The Mammalian DNA Polymerase δ−Proliferating Cell Nuclear Antigen−Template-Primer Complex: Molecular Characterization by Direct Binding†

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ABSTRACT: Three direct assays, polyacrylamide gel electrophoresis-band mobility shift, agarose gel electrophoresis-band mobility shift, and nitrocellulose filter binding, were established to study complexes formed among mammalian DNA polymerase δ (pol δ), proliferating cell nuclear antigen (PCNA), and synthetic oligonucleotide template-primers. In all contexts, complex formation requires simultaneous presence of pol δ, PCNA, and template-primer. Moreover, we showed in one such assay that the complex formed contains each molecular component. Nuclease protection experiments demonstrate that complex formation protects template from degradation by DNase I. The mass determined for the pol δ·PCNA·template-primer complex was about 267 kDa, consistent with the participation of one molecule of pol δ, two or three molecules of PCNA and one molecule of template-primer. PCNA alone behaved as a trimer (mass determined to be about 87 kDa). Complex could be manipulated enzymologically. Measurement of off rates demonstrates directly that PCNA stabilizes the pol δ·template-primer complex.

Proliferating cell nuclear antigen (PCNA), an auxiliary factor for DNA polymerase δ (pol δ), is active in both DNA replication [see e.g. Prelich and Stillman (1988) Waga and Stillman (1994)] and repair (Nichols & Sancar, 1992; Shivji et al., 1992; Stivala et al., 1993; Kvam & Stokke, 1994; Matsumoto et al., 1994). With respect to replication, it was suggested that the role of PCNA, though complex, was related to its marked ability to enhance the processivity of pol δ [see e.g. Tan et al. (1986), Bravo et al. (1987), and Prelich et al. (1987); see also Downey et al. (1988) and Prelich and Stillman (1988)]. The precise role of PCNA in DNA repair is currently obscure.

Theoretically, stimulation of pol δ processivity by PCNA could be due to either increased rates of individual nucleotide incorporation, enhanced template-primer binding, or both. Initial kinetic analyses demonstrated that the effect of PCNA on pol δ was due in part to acceleration of single nucleotide incorporation (k_{cat}) by the polymerase but suggested significant binding effects as well (Ng et al., 1991). To explore further the binding interactions among pol δ, PCNA, and nucleic acid, a polyacrylamide gel electrophoresis (PAGE)-band mobility shift assay was developed (Ng et al., 1993). In this assay, complex formation among these components required that all be present simultaneously. In addition, unlike cell-free SV40 DNA synthesis (Ng et al., 1990), *Drosophila* PCNA could not substitute for mammalian PCNA. This confirmed qualitatively that enhancement of pol δ processivity by PCNA was also due to stabilization of the binding between the polymerase and the nucleic acid substrate. Moreover, both nucleic acid template and complementary primer were required.

It was also noted in kinetic studies that, like other eukaryotic polymerases (Fisher & Korn, 1981a,b; Wong et al., 1986; Ng et al., 1989; Pelletier et al., 1994; Mozzherin & Fisher, 1995), pol δ could bind effectively to a 2′,3′-H (dideoxy)-terminated primer. This observation, in conjunction with results of enzyme inhibition studies, suggested that pol δ followed an ordered sequential ter-reactant mechanism of substrate recognition and binding. Like both pol α [for a review, see Fisher (1994)] and pol ϵ (Mozzherin & Fisher, 1995), pol δ alone binds template first followed by template-complementary primer and finally template-directed dNTP. It is not known what effect, if any, PCNA has on this mechanism.

To understand better the potential role of PCNA in DNA replication and repair, complex formation among pol δ, PCNA, and synthetic oligonucleotide template-primers was quantified using three different assay systems, all of which measure complex by direct binding. In all systems, complex detection required simultaneous presence of pol δ, PCNA, and template-primer; the complex formed included all molecular components. Complex formation protects the nucleic acid template from degradation by nucleases and could be manipulated enzymologically. The mass of the complex was estimated by polyacrylamide gel electrophoresis (a form of gel filtration). Our results demonstrate for the first time direct stabilization of the pol δ·template-primer complex by PCNA.

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EXPERIMENTAL PROCEDURES

Most of the materials and methods were described previously (Ng et al., 1989, 1991, 1993). SDS−PAGE was according to Laemmli (1970). Calf thymus pol δ was purified according to Lee et al. (1984) as modified (Ng et al., 1991). Calf thymus PCNA was purified according to Tan et al. (1986). Nondenaturing PAGE-band mobility shift assays were performed in either $MgCl_2$ or EDTA, exactly as previously (Ng et al., 1993) and as specified in the figure legends; Ferguson plot analyses (Ferguson, 1964) were performed on gels of varying polyacrylamide concentration (5.0, 5.5, 6.0, 6.5, and 7.0%) at a constant acrylamide to bis-acrylamide ratio. Nondenaturing agarose gel electrophoresis (AGE)-band mobility shift assays were performed on 2% agarose gels and otherwise as described for PAGE-band mobility shift assays. Agarose gels were not prerun. Further details are provided in the figure legends. Immunoblot analyses after nondenaturing PAGE using either colorimetric detection of alkaline phosphatase-conjugated secondary antibodies or enhanced chemiluminescence detection of horseradish peroxidase-conjugated secondary antibodies were otherwise as recently described [see Berrios et al. (1995), Maus et al. (1995), and Stuurman et al. (1995)].

Materials. Bovine serum albumin was from Pierce Chemical Co. (Rockford, IL); DNase I and urease were from Sigma Chemical Co. (St. Louis, MO). Nitrocellulose was from Schleicher and Schuell (Keene, NH). Immobilon-P was from Millipore (Bedford, MA). Mouse monoclonal antibody PC10, directed against mammalian PCNA, was obtained from Oncogene Sciences (Uniondale, NY) and was used at a final dilution of 1:1000. Two rabbit antisera, directed against the large subunit (p125) of mammalian pol δ, were prepared against two different peptides. The peptide antigens were KRRPGPGGVPPKRARC (the NH_2 terminal region of the pol δ large subunit) and CDQEQLLRRFGPPGP (the COOH terminal region of the pol δ large subunit), each linked to maleimide-activated keyhole-limpet hemocyanin (Pierce Chemical Co.) through the C residue as specified by the manufacturer. Rabbit antiserum directed against the small subunit (p48) of mammalian pol δ (Zhang et al., 1995) was prepared against recombinant human protein which had been expressed in bacteria using a pET vector. The recombinant protein was recovered in inclusion bodies, solubilized in 6 M urea, purified by chromatography on DEAE-cellulose, and renatured by sequential dialysis against solutions with decreasing urea concentrations. Each antiserum was diluted 1:1000; the three diluted sera were pooled for probing blots.

Preparation of Nucleic Acids for Binding Studies. Synthetic oligonucleotides of defined sequence were prepared using conventional phosphoramidite chemistry (Takeshita et al., 1987). Terminal deoxynucleotidyltransferase (Life Sciences Inc., St. Petersburg, FL) was used to add dideoxynucleoside monophosphate (ddNMP) residues to synthetic 20-mer primers, essentially as previously (Fisher & Korn, 1981a; Ng et al., 1989, 1991; Weiss & Fisher, 1992). After ddNMP addition, ddNMP-terminated 21-mers were separated from unreacted 20-mers by denaturing PAGE performed preparatively. Typically, 65−100% of the 20-mer starting material was converted to ddNMP-terminated 21-mer during the incubation with terminal deoxynucleotidyltransferase.

Nitrocellulose Binding Assays. For nitrocellulose binding assays of protein−nucleic acid interaction, nucleic acids were 5′-^{32}P-labeled and incubations were formulated exactly as for the gel mobility shift. Instead of being subjected to electrophoresis, samples were applied directly to a wet nitrocellulose membrane in a BRL Hybri·Dot Manifold. The nitrocellulose had been prewashed in H_2O, followed by 1 × 100 μL of 40 mM Bis-Tris (pH 6.8), and 1 mM DTT, and 1 mM EDTA (wash buffer). Care was taken not to subject the nitrocellulose to any physical pressure during handling, and the apparatus was not tightened beyond "finger-tight" as specified by the manufacturer. Physical pressure or overtightening resulted in unacceptably high binding of DNA alone (background). After sample application, the nitrocellulose was washed with 2 × 100 μL per sample of wash buffer. The nitrocellulose was then removed from the apparatus, dried, and exposed to X-ray film and/or subjected to quantitative analysis. k_{off} and $t_{1/2}$ were calculated from quantitative data according to Fersht (1985).

Quantification. Quantification of protein−nucleic acid complexes was by analysis using the BioRad Molecular Imager. Further details are provided in the figure legends.

In Situ Determination of DNA Polymerase Activity. Determination of DNA polymerase activity in polyacrylamide gels *in situ* was performed after standard nondenaturing PAGE-band mobility shift assays except that gels contained 40 μg/mL BSA and nucleic acid template-primers were unlabeled. After electrophoresis, entire gels were incubated for 30 min at 37 °C in a standard pol δ reaction mixture (Ng et al., 1991, 1993) containing 6 mM $MgCl_2$, 40 μM dCTP, and 60 μCi of [α-^{32}P]dTTP. After incubation, gels were washed extensively (24−48 h) in cold 5% trichloroacetic acid to remove unincorporated dTTP, after which they were subjected to autoradiography.

RESULTS

Formation of a PCNA-Dependent Complex between pol δ and Oligonucleotide Template-Primers. To study the interaction of pol δ with template-primers, PAGE-band mobility shift assays were initially performed as previously (Ng et al., 1993) and results were compared with those obtained by AGE and nitrocellulose filter binding. PAGE-stable complex formation with the typical 30−21-mer synthetic oligonucleotide template-primer was detected by autoradiography after 5′-^{32}P-labeling of nucleic acid and was dependent on the simultaneous presence of pol δ, PCNA, and 30−21-mer (Figure 1 inset). Moreover, the amount of complex detected was linearly dependent upon the amount of pol δ added to the incubation (Figure 1).

Because of the limited mobility of pol δ·PCNA·template-primer complexes on polyacrylamide gels, complex formation was studied on agarose gels. The pol δ·PCNA·template-primer complex was readily detectable after AGE; as in PAGE-band mobility shift assays [see Ng et al. (1993) and Figure 1 inset], formation of an AGE-detectable complex was dependent on the simultaneous presence of pol δ, PCNA, and labeled template-primer, was comparable with dideoxy-terminated and 3′-OH-terminated primers, and occurred equally well in EDTA or $MgCl_2$ (not shown).

Both PAGE, and AGE-band mobility shift assays depend upon electrophoresis; to perform electrophoresis requires a minimum of 2 h and by placement of the complex in an

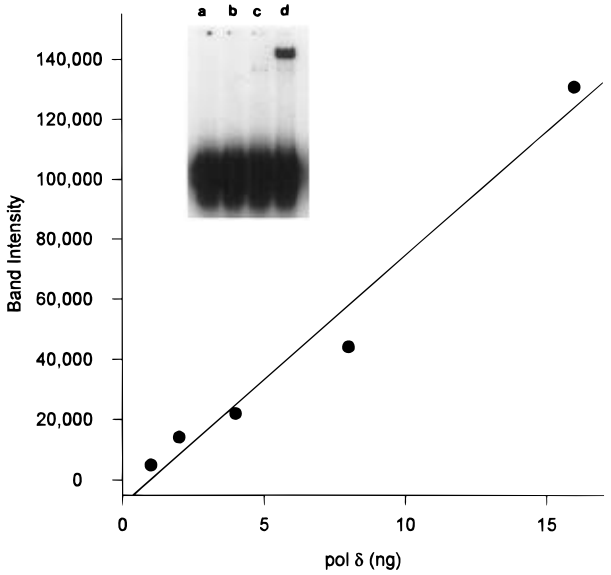

FIGURE 1: Complex detection requires the simultaneous presence of pol δ, PCNA, and oligonucleotide template-primer and is linearly dependent on the amount of pol δ; nondenaturing 7% PAGE analysis. Autoradiogram (inset): lane a, ^{32}P-labeled template-primer alone; lane b, labeled template-primer plus PCNA; lane c, labeled template-primer plus pol δ; and lane d, labeled template-primer plus PCNA plus pol δ. Graph: labeled template-primer plus PCNA plus various amounts of pol δ as indicated, quantified by phosphorimager.

electric field involves strong physical forces that may induce dissociation of interacting macromolecules. In contrast, nitrocellulose filter binding occurs virtually instantaneously and does not involve any physical force other than relatively gentle suction. Accordingly, a nitrocellulose filter binding assay was developed to investigate pol δ·PCNA·template-primer complex formation and stability. This assay is based on the fact, that under the conditions used, ^{32}P-labeled template-primer will not bind to nitrocellulose whereas template-primer bound to protein(s) will. As with both PAGE- and AGE-band mobility shift assays, complex formation requires simultaneous presence of pol δ, PCNA, and template-primer (Figure 2A). Also like PAGE assays (Figure 1), the intensity of binding was linearly related to the amount of pol δ initially present in the incubation (Figure 2B,C).

Molecular Characterization of the pol δ·PCNA·Template-Primer Complex. To determine if the ^{32}P-labeled complex seen after nondenaturing PAGE contained PCNA, immunoblot analyses were performed with monoclonal anti-PCNA antibody, PC10. Detection was with alkaline phosphatase-conjugated goat anti-mouse IgG and a one-solution alkaline phosphatase substrate (Kirkegaard and Perry, Gaithersburg, MD). Before nondenaturing PAGE, 30−21-mer template-primer was 5′-^{32}P-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP. After electrophoresis, macromolecules were transferred electrophoretically from the gel to Immobilon-P. The resulting blot replica of the gel was subjected to autoradiography to detect ^{32}P and immunoblot analysis to detect PCNA.

As expected, only the complete reaction mixture resulted in the characteristically shifted species seen after nondenaturing PAGE (Figure 3A, lane a, arrowhead). We think it noteworthy that the autoradiogram shown (Figure 3A) was made from a blot replica of a gel rather than from the gel

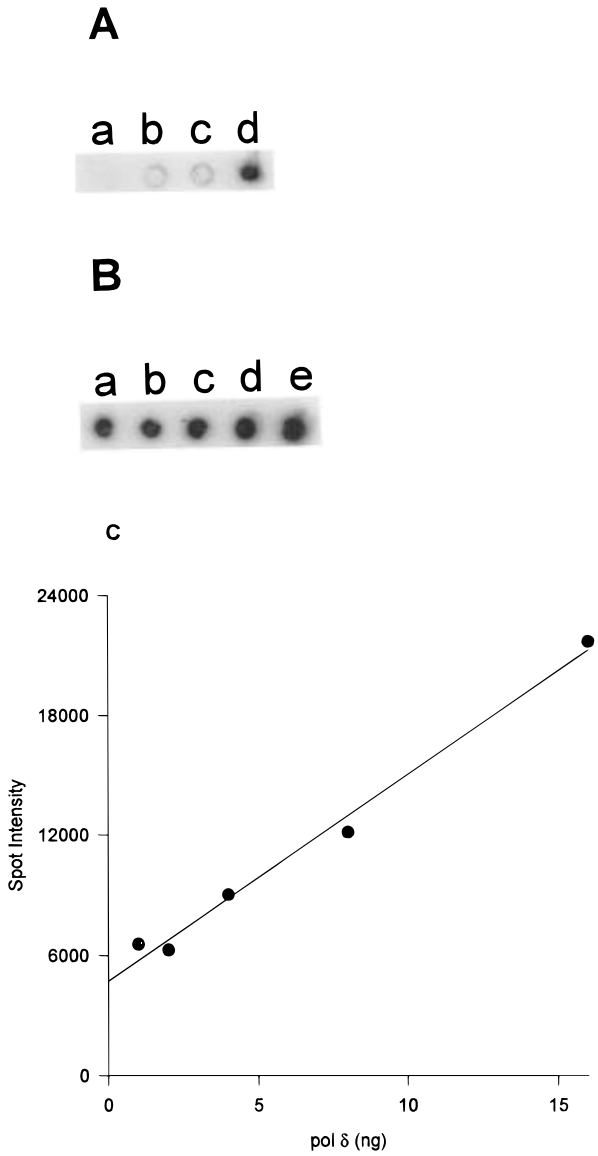

FIGURE 2: Complex detection requires the simultaneous presence of pol δ, PCNA, and oligonucleotide template-primer and is linearly dependent on the amount of pol δ; nitrocellulose filter binding analysis. (A) Spot a, ^{32}P-labeled template-primer alone; spot b, labeled template-primer plus PCNA; spot c, labeled template-primer plus pol δ; and spot d, labeled template-primer plus PCNA plus pol δ. (B) Labeled template-primer plus PCNA plus various amounts of pol δ: spot a, 1 ng; spot b, 2 ng; spot c, 4 ng; spot d, 8 ng; and spot e, 16 ng. (C) Data in B quantified by phosphorimager.

itself. Immunoblot analysis with anti-PCNA antibody revealed that this shifted species contained PCNA (Figure 3B, lane a, arrowhead). Large amounts of faster-migrating immunoreactive material were also detected in all lanes (Figure 3B).

To demonstrate the migration of pol δ, the blot replica shown in Figure 3A was probed with rabbit anti-pol δ antibodies. Detection was by enhanced chemiluminescence (Amersham, Arlington Heights, IL). The shifted species seen by ^{32}P autoradiography after nondenaturing PAGE (Figure 3A, lane a) contained most or all of the immunoreactive material loaded (Figure 3C, lane a, arrowhead). When pol δ and PCNA were mixed without template-primer, a species with different PAGE mobility was seen (Figure 3C, lane b). The migration of this species was indistinguishable from that seen when pol δ was subjected to electrophoresis alone (not

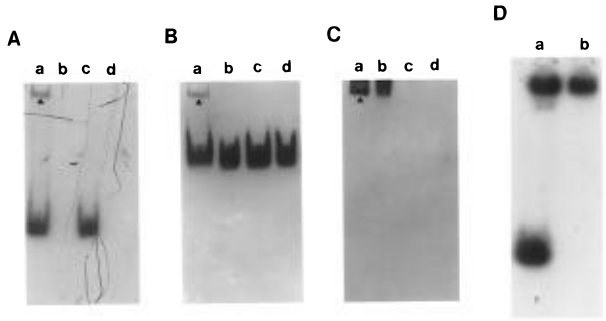

FIGURE 3: Triple-label experiment demonstrating that the complex detected by nondenaturing PAGE contains both PCNA and pol δ; the pol δ in the complex is active. For panels A−C, incubations contained the following: lanes a, ^{32}P-labeled template-primer plus PCNA plus pol δ ("complete"); lanes b, PCNA plus pol δ (no template-primer); lanes c, PCNA plus template-primer; and lanes d, PCNA alone. Nondenaturing PAGE was performed, and macromolecules were transferred to Immobilon-P: (A) autoradiogram, (B) PCNA detected colorimetrically with mouse anti-PCNA antibodies and alkaline phosphatase-conjugated anti-mouse IgG, (C) pol δ detected by enhanced chemiluminescence (ECL) with rabbit anti-pol δ antibodies and horseradish peroxidase-conjugated anti-rabbit IgG, and (D) pol δ activity detected by an *in situ* DNA polymerase assay. Assays were performed as described (Experimental Procedures). After nondenaturing PAGE, the entire gel was incubated in standard pol δ reaction mixture. Incubations before PAGE contained the following: lane a, ^{32}P-labeled template-primer plus PCNA plus pol δ; and lane b, unlabeled template-primer plus PCNA plus pol δ.

shown). Although this difference was minimal, it was observed reproducibly. Thus, it appears that pol δ and PCNA can only interact in the presence of nucleic acid template-primer.

The complex formed among pol δ, PCNA, and template-primer is presumably poised to replicate DNA. When a 3′-OH-terminated primer is used and the pol δ·PCNA·template-primer complex is formed in EDTA, the only additional components required to visualize DNA synthesis are $MgCl_2$ and radiolabeled dNTP. Accordingly, complex was formed with unlabeled template-primer; to monitor the formation and mobility, complex was also formed with ^{32}P-labeled template-primer. Both were subjected to nondenaturing PAGE, and the portion of the gel containing complex formed with unlabeled template-primer was incubated in [α-^{32}P]dTTP, dCTP, and 6 mM $MgCl_2$. Nucleotide incorporation was readily detected, coincident in PAGE mobility with the complex formed among pol δ, PCNA, and labeled template-primer (Figure 3D, compare lanes a and b). Thus, we conclude that the pol δ·PCNA·template-primer complex detectable after nondenaturing PAGE contains active pol δ.

The observation that the pol δ·PCNA·template-primer complex could be formed with limiting nucleic acid (i.e. at very low template-primer concentrations; Ng et al., 1993) suggested conditions under which protection of DNA from digestion by DNase I ("footprinting") would be demonstrable. Although results were complex, pol δ combined with homologous PCNA clearly protected 5′-^{32}P-labeled 30-mer present as a 30−21-mer template-primer from digestion by DNase I (Figure 4). DNase I is a double-stranded specific nuclease. Hence, it is impossible to draw any conclusions regarding protection of the single-stranded region of the 30−21-mer. However, in the duplex region, pol δ and PCNA protected virtually all of the available DNA (Figure 4, lane b).

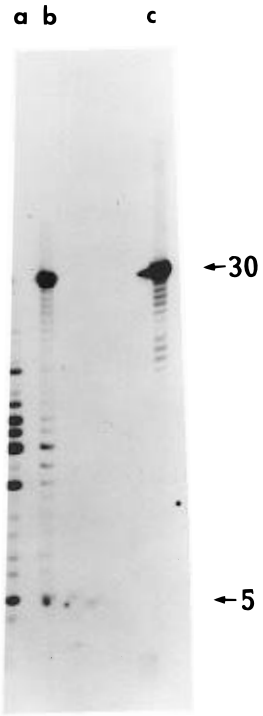

FIGURE 4: Footprinting demonstrates that pol δ and PCNA protect template from degradation by nuclease. Digestion was with DNase I; template was 5′-^{32}P-labeled. Autoradiogram: lane a, labeled template-primer alone; lane b, PCNA plus pol δ plus labeled template-primer; and lane c, labeled template-primer without DNase I treatment.

To corroborate these observations, ^{32}P-labeled material was extracted from the putative protein−nucleic acid complex detected after nondenaturing PAGE and subjected to standard denaturing PAGE (Ng et al., 1989). Both 30-mer (template) and 21-mer (primer) were detected in approximately equimolar ratios (not shown).

Ferguson plot (Ferguson, 1964) analysis was used to estimate the size of the complex formed among pol δ, PCNA, and template-primer. Gels of five different polyacrylamide concentrations were used, and the mass of the complex relative to known standards was determined. On the basis of data shown (Figure 5) as well as one complete additional experiment (not shown), the mass of the pol δ·PCNA·-template-primer complex was estimated to be 267 kDa (mean of two independent determinations with masses measured at 265 and 268 kDa, respectively). The mass of PCNA alone determined on the same gels was 87 kDa (mean of two independent determinations with masses measured at 85 and 88 kDa, respectively); identical results were obtained with both mammalian and *Drosophila* PCNA (not shown). These data, in conjunction with those presented on complex composition (Figure 3), suggest that the pol δ·PCNA·-template-primer complex is formed by one molecule of pol δ (175 kDa), two to three molecules of PCNA (58.5−87.8 kDa), and one molecule of template-primer (16.8 kDa). These data are also consistent with the conclusion that PCNA alone is present as a trimer.

Effect of Template-Complementary dNTP on Detection of Complex Formed by pol δ and PCNA with Template-Primers. In the presence of Mg^{2+}, addition of template-specified dNTP led to destabilization of complex with template annealed to 3′-OH-containing primers (Ng et al., 1993; see also Figure 6, lanes a versus c) and stabilization

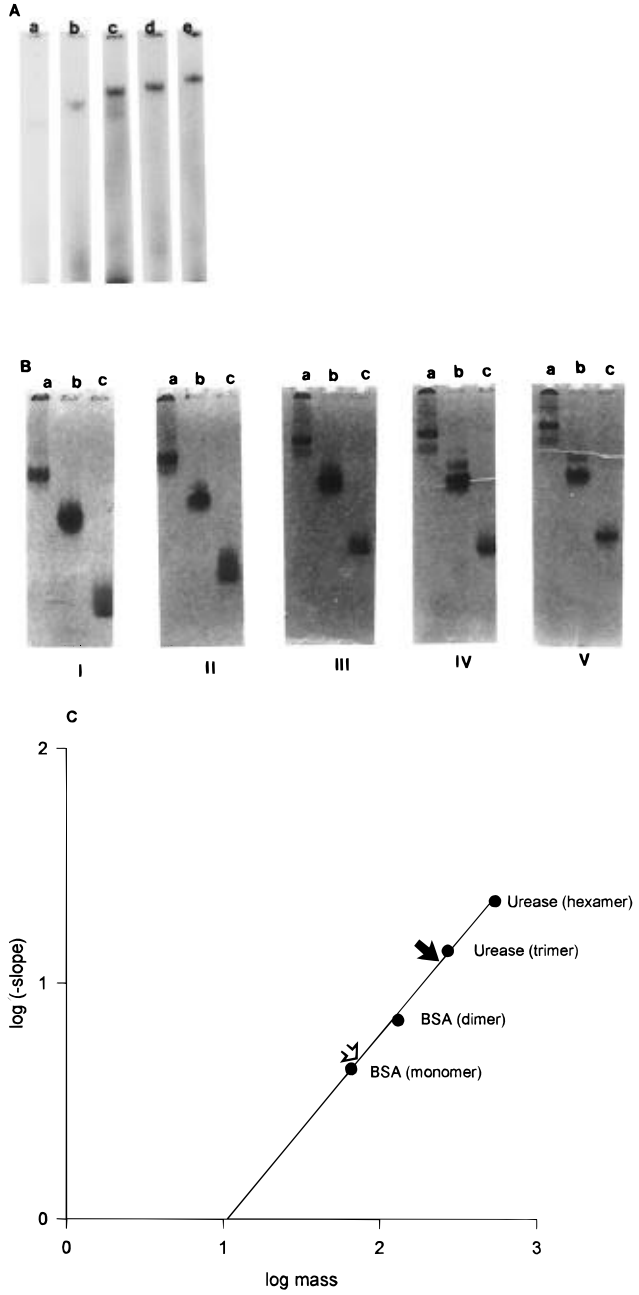

FIGURE 5: Ferguson plot analysis demonstrates that the pol δ·PCNA·template-primer complex has a mass of about 267 kDa; PCNA alone has a mass of about 87 kDa. (A) pol δ·PCNA·^{32}P-labeled 30−21-mer complex formed on each of five gels of varying polyacrylamide concentration: lane a, 5.0%; lane b, 5.5%; lane c, 6.0%; lane d, 6.5%; and lane e, 7.0%. Lanes are aligned such that the origins of each are positioned identically. (B) Coomassie blue-stained proteins including standards and PCNA on each of five gels: I, 5.0%; II, 5.5%; III, 6.0%; IV, 6.5%; and V, 7.0%. Lanes a, urease; lanes b, bovine serum albumin; and lanes c, calf thymus PCNA. (C) Ferguson plot of log(−slope) versus log mass. The solid arrow designates the value determined for the pol δ·PCNA·30−21-mer complex; the open arrow designates the value determined for free PCNA.

of PAGE-detectable complexes with template annealed to 2′,3′-H-containing primers (Figure 6, lanes b versus d). Thus, the interaction with a 2′,3′-dideoxy primer documented for pol δ alone (Ng et al., 1991) apparently occurs similarly in the presence of homologous PCNA. Moreover, PAGE-band mobility shift can be used for enzymologic study.

PCNA Stabilizes Directly the Complex Formed between pol δ and Template-Primer. The nitrocellulose filter binding

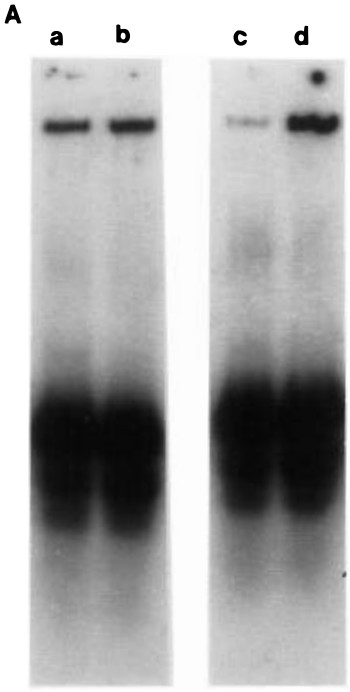

FIGURE 6: Effect of template-specified dNTP on PCNA-dependent complex formed by pol δ with 30−21-mer; comparison of 3′-OH-terminated primers with 2′,3′-H-terminated primers. Effect of template-specified dNTP on complex formation with template-primers containing 3′-OH versus 2′,3′-H-terminated primers: lane a, 3′-OH; lane b, 2′,3′-H; lane c, 3′-OH plus dTTP; and lane d, 2′,3′-H plus dTTP. $MgCl_2$ (6 mM) was included in all solutions and was required for all dNTP-related effects.

assay was used to compare directly the stability of the pol δ·PCNA·template-primer complex with that of the pol δ·-template-primer complex. Complex was formed with ^{32}P-labeled 30−21-mer, a 100-fold excess of unlabeled 30−21-mer was added, and the rate of exchange (loss of detectable complex) was measured. This technique allowed precise determination of $t_{1/2}$ for both the pol δ·PCNA·template-primer complex and the pol δ·template-primer complex. With PCNA present, $t_{1/2}$ was measured at 2.65 h (mean of two independent determinations with $t_{1/2}$ measured at 2.4 and 2.9 h, respectively); data from one such experiment are shown (Figure 7). In the absence of PCNA, $t_{1/2}$ was less than 5 s (no complex was detectable 30 s after addition of the unlabeled 30−21-mer). These data demonstrate directly that PCNA stabilizes the pol δ·template-primer complex greater than 1900-fold.

DISCUSSION

Three different binding assays were optimized for characterization of mammalian pol δ·PCNA·nucleic acid complexes. One, PAGE-band mobility shift, was described previously (Ng et al., 1993) and was used in the current work to determine complex composition (Figure 3), to estimate the mass of the pol δ·PCNA·template-primer complex (Figure 5), and to study the complex enzymologically (Figure 6). The second assay, AGE-band mobility shift, is novel and will be particularly useful for identification and characterization of additional proteins that interact with pol δ, PCNA, and/or nucleic acid template-primer in facilitating nuclear DNA synthesis in mammals. This is because, in our estimation, agarose offers greater resolution than polyacrylamide for protein−nucleic acid complexes larger than that

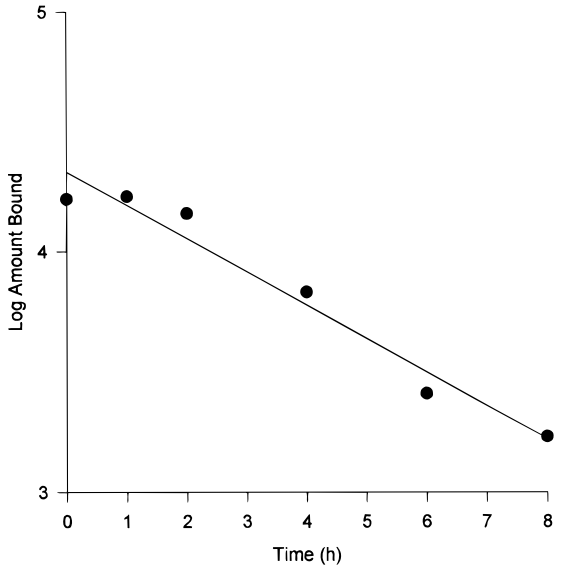

FIGURE 7: PCNA stabilizes directly the pol δ·template-primer complex more than 1900-fold. ^{32}P-labeled template-3′-OH-containing primer was mixed with pol δ and PCNA, incubated for 2 min at 37 °C to form the pol δ·PCNA·labeled template-primer complex, and then mixed with a 100-fold excess of unlabeled but otherwise identical template-primer. At the times indicated, the complex remaining after incubation at 4 °C was spotted on nitrocellulose and spots were quantified using a phosphorimager. k_{off} was determined by fitting the data to a single exponential curve. $t_{1/2}$ was calculated from this curve. $t_{1/2}$ for the pol δ·template-primer complex was estimated identically (not shown).

formed among pol δ, PCNA, and template-primer. The third, nitrocellulose filter binding, is the most amenable of the three to rigorous quantitative analysis. Accordingly, it was used in the current work to establish the effect of PCNA in reducing the k_{off} for the pol δ·template-primer complex (Figure 7).

In all three contexts, complex detection requires simultaneous presence of pol δ, PCNA, and nucleic acid template-primer. Although this observation could be most easily explained if all three macromolecules participated directly in the complex formed, the possibility of a catalytic role in complex formation or stabilization for either pol δ or PCNA could not be excluded. Moreover, since in previous studies both template and primer were ^{32}P-labeled, it was also possible that only one of them was present in the radiolabeled complex detected after PAGE. Immunoblot analyses with an anti-PCNA antibody (Figure 3B) demonstrated that the protein−nucleic acid complex detected did indeed contain PCNA. A large amount of apparently free PCNA was also detected (Figure 3B). PCNA titration experiments (M. McConnell and P. A. Fisher, unpublished) established that excess (i.e. free) PCNA was in fact required to promote PAGE-stable complex formation.

Immunoblot analyses with anti-pol δ antibodies confirmed the presence of pol δ in the protein−nucleic acid complex detected after nondenaturing PAGE (Figure 3C). Moreover, this pol δ was active as a DNA polymerase (Figure 3D). In contrast with results obtained for PCNA, the amount of complex detected was linearly dependent on the amount of pol δ included in the incubation as judged by both the PAGE-band mobility shift assay (Figure 1) and the nitrocellulose filter binding assay (Figure 2B and C). Consistent with these observations, most or all of the pol δ detected by immunoblot analysis was in the pol δ·PCNA·template-primer complex (Figure 3C, lane a). Hence, we can conclude that, under the conditions used (excess PCNA and template-primer), the amount of complex detected is limited primarily by the amount of pol δ provided.

Previously, we reported that both template and complementary primer were required to promote complex formation (Ng et al., 1993). During the current study, we noted that the complex detected was of identical gel mobility when only the template was radiolabeled, when only the primer was radiolabeled, and when both were radiolabeled (M. McConnell and P. A. Fisher, unpublished). Finally, pol δ and PCNA protect template from the actions of DNase I (Figure 4); after complex extraction and re-electrophoresis of ^{32}P-labeled nucleic acid on denaturing gels, both template and primer were detected in approximately equivalent ratios (Results). These observations establish the presence of both template and primer in the putative pol δ·PCNA·template-primer complex.

Ferguson plot analysis performed by nondenaturing PAGE-band mobility shift assay (Figure 5) is to the best of our knowledge the first estimation of mass for the pol δ·PCNA·-nucleic acid complex. The primary plot (mobility versus polyacrylamide concentration) of data shown for the complex (Figure 5A) was perfectly linear, suggesting normal behavior under the conditions used. From this analysis, we estimate that the biologically active form of pol δ core enzyme is monomeric (two dissimilar subunits with a total mass of 175 kDa) while biologically active PCNA is either dimeric or trimeric (58.5−87.8 kDa). By similar Ferguson plot analysis, free authentic mammalian PCNA was apparently trimeric in aqueous solution, consistent with results of X-ray crystallography performed with recombinant yeast PCNA (Krishna et al., 1994). Our results are also consistent with those of Kong et al. (1992), who suggested on the basis of X-ray crystallography of the β subunit of *Escherichia coli* DNA polymerase III holoenzyme that free PCNA was trimeric.

Results of footprinting experiments indicated that pol δ and PCNA together protected virtually the entirety of a 21-nucleotide duplex region of the oligonucleotide template-primer from digestion by DNase I (Figure 4). This constitutes more than two turns of the DNA helix totaling about 7 nm. The reported size (twice the Stokes' radius) of pol δ is 10.6 nm (Lee et al., 1984); the reported size (twice the Stokes' radius) of PCNA is 7.3 nm (Tan et al., 1986). Our data are thus consistent with the notion that, together pol δ and PCNA contact the DNA duplex extensively in forming the catalytically active protein−nucleic acid complex. Because DNase I is a double-stranded specific nuclease, no information is available on protein single-stranded template contacts in the pol δ·PCNA·template-primer complex. Presumably, footprinting experiments performed with single-stranded specific nucleases and/or cleavage reagents will allow definitive conclusions in this regard. Longer templates and primers may also be required to enhance conclusions from future studies.

The ordered sequential mechanism of substrate recognition and binding by mammalian pol δ was elucidated through a combination of steady state kinetics and sedimentation binding (Ng et al., 1991). Crucial to the establishment of this mechanism was the observation in enzymologic analyses of induced dNTP inhibition in the presence of a 2′,3′-dideoxy primer. Current results (Figure 6) confirm this mechanism by direct binding and as such establish for the first time the

utility of 2′,3′-dideoxy primer-templates as mechanism-based affinity ligands for the purifcation of mammalian pol δ. Such a strategy was used successfully for the purification of herpes virus DNA polymerase (Reardon & Spector, 1989).

Current results also quantitate, for the first time, the enhancement of template-primer binding by pol δ brought about by addition of homologous PCNA. It should be stressed that this is under static conditions (no dNMP incorporation) and in the absence of dNTPs. We previously presented evidence which was interpreted to suggest that dNTPs substantially destabilize the pol δ·PCNA·template-primer complex (Ng et al., 1993). Experiments leading to these results need to be repeated in the context of the nitrocellulose filter binding assay. These caveats notwithstanding, our data demonstrate that PCNA reduces dissociation of pol δ from the model template-primer by more than 1900-fold (Figure 7). The processivity of pol δ alone on poly(dA)-$(dT)_{10}$ was determined to be 7−10 nucleotides (Downey et al., 1988). Hence, we can caculate that, on the basis of decreased k_{off} alone, the processivity of the pol δ·PCNA complex would be at least 13 300 nucleotides incorporated per binding event. For a typical mammalian chromosome, this, in conjunction with the increase in the rate of single-nucleotide incorporation determined previously, could result in complete leading strand replication in a single binding event. This, in our estimation, is consistent with the efficiency of *in vivo* DNA synthesis.

Current models imply that PCNA is first loaded onto the double-stranded DNA by the action of RF-C [see e.g. Fukuda et al. (1995)]. There it has been proposed to recruit pol δ to form a specific pol δ·PCNA·template-primer complex. We here offer a different interpretation of available data, based largely on two observations. First, free pol δ is active as a DNA polymerase in the absence of PCNA [see Ng et al. 1991)]; this indicates that it alone can recognize template, primer, and dNTP. Second, in the absence of template-primer, pol δ and PCNA do not form a complex detectable by nondenaturing PAGE (Figure 3B,C, lanes b). As we proposed previously [see Figure 9 of Ng et al. (1993)], these results suggest that free pol δ (i.e. lacking PCNA) binds first to template and then to template-complementary primer to form the pol δ·template-primer complex. Only then is pol δ competent to bind homologous PCNA. PCNA, which may indeed be resident on the DNA duplex, interacts with the pol δ·template-primer complex to form the pol δ·PCNA·template-primer complex. As documented (Figure 7), this last interaction results in nearly a 2000-fold stabilization of the pol δ·template-primer complex prior to the onset of catalysis.

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