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Enzymatic Incorporation of LNA Nucleotides into DNA Strands

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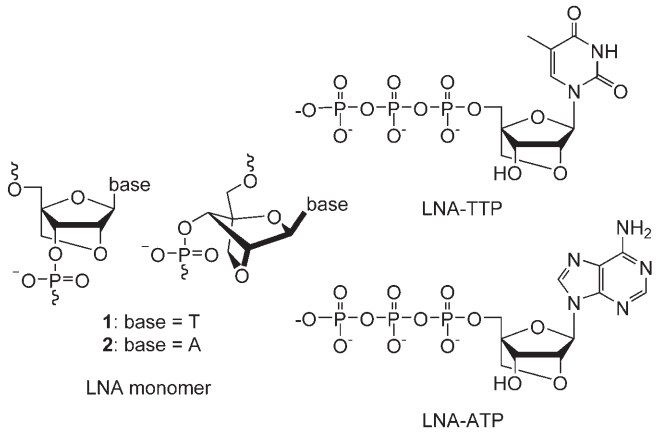
Oligonucleotides composed of naturally occurring nucleic acids are unsuitable for many therapeutic and biotechnological applications because of their poor biostability. Numerous nucleic acid analogues have therefore been synthesized for applications related to target-validation studies or therapeutic molecules.[1,2] Locked nucleic acid (LNA) nucleotide monomers are nucleic acid analogues with a fixed C3′-*endo*/N-type furanose conformation that mimics the ribose conformation of A-type helical RNA (Scheme 1).[3–5] LNA-modified DNA/RNA oligonucleotides display unprecedented hybridization affinities towards complementary single-stranded RNA and single- or double-stranded DNA, and improved mismatch discrimination compared to natural nucleic acids.[3–7] Moreover, LNA-modified oligonucleotides are characterized by high biostability, low toxicity in biological systems and good aqueous solubility, and they have shown their significance in antisense studies both in vitro and in vivo.[8,9]



Scheme 1. Structural representations of LNA monomers and LNA nucleoside 5′-triphosphates.

The remarkable properties of LNA-modified oligonucleotides led us to synthesize LNA nucleoside 5′-triphosphates and evaluate these as substrates for different polymerases. So far, synthesis of LNA-modified oligonucleotides has been achieved by standard machine-aided automated DNA synthesis. DNA/RNA polymerases incorporate their natural substrates with high specificity and fidelity; however, different research groups have shown that polymerases can also accept a range of sugar-modified nucleotides as substrates.[10–21] Although LNA monomers structurally mimic RNA, they mediate high-affinity recognition of complementary RNA and DNA as constituents of either RNA or DNA strands. We therefore decided to study the enzymatic incorporation of LNA monomers into both RNA (transcription experiments) and DNA (primer extension assays) strands by using a variety of DNA/RNA polymerases. We have so far successfully achieved template-directed enzymatic synthesis of DNA strands containing LNA nucleotides, and our preliminary results in this direction are reported herein.

The LNA nucleotides LNA-TTP and LNA-ATP (Scheme 1) were synthesized by minor modification of a published procedure for nucleotide synthesis.[22] We designed appropriate primer and template sequences for primer extension assays by using DNA polymerases to extend a DNA strand. The primer sequences were 5′-labelled by treatment with [γ-^{32}P]-ATP. End-labelled primers were then annealed to their templates and extended by incubation at 72 °C for 2 h by using Phusion high-fidelity DNA polymerase (Finnzymes). In addition, the following enzymes were tested for LNA nucleotide incorporation: *Taq* DNA polymerase (Ampliqon), Klenow Enzyme, large fragment (New England Biolabs), T4 DNA polymerase (New England Biolabs), *pfu* DNA polymerase (Invitrogen), *pfx* DNA polymerase (Invitrogen), Speed STAR HS DNA polymerase (TaKaRa Bio), T7 RNA polymerase (Promega), *E. coli* RNA polymerase (Sigma), AMV reverse transcriptase (Finnzymes) and mutant T7 R&DNA polymerase (Epicenter). Phusion high-fidelity DNA polymerase was the only enzyme found to be efficient for the incorporation of LNA nucleotides.

