# Polyamide Nucleic Acid−DNA Chimera Lacking the Phosphate Backbone Are Novel Primers for Polymerase Reaction Catalyzed by DNA Polymerases †

**ARTICLE** *in* BIOCHEMISTRY · MARCH 1998

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# Polyamide Nucleic Acid−DNA Chimera Lacking the Phosphate Backbone Are Novel Primers for Polymerase Reaction Catalyzed by DNA Polymerases†

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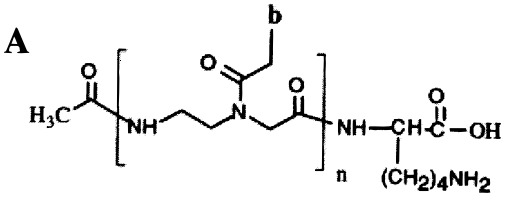
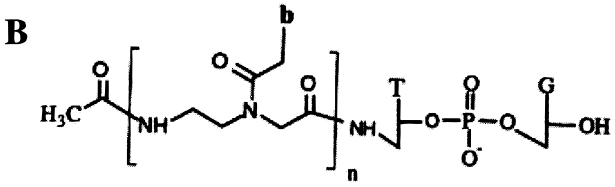
*Received June 24, 1997; Revised Manuscript Received December 12, 1997*

ABSTRACT: A peptide nucleic acid (PNA) oligomer, an analogue of DNA, was examined for its ability to function as a primer or a template to support DNA synthesis catalyzed by DNA polymerases. The analogue, $(PNA)_{19}$-TpG-OH, comprised of 19 bases in the form of PNA followed by a dinucleotide (TpG-OH) with a single phosphate and a free 3′OH terminus, was recognized as a bona fide primer by 2 reverse transcriptases and also by the Klenow fragment of *E. coli* DNA polymerase I. The 21-mer PNA chimera is extended on both RNA and DNA templates by all three polymerases. The specificity of binding of the PNA chimeric primer/DNA template at the template-primer binding site of the enzyme was shown by its photo-cross-linking ability to the enzyme which could be effectively competed out by another TP but not by template or primer alone. Furthermore, the chimeric TP−enzyme covalent complex was found to be catalytically active as judged by its ability to incorporate one nucleotide onto the 3′OH terminus of the immobilized primer. PNA sequences were also recognized as template when annealed with a DNA primer. These observations are in variance with the conventional suggestion that the phosphate backbone in the duplex region is essential for recognition and binding by DNA polymerases. The efficient extension of $(PNA)_{19}$-TpG-OH suggests that the diameter of the duplex region of template primer rather than the phosphate backbone may be sufficient for recognition by DNA polymerases.

In 1991, Nielsen and his colleagues described the synthesis of a new type of DNA analogue which was named peptide nucleic acid (*1*). These analogues are structural homologs of DNA **(**Figure 1) with a peptide backbone of 2-aminoethylglycine units to which purine and pyrimidine bases are linked (*2*, *3*). Although PNAs[1] lack phosphate backbone, they hybridize with the complementary sequences in DNA or RNA. The oligomeric PNAs have been shown to recognize their complementary sequences even in the double-stranded DNA and are shown to form exceptionally stable complexes by strand displacement (*1*−*3*). These analogues can also form stable triplexes with dsDNA which are resistant to denaturation at temperatures where DNA duplexes of identical sequence exhibit melting (*4*). Despite the high stability of the PNA−DNA complexes, single base pair mismatches greatly reduce the melting temperature, suggesting that the PNA is able to recognize dsDNA in a sequence




General structure of PNA




General structure of PNA-DNA chimera

FIGURE 1: Structure for $(PNA)_{21}$ and $(PNA)_{19}$-TpG-OH chimera. The sequences of $(PNA)_{21}$ and $(PNA)_{19}$-TpG-OH chimera are shown in Chart 1. In the figure, b represents A, T, G, or C purine and pyrimidine bases linked with polyamide backbone and n represents the number of these bases in the molecule. $(PNA)_{21}$ has a single Lys amino acid at the end of (pseudo 3′ terminus) the sequence while the $(PNA)_{19}$-TpG-OH chimeric molecule is comprised of 19 bases in the form of PNA followed by a normal dinucleotide TpG with G having a free 3′OH terminus.

†This research was supported in parts by a grant from the National Cancer Institute and the National Institute of General Medical Science (CA72821 to V.N.P.; GM36307 to M.J.M.).

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[1] Abbreviations: PNA, polyamide (peptide) nucleic acid; DiPEA, diisopropylethylamine; DMF, dimethylformamide; HBTU, benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate; HIV-1 RT, human immunodeficiency virus type 1 reverse transcriptase; IMAC, immobilized metal affinity chromatography; IDA−Sepharose, iminodiacetic acid−Sepharose; MuLV, murine leukemia virus; U5-PBS RNA template, 5′ non translated region of HIV-1 genomic RNA template containing the primer binding site (PBS); PBS−DNA template, HIV-1 genomic DNA template corresponding to the U5-PBS RNA sequence; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; DTT, dithiothreitol; TP, template primer; dATP, dGTP, dCTP, and dTTP, nucleoside triphosphate of deoxyadenosine, deoxyguanosine, deoxycytidine, and thymidine, respectively.

S0006-2960(97)01524-9 CCC: $15.00 
Published on Web 01/30/1998

specific manner (*5*). PNA has been used as a sequence-specific blocker of DNA-recognizing enzymes, mostly restriction endonucleases (*6*). PNA−DNA chimera has also been shown to exhibit similar sequence specificity and binding affinity for complementary DNA/RNA sequences (*7*). It was interesting to examine the possibility that PNA-chimera annealed with the complementary RNA or DNA templates could be recognized as a normal template primer by HIV-1 RT and other DNA polymerases. We found in the case of HIV-1 RT that, the PNA chimera targeted to the primer binding site of the viral RNA genome is able to block the initiation of reverse transcription, suggesting that the PNA chimera bound to RNA template may be oriented in the polymerase mode of the enzyme (*7*). In the present study, we show that a chimeric PNA annealed with a complementary RNA or DNA strand as template is efficiently used as a primer to catalyze the nucleotide addition reaction by all three polymerase enzymes so far tested. The photo-cross-linking of the hybrid complex to the enzymes was also found to be sensitive to high salt media and was significantly reduced in the presence of regular template primer. Based on these results, we propose that the phosphate backbone of the TP may not be essential for recognition and binding by the polymerase class of enzymes and that the enzyme binding may be dependent on the diameter of the duplex region of TP to be accommodated in the TP binding groove.

## MATERIALS AND METHODS

DNA-modifying enzymes were from Promega or Boehringer Mannheim. Tritiated dNTPs, [$\gamma$-$^{32}$P]ATP, and [$\alpha$-$^{32}$P]dNTPs were the products of Dupont-New England Nuclear Inc. Synthetic oligomeric primers were obtained from the Molecular Resource Facility of UMD−New Jersey Medical School and were further purified by polyacrylamide gel electrophoresis (*8*). Diisopropylethylamine (DiPEA), dimethylformamide (DMF), and benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU) were obtained from Applied Biosystems. All other reagents were of the highest available purity grade and purchased from Fisher, Millipore Corp., Boehringer Mannheim, and Bio-Rad. Terminal deoxynucleotidyltransferase was purified from calf thymus glands (*9*).

*Enzymes.* Preparations of the Klenow fragment of *E. coli* DNA polymerase I (*10*, *11*), Moloney murine leukemia virus reverse transcriptase (*12*), and human immunodeficiency virus reverse transcriptase (*13*, *14*) were carried out from recombinant clones according to published protocols. Protein concentrations were determined by using the Biorad colorimetric kit.

*Synthesis of (PNA)$_{19}$-T$_p$G-OH Chimera.* The synthesis of the 21-mer PNA−DNA chimera [5′(N)>gtc cct gtt cgg gcg cca T*G-3′OH] corresponding to the primer binding site (PBS) of the HIV genome was carried out on an ABI 394 DNA/RNA synthesizer using reported protocols (*15*, *16*). The PNA bases are designated by lower case while DNA sequences are represented as capital letters. In the first step of synthesis of the chimera, 3′-*O*-phosphoramidite of 5′-deoxy-5′-[*N*-(monomethoxytrityl)amino]thymidine (T*) (*17*) was dissolved in acetonitrile to a concentration of 0.12 M and coupled onto the 3′-DNA support (G) using standard DNA synthesis cycles. Next, the PNA monomers (*Applied Biosystems*) were dissolved in 1:1 DMF/acetonitrile to a concentration of 0.2 M. To each of these monomer solutions was added an equal volume of a 0.2 M solution of HBTU (*O*-benzotriazol-1-yl-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate) in 1:1 DMF/acetonitrile. The coupling was effected in the presence of a 0.2 M solution of diisopropylethylamine (DiPEA) in 1:1 dimethylformamide (DMF)/acetonitrile. Finally, the 5′ (N) end of the PNA portion was capped by acetylation using standard capping reagents, and the chimera was cleaved from the support (1 h, ambient temperature, ammonium hydroxide). The exocyclic amino groups were deprotected by heating the ammonium hydroxide solution at 55 °C for 12−16 h. The solution was then concentrated to half the original volume to remove most of the ammonium hydroxide and then desalted by gel filtration.

*$^{32}$P-Labeling of Oligomeric (PNA)$_{19}$-T$_p$G-OH Chimeric Primer.* A typical reaction mixture (20 $\mu$L) for labeling the 3′ end of the chimeric primer contained 1 $\mu$M primer termini, 0.33 $\mu$M [$\alpha$-$^{32}$P]dCTP (3 $\mu$Ci/pmol), 20 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 1 mM DTT, and 50 ng of calf thymus terminal deoxynucleotidyl transferase. The reaction mixture was incubated at 25 °C for 2 h and was applied to a C18 SEP-PAK cartridge preequilibrated with a buffer solution containing 100 mM triethylamine acetate, 10 mM Tris-HCl, pH 7.7, and 1 mM DTT. The column was washed with 20 mL of the equilibration buffer to remove unincorporated [$\alpha$−$^{32}$P]dCTP, and the labeled PNA was eluted with 35% acetonitrile and lyophilized. The sample was dissolved in 7% acetonitrile by heating to 70 °C for 10 min and stored at room temperature. Two major labeled products representing primer+1 and primer+2 nucleotides were obtained by this procedure. The labeled chimera was then annealed with complementary DNA or RNA oligomers and used as the template primer for the polymerase reaction. In some experiments, the chimeric primer was annealed with the complementary DNA template strand and then labeled at the 3′ end of the chimera by incubating it with 50 ng of either the Klenow fragment, HIV-1 RT, or MuLVRT in the presence of [$\alpha$-$^{32}$P]dCTP in a standard polymerase reaction (*18*, *19*). The labeled TP was then purified using a C18 SEP-PAK cartridge as described above and directly used in the extension reaction.

*Cross-Linking of DNA Template/(PNA)$_{19}$-T$_p$GpC-OH Chimeric Primer to DNA Polymerase Enzymes.* The $^{32}$P-labeled 22/23-mer PNA chimeric primer was annealed to the 49-mer complementary DNA template by mixing equimolar amounts of PNA chimera and 49-mer DNA in an annealing mixture containing 20 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, and 1 mM EDTA followed by heating the mixture at 100 °C for 5 min and then cooling to room temperature. It was then used as the template primer for determining its binding to the desired enzyme. The binding of TP to the enzyme was assessed by UV-mediated cross-linking of the E−TP binary complex. For cross-linking experiments, 512 nM enzyme and 50 nM labeled TP ($4 \times 10^4$ Cerenkov cpm/pmol) were incubated on ice for 5 min in a reaction mixture containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM $MgCl_2$, and 5% glycerol in a final volume of 50 $\mu$L. The mixture was irradiated in a Spectrolinker (Spectronic Corp.) at a UV dose of 300 mJ/cm$^2$. Measurement of the covalent attachment of labeled TP to the enzyme protein was assessed by SDS−8% polyacrylamide gel electrophoresis, followed

Chart 1: Sequences of PNA, PNA Chimera, DNA, and RNA Used as Primer and/or Template

**1.** 18 mer DNA primer complementary to U5-PBS RNA template.

**5'-GTCCCTGTTCGGGCGCCA-3'**

**2.** 22 mer DNA primer complementary to U5-PBS RNA template.

**5'-GTCCCTGTTCGGGCGCCACTGC-3**

**3.** 21 mer **$(PNA)_{19}$-TpG-OH** chimera complementary to U5-PBS RNA template.

**<------$(PNA)_{19}$----><-$(DNA)_2$->**
**gtccctgttcgggcgccac-TG-3'OH**

**4.** 21 mer PNA complementary to complementary to PBS region of U5-PBS RNA template.
**<------(PNA)21--------->**
**gtccctgttcgggcgccactg-Lys**

**5.** 15 mer DNA primer complementary to 21 mer PNA, 21 mer PNA chimera, 22 mer DNA, and 18 mer DNA.

**5'-CAGTGGCGCCCGAAC-3'**

**6.** 30 mer RNA corresponding to U5-PBS region of HIV-1 RNA genome.

**3'-CAGGGACAAGCCCGCGGUGACGAUCUCUAA**-5'

**7** 49 mer DNA template corresponding to U5-PBS sequences.

**3'-CAGGGACAAGCCCGCGGTGACGATCTCTAAAAGGTGTGACTG**
**ATTTTCC-5'**

by autoradiography (*11*).

