The relative positions of components of the DNA-dependent DNA polymerase δ (pol δ)–proliferating cell nuclear antigen (PCNA)–DNA complex were studied. We have shown that pol δ incorporates nucleotides close to a template biotin-streptavidin complex located 5′ (downstream) to the replicating complex in the presence or absence of PCNA. PCNA-dependent synthesis catalyzed by pol δ was nearly totally (95%) inhibited by a biotin-streptavidin complex located at the 3′-end of a template with a 15-mer primer (upstream of the replicating complex), but was only partially inhibited with a 19-mer primer. With either primer, PCNA-independent template with a 15-mer primer (upstream of the replicating complex) was not affected by the biotin-streptavidin complex located at the 3′-OH primer terminus. Using UV photocross-linking, we determined that the 125-kDa subunit of pol δ, but not the 50-kDa subunit, interacted with a photosensitive residue of a substrate oligonucleotide. Interaction apparently takes place through the C terminus of p125. Based on these results, we conclude that PCNA is located “behind” pol δ in the polymerization complex during DNA synthesis and that only the large subunit of pol δ (two-subunit form) interacts directly with DNA. A detailed model of the enzymatically active complex is proposed.

DNA polymerase δ (pol δ) is an essential eukaryotic enzyme participating in both DNA replication and repair (for a review, see, for example, Ref. 1). Mammalian pol δ is a heterodimer with molecular masses of 125 kDa for the large subunit and 50 kDa for the small subunit. Proliferating cell nuclear antigen (PCNA), a multifunctional protein, acts as an auxiliary factor of pol δ and dramatically increases the processivity of pol δ-catalyzed DNA synthesis (2, 3). Processivity is defined as the number of nucleotides incorporated by the polymerase/binding event. The 29-kDa mammalian PCNA monomer forms a homotrimer (three monomers) of ring-like shape (homotrimeric toroid) that loads onto linear DNA without additional cofactors or that can be loaded onto closed circular or otherwise topologically constrained DNA by an another essential replication protein, replication factor C (RF-C) (4, 5). Loading by RF-C is ATP-dependent (5). PCNA is thought to encircle DNA and act as a sliding clamp for pol δ, facilitating highly processive synthesis of DNA (6, 7). Reconstitution of SV40 DNA replication with purified proteins (8) has allowed definition of many components of the eukaryotic replication machinery. However, the relative positions of proteins in the replication complex, their interactions with DNA, and their interactions with each other are just beginning to be determined.

Despite much interest, the position of PCNA relative to pol δ during DNA synthesis is still unknown. Mutation analyses and other methods showed that the PCNA trimer has polarity; several proteins, including pol δ (9), RF-C (9, 10), flap endonuclease 1 (FEN-1) (11) and DNA ligase I (12), all bind to the same side of the PCNA trimer. This region of the PCNA trimer is usually designated as the carboxyl-terminal side.

A functional analog of PCNA in the phage T4 replication holoenzyme complex, gp45, also forms a homotrimeric toroid and acts as a sliding clamp for gp43 DNA polymerase. Recent experiments showed that gp43 DNA polymerase binds to the side of the gp45 ring containing the C termini of gp45 monomers (13). Cross-linking experiments with the holoenzyme of DNA polymerase III from Escherichia coli showed that another functional and structural analog of PCNA, the β-subunit, binds “behind” the polymerase catalytic subunit (α) in the initiation complex of the polymerase with an RNA-primed DNA template (14).

In this work, we describe a novel dynamic mapping method that was used to elucidate the relative positions of PCNA and pol δ during DNA synthesis. Here we show that PCNA is located behind pol δ when polymerization occurs. We think it likely that this methodology can also be used to study the relative positions of other proteins of the replication machinery. Results of photocross-linking experiments suggest that only the large subunit of pol δ (p125) interacts closely with DNA. The C-terminal domain of p125 is apparently responsible. We propose a detailed model of the pol δ-PCNA-DNA complex present during the polymerization reaction.