We first tested the incorporation of LNA monomers **1** and **2** separately. All experiments included positive and negative control reactions in parallel to the reactions involving the incorporation of LNA monomers. The positive control mixture consisted of all four natural dNTPs and, hence, resulted in extension to full length. The negative control mixture lacked the nucleoside triphosphates similar to the LNA nucleotides that were to be tested for enzymatic incorporation, and thus contained three (incorporation of LNA monomers **1** or **2**) or two (in the case of incorporation of both **1** and **2**) natural nucleoside triphosphates. Thus, the negative control experiments were expected to result in extension only up to the nucleotide before the first site of LNA incorporation.

Template 1, designed to study incorporation of LNA-T monomer **1**, contains three incorporation sites opposite DNA-A monomers at positions 37, 39 and 41; template 2 was designed to study incorporation of LNA-A monomer **2**, and contains two incorporation sites at positions 38 and 41 (Table 1). The experiments showed that Phusion high-fidelity DNA polymerase efficiently extended the primers to full length (Figure 1). Template 3 was designed to enforce incorporation of first three LNA-T monomers **1** and then three LNA-A monomers **2** into

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Table 1. Primer and template sequences used for studying enzymatic incorporation of LNA nucleotides **1** and **2.**

		Length
Incorporation of LNA-T, **1**	5′-TAATACGACTCACTATAGG-3′	19
Primer template 1	3′-ATTATGCTGAGTGATATCCGTTCTGCCTTTCTGGGG**A**^{37}T**A**G**A**CGG-5′	44
Incorporation of LNA-A, **2**	5′-TAATACGACTCACTATAGG-3′	19
Primer template 2	3′-ATTATGCTGAGTGATATCCGACCGGCGACGCGCCAGG**T**^{38}AC**T**G-5′	42
Multiple incorporation of **1** and **2**	5′-TAATACGACTCACTATAGG-3′	19
Primer template 3	3′-ATTATGCTGAGTGATATCCGGGGCCG**A**^{27}CCC**A**C**A**CC**T**GG**T**C**T**GG-5′	43
Successive incorporation of LNA-T, **1**	5′-TAATACGACTCACTATAGG-3′	19
Primer template 4	3′-ATTATGCTGAGTGATATCCGGGGCCC**A**^{27}**AAAAAAA**TGGTCGCC-5′	42
Successive incorporation of LNA-A, **2**	5′-TAATACGACTCACTATAGG-3′	19
Primer template 5	3′-ATTATGCTGAGTGATATCCGGGGCCG**T**^{27}**TTTTTTT**AGACGG-5′	40
Successive multiple incorporation of **1** and **2**	5′-TAATACGACTCACTATAGG-3′	19
Primer template 6	3′-ATTATGCTGAGTGATATCCGGGGCCG**T**^{27}**TTAATAA**GCGCC-5′	39

[a] The sites of the incorporation of LNA nucleotides are underlined in the template sequences, and the first site of incorporation is indicated by the corresponding position number next to the nucleotide in the template strands.

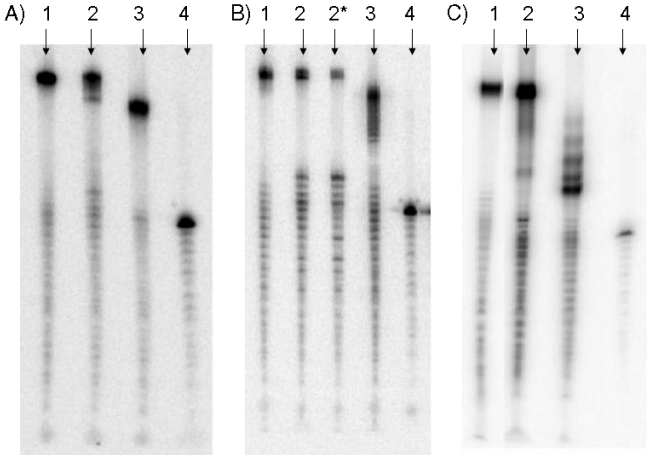

Figure 1. Enzymatic incorporation into DNA strands of A) LNA-T nucleotide **1**, B) LNA-A nucleotide **2**, and C) both **1** and **2**. Bands 1: positive control using all four natural deoxynucleotides (0.5 mM of each), bands 2: incorporation of LNA nucleotides (natural nucleotide was replaced with analogous LNA-modified nucleotide (5 mM of all nucleotides)), band 2*: as band 2 but three times less concentrated in nucleotides, bands 3: negative control reactions with only three (A and B) or two (C) nucleotides present, bands 4: primer strand.