*Cross-Linking of 21-mer PNA Template/15-mer DNA Template Primer to DNA Polymerases.* To determine the binding specificity of PNA and PNA chimera as a template, a complementary $^{32}$P-labeled 15-mer DNA primer (see Chart 1) annealed with 21-mer PNA and PNA chimera were used to cross-link to the enzyme. The enzyme−TP complexes were incubated in the absence and presence of poly(dC), oligo(dG), or poly(dC)·$(dG)_{18}$ and UV-irradiated as described above and resolved by SDS−polyacrylamide gel electrophoresis. The effect of increasing ionic strength in the irradiation mixture on the extent of PNA−enzyme cross-linking was similarly assessed.

*Nucleotidyltransferase Activity of the Enzyme Covalently Cross-Linked to DNA Template/PNA Chimeric Primer.* Nucleotidyltransferase activity of the enzyme containing covalently cross-linked 49-mer template/$(PNA)_{19}$-$T_pG$-OH chimeric primer was carried out essentially as described previously for normal template primer *(11, 13).* Forty picomoles of the enzyme was cross-linked with 40 pmol of unlabeled 49-mer DNA/$(PNA)_{19}$-$T_pG$-OH chimeric template primer as described above in a final volume of 50 $\mu$L. The nucleotidyltransferase reaction was initiated by the addition of 0.5 $\mu$M complementary [$\alpha$-$^{32}$P]Mg·dCTP (5 $\mu$Ci). The reaction mixture was incubated for 60 min at room temperature and terminated by the addition of 1% SDS and 20 mM EDTA. An aliquot of the reaction mixture was subjected to SDS−polyacrylamide gel electrophoresis followed by autoradiography. The radioactivity associated with the E−TP covalent complex was determined by Cerenkov counting after excising the radioactive band from the gel.

*Nucleotidyltransferase Activity of the Enzyme Covalently Cross-Linked to PNA Template/DNA Primer.* The 21-mer PNA template or the PNA chimeric template annealed with 15-mer DNA primer was covalently cross-linked with the individual enzymes as described above. The enzyme−TP covalent complexes were examined for their catalytic competence to incorporate a single nucleotide on the 3′ terminus of the immobilized 15-mer DNA primer as described above. The labeled E−TP covalent complexes were separated by SDS−polyacrylamide gel electrophoresis.

*Extension of PNA Chimeric Primer Annealed with Complementary RNA or DNA Template.* The $^{32}$P-labeled 22-mer $(PNA)_{19}$-$T_pG^{32}pC$-OH chimera complementary to the PBS region contained 19 bases in the form of PNA and 3 bases in the form of DNA with a free 3′OH end. The PNA chimera was annealed with either synthetic 30-mer U5-PBS HIV-1 RNA template or 49-mer U5-PBS HIV-1 DNA template (see Chart 1) in the molar ratio of 2 template:1 primer in a solution containing 50 mM Tris-HCl, pH 7.5, 1 mM DTT, and 2 mM $MgCl_2$ and used in the extension reaction. The extension reactions contained 50 mM Tris-HCl, pH 7.5, 10 mM DTT, 100 nM chimeric TP, 5 mM $MgCl_2$, 50 $\mu$M each of dATP, dTTP, dGTP, and dCTP, and 40 nM each of HIV-1 RT, MuLVRT, or Klenow fragment in a total volume of 10 $\mu$L. The reaction mixture was incubated for 30 min at room temperature and terminated by the addition of an equal volume of Sanger's gel loading solution (*20*). The samples were heated at 100 °C for 5 min and resolved by denaturing 12% polyacrylamide−urea gel electrophoresis followed by autoradiography.

## RESULTS

*Extension of PNA Chimeric Primer Annealed with DNA or RNA Template by DNA Polymerases.* The sequence of $(PNA)_{19}$-$T_pG$-OH chimeric primer used in this experiment is shown in Figure 1. The chimera contains 19 bases in the form of PNA lacking a phosphate backbone and 2 bases in the form of a dinucleotide with a common phosphate group linking both nucleosides through a single phosphodiester bond. Footprinting studies demonstrated that the Klenow fragment, HIV-1 RT, and MuLVRT, respectively, cover a minimum of 8, 24, and 26 bp in the duplex DNA upstream of the primer terminus (*21, 22*). Since contact between DNA polymerases and duplex DNA has been suggested to be exclusively through protein interactions with the DNA phosphate backbone (*23*), it was of interest to examine if the PNA chimera without the phosphate backbone could be recognized as a primer and extended by DNA polymerases. To monitor the extension, we first examined whether the 21-mer PNA chimera annealed with the respective RNA or DNA templates could be labeled with [$\alpha$-$^{32}$P]dCTP by either of these polymerases under standard reaction conditions. It was observed that 21-mer PNA chimera could be effectively labeled when incubated with these polymerases in the presence of [$\alpha$-$^{32}$P]dCTP. Thus, the resulting $^{32}$P-labeled $(PNA)_{19}$-$T_pGpC$−OH chimera with two phosphate groups was used to monitor its extension in a template primer fashion. The results shown in Figure 2 (A, B) indicate that the PNA chimeric primer is efficiently labeled and extended on both DNA and RNA templates by HIV-1 RT, MuLVRT, and the Klenow fragment. As shown in the figure $^{32}$P-labeled PNA chimeric primer could be extended up to its full-length product by all three polymerases. A significant amount of product accumulation was observed at the 24-mer position. This was due to extension of a portion of $^{32}$P-labeled 23-mer PNA chimeric primer present in the reaction mixture that contained a mismatch dC nucleotide at the 3′ end (lane P in panel B)**.** The 23-mer chimeric primer was generated due to incorporation of two dC nucleotides during the