EXPERIMENTAL PROCEDURES

Materials—A photosensitive analog of dTTP, 5-(N-(p-azidobenzyloxy)-3-aminomethyl)-dUTP (AB-dUTP), was synthesized as described (15). Unlabeled dNTPs and micrococcal nuclease were from Roche Molecular Biochemicals. [γ-32P]dATP and [ε-32P]dCTP were from Amersham Pharmacia Biotech. Oligonucleotides of defined sequences were synthesized by Dr. F. Johnson and colleagues (State University of New York, Stony Brook, NY) and purified by PAGE in the presence of 7 M urea. N6-(N-Biotinyl-6-aminohexyl)-2′-deoxyadenosine 5′-triphosphate (bio-7dATP),...
streptavidin, and terminal deoxynucleotidyltransferase were from Sigma. Rabbit polyclonal antibodies against either the N or C terminus of the 125-kDa subunit of pol δ or against the 50-kDa subunit of pol δ were developed as described (16). E. coli DNA polymerase I Klonev fragment, without 3'-5' exo activity, was a generous gift of Dr. H. Miller (State University of New York, Stony Brook, NY). Pfu DNA polymerase was from Stratagene. PCNA and DNA polymerase δ were both purified from calf thymus according to published protocols (2, 17, 18). Acrylamide and methylenebisacrylamide were from Eastman Organic Chemicals and were further purified by adsorption of impurities to activated charcoal. All other materials were of reagent grade and were used without further purification

### Synthesis of Biotinylated Oligonucleotide Template—A 50-mer template with bio-7-dAMP at position 11 (CTCTCGCGTC TCGTCGTA) was annealed to the 50-mer TTGACGACCC (primer) was annealed to the 50-mer TTGACGACCC (without bio-7-dAMP) was extended by terminal deoxynucleotidyltransferase to a 51-mer with bio-7-dAMP at position 11 (CTCTCGCGTC (bio-7-dA)CGCAGGGGAG GTGGATGGGGG). The 55-mer contained 10 dGMP residues at the 3'-end. The 18-mer ATCCAACCTCGGCGTTGTGGC (with 3'-OH ends), 2 nmol of bio-7-dATP, and 34 units of terminal deoxynucleotidyltransferase. Incubation was for 1 h at 37 °C. The desired product was purified by PAGE in the presence of 7 M urea.

A 51-mer with bio-7-dAMP at position 51 was synthesized as follows. The 50-mer CTCTCGCGTC ACGCCGGTC TCGTCGTA (without bio-7-dAMP) was extended by terminal deoxynucleotidyltransferase to a 51-mer with bio-7-dATP as a substrate. The reaction mixture (30 μl) contained 500 mm sodium cacodylate (pH 7.2), 1 mm 2-mercaptoethanol, 10 mm CoCl₂, 100 pmol of 50-mer (3'-OH ends), 2 nmol of bio-7-dATP, and 34 units of terminal deoxynucleotidyltransferase. Incubation was for 1 h at 37 °C. The desired product was purified by PAGE in the presence of 7 M urea.

### Synthesis of a 45-mer with Photoreactive AB-dUMP—At Position 19 and [32P]dCMP at Position 20—Before UV treatment, all operations with photoreactive materials were performed in a room illuminated only by a 10-watt incandescent lamp. The 19-mer ATCCAACCTCGGTCTTGTGGC (primer) was annealed to the 55-mer TTGACGACCC. The same procedure before the biotin residue (Fig. 2, lane 2) was opened and subjected to 1 min of UV irradiation (~254 nm) at 0 °C with the UV lamp (UVGL-58) placed 5 cm from the surface of the sample. Then CaCl₂ was added to a final concentration of 1 mm, and 7 units of micrococcal nuclease were added. The reaction volume was 12 μl. The sample was incubated for 5 min at 37 °C. Afterward, products were subjected to SDS-PAGE (19) followed by silver staining or immunoblotting and PhosphorImager analysis.

### Results

**PCNA Does Not Prevent Nucleotide Incorporation by pol δ Close to a Downstream Biotin-Streptavidin Complex—**Based on x-ray crystallographic analysis (4), it was suggested that PCNA functions as a trimer to encircle DNA and slide along it. Other studies indicate that pol δ binds to the C-terminal side of PCNA (9, 11). A priori, one could envision two possible arrangements for pol δ and PCNA on the DNA template-primer. In the first model (Fig. 1A), PCNA is situated behind pol δ. This is similar to the way in which the β-subunit of E. coli pol III is believed to function (14) and is supported by some experimental data reported to date (20, 21). Moreover, the central “hole” formed by the PCNA trimer is of sufficient caliber to accommodate a DNA duplex as would be required by this model. In the second model (Fig. 1B), PCNA is situated “ahead” of pol δ.

