spectra, we were able to show that for the former case, rates between the poorest and the best template sequences (as defined by the 64 possible combinations of templating base and upstream and downstream flanking nucleotide) differ by less than 2 orders of magnitude.[7] Mechanistic details that had remained enigmatic (fast reactions with monoexponential, slow reactions with biphasic kinetics) could be explained with a model that takes binding equilibrium, deprotonation, nucleophilic attack, and pseudorotation/leaving group expulsion into account.[7] Mass spectrometric monitoring with isotopic resolution showed very clean conversions with minimal side reactions, despite the plethora of nucleophilic sites that primers offer. For the consequences of poor sequence selectivity, a problem that will not be covered in this Synopsis,

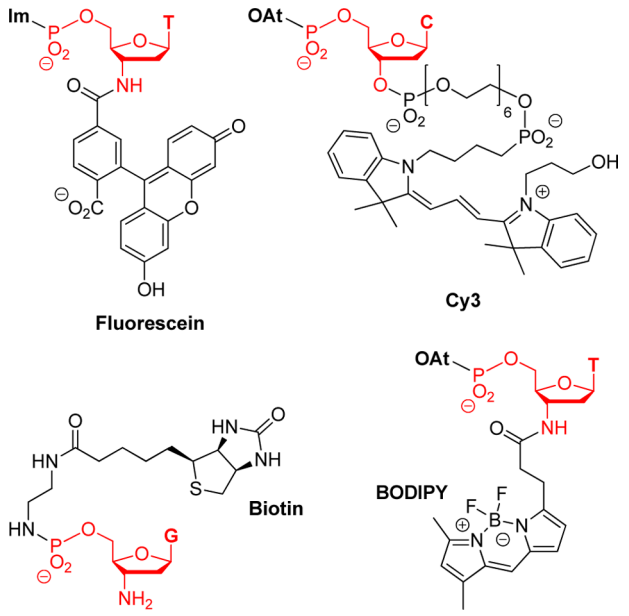

Figure 3. Structure of nucleotides developed for read-out of genetic information. Nucleotides labeled with fluorescein,[74] cyanine dyes, like Cy3,[75] or BODIPY dyes[76] may be used for direct optical read-out. Biotin-bearing nucleotides[73] require staining with labeled streptavidin.

Chen et al. found "stalling" after misincorporation.[9,77] But, one problem stubbornly remained: atom economy.

Typical assays with aminoterminal primers use low millimolar concentrations of nucleotides, meaning that 100 equiv or more of the activated nucleotide is used to induce full conversion. For RNA, monomers are at least 20 mM, again with primer concentrations in the μM range. Further, multiple extensions in a row are difficult to induce on templates with mixed sequences,[6,46] if one wants to read out sequence in the process, meaning that every step has to occur in controlled fashion, with read-out in between.[11,75] In situ activation with high carbodiimide concentration is an option, but the potential for side reactions (carbodiimde adducts, acylation products resulting from traces of carboxylic acids, pyrophosphates etc.) and the slow rates (activation and primer extension have different pH optima, the compromise pH slows down either) are a concern. High monomer concentrations are costly, particularly when labeled nucleotides are used (Figure 3), and can cause problems with unspecific adsorption and unwanted reactivity on beads and microarray surfaces.[76] Whatever was subduing a good part of the intrinsic reactivity of the monomers that had to be overcome!

Despite an immense body of literature on prebiotic chemistry,[23] no experimental proof existed of what "invisible hand" was making chemical copying inefficient. With other side reactions minimized (pyrophosphate formation and other side reactions of monomers can be detected by NMR), it was likely that this was, in fact, hydrolysis. Hydrolysis rates of activated monomers had been measured,[3,22,59] but, being on an "hours to days" time scale, depending on temperature and salt content, they were not sufficient to explain incomplete conversions in RNA-based systems.[19,37−40] We and others suspected that the hydrolyzed monomers actively inhibited primer extensions by acting as competitive inhibitors at the primer extension site (Figure 4),[8,21,54] but no proof existed until this effect was demonstrated experimentally for RNA.[22] The clear drop in yield upon spiking assay solutions with unactivated nucleotide

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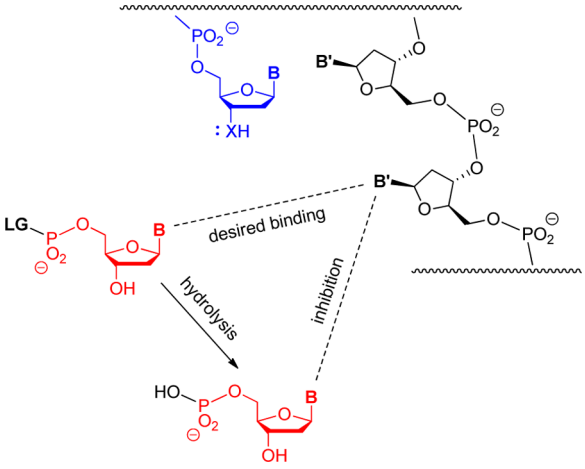


Figure 4. Inhibitory effect of hydrolyzed monomers on chemical primer extension.

showed that hydrolysis not only reduced the concentration of the electrophile but also produced a competitive inhibitor that further slowed extension as time progressed, often stalling the reaction before complete conversion had occurred, even at high initial concentrations of monomers.[22]

Two approaches presented themselves as options for overcoming hydrolysis: efficiently reactivating in situ or periodically removing the spent monomer and replacing it with active monomer. The former is currently being revisited by us for RNA, after what we consider a successful implementation for amino-terminal DNA.[8] The latter became feasible after suitable immobilization methodologies were found.