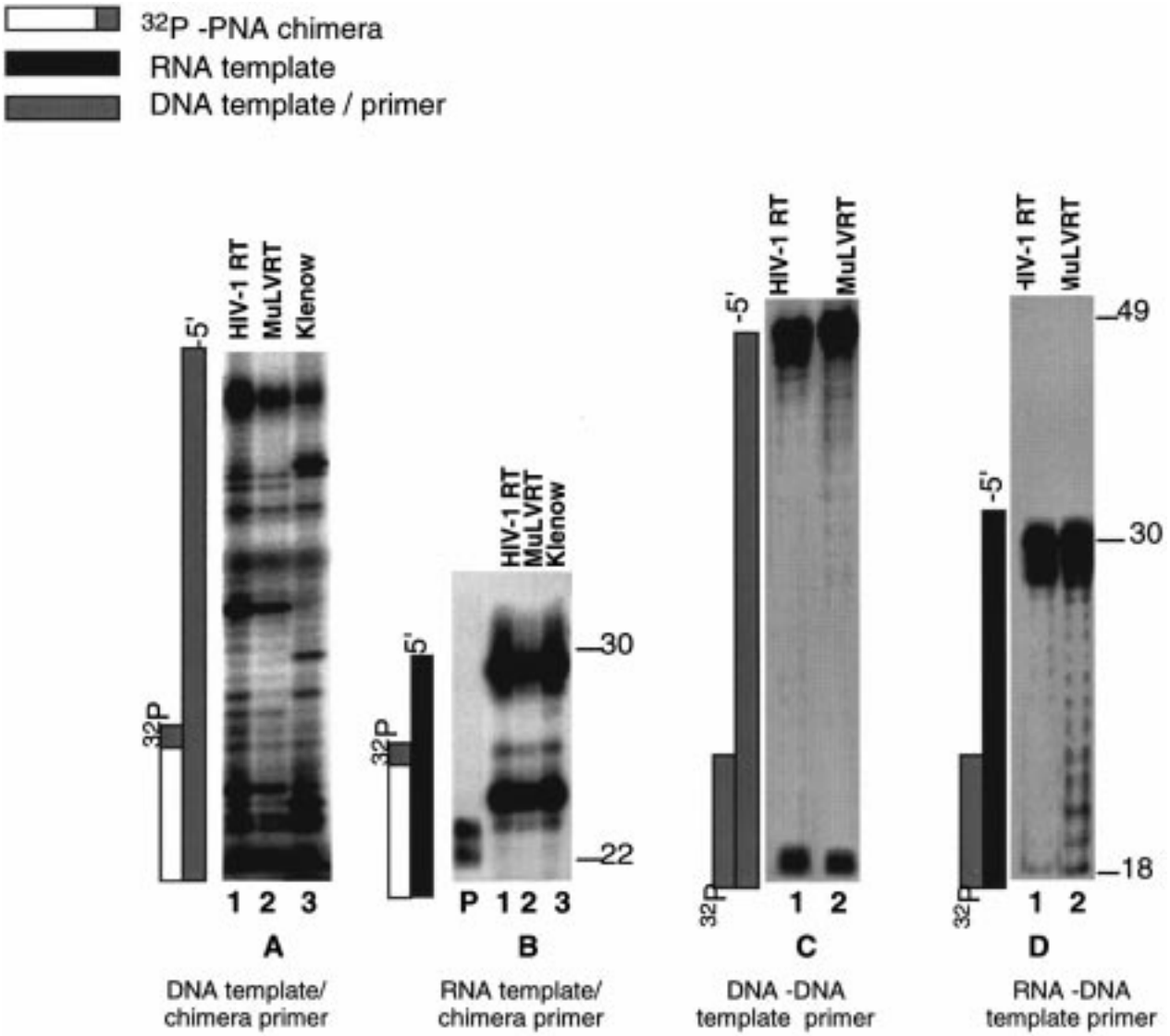


FIGURE 2: Extension of $(PNA)_{19}$-TpG$^{32}$pC-OH chimeric primer annealed separately with RNA and DNA templates. The $^{32}$P-labeled PNA chimera annealed either with 49-mer DNA template (panel A) or with 30-mer RNA template (panel B) was used as template primer for polymerase reactions catalyzed by HIV-1 RT, MuLVRT, and the Klenow fragment. For comparison, 5′-$^{32}$P-labeled 18-mer DNA primer annealed with the DNA template (panel C) or RNA template (panel D) was used as a control for the extension reaction catalyzed by HIV-1 RT and MuLVRT. Lanes 1 through 3, reaction products resulting from the extension of PNA chimera catalyzed by HIV-1 RT, MuLVRT, and the Klenow fragment, respectively. Lane P, $^{32}$P-labeled PNA chimera alone.

labeling experiment. For comparison, control experiments with DNA−DNA and RNA−DNA template primers are also shown in Figure 2 (C, D).

*Binding Specificity of $(PNA)_{19}$-$T_pG$-OH Chimera to DNA Polymerases as a Primer Annealed with 49-mer DNA Template.* The results of the extension reaction with PNA chimeric primer by reverse transcriptases and the Klenow fragment clearly suggest that the PNA chimera annealed with the complementary DNA template in all probability binds in the template primer binding track of these enzymes. To substantiate this possibility, we resorted to determining the specificity of binding of the PNA chimera, annealed with the 49-mer DNA template to HIV-1 RT, MuLV RT, and the Klenow fragment by photo-cross-linking. The results are shown in Figure 3B. For purpose of comparison, identical cross-linking experiments carried out with DNA−DNA template primer as control are shown in Figure 3A. As shown in the figure, all three DNA polymerases bind and cross-link with the chimeric template primer. The cross-linked $^{32}$P-labeled E−TP covalent complex migrates as the larger species, as a molecular mass greater than 73 kDa. It was further observed that cross-linking to the chimeric template primer is competed out by the inclusion of other template primers, such as poly(dC)·$(dG)_{18}$, but not by template or primer strand alone. Only the data obtained with the Klenow fragment are shown as a representative of this set of experiments (Figure 3C). The ionic strength of the medium, which influences the binding of the template primer to the enzyme, also exhibits a negative impact on the extent of cross-linking as judged by a decrease or near-complete loss of cross-linking at increasing salt concentrations in the reaction mixture (Figure 3D). In the cross-linking experiments, some enzymes show more than one cross-linked species. This may possibly be due to the enzyme cross-linking either to the primer strand alone or to both the template and primer strands. The covalent labeling of the enzyme to the template primer required the presence of an active enzyme species since the heat-denatured enzyme did