A 20-mer primer was annealed to a 50-mer template that had a biotinylated base at position 11 (Fig. 2A). pol δ alone can elongate the primer up to 39 nucleotides and stops 1 base before the biotin residue (Fig. 2B, lane 1). If streptavidin is added before incubation, pol δ can extend the primer only up to 37 nucleotides, presumably due to steric interference by the bulky biotin-streptavidin complex (Fig. 2B, lane 3). As expected (22), addition of PCNA allowed pol δ to bypass the biotinylated base in the absence of streptavidin and to elongate the primer to the end of the template (50 nucleotides) (Fig. 2B, lane 2). Nevertheless, there was an apparent slowing of elongation at a length of 39 nucleotides. PCNA-dependent bypass by pol δ was previously reported for other template lesions (22–24). If PCNA is located ahead of pol δ in the complex (see Fig. 1B), it should block primer elongation close to the biotin-streptavidin complex. Based on the x-ray crystallographically determined size of PCNA, we can estimate that pol δ should stop synthesis at or near the spot marked by the arrow to the left of Fig. 2B. But with streptavidin present, pol δ-PCNA can extend the primer even further than pol δ alone (Fig. 2B, compare lanes 4 and 3). These data are therefore consistent with the notion that PCNA is located behind pol δ during DNA synthesis.
Fig. 2. PCNA does not prevent nucleotide incorporation by pol $\delta$ close to a downstream biotin-streptavidin complex. A, shown are the nucleic acid substrates used to elucidate the relative locations of PCNA and pol $\delta$ during DNA synthesis. The position of the biotinylated dAMP (bio-7-dAMP; Bio) is indicated. B, standard reaction mixtures (5 $\mu$l) all contained 5 ng of calf thymus pol $\delta$, the 50-mer template with bio-7-dAMP incorporated at position 11 and the 20-mer primer (0.005 pmol of functional 3'-OH ends), as shown in A, and a 50 $\mu$m concentration of each dNTP. When PCNA was present, 70 ng were added. When streptavidin was present, 0.05 pmol were added to the reaction mixtures prior to addition of pol $\delta$ and, where indicated, prior to addition of PCNA. Polymerization reactions were for 5 min at 37 °C, after which gels were dried and subjected to PhosphorImager analysis. The arrow in B indicates the point at which synthesis (primer extension) would be expected to stop if PCNA were situated in front of pol $\delta$ during DNA synthesis.

A Biotin-Streptavidin Complex Upstream (in Back) of the Primer 5' Terminus Inhibits PCNA-mediated Stimulation of pol $\delta$ Much More on Short than on Long Primers—To demonstrate the relative positions of pol $\delta$ and PCNA in another way, we placed the biotin-modified nucleotide on the 3'-end of the template. We then annealed either a 15-mer primer or a 19-mer primer as shown (Fig. 3, A and B). Without streptavidin, either pol $\delta$ alone or pol $\delta$-PCNA can extend both primers efficiently. The dramatic effect of PCNA is demonstrated by the increased processivity and elongation of primers to the full length of the template provided (Fig. 3C, compare lanes 3 and 2 for the 15-mer primer and lanes 8 and 7 for the 19-mer primer). When streptavidin was added, PCNA-dependent synthesis was almost totally inhibited on the 15-mer (Fig. 3, C, compare lanes 5 and 3; and D), but synthesis by pol $\delta$ alone was unaffected (Fig. 3C, compare lanes 4 and 2). When streptavidin was present, PCNA-dependent synthesis on the 19-mer primer was only ~50% inhibited (Fig. 3, C, compare lanes 10 and 8; and D). Synthesis by pol $\delta$ alone was unaffected (Fig. 3C, compare lanes 9 and 7). These results are consistent with only the model in which PCNA is behind, as shown (Fig. 3, A and B). Similarly consistent, the activity of pol $\delta$ alone was not inhibited by streptavidin in a reaction mixture containing 10-mer or 13-mer primers annealed to the biotinylated template (data not shown). In contrast to results with larger primers (Fig. 3C, compare lanes 2, 7, 11, 13, 15, and 17 with lanes 3, 8, 12, 14, 16, and 18, respectively), elongation of the 10-mer or 13-mer by pol $\delta$ was also not affected by PCNA addition (data not shown).

