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Polymerase-directed synthesis of C5-ethynyl locked nucleic acids

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ABSTRACT

Modified nucleic acids have considerable potential in nanobiotechnology for the development of nanomedicines and new materials. Locked nucleic acid (LNA) is one of the most prominent nucleic acid analogues reported so far and we herein for the first time report the enzymatic incorporation of LNA-U and C5-ethynyl LNA-U nucleotides into oligonucleotides. Phusion High Fidelity and KOD DNA polymerases efficiently incorporated LNA-U and C5-ethynyl LNA-U nucleotides into a DNA strand and T7 RNA polymerase successfully accepted the LNA-U nucleoside 5′-triphosphate as substrate for RNA transcripts.

Functional nucleic acids have attracted considerable attention because of their potential applications in chemical biology, bioanalysis, nanotechnology, and material science.[1–4] A variety of chemically modified nucleotide monomers can be introduced into DNA or RNA to provide additional functions.[5] Locked nucleic acid (LNA) is one of the most prominent nucleic acid analogues reported in recent years.[6–10] The sugar ring in LNA is locked by a O2′–C4′-methylene linkage which conformationally restricts LNA monomers into an *N*-type sugar (Fig. 1).[6,7] LNA-modified oligonucleotides display extraordinary binding affinity towards complementary DNA or RNA and improved mismatch discrimination. Moreover, LNA-modified oligonucleotides are characterized by a high degree of nuclease resistance and low toxicity.[8,9]

Recently, Hrdlicka and co-workers reported the synthesis of C5-derivatized LNA-U monomers and their incorporation into DNA oligonucleotides via standard phosphoramidite chemistry using a DNA synthesizer.[11,12] Compared to corresponding conventional LNA, C5-ethynyl-modified LNA (Fig. 2) displays even more pronounced binding affinity toward RNA and double stranded DNA targets, improved mismatch discrimination and markedly enhanced stability against 3′-exonucleases. Enzymatic incorporation of nucleobase-modified nucleoside triphosphates is a particularly popular approach toward increasing the function of nucleic acids. In 1981 Langer et al. published the very first polymerase incorporation of base-modified dNTPs.[13] They prepared dUTP biotinylated at position 5 and found that it is a substrate for several DNA polymerases. Thereafter, several groups have investigated the polymerase incorporations of base modified dNTPs and some even went on to demonstrate their functional involvement in catalysis and binding.[14]

In the present work, we study the enzymatic incorporation of LNA-U and C5-ethynyl LNA-U nucleotides. For this purpose, we synthesized LNA-U and C5-ethynyl LNA-U nucleoside 5′-triphosphates (Fig. 2) from known intermediates[12,15] using protocols similar to those previously described[16] (see Supplementary data for details). To investigate the substrate specificity of LNA-UTP and C5-ethynyl LNA-UTP for polymerases, we designed primer and template DNA sequences to perform primer extension (DNA synthesis using DNA polymerase) and transcription reactions (RNA

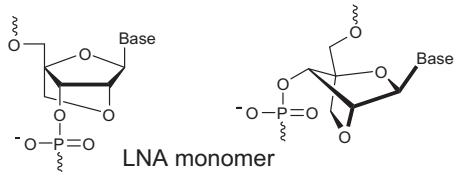

Figure 1. Structural representation of an LNA nucleotide monomer.

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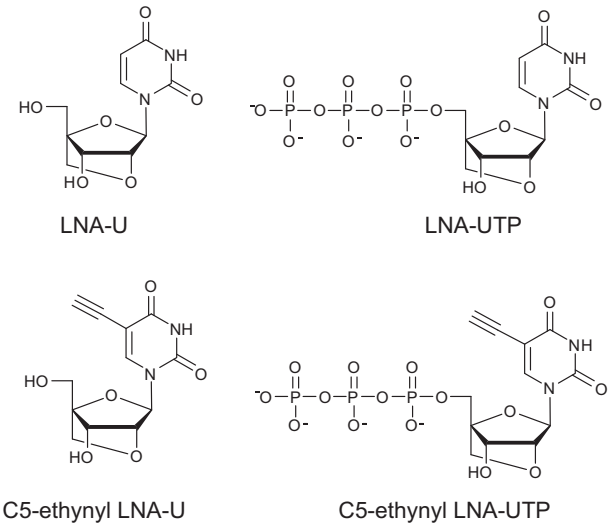

Figure 2. Structural representation of LNA-U and C5-ethynyl LNA-U nucleosides and their corresponding 5′-triphosphates.

synthesis using RNA polymerase) (see Supplementary data for details).