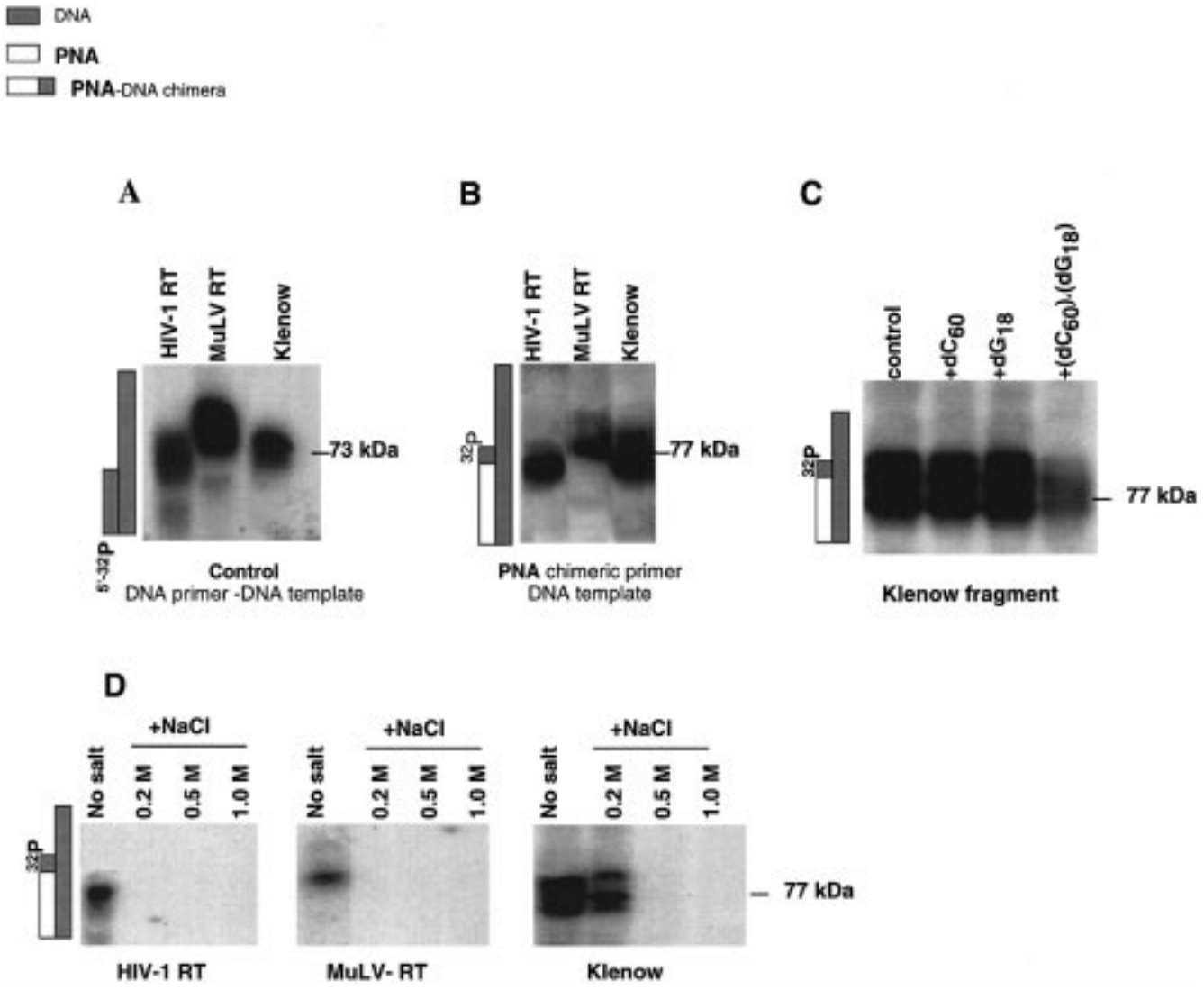


FIGURE 3: (A) Binding/photochemical cross-linking of $(PNA)_{19}$-TpG-OH chimeric primer/DNA template to HIV-1 RT, MuLVRT, and Klenow fragment. The $(PNA)_{19}$-TpG-OH chimera was labeled at the 3′ end using [α-$^{32}$P]dCTP and calf thymus terminal deoxynucleotidyltransferase as described under Materials and Methods. The labeled chimera annealed with 49-mer DNA template was incubated with HIV-1 RT, MuLVRT, and the Klenow fragment and photo-cross-linked by UV irradiation as described under Materials and Methods. The cross-linked samples were subjected to SDS−polyacrylamide gel electrophoresis, and the cross-linked radioactive species were detected by autoradiography (*11*). Panel A, cross-linking experiment with 49/22-mer DNA−DNA template primer (control); panel B, with the PNA chimeric primer/DNA template; panel C, cross-linking of PNA chimeric primer/DNA template with the Klenow fragment in the absence (control) or presence of $dC_{60}$, $dG_{18}$, or poly(dC)·$(dG)_{18}$; panel D, cross-linking in the presence of the indicated salt concentrations.

not show any cross-linking. These observations suggest that the $(PNA)_{19}$-$T_pGpC$-OH chimera devoid of phosphate backbone is well recognized by all three enzymes as normal template primer.

*Binding/Cross-Linking of the $(PNA)_{19}$-$T_pG$-OH Chimeric Primer to the Enzyme Is Productive and Specific to the Polymerase Mode of the Enzyme.* The most compelling evidence that indicates that the cross-linking of the enzyme to the PNA chimeric primer represents true binding (i.e., in the polymerase mode) was obtained by the experiments which show the ability of the cross-linked enzyme to catalyze the nucleotidyltransferase reaction on the cross-linked PNA chimeric primer. Figure 4 B depicts the catalytic competence of the enzyme−DNA/PNA chimeric TP [49-mer DNA/$(PNA)_{19}$-$T_pG$-OH] cross-linked covalent complexes of HIV-1 RT, MuLVRT, and the Klenow fragment. For comparison, the catalytic competence of these enzymes cross-linked with the normal DNA−DNA template primer is also shown in Figure 4A. The covalently linked E−TP complex is catalytically active and can effectively incorporate [α-$^{32}$P]-dCTP onto the 3′-OH terminus of the cross-linked PNA chimeric primer. This indicates that the PNA chimeric primer is covalently cross-linked in the TP binding domain and is oriented in the polymerase mode. It was further observed that only a single nucleotide could be added on the primer terminus of the cross-linked chimeric primer. The addition of a second nucleotide corresponding to the second template base was not observed, suggesting that the enzyme immobilized on the TP is unable to translocate across the template strand. The two bands seen in the autoradiogram probably represent the product synthesized by the enzyme species cross-linked either to the primer strand alone or to both the primer and template strands during in situ labeleing with [$^{32}$P]dNTP. Two species of the E−TP covalent complexes labeled in this manner separate from each other on an SDS−polyacrylamide gel. It was observed that the

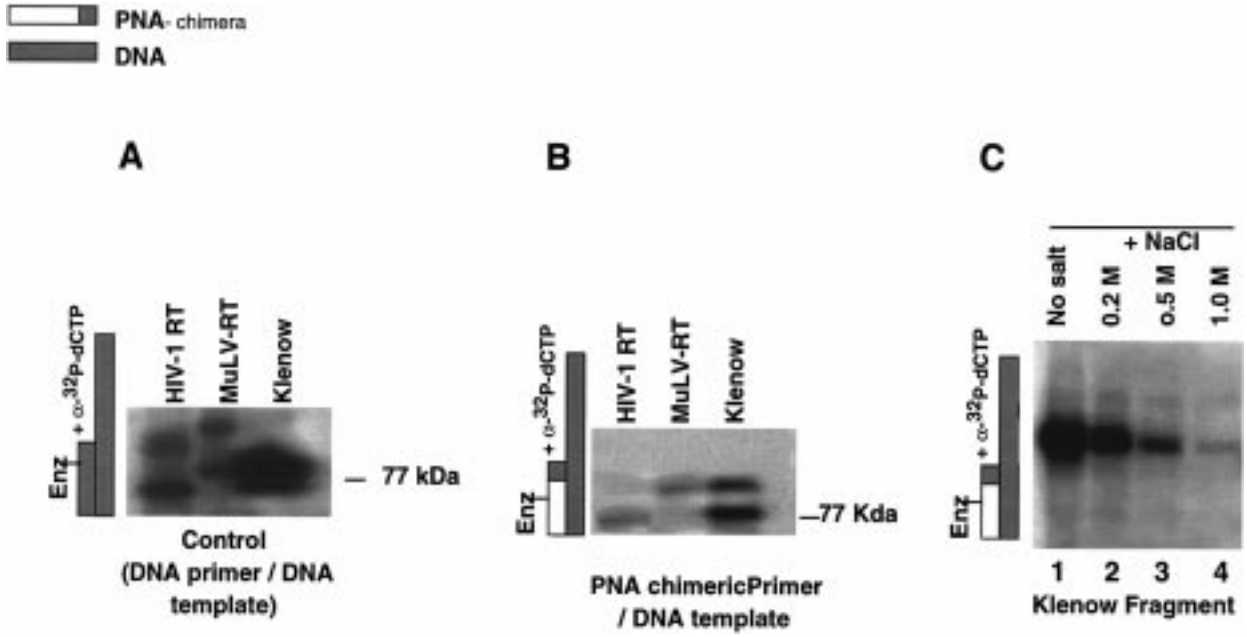


FIGURE 4: Addition of nucleotide on the 3′OH terminus of 21-mer PNA chimeric primer/49-mer DNA template covalently immobilized in the polymerase mode of the enzyme. The unlabeled $(PNA)_{19}$-TpG-OH chimeric primer annealed with 49-mer DNA template was cross-linked with HIV-1 RT, MuLVRT, or the Klenow fragment as described under Materials and Methods. The enzyme−TP covalent complex was supplemented with α-$^{32}$P-labeled Mg·dCTP (complementary to the first template base). After incubation for 30 min at room temperature, the mixture was subjected to SDS−PAGE, and the radioactive enzyme−TP covalent complex migrating as species greater than 73 kDa was detected by autoradiography. Panel A shows the control cross-linking experiment of 49/22 DNA−DNA template primer (control) with these enzymes followed by in situ addition of [α-$^{32}$P]Mg·dNTP on the cross-linked 3′OH primer terminus. Panel B shows in situ addition of a nucleotide on the 3′OH terminus of PNA chimeric primer. Panel C shows cross-linking of PNA chimeric primer/DNA template cross-linked with the Klenow fragment and in situ addition of a nucleotide on the immobilized 3′OH terminus of the chimeric primer in the presence of the indicated salt concentrations.

nucleotidyltransferase activity of the enzyme−chimeric template primer covalent complex was only slightly affected by increasing salt concentration. Activity was detected even at 1 M salt concentration. The results obtained with the Klenow fragment are shown in Figure 4B as representative of this set of experiments. The absence of any effect of high salt on the nucleotidyltransferase reaction shows that the binding of dNTP in the ternary complex may be solely guided by hydrophobic interactions and not by ionic interaction. In contrast, the incorporation of a nucleotide onto the DNA−DNA template primer by the free enzyme is significantly sensitive to high salt concentration due to the salt-induced dissociation of TP from the enzyme (*11*). The polymerase activity of free enzyme (HIV-1 RT, MuLVRT, and Klenow fragment) is reduced to background levels in the presence of salt concentration above 500 mM. As expected, the nucleotidyltransferase reaction by chimeric TP−enzyme covalent complex is also $Mg^{2+}$-dependent (data not shown).

*Specificity of Binding/Cross-Linking of DNA Polymerases to $(PNA)_{19}$-$T_pG$-OH Chimera as Template Annealed with 15-mer DNA Primer.* Since the binding/cross-linking of PNA chimeric primer/DNA template to the enzyme was found to occur in the TP binding site of DNA polymerases, it was expected that the binding of the PNA chimera as a template should also occur within the same TP binding site. To examine this, 5′-$^{32}$P-labeled 15-mer DNA primer was annealed with either 21-mer PNA or 21-mer PNA chimeric template and cross-linked with HIV-1 RT, MuLVRT, and the Klenow fragment in the absence or presence of $dC_{60}$, $dG_{18}$, and poly(dC)·$(dG)_{18}$ as described above. Identical cross-linking experiments were also carried out with DNA−DNA template primer (22/15-mer TP) as control for comparison (Figure 5A). The results depicted in Figure 5B and Figure 5C indicate that all three enzymes are able to bind/cross-link to the PNA-chimeric template/DNA primer or PNA template/DNA primer similar to the control DNA−DNA template primer. Further, binding/cross-linking to the PNA-chimeric template/DNA primer was influenced by ionic strength and could be effectively competed out by poly(dC)·$(dG)_{18}$ TP but not by $dC_{60}$ template or $dG_{18}$ primer alone. Results obtained with the Klenow fragment are shown in Figure 5D,E as representative of this set of experiments.