Addition of streptavidin to a control mixture with the non-
biotinylated template did not affect the activity of pol δ either in the presence or absence of PCNA (Fig. 3C, lanes 11–18). When a 17-mer was used in this assay, PCNA-dependent synthesis was inhibited 72% in the presence of streptavidin (Fig. 3D). A 21-mer and 23-mer both gave results close to those obtained with the 19-mer, 52% and 51%, respectively, versus 47% inhibition (Fig. 3D). Experiments performed with longer primers (and templates) gave complex results (data not shown) that could not be interpreted in the current context.

As noted above, pol δ alone was active with short primers (10-mer and 13-mer). Moreover, comparison with longer primers (e.g., a 15-mer) suggested that it was fully active (data not shown). When primers were annealed to a biotinylated template, addition of streptavidin had no effect on pol δ-catalyzed DNA synthesis (data not shown). This suggests that in interacting with primer, pol δ contacts closely <10 base pairs of duplex DNA.

The 125-kDa Subunit of pol δ, But Not the 50-kDa Subunit, Interacts Closely with Single-stranded DNA—To elucidate how the pol δ subunits are located relative to DNA, we analyzed the photocross-linking of pol δ to a single-stranded 45-mer oligonucleotide containing a photoreactive analog of dTTP (AB-dUMP) at position 19 and [32P]dCMP at position 20 (Fig. 4A). Silver staining of an SDS-10% polyacrylamide gel loaded with 100 ng of pol δ UV-photocross-linked with the oligonucleotide shown in A, followed by treatment with micrococcal nuclease (lane 1), and PhosphorImager scan of the gel shown in lane 1 (lane 2). The molecular weights (×10−3) of the markers are shown to the right.

![Fig. 4](http://www.jbc.org/) The 125-kDa subunit of pol δ, but not the 50-kDa subunit, interacts closely with single-stranded DNA; analysis by UV photocross-linking. A, the 45-mer oligonucleotide with the photoreactive AB-dUMP nucleotide analog at position 19 and [32P]dCMP at position 20; B, silver staining of an SDS-10% polyacrylamide gel loaded with 100 ng of pol δ UV-photocross-linked with the oligonucleotide shown in A, followed by treatment with micrococcal nuclease (lane 1), and PhosphorImager scan of the gel shown in lane 1 (lane 2). The molecular weights (×10−3) of the markers are shown to the right.

lane 1) shows a polypeptide of ~21 kDa, corresponding to micrococcal nuclease. All larger polypeptides were derived from the pol δ preparation (data not shown). Comparison of silver stain patterns with PhosphorImager analysis revealed that the major radioactive band corresponded to the 125-kDa subunit of pol δ (p125) and that no [32P] labeling of the small subunit of pol δ (p50) could be detected.

Photocross-linking experiments were repeated with a mixture of primed templates as shown (Fig. 5A). Each of the templates had a single photoreactive analog (AB-dUMP) incorporated in one of eight possible positions followed by [32P]dCMP; a 21-mer primer was annealed also as shown (Fig. 5A). After standard photocross-linking and micrococcal nuclease treatment, the sample was subjected to SDS-PAGE and analyzed by PhosphorImager analysis and immunoblot assay with antibodies against p125 and p50 (Fig. 5B). Again, [32P] labeling of the 125-kDa subunit of pol δ, but not p50, was detected.

We noted that three bands immediately below p125 were not labeled in our original photocross-linking analysis with the single-stranded 45-mer (Fig. 4B). To elucidate the nature of p125 degradation that abrogates DNA binding, we analyzed a
preparation of pol δ that, based on protein staining, exhibited significant degradation of p125 (data not shown). We performed an experiment with antibodies against either the N or C terminus of p125 in conjunction with standard photocross-linking and micrococcal nuclease digestion (Fig. 5C). Cross-linking of the template to a degraded large subunit with a preserved C terminus was clearly visible. Cross-linking to a degraded large subunit with a preserved N terminus was undetectable (Fig. 5C).