First, we tested LNA-U nucleoside triphosphate as a substrate for T7 RNA polymerase in transcription reactions in vitro. In this case, a T7 promoter DNA strand (P1, Fig. 3A) was annealed to its template (T1, Fig. 3A) and incubated at 37 °C together with T7 RNA polymerase in transcription buffer to synthesize a single stranded RNA product. CTP was complemented with (α-^{32}P) CTP in the rNTP mixture (see Supplementary data) and the products were analyzed by denaturing polyacrylamide gel electrophoresis (PAGE). The designed template (Fig. 3A) directs three incorporations of LNA-U nucleotides in the resulting 26nt long RNA transcript. The experiment revealed that T7 RNA polymerase readily incorporates LNA-U nucleotides at the expected positions to yield the desired full-length RNA transcript. Product degradation was observed both in the case of positive control and reaction involving LNA-U nucleotides. This may be due to RNases as reaction is performed overnight without the addition of RNase inhibitor.

Although LNA is considered an RNA mimic, it was reported that LNA nucleoside triphosphates can be accepted as substrates by DNA polymerases.[16–21] This motivated us to examine LNA-U nucleoside 5′-triphosphates as substrates for DNA polymerases. In this experiment, a 19nt long 5′-^{32}P labeled primer DNA (P1, Fig. 3B) was annealed to a 43nt long template DNA (T2, Fig. 3B) strand and extended using KOD DNA polymerase at 72 °C. Positive and negative control experiments were performed in parallel. The positive control reaction mixture includes all four natural dNTPs and full-length extension is observed as expected (lane 3, Fig. 3D). The negative control reaction mixture lacks dTTP, and extension is observed to stop at position 26nt (lane 2, Fig. 3D) as expected. The template is designed such that the first site of incorporation at position 27nt is opposite to a natural DNA-A nucleotide whereas the two remaining incorporations at positions 31 and 33nt are opposite to LNA-A nucleotides. KOD DNA polymerase successfully incorporated LNA-U nucleotides opposite to DNA-A and LNA-A of the template strand and afforded the desired full-length product (Fig. 3D).

Encouraged from our initial results we then investigated C5-ethynyl LNA-U nucleoside triphosphate as a substrate for the polymerases. First, we studied the incorporation into DNA oligonucleotides. The designed template sequence (T1, Fig. 4A) directs