*Binding/Cross-Linking of $(PNA)_{19}$-$T_pG$-OH Chimeric Template to the Enzyme Is Productive and Occurs Specifically in the Polymerase Mode of the Enzyme.* The binding specificity of PNA template to DNA polymerases also raised a pertinent question about the ability of these enzymes to copy the PNA template sequences. To ascertain this possibility, all three enzymes were cross-linked individually with the template primers consisting of $(PNA)_{21}$ template/15-mer DNA primer. The E−TP covalent complexes were examined for their ability to catalyze nucleotidyltransferase activity onto the immobilized primer terminus in the presence of 0.5 M salt concentration. Since the template sequence to be copied is that of the PNA base, any addition of nucleotide onto the primer terminus should be considered as template-dependent. A representative result obtained with the Klenow fragment is shown in Figure 6. The addition of nucleotide on the cross-linked primer terminus is dependent on the PNA template base, and does not take place in its absence.

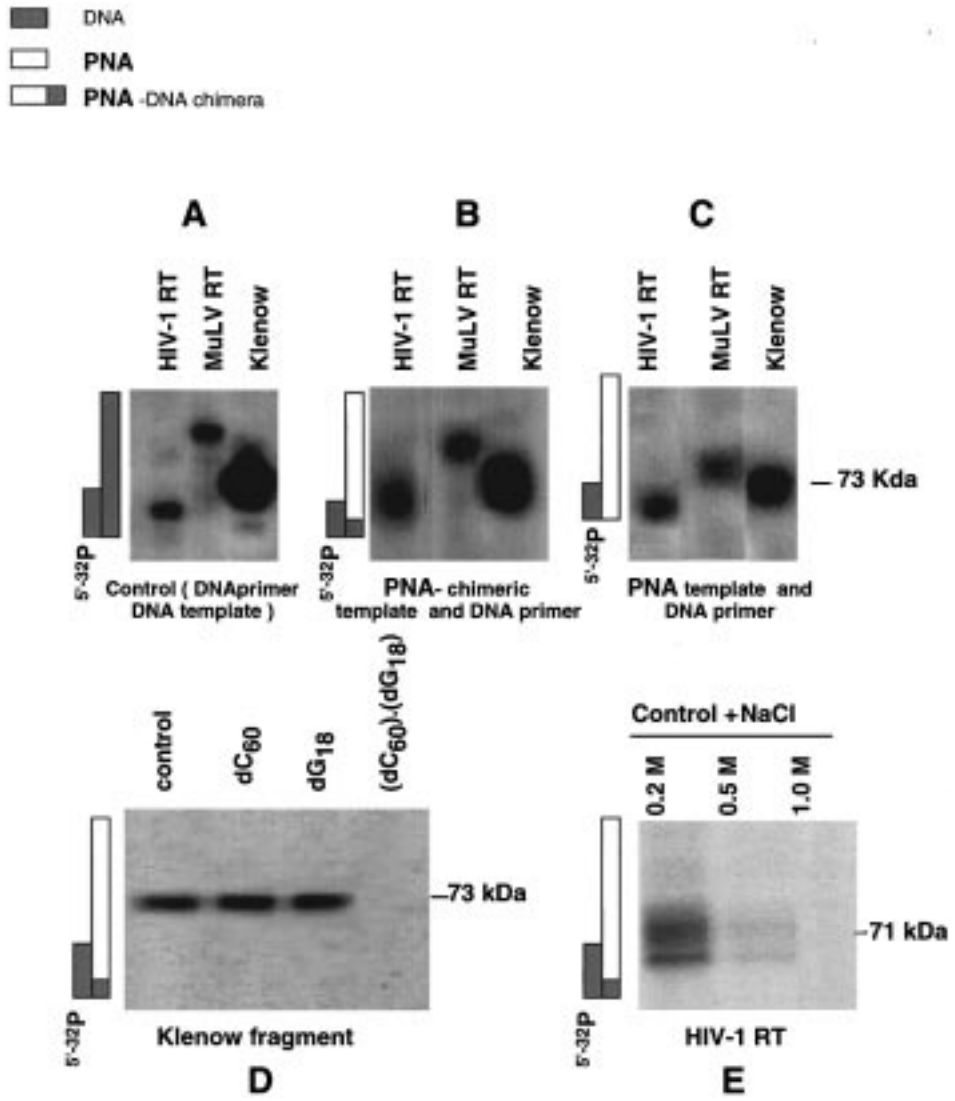


FIGURE 5: Specificity of binding/photochemical cross-linking of 21-mer PNA or PNA−DNA chimera as template to HIV-1 RT, MuLVRT, and the Klenow fragment. 5′-$^{32}$P-Labeled 15-mer DNA primer annealed with 21-mer PNA, $(PNA)_{19}$-TpG-OH chimera, or 22-mer DNA template was incubated with the enzymes at room temperature, and the complex was UV-irradiated as described under Materials and Methods. The cross-linked species were resolved by SDS−PAGE followed by autoradiography. Panel A represents a control cross-linking experiment with 22/15 mer DNA−DNA template primer; panel B, with 21-mer PNA chimeric template/15-mer DNA primer; panel C, with 21-mer PNA template/15-mer DNA primer; panel D, cross-linking of the Klenow fragment with 21-mer PNA chimeric template/15-mer DNA primer in the absence (control) and presence of $dC_{60}$, $dG_{18}$, or poly(dC)·$(dG)_{18}$; panel E, cross-linking of the Klenow fragment with 21-mer PNA chimeric template/15-mer DNA primer in the presence of the indicated salt concentrations.

## DISCUSSION

This is the first report to suggest that a nucleic acid analogue lacking a phosphate backbone can be utilized as both primer and template in the nucleotidyltransferase reaction catalyzed by DNA- and RNA-dependent DNA polymerases. The results presented here suggest that the phosphate backbone in the duplex region of the DNA template primer is not essential for interaction with DNA polymerases. The 21-mer PNA chimera used in this study contains 19 bases linked with peptide bonds lacking the phosphate backbone and a dinucleotide with a single phosphate group. We have also used a 21-mer PNA without any DNA nucleotides as a template lacking the phosphate backbone. The 21-mer PNA chimera annealed with a complementary DNA or RNA template was used as template primer for 3′ labeling and subsequent extension of the chimeric primer by DNA polymerases including reverse transcriptases. The result obtained was surprising, since it has conventionally been argued that the protein−DNA interactions occur via the phosphate backbone of the duplex DNA and positive electrostatic potential at the interacting surface of the protein (*23*). The present results suggest that the size in terms of the diameter of the duplex region rather than the phosphate backbone may be the factor responsible for accommodating TP in the TP binding track of the enzyme.