Immobilization. For RNA, finding a support proved reasonably straightforward. Conventional streptavidin-coated magnetic beads, combined with biotin-bearing oligodeoxynucleotides that act as "capture strands" and hybridize to free template regions of template/primer duplexes gave a high-yielding primer extension system.[22] It was with this system that the inhibitory effect of spent monomers was first overcome.[22] When supernatants containing active monomers and micro-helpers were replenished periodically, near-quantitative conversion was observed for any of the four different nucleobases (A/C/G/U). Assays spiked with increasing concentrations of the inhibitor (unactivated nucleotides) showed the decrease in yield mentioned above.[22]

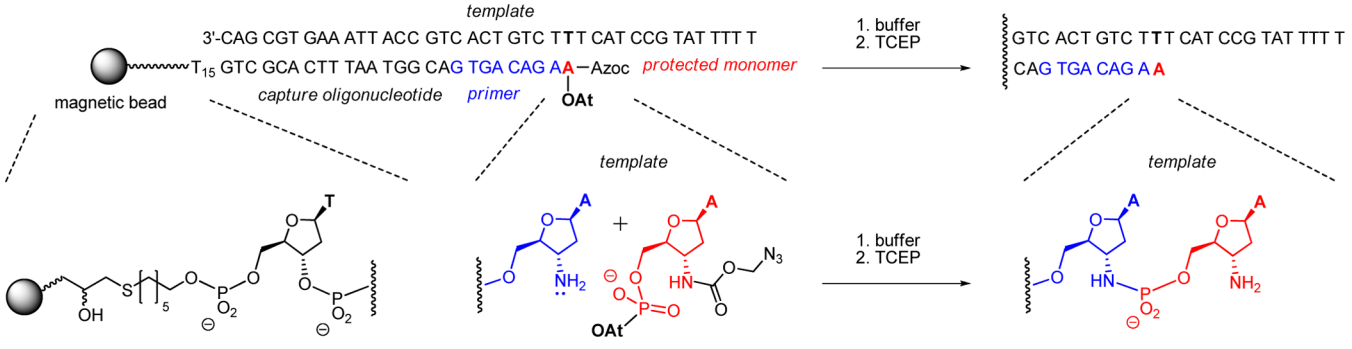
Establishing a solid-phase-based system for aminoterminal DNA was more challenging. One problem was identifying beads whose surface coating did not react with amino groups of primers under extension conditions. Streptavidin and a number of other surface chemistries did not fulfill this criterion,[11] leading to progressive loss of signal when samples were denatured in the heat and primers were detected by MALDI-TOF MS.[78] Epoxy-terminated magnetic beads were most promising, but early experiments were plagued by low reactivity (resulting in low loading) and difficulties in finding a good supplier. Even more challenging was developing protected aminonucleotide monomers whose protecting group is cleavable under nondenaturing conditions. Scheme 3 shows the system that led to a break-through after approximately 10 man-years of experimental work.[11]

Reversible Termination. In the sequencing field, the approach of using protecting groups to limit primer extension to individual steps is called "reversible termination". In our case, both 3′-azides as latent amines and amines with photolabile protecting groups[4,75] were tested. Either had too high a potential for side reactions or incomplete conversion to be useful for multiple rounds of extension. Only when Winssinger's azidomethyloxycarbonyl (Azoc) protecting group[79] was adapted for aminonucleotides did we see near-quantitative yields for extension *and* deprotection. Protected monomers (Scheme 3) became accessible in three steps[11] after a reagent was developed for introducing the Azoc group in one step.[80] Assays relying on such monomers and tris(carboxyethyl)-phosphine (TCEP) as deprotection reagent allowed for controlled, 10-fold extension of aminoterminal primers, both in the conventional 3′-to-5′ direction and in 5′-to-3′ direction.[11] The magnetic support facilites handling and monitoring by mass spectrometry allows for miniaturized assays.[11,22] Any of the four bases (A/C/G/T) is incorporated opposite its complementary base in the template within 12 h at room temperature. The length of sequence that can be read out is currently limited by the mass spectrometric detection method, not the extent to which side reactions occur.

■ CONCLUSIONS AND PERSPECTIVES

Some problems of enzyme-free copying of nucleic acid sequences have been addressed. The direct and indirect effects of hydrolysis of monomers now look more manageable, and no fundamental barriers to successful copying look insurmountable. But, a very substantial amount of work remains to be done

Scheme 3. Template-Directed Solid-Phase Synthesis on Support via Extension and Deprotection[a]



[a]The capture oligonucleotide is linked to magnetic beads by reacting thiols with surface-bound epoxides. The template/primer duplex reacts with Azoc-protected monomers, followed by deprotection with tris(carboxyethyl)phosphine (TCEP).[11]

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before artificial replication systems or enzyme-free sequencing based on chemical primer extension become a reality. Among the issues that we hope to address in coming years are establishing assays for successive rounds of copying to better mimic replication, monitoring sequence drift and sequence evolution, and realizing in situ activation-based assays in RNA systems. At the same time, we are continuing our mechanistic studies, with one focus on better quantifying the template effect as well as the inhibitory effect of unactivated monomers. Progress in these areas will be reported in due course.

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Notes

The authors declare no competing financial interest.

Biographies

Andreas Kaiser obtained his diploma in Chemistry from the University of Karlsruhe (TH). He will soon complete his Ph.D. work in Clemens Richert's laboratory at the University of Stuttgart.

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