**DISCUSSION**

Based both on our results and those of others, a model may be proposed for the actively polymerizing pol δ-PCNA-DNA complex (Fig. 6). As evidenced by de novo DNA synthesis, we suggest that, by itself, the two-subunit form of pol δ can bind the DNA template-primer (see, for example, Ref. 17). Although composed of a 125-kDa polypeptide (p125, the “catalytic” subunit) and a 50-kDa polypeptide (p50), photocross-linking results (Figs. 4 and 5) suggest that only the catalytic subunit (p125) interacts directly with DNA; p50 does not. Consistent with this, the work of others suggests that only p125 is necessary for primer-dependent, template-directed DNA synthesis; however, p50 is required for pol δ-catalyzed incorporation to be stimulated by PCNA (25–27). There is also evidence to suggest that p125 interacts significantly with PCNA (28, 29). Our results do not in any way preclude such an interaction.

When PCNA is included in incubations, it encircles the duplex DNA behind pol δ and, by simultaneously binding pol δ, enhances template-primer binding by pol δ (decreases $k_{cat}$) by nearly 2000-fold (16). Moreover, since PCNA functions as a trimer, each binding site is present in triplicate. Thus, it can interact with other molecules (e.g. RF-C, DNA ligase I, and FEN-1) at the same time as pol δ. The model of the pol δ-PCNA complex is consistent with the structure of PCNA derived from x-ray crystallography (4, 30) and with the sliding clamp model for DNA synthesis proposed by others (6). This model proposes a general mechanism of DNA polymerase processivity factor action. The suggestion that, during synthesis, PCNA is situated behind, not in “front” of, pol δ is based on our current observation that PCNA-dependent DNA synthesis by pol δ proceeds to a point immediately adjacent to a streptavindin-bound biotinylated template nucleotide (Fig. 2). If PCNA was situated in front of pol δ, we reasoned that since PCNA is thought to encircle the DNA, steric hindrance from the streptavindin would render PCNA-dependent DNA synthesis by pol δ impossible in its immediate vicinity. In other words, PCNA itself would get in the way. In empirical terms, the crystal-derived size of PCNA (4, 30) suggests that if PCNA were located in front of pol δ, synthesis would stop at or near nucleotide 30. Instead, it continues to nucleotide 39; the streptavindin-bound biotinylated template residue would be opposite nucleotide 40. That such hindrance does indeed occur is evidenced by the near-total inhibition of PCNA-dependent synthesis catalyzed by pol δ on a 15-mer primer (Fig. 3; also see below).

We considered the alternative possibility that a complex of pol δ and PCNA was responsible for extension of the primer up to about nucleotide 30 and that pol δ alone was responsible for synthesis up to nucleotide 39. For at least two reasons, we think this a very unlikely explanation for the results shown (Fig. 2). First, our interpretation of the results shown (Fig. 2), which include DNA synthesis largely or entirely by a pol δ-PCNA complex, is fully consistent with and thus corroborated by the effect of streptavindin placement behind the primer terminus (Fig. 3; also see below). Second, in comparing lanes 3 and 4 in Fig. 2B, we noted that synthesis by pol δ alone (lane 3, without PCNA) resulted in a distribution of synthesis products that was very different from that seen when PCNA was added (lane 4, with PCNA). This observation suggests that synthesis near the template streptavindin in the presence of PCNA is not by pol δ alone, but rather by a complex, the presence of which was dependent on PCNA addition. We presume this to be the pol δ-PCNA complex.

The placement of PCNA behind pol δ in the catalytically active pol δ-PCNA-DNA complex was further supported by results demonstrating that streptavindin-mediated inhibition of PCNA-dependent, pol δ-catalyzed DNA synthesis was inversely related to the size of the oligonucleotide used to prime incorporation (Fig. 3). By using a modification of a method described previously (31), we found that with a 15-mer primer, streptavindin-mediated inhibition of PCNA-dependent DNA synthesis was almost complete (Fig. 3C, compare lanes 5 and 3); in contrast, we saw no streptavindin-mediated inhibition of PCNA-independent synthesis (Fig. 3C, compare lanes 4 and 2). Use of a 19-mer primer on the same template resulted in much less streptavindin-mediated inhibition of PCNA-dependent DNA synthesis (Fig. 3C, compare lanes 10 and 8). Again, no inhibition of PCNA-independent DNA synthesis was observed (Fig. 3C, compare lanes 9 and 7).