Earlier we have reported the binding affinity of the chimeric PNA to its complementary RNA or DNA template as assessed by measuring the melting temperature ($T_m$) of the duplex compared with the corresponding oligomeric DNA annealed to the same region. An increase in the $T_m$ by 15 °C for duplexes of RNA−PNA chimera and DNA−PNA chimera revealed a very tight association of the chimera with the complementary template strand (*2*, *7*). The binding of chimeric PNA−DNA template primer to HIV-1 RT, MuLVRT, and Klenow fragment was found to be specific

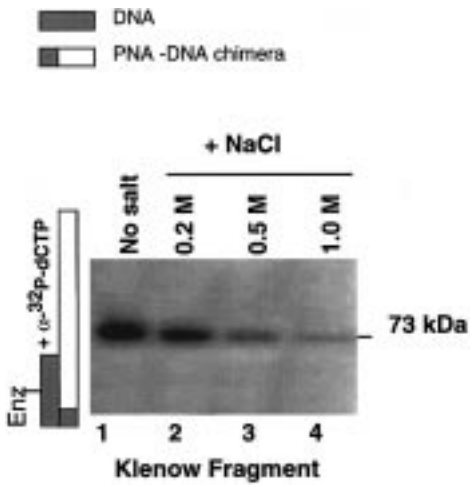


FIGURE 6: Addition of a nucleotide on the 3′OH of DNA primer/PNA chimeric template covalently cross-linked with the Klenow fragment. The $(PNA)_{19}$-TpG-OH chimeric template annealed with 15-mer DNA primer was cross-linked with the Klenow fragment as described under Materials and Methods. The enzyme−TP covalent complex was supplemented with α-$^{32}$P-labeled Mg·dATP (complementary to the first template base). After incubation for 30 min at room temperature, the mixture was subjected to SDS−PAGE, and the radioactive enzyme−TP covalent complex migrating as the 73 kDa species was detected by autoradiography. Lane 1, additions of [α-$^{32}$P]dATP under standard conditions in the absence of salt; lanes 2 through 4, in the presence of the indicated salt concentrations.

to the template primer binding site as judged by the observation that enzyme−TP cross-linking is effectively competed out by poly(dC)·$(dG)_{18}$ including other template primers such as poly(rA)·$(dT)_{18}$, but remains unaffected by single-stranded DNA oligomer serving as template or primer strand (Figure 3B).

Furthermore, at high salt concentration, no template primer cross-linking to the enzyme is apparent, which is consistent with the biochemical observation that the template primer binding to the enzyme is severely affected in a high-salt medium. This salt effect could be attributed to the fact that one of the strands of the PNA chimeric TP contains a phosphate backbone, binding of which to the enzyme may be sensitive at high salt concentration. The most interesting observation, however, is that the covalent complex of enzyme−(PNA chimeric primer/DNA template) is catalytically competent to catalyze the nucleotidyltransferase reaction onto the immobilized 3′OH terminus of the cross-linked PNA chimeric primer. The possibility that the nucleotidyltransferase activity observed with the E−TP complex may be due to the free enzyme which may catalyze the polymerase reaction on the TP of the enzyme−TP cross-linked complex was ruled out based on the following observations: (1) Only a single nucleotide could be incorporated onto the chimeric primer terminus of the cross-linked chimeric TP, suggesting that the covalently immobilized enzyme on the TP molecule could not translocate to add the next nucleotide. (2) Nucleotidyltransferase reaction of E−chimeric TP covalent complex could occur in high-salt medium (up to 1 M), ruling out the possibility that the free enzyme was catalyzing this reaction. Under similar reaction conditions, the polymerase activity of the free enzyme is undetectable due to the inability of the enzyme to bind to the template primer. These results suggest that both chimeric PNA−DNA and chimeric PNA−RNA template primers bind to the enzyme in the polymerase mode, resulting in a catalytically active covalent enzyme−template primer binary complex. Surprisingly, 21-mer PNA annealed with 15-mer DNA was also recognized by the polymerases as a bona fide template primer. The binding/cross-linking of such PNA−DNA template primer was specific and could be competed out by natural template primer but not by template or primer alone. Furthermore, the polymerase enzyme covalently bound to PNA template−DNA primer is able to incorporate a single nucleotide onto the primer terminus suggesting that the PNA lacking the phosphate backbone is recognized as template for the polymerase reaction.

The observation that a nucleotidyltransferase reaction catalyzed by the enzyme−TP covalent complexes with PNA chimera as primer was further confirmed when the PNA chimeric primer annealed with a DNA or RNA template could be fully extended by all three DNA polymerases (Figure 2). However, our attempts to use PNA template primed with $^{32}$P-labeled DNA primer in normal polymerase reaction yielded a smear on gel analysis. Neverthless, it was possible to demonstrate the addition of a single nucleotide on the immobilized enzyme−template primer covalent complex. The smear observed in the product analysis could be due to the lack of separation of the extended DNA species from the PNA template. Such a hybrid may not be fully denatured upon heating at 100 °C in the formamide-containing dye. The binding of PNA to complementary ssDNA or dsDNA has been shown to be so tight that it is virtually irreversible under standard conditions (*24*). In contrast, extension products of PNA chimera annealed with DNA or RNA template yielded discrete bands upon gel analysis (Figure 2).

These observations suggest that a phosphate backbone in one of the strands in the duplex region of template primer is sufficient for recognition by DNA polymerases provided the diameter of the duplex region is equivalent to that of the natural duplex DNA−DNA or RNA−DNA structure. Although PNA molecules lack the phosphate backbone, they mimic the structure of DNA. The optimal number of bonds between the nucleobases in a PNA molecule is six, which corresponds to that found in DNA (*2*). It would be interesting to see whether a template primer lacking a phosphate backbone in both strands of the duplex region but having a phosphate backbone in the single-stranded template overhang and at the 3′OH terminus on the primer strand is capable of supporting DNA synthesis.

## ACKNOWLEDGMENT

We thank Neerja Kaushik and Cindy Arrigo for critical reading of the manuscript.

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BI971524N