corporation of eight continuous LNA nucleotides in the primer strand (Table 1, templates 4, 5 and 6). These experiments showed that the enzyme is able to incorporate up to three LNA-T nucleotides **1** successively. However, in the case of LNA-A nucleotide **2**, all eight modified nucleotides were incorporated, whereupon no further extension was observed (Figure 2). The preferential incorporation of LNA-A relative to LNA-T

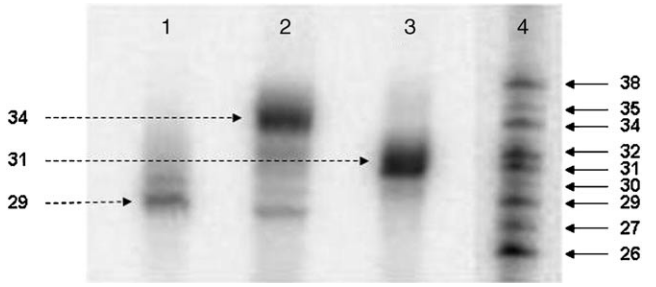

Figure 2. Enzymatic incorporation into DNA strands of contiguous stretches of 1: LNA-T nucleotide **1**, 2: LNA-A nucleotide **2**, and 3: both **1** and **2**. Band 4: 5′-^{32}P end-labelled DNA strands of the indicated lengths used as markers. Template and primer sequence are listed in Table 1.

one DNA strand (Table 1). Primer extension to full length was again observed; this demonstrated the capability of the enzyme to incorporate two different LNA-modified nucleotides in one reaction to lead to a substantially LNA-modified DNA strand. Elongation to full length was not observed for template 1 in the presence of only LNA-T triphosphate, dCTP and dGTP, or for template 3 in the presence of only LNA-A triphosphate, dCTP and dGTP (data not shown). Chain extension was arrested at the expected positions, and is thus the first indication of satisfactory fidelity of LNA incorporation.

Multiple and successive incorporation of LNA nucleotides **1** and **2** was likewise attempted by using Phusion high-fidelity DNA polymerase. For these experiments, we designed template DNA strands containing segments that encoded for in-

might be due to the relatively stronger base–base stacking interactions of the former. In the case of attempted incorporation of a mixed contiguous segment of LNA nucleotides **1** and **2**, it was observed that the enzyme could incorporate up to five LNA nucleotides successively (Figure 2). However, this number might be sequence dependent.

In all primer-extension experiments, the reaction mixture was supplemented with $MnCl_2$, as Mn^{2+} is known to increase the tolerance of polymerases for the incorporation of nucleotides with modifications in the furanose ring, the base moiety or the phosphate linkage.[17,18,23–25] Betaine enhancer solution was also added as it is known to be an effective additive for templates that are difficult to amplify.[26] In this preliminary study we used 100 mM LNA 5′-triphosphates, which corre-

sponds to a tenfold excess relative to the concentration used for the natural deoxynucleoside 5′-triphosphates. For full-length products, we observed comparable synthesis efficiencies for LNA-modified and unmodified strands.

This is the first report on efficient enzymatic synthesis of LNA-modified DNA strands by using Phusion high-fidelity DNA polymerase and LNA-T and LNA-A nucleoside 5′-triphosphate substrates. Primer extension to full length was achieved for sequences with mixed DNA/LNA compositions. Furthermore, incorporation of up to eight contiguous LNA-A nucleotides was achieved. These preliminary results demonstrate that Phusion high-fidelity DNA polymerase, contrary to all other polymerases tested, readily accepts LNA 5′-triphosphates as substrates. Further studies involving LNA-modified primers and templates are underway.

Experimental Section

Synthesis of LNA 5′-triphosphates: LNA nucleoside 5′-triphosphates **1** and **2** were synthesised according to the one-pot synthesis method described by Ludwig.[22] After the addition of tributylammonium pyrophosphate, the reaction mixture was stirred for 2.5 h before the reaction was quenched by the addition of triethyl ammonium bicarbonate. Purification and isolation involved gravity chromatography of the crude product on WHATMAN DEAE cellulose-D50 anion-exchange resin by eluting with an increasing concentration of triethyl ammonium bicarbonate in water. LNA nucleoside 5′-triphosphate **1**: ^{1}H NMR (200 MHz, D_2O): $\delta = 1.77$ (s, 3H; CH_3), 3.50 (m, 2H; 5′-Ha and 5′-Hb), 3.55 (dd, $J = 8$ Hz, 2H; 5″-Ha and 5″-Hb), 4.16 (s, 1H; 3′-H), 4.29 (s, 1H; 2′-H), 5.49 (s, 1H; 1′-H), 7.58 (s, 1H; 6-H); ^{31}P NMR (80 MHz, D_2O): $\delta = -4.95$ (d, γ-P), −10.65 (d, α-P), −22.17 (d, β-P); ESI-MS calcd for $C_{11}H_{16}N_2O_{15}P_3$: 508.968 $[M+H]^+$, found 508.938. LNA nucleoside 5′-triphosphate **2**: ^{1}H NMR (300 MHz, D_2O): $\delta = 3.86$ (dd, $J = 8.7$ Hz, 2H; 5′-Ha and 5′-Hb), 4.19 (m, 2H; 5″-Ha and 5″-Hb), 4.37 (s, 1H; 2′-H), 4.40 (d, $J = 5.1$ Hz, 1H; 3′-H), 5.88 (s, 1H; 1′-H), 7.98 (s, 1H; 2-H), 8.12 (s, 1H; 8-H); ^{31}P NMR (80 MHz, D_2O): $\delta = -7.73$–8.78 (m, γ-P), −10.62 (d, α-P), −22.37 (d, β-P); ESI-MS calcd for $C_{11}H_{15}N_5O_{13}P_3$: 517.990 $[M+H]^+$, found 518.001.

Primer extension assays: The DNA primer sequences were purchased from DNA Technology, and the template sequences from Sigma-Genosys. Phusion high-fidelity DNA polymerase was purchased from Finnzymes. The primer sequences were 5′-^{32}P labelled by treatment with [γ-^{32}P]-ATP (~6000 Ci mmol^{-1}, Amersham Biosciences) by using T4 polynucleotide kinase (New England Biolabs) according to manufacturers' recommendations. The 5′-labelled primers were annealed to the templates by combining primer and template in a molar ratio of 1:1 and heating the mixture to 80 °C for 2 min, followed by slow cooling to room temperature. The reaction mixtures were prepared in a total volume of 20 μL by adding a solution containing 5′-^{32}P-labelled primer template (0.6 μL, 5 pmol) complex, 5×Phusion HF buffer (4 μL; included in the polymerase kit), $MnCl_2$ (1 μL, 50 mM), betaine enhancer solution (1 μL, 2 M, Ampliqon), dNTP mixture (1 μL, 10 mM in each dNTP; for the preparation of nucleoside triphosphate mixtures containing LNA 5′-triphosphates, 100 mM of LNA triphosphates were used), Phusion high-fidelity DNA polymerase (1 μL, 2 U μL^{-1}) and doubly distilled water (11.6 μL). The reaction mixtures were gently vortexed and heated at 72 °C for 2 h. The polymerase reactions were quenched by the addition of a double volume of loading buffer (95% formamide, 0.05% bromophenol blue, 0.05% xylene cyanol and 20 mM EDTA). Analysis was performed by gel electrophoresis for 30 min on a 13% urea polyacrylamide gel (7 M) in the presence of a TBE buffer (100 mM Tris, 90 mM boric acid, 1 mM EDTA; pH 8.4). The products were visualized by phosphor imaging.

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