Upon examination of the results in Fig. 3D, we noted that even at apparently optimal primer length, maximal synthesis in the presence of streptavindin was only ~50% of that seen in its absence. This was clearly not a direct effect of streptavindin since streptavindin did not in any way inhibit pol δ-catalyzed DNA synthesis on a control (non-biotinylated) template (Fig. 3C, lanes 11–18). Rather, we think that this inhibition provides specific insight into the mechanism by which PCNA stimulates pol δ in the absence of RF-C and ATP. In the absence of streptavindin, PCNA can load and/or unload passively onto the synthetic oligonucleotide template-primer from either end. In the presence of streptavindin, loading and/or unloading is blocked from the primer end and can only occur from the template end. Moreover, loading of PCNA must occur randomly, such that it can assume either of two orientations on the DNA. PCNA has polarity. In one orientation, the C-terminal (pol δ-interacting) side would be pointed away from the 3’-OH primer terminus, and loading would hence be nonproductive. For PCNA to stimulate pol δ, unloading and reloading in the opposite orientation would have to occur. Since RF-C is always present in vivo, this inhibition is likely of no biological significance. However, from a mechanistic perspective, our results suggest that PCNA can be loaded onto DNA from either the template (single-stranded) or primer (double-stranded) side of the 3’-OH terminus. This conclusion may have biological
implications.

Because the length of the DNA double helix is known precisely, it may be used, in conjunction with quantitative analysis of data such as those shown (Fig. 3D), as a molecular “ruler” to estimate the size of the pol δ-PCNA complex as it interacts with the DNA. With a 15-mer primer and streptavidin present, PCNA-dependent synthesis was negligible. With a 19-mer primer, PCNA-dependent synthesis was maximal (evidenced by the observation that synthesis did not increase when a 21-mer primer was used). A 17-mer primer gave an intermediate amount of PCNA-dependent DNA synthesis, thereby indicating a significant increase between a 17-mer and a 19-mer. Together, these data suggest that the pol δ-PCNA complex occupies a region of the duplex behind the primer terminus of between 58 and 65 Å. Since results of x-ray crystallographic analysis indicate that PCNA is ~30 Å thick, this would suggest that pol δ occupies between 28 and 35 Å or between 8 and 10 nucleotides of the primer duplex immediately distal to (behind) the 3′-OH terminus. This last conclusion is also consistent with results of photocross-linking inhibition of pol δ-alone activity with short primers (10-mer and 13-mer). Previously, we concluded, based on results of steady-state analyses, that pol α, another essential eukaryotic DNA polymerase, interacted with eight primer nucleotides, four of which had to be base-paired, during enzymatic DNA synthesis (Ref. 32; see also Ref. 33). Our present conclusions for pol δ, derived from entirely different approaches, are remarkably similar.

Photocross-linking to p125 occurs independently of whether single-stranded or template-primer DNA is used. Only the template-primer is a substrate for pol δ-catalyzed DNA synthesis. However, single-stranded DNA competes with the template-primer for binding to pol δ (17). This suggests that the interaction of pol δ with single-stranded DNA occurs through the active site of the enzyme. Current photocross-linking results are consistent with this notion.

Analyses of p125 sequence demonstrated the presence of zinc finger DNA-binding motifs located near the C terminus of the pol δ catalytic subunit (34). Similarly, deletion mutagenesis revealed that the C terminus of pol δ p125 is essential for DNA polymerase activity (35). Our current photocross-linking results, performed in conjunction with immunoblot analyses (Fig. 5), demonstrate that DNA binding, as measured in our assay, is mediated largely or exclusively by this region of p125.
Architecture of the Active DNA Polymerase δ-Proliferating Cell Nuclear Antigen-Template-Primer Complex
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