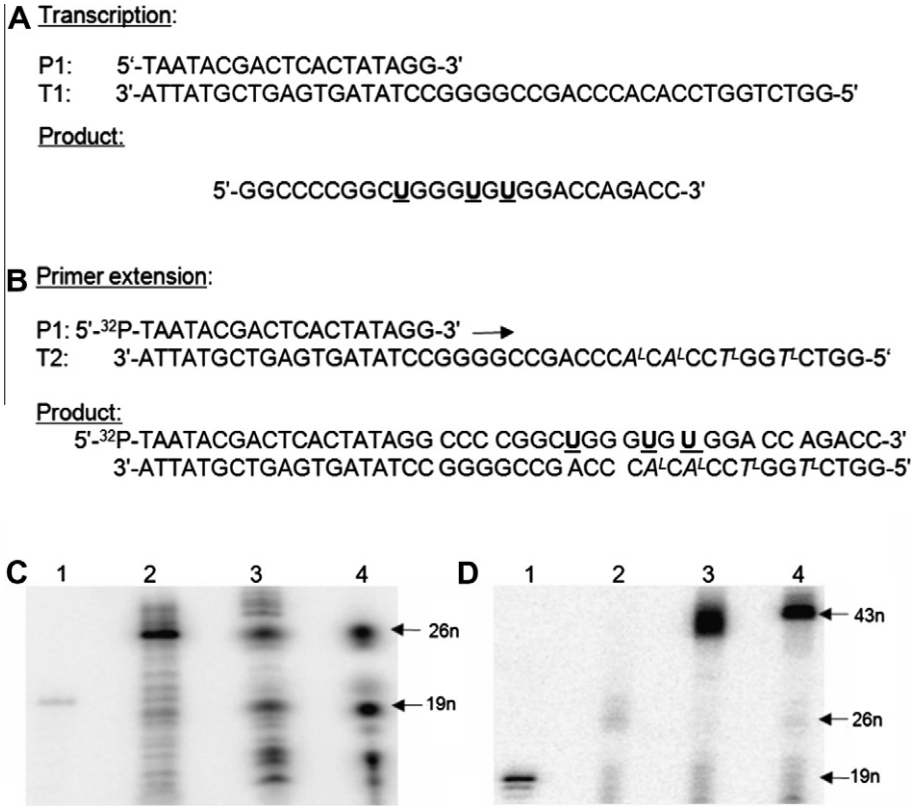

Figure 3. (A and C) T7 RNA polymerase catalyzed incorporation of LNA-U nucleotides into an RNA strand. Lane 1: primer P1; lane 2: 26nt RNA marker; lane 3: positive control (ATP, GTP, CTP, and UTP); lane 4: Incorporation of LNA-U nucleotides (ATP, GTP, CTP, and LNA-UTP). (B and D) Enzymatic incorporation of LNA-U nucleotides into a DNA strand using KOD DNA polymerase. Lane 1: primer P1; lane 2: negative control (dATP, dGTP, and dCTP); lane 3: positive control (dATP, dGTP, dCTP, and TTP); lane 4: incorporation of LNA-U nucleotides (dATP, dGTP, dCTP, and LNA-UTP). LNA-U nucleotides are denoted by the letter 'U' and represented in bold underlined.

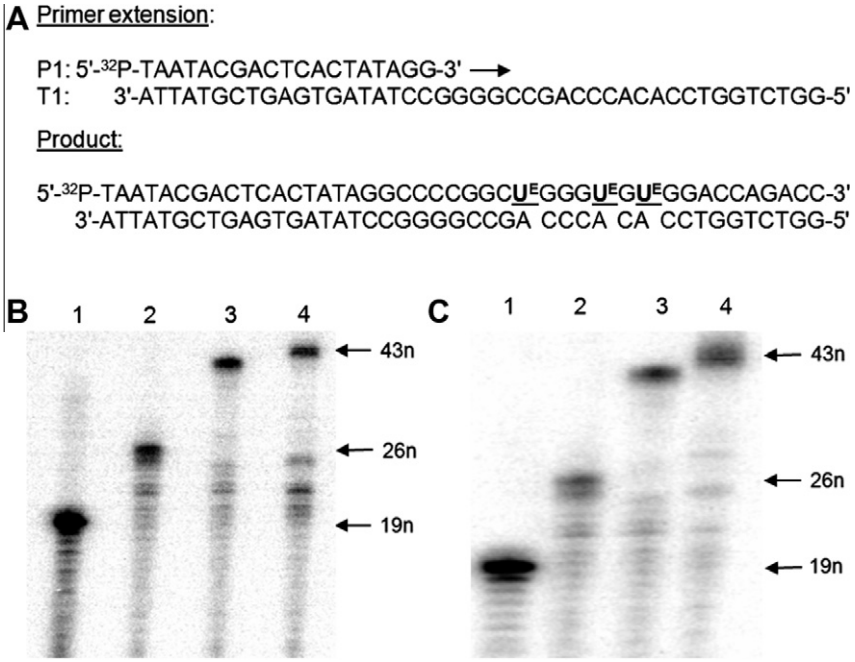

Figure 4. Enzymatic incorporation of C5-ethynyl LNA-U nucleotides into a DNA strand using Phusion High Fidelity DNA polymerase (A and B) and KOD DNA polymerase (A and C). Lane 1: primer P1; lane 2: negative control (dATP, dGTP, and dCTP); lane 3: positive control (dATP, dGTP, dCTP, and TTP); lane 4: incorporation of C5-ethynyl LNA-U nucleotides (dATP, dGTP, dCTP, and C5-ethynyl LNA-UTP). C5-ethynyl LNA-U nucleotides are denoted by the letter 'U^E' and represented in bold underlined.

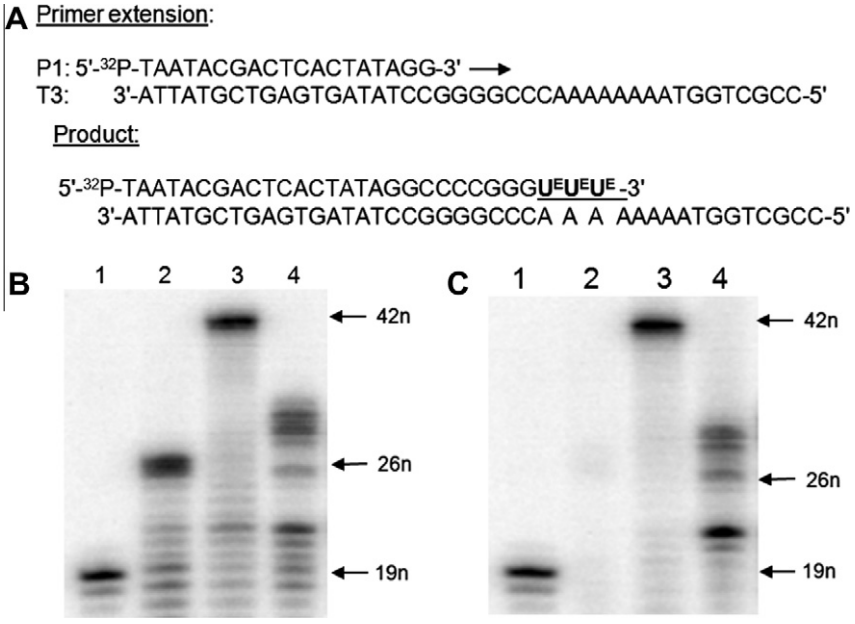

Figure 5. Enzymatic incorporation of successive C5-ethynyl LNA-U nucleotides into a DNA strand using Phusion High Fidelity DNA polymerase (A and B) and KOD DNA polymerase (A and C). Lane 1: primer P1; lane 2: negative control (dATP, dGTP, and dCTP); lane 3: positive control (dATP, dGTP, dCTP, and TTP); lane 4: incorporation of C5-ethynyl LNA-U nucleotides (dATP, dGTP, dCTP, and C5-ethynyl LNA-UTP. C5-ethynyl LNA-U nucleotides are denoted by the letter 'U^E' and represented in bold underlined.

three incorporations of C5-ethynyl LNA-U nucleotides at positions 27, 31, and 33nt, respectively. The data clearly demonstrate that both Phusion High Fidelity DNA polymerase and KOD DNA polymerase efficiently incorporate C5-ethynyl LNA-U nucleotide at the expected positions and furnishes the desired full-length products (Fig. 4B and C). The slight difference in mobility observed between the bands from the positive control reaction and the C5-ethynyl LNA-U reaction (lanes 3 and 4, respectively, Fig. 4B and C) is likely due to the slightly increased hydrophobic nature of the product of the latter reaction.

Next, we investigated the ability of DNA polymerases to catalyze successive incorporations of C5-ethynyl LNA-U nucleotides. For this experiment, the template (T3, 42nt, Fig. 5A) was designed to encode up to eight consecutive incorporations opposite to DNA-A nucleotides. This experiment showed that both Phusion High Fidelity DNA polymerase and KOD DNA polymerase failed to yield the full-length product, although both polymerases incorporated at least three consecutive C5-ethynyl LNA-U nucleotides (Fig. 5B and C). These findings are in line with our previous report on enzymatic incorporation of consecutive LNA-T nucleotides.[16]

We have also investigated the incorporation of C5-ethynyl LNA-U nucleotides into RNA strands using T7 RNA polymerase. The experiment revealed that unlike LNA-U nucleotides, T7 RNA polymerase failed to accept C5-ethynyl LNA-U nucleoside triphosphate as a substrate (data not shown).

In summary, we have synthesized LNA-U and C5-ethynyl LNA-U nucleoside 5′-triphosphates. Phusion High Fidelity DNA polymerase and KOD DNA polymerase efficiently utilized LNA-U and C5-ethynyl LNA-U as substrates in primer extension reactions yielding full-length products. In addition, LNA-U nucleoside triphosphate was found to be a substrate of T7 RNA polymerase. These findings will be useful in deriving functional nucleic acids such as aptamers with high stability and added functionality.

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Supplementary data

Supplementary data (protocols for synthesis of triphosphate precursors, primer extension reactions and transcription reactions) associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.09.044.

References and notes

1. Condon, A. *Nat. Rev. Genet.* **2006**, *7*, 565.
2. Alemdaroglu, F. E.; Herrmann, A. *Org. Biomol. Chem.* **2007**, *5*, 1311.
3. Feldkamp, U.; Niemeyer, C. M. *Angew. Chem., Int. Ed.* **2006**, *45*, 1856.
4. Wengel, J. *Org. Biomol. Chem.* **2004**, *2*, 277.
5. Weisbrod, S. H.; Marx, A. *Chem. Commun.* **2008**, *44*, 5675.
6. (a) Koshkin, A. A.; Singh, S. K.; Nielsen, P.; Rajwanshi, V. K.; Kumar, R.; Meldgaard, M.; Olsen, C. E.; Wengel, J. *Tetrahedron* **1998**, *54*, 3607; (b) Singh, S. K.; Nielsen, P.; Koshkin, A. A.; Wengel, J. *Chem. Commun.* **1998**, 455.
7. Obika, S.; Nanbu, D.; Hari, Y.; Andoh, J.; Morio, K.; Doi, T.; Imanishi, T. *Tetrahedron Lett.* **1998**, *39*, 5401.
8. Wengel, J. *Acc. Chem. Res.* **1999**, *32*, 301.
9. Jepsen, J. S.; Sørensen, M. D.; Wengel, J. *Oligonucleotides* **2004**, *14*, 130.
10. (a) Veedu, R. N.; Wengel, J. *Chem. Biodivers.* **2010**, *7*, 536; (b) Veedu, R. N.; Wengel, J. *RNA Biol.* **2009**, *6*, 321.
11. Sau, S. P.; Kumar, P.; Anderson, B. A.; Østergaard, M. E.; Deobald, L.; Paszczynski, A.; Sharma, P. K.; Hrdlicka, P. J. *Chem. Commun.* **2009**, 6756.
12. Østergaard, M. E.; Kumar, P.; Baral, B.; Raible, D. J.; Kumar, T. S.; Anderson, B. A.; Guenther, D. C.; Deobald, L.; Paszczynski, A. J.; Sharma, P. K.; Hrdlicka, P. J. *ChemBioChem* **2009**, *10*, 2740.
13. Langer, P. R.; Waldrop, A. A.; Ward, D. C. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6633.
14. (a) Gebeyehu, G.; Rao, P. Y.; Soon Chan, P.; Simms, D. A.; Klevan, L. *Nucleic Acids Res.* **1987**, *15*, 4513; (b) Switzer, C. Y.; Moroney, S. E.; Benner, S. A. *J. Am. Chem. Soc.* **1989**, *111*, 8322; (c) Piccirilli, J. A.; Krauch, T.; Moroney, S. E.; Benner, S. A. *Nature* **1990**, *343*, 33; (d) Petrie, C. R.; Adams, A. D.; Stamm, M.; Van Ness, J.; Watanabe, S. M.; Meyer, R. B., Jr. *Bioconjugate Chem.* **1991**, *2*, 441; (e) Latham, J. A.; Johnson, R.; Toole, J. J. *Nucleic Acids Res.* **1994**, *22*, 2817; (f) Dewey, T. W.; Mundt, A. A.; Crouch, G. J.; Zyznewski, M. C.; Eaton, B. E. *J. Am. Chem. Soc.* **1995**, *117*, 8474; (g) Nieuwlandt, D.; Wiegand, T. W.; Janssen, R. C.; Eaton, B. E. *Chem. Biol.* **1997**, *4*, 675; (h) Tarasow, T. M.; Tarasow, S. L.; Eaton, B. E. *Nature* **1997**, *389*, 54; (i) Sakthivel, K.; Barbas, C. F., III *Angew. Chem., Int. Ed.* **1998**, *37*, 2872; (j) Battersby, T. R.; Ang, D. N.; Burgstaller, P.; Jurczyk, S. C.; Bowser, M. T.; Buchanan, D. D.; Kennedy, R. T.; Benner, S. A. *J. Am. Chem. Soc.* **1999**, *121*, 9781; (k) Perrin, D. M.; Garestier, T.; Helene, C. *Nucleosides Nucleotides Nucleic Acids* **1999**, *18*, 377; (l) Tarasow, T. M.; Tarasow, S. L.; Tu, C.; Kellogg, E.; Eaton, B. E. *J. Am. Chem. Soc.* **1999**, *121*, 3614; (m) Santoro, S. W.; Joyce, G. F.; Sakthivel, K.; Gramatikova, S.; Barbas, C. F., III *J. Am. Chem. Soc.* **2000**, *122*, 2433; (n) Vaish, N. K.; Fraley, A. W.; Szostak, J. W.; McLaughlin, L. W. *Nucleic Acids Res.* **2000**, *28*, 3316; (o) Gourlain, T.; Sidorov, A.; Mignet, N.; Thorpe, S. J.; Lee, S. E.; Grasby, J. A.; Williams, D. M. *Nucleic Acids Res.* **2001**, *29*, 1898; (p) Sawai, H.; Ozaki, A. N.; Satoh, F.; Ohbayashi, T.; Masud, M. M.; Ozaki, H. *Chem. Commun.* **2001**, 2601; (q) Held, H. A.; Benner, S. A. *Nucleic Acids Res.* **2002**, *30*, 3857; (r) Thoresen, L. H.; Jiao, G. S.; Haaland, W. C.; Metzker, M. L.; Burgess, K. *Chem. Eur. J.* **2003**, *9*, 4603; (s) Vaught, J. D.; Dewey, T.; Eaton, B. E. *J. Am. Chem. Soc.* **2004**, *126*, 11231; (t) Jager, S.; Rasched, G.; Kornreich-Leshem, H.; Engeser, M.; Thum, O.; Famulok, M. *J. Am. Chem. Soc.* **2005**, *127*, 15071; (u) Srivatsan, S. G.; Tor, Y. *J. Am. Chem. Soc.* **2007**, *129*, 2044; (v) Burley, G. A.; Gierlich, J.; Mofid, M. R.; Nir, H.; Tal, S.; Eichen, Y.; Carell, T. *J. Am. Chem. Soc.* **2006**, *128*, 1398; (w) Hocek, M.; Fojta, M. *Org. Biomol. Chem.* **2008**, *6*, 2233; (x) Hollenstein, M.; Hipolito, C. J.; Lam, C. H.; Perrin, D. M. *ChemBioChem* **2009**, *10*, 1988; (y) Vaught, J. D.; Bock, C.; Carter, J.; Fitzwater, T.; Otis, M.; Schneider, D.; Rolando, J.; Waugh, S.; Wilcox, S. K.; Eaton, B. E. *J. Am. Chem. Soc.* **2010**, *132*, 4141.
15. Obika, S.; Nanbu, D.; Hari, Y.; Morio, K.; In, Y.; Ishida, T.; Imanishi, T. *Tetrahedron Lett.* **1997**, *38*, 8735.
16. Veedu, R. N.; Vester, B.; Wengel, J. *ChemBioChem* **2007**, *8*, 490.
17. Veedu, R. N.; Vester, B.; Wengel, J. *J. Am. Chem. Soc.* **2008**, *130*, 8124.
18. Veedu, R. N.; Vester, B.; Wengel, J. *Org. Biomol. Chem.* **2009**, *7*, 1404.
19. Veedu, R. N.; Vester, B.; Wengel, J. *New J. Chem.* **2010**, *34*, 877.
20. Veedu, R. N.; Wengel, J. *Mol. BioSyst.* **2009**, *5*, 787.
21. Kuwahara, M.; Obika, S.; Nagashima, J.; Ohta, Y.; Suto, Y.; Ozaki, H.; Sawai, H.; Imanishi, T. *Nucleic Acids Res.* **2008**, *36*, 4257.