Recognition of Uncharged Polyamide-Linked Nucleic Acid Analogs by DNA Polymerases and Reverse Transcriptases

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Polyamide-linked nucleic acid analogs (PNAs) are DNA mimics in which the deoxyribose phosphate backbone is replaced by uncharged *N*-(2-aminoethyl)glycine units.[1] PNA is the only example of a biopolymer whose backbone structure differs significantly from that of natural nucleic acids which nevertheless follows Watson−Crick base-pairing rules with complementary strands.[2] Initially, PNAs were investigated as potential antisense compounds. They block both translation and transcription.[3] More recently, their improved affinity to complementary DNA has suggested that PNAs may have further value as diagnostic tools for detecting genetic mutations.[4] PNAs have not, however, been found to serve as substrates for nucleic acid processing enzymes, in particular, for polymerases.[3] The search has been discouraged in part by crystallographic[5] and biochemical studies[6] that suggested that polymerases bind to the negative charges of the phosphate backbone of the nucleic acid substrates via highly conserved amino acid residues. Here, we show for the first time that PNAs can at least serve as primers for certain DNA polymerases and reverse transcriptases, even though no phosphate residues are present in the primer to interact with the polymerase.

A derivatized PNA carrying a 5′-amino-5′-deoxythymidine at the carboxyl terminal end (Figure 1) was prepared by elongation of a solid support carrying *N*-(monomethoxytrityl)-5′-amino-5′-deoxythymidine 3′-*O*-succinate following methods described elsewhere.[7] DNA oligomers to serve as templates and control primers were prepared by automated synthesis,

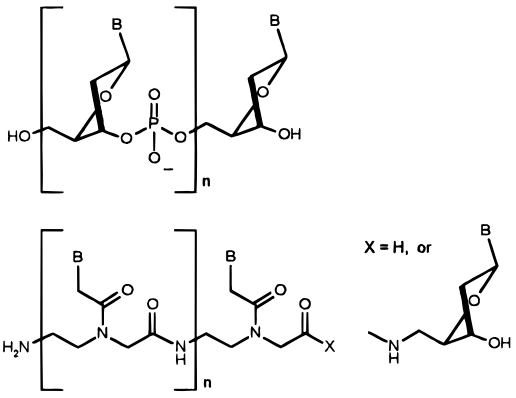

Figure 1. Chemical structure of DNA (upper) and PNA (lower; X = H). For PNA primer **1**, X is 5′-amino-5′-deoxythymidine.

labeled, and annealed.[8] In a typical experiment,[9] primer/template complex (containing either labeled DNA primer or unlabeled PNA primer) was incubated with one of the various commercially available polymerases. Elongation of the PNA primer was detected by incorporation of radioactively labeled [α-^{32}P]-dCTP[10] into oligonucleotides as visualized by both denaturing and native polyacrylamide gel electrophoresis (PAGE) followed by autoradiography (Figure 2, parts a and b). On native gels, PNA-containing duplex shows considerably higher mobility than the corresponding DNA duplex. This result may indicate that the PNA−DNA duplex has a smaller "effective size" in accordance with a NMR solution structure of a PNA−DNA duplex where 13 nucleotides per helical turn were found compared to 11 in B-DNA.[11]

Most of the DNA polymerases examined in this study, did not elongate the PNA primer to yield a PNA−DNA chimera, such as phage T4, phage T7 exo (Sequenase 2.0), *Thermus aquaticus, Thermus flavus, Thermus "ubiquitos"* (HotTub), *Pyrococcus furiosus*, and Deep Vent exo DNA polymerases, as well as HIV-1 reverse transcriptase. However, several DNA polymerases and reverse transcriptases were found to use the PNA primer for DNA synthesis, including the Klenow fragment (KF) of DNA polymerase I (*Escherichia coli*) and Vent DNA polymerase (*Thermococcus litoralis*). The ability to elongate the PNA primer 1 was weaker for Tth Polymerase (*Thermus Thermophilus*), *Pyrococcus woseii*, and the reverse transcriptases from avian myeloblastosis virus (AMV) and moloney murine leukemia virus (M-MuLV).

Linear amplification of the template strand was possible using an excess of PNA primer. Exponential amplification of both the template and the copied strand could not be obtained. When

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(8) The PNA primer **1** (16-mer) has the sequence H-taa tac gac tca cta-(5′-amino-T)-OH 3′. DNA primer **1** (16-mer, 5′ TAA TAC GAC TCA CTA T-3′), primer **2** (18-mer, 5′-GCC CCA GGG AGA AGG CAA-3′), the DNA template (81-mer, 5′-GCC CCA GGG AGA AGG CAA CTG GAC CGA AGG CGC TTG TGG AGA AGG AGT TCA TAG CTG GGC TCC CTA TAG TGA GTC GTA TTA-3′), and the 65-mer DNA marker (5′-GCC CCA GGG AGA AGG CAA CTG GAC CGA AGG CGC TTG TGG AGA AGG AGT TCA TAG CTG GGC TCC CT-3′) were synthesized and purified by standard methods. Annealing was performed in a total volume of 500 μL containing 1.8 mM Tris-HCl (pH 7.0), 0.5 mM $MgCl_2$, and 23 mM NaCl with 15 pmol primer (either 5′-[^{32}P]-labeled DNA primer **1** or unlabeled PNA primer **1**) and 50 pmol template.

(9) Enzymatic reactions were carried out in the appropriate reaction buffer (25 μL) containing 0.15 pmol primer/template complex and all required dNTPs at a final concentration of (a) 5 μM for thermolabile and (b) 50 μM for thermostable enzymes. In the case of PNA primer, unlabeled dCTP was replaced by [α-^{32}P]-dCTP (400 Ci/mmol).[10] The reaction buffers were supplemented with $MgCl_2$ or $MgSO_4$ (2 mM) if needed. Reactions were started by adding either 2 or 0.2 units of the enzyme and incubated for 15 min either at 37 or 75 °C. Aliquots of each reaction (5 μL) were analyzed by both native (12% acrylamide, 10 Watts, 4 h) and upon heating for 20 min at 95 °C by denaturing (15% acrylamide, 35 Watts, 2 h) PAGE. Gels were fixed, dried, and autoradiographed (PhosphorImager, Molecular Dynamics).
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(12) To detect whether any polymerase is able to read through a template containing PNA, 50 pmol primer **1** (PNA or DNA), 5 pmol template (81-mer), and 200 μM dNTPs were mixed in a final volume of 50 μL containing the Vent DNA polymerase reaction buffer and 2 units of Vent DNA polymerase. The linear amplification was performed as follows: 30 cycles, 1 min 94 °C, 2 min 58 °C, 50 s 72 °C. Finally, the nucleic acid templates were isolated by standard methods, annealed with 15 pmol of 5′-labeled DNA primer **2**, and the following enzymatic reactions were performed as described (in ref 9).

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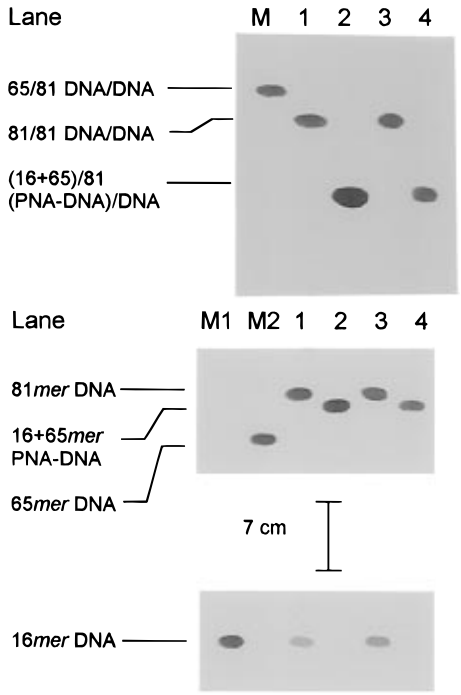

Figure 2. Primer extension reaction by Klenow fragment (KF) from DNA polymerase I (*Escherichia coli*) and Vent DNA Polymerase (*Thermococcus litoralis*): (A, top) Analysis of elongation of primer **1** with KF or Vent by native PAGE. Deoxynucleoside triphosphates were incubated at 37 (KF) or 75 °C (Vent DNA polymerase) with 0.15 pmol primer/template complex, either containing labeled DNA primer **1** (lanes 1 and 3) or unlabeled PNA primer **1** (lanes 2 and 4) and 2 units of KF (lanes 1 and 2) or Vent DNA polymerase (lanes 3 and 4). M contains the 65-mer hybridized to the 81-mer DNA. (B, bottom) Analysis of elongation of primer **1** with KF or Vent by denaturing PAGE. Lane M1 contains only the labeled DNA primer **1**, lane M2 contains the labeled 65-mer DNA.

a PNA−DNA chimera was presented as a template,[12] all polymerases examined in this study evidently stopped near the PNA−DNA linkage (data not shown).

These results show that neither a negative charge nor a standard backbone geometry is necessary for an oligonucleotide to serve as primer for the DNA polymerization reaction. This finding is quite unexpected, especially for Klenow fragment, as recent X-ray studies with this and Taq DNA polymerase identified highly conserved amino acid residues that interact with phosphate groups of the primer backbone.[5] It is difficult to imagine what contacts are made to the PNA primer in those polymerases where PNA is accepted as substrate. As DNA polymerases accepting PNAs as primer fall into two different evolutionary classes,[13] class A (Klenow fragment and Tth) and class B (Vent DNA polymerase), some general mode of recognition between polymerases and uncharged PNA primers must be possible.

The results presented here have several practical implications. For example, when screening for genetic mutations exploiting the higher affinity and sequence specificity of PNA for DNA than DNA itself, polymerase extension of a PNA primer to incorporate radioactively, fluorescently, or biotin labeled mononucleotides should provide much faster and more sensitive detection tools than presently available.[4] By using PNA primers in reverse transcriptase or DNA polymerase reactions, unwanted side products can be eliminated by successive 5′-exonuclease digestion, as the PNA-primed product is stable to this nuclease. PNA−DNA chimeras (synthesized either enzymatically or chemically[14]) should also be useful for studying structure−function relationships with a variety of nucleic acid processing enzymes.

Lastly, these results are relevant to models for the origin of life, where the possibility of nonnucleotidic polymers as nascent informational macromolecules is being considered.[15] Recently, Böhler *et al*. suggested on the basis of template-directed nonenzymatic polymerization reactions that PNA-type oligomers were possible alternatives for DNA and RNA during early episodes of life on earth.[16] If so, this would require at some point a linkage between PNA and DNA dependent polymerization. The results reported here establish that such a transition is basically possible.

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