Interactions of DNA with Human DNA Primase Monitored with Photoactivatable Cross-Linking Agents: Implications for the Role of the p58 Subunit†

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ABSTRACT: Regulation of the p49−p58 primase complex during primer synthesis and the interaction of the primase subunits with DNA were examined. After primase synthesizes a primer that DNA polymerase α (pol α) can readily elongate, further primase activity is negatively regulated. This occurs within both the context of the four-subunit pol α−primase complex and in the p49−p58 primase complex, indicating that the newly generated primer−template species need not interact with pol α to regulate further primase activity. Photo-cross-linking of single-stranded DNA−primase complexes revealed that whereas the isolated p49 and p58 subunits both reacted with DNA upon photolysis, only the p58 subunit reacted with the DNA when photolysis was performed using the p49−p58 primase complex. After primer synthesis by the complex, p58 was again the only subunit that reacted with the DNA. These results suggest a model for regulation of primer synthesis in which the newly synthesized primer−template species binds to p58 and regulates further primer synthesis. Additionally, the ability of p58 to interact with primer−template species suggests that p58 mediates the transfer of primers from the primase active site to pol α.

Eukaryotic DNA primase catalyzes the de novo synthesis of short RNA oligomers that are required for the initiation of new strands of DNA. DNA polymerase α (pol α)[1] then elongates these primers to complete the initiation process (*1*). Primase consists of two subunits with molecular masses of 58 and 49 kDa, and normally exists in a complex with the 180 kDa pol α subunit and a 70 kDa subunit whose function is still obscure (*2*, *3*). The p70 subunit may serve to tether the pol α−primase complex to replication forks as well as to help the production and nuclear translocation of p180 (*4*, *5*).

At present, there is little information about the catalytic organization of primase. Previous studies showed that the p49−p58 primase complex can be separated from the p180−p70 complex and retain primase activity (*3*, *6*, *7*). The p49 subunit can bind single-stranded DNA, contains phosphodiester bond forming activity, and if purified under the appropriate conditions can catalyze complete primer synthesis (*8*−*11*). Relatively little is known about the roles of p58, although it clearly helps stabilize p49, and may be involved in nucleotide binding, primer initiation, and processivity (*8*). Immunoprecipitation studies suggest that p58 binds to both the p180 and p49 subunits, whereas p49 only binds to p58 (*8*). Thus, the apparent location of p58 between p180 and p49 implies that it may help transfer the newly generated primer−template species between the primase active site on p49 and pol α. Deletion studies by Copeland indicate that contacts between the p49 subunit and the p58 subunit involve amino acids in both the N-terminal and C-terminal halves of p58 (*12*).

Kinetic experiments showed that the primase and pol α active sites are independent prior to primer synthesis (*13*). Once primase has generated a primer−template species, however, the two activities become coordinated such that further primer synthesis is negatively regulated (*13*). Negative regulation requires that the new primer remain stably bound to the template and be at least seven nucleotides long (i.e., a unit length primer). Reactivation of primase occurs when either pol α elongates the primer via dNTP polymerization or the primer−template species dissociates from the enzyme complex.

We have examined the kinetics of primer synthesis by the p49−p58 primase complex and the location of template DNA during the catalytic cycle. Negative regulation of further primer synthesis after generation of a unit length primer occurs with both the p49−p58 complex and the four-subunit pol α−primase complex. Cross-linking studies with primase−DNA complexes revealed that both prior to and after primer synthesis, only the p58 subunit reacted with the DNA. The implications of these results for the regulation of primase activity and initiation of new DNA strands by the pol α−primase complex are discussed.

EXPERIMENTAL PROCEDURES

Materials

Prestained protein markers and T4 polynucleotide kinase were from Gibco BRL. Ni−NTA resin was from Qiagen. Poly(dT) and azidophenacyl bromide were from Sigma.

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[1] Abbreviations: pol α, DNA polymerase α; 5-IdU, 5-iodo-2′-deoxyuridine; 5-BrdU, 5-bromo-2′-deoxyuridine; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane (HCl salt).

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Table 1: Synthetic Templates[a]

D_{43}:	5′-CTGGGTAAAATGGGGTAAAATGGGGGTCTCTCTCTCTCTCAAA
D_{43}-2I:	5′-C**I**GGGTAAAATGGGGTAAAATGGGGGTCTCTCTCTCTCTCAAA
D_{43}-11I:	5′-CTGGGTAAAA**I**GGGGTAAAATGGGGGTCTCTCTCTCTCTCAAA
D_{43}-33I:	5′-CTGGGTAAAATGGGGTAAAATGGGGGTCTCTC**I**CTCTCTCAAA
D_{43}-39I:	5′-CTGGGTAAAATGGGGTAAAATGGGGGTCTCTCTCTCTC**I**CAAA
R_{15}:	3′-AGAGAGAGAGAGUUU
D_{43}a:	5′-CTGGGTAAAATGGGGTAAAATGGGGGTTCTTCACTCTCTCAAA
R_{15}I:	3′-AGAG**I**GAGAGAGUUU
TT_{43}-33I:	5′-CTGGGTAAAATGGGGTAAAATGGGGGTTTTTT**I**TTTTTTTAAA
D_{43}-33AA:	5′-CTGGGTAAAATGGGGTAAAATGGGGGTCTCTCT-S-CTCTCTCAAA
$d(TC)_{30}$:	5′-TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
DNA_G:	TCCATATCACAT-3′ AGGTATAGTGTAGATCTTATCATCT

I= 5-Iodo-2′-deoxyuridine

[a] The relative orientation and position of the primers and templates are arranged to facilitate viewing of how they will hybridize to each other.

Synthetic oligonucleotides that contained 5-IdU were purchased from Bio-Synthesis, while all other oligonucleotides were from Oligos, Etc. (Table 1). Unless noted otherwise, DNA concentrations are given in terms of 5′-termini. Protein G−Sepharose was from Pharmacia Biotech. [γ-^{32}P]ATP and [α-^{32}P]NTPs (3000 Ci/mmol) were from New England Nuclear. All other reagents were of the highest quality available.

Methods

Purification of Recombinant Human Primase. Recombinant human primase was expressed in *Escherichia coli* JM105 transformed cells and purified as described previously (*8*) with minor modifications. A single colony from a fresh plate of cells (2X YT medium) was transferred to 50 mL of Terrific broth containing the required antibiotic(s) and incubated for 10−12 h at 37 °C. This culture was diluted 10-fold in Terrific broth containing the required antibiotic(s) and grown for 1.5 h at 37 °C, at which point 1 mM IPTG, 1 mM $MgCl_2$, and 0.1 mM $MnCl_2$ were added. Cells were harvested after growth for 3 h at 23 °C, lysed, and protein purified by Ni−NTA affinity chromatography as described previously (*8*), except that all buffers contained 1 mM $MgCl_2$ and a second wash consisting of 100 mM $(NH_4)_2SO_4$ (pH 8) and 5 mM imidazole was included (*11*). Primase complexes containing truncated forms of p58 were purified as previously described (*12*).

Primase Assays. Assays were performed and quantified as described previously (*14*). When present in assays, DNA_G and dNTPs were at concentrations of 0.5 (3′-end) and 5 μM, respectively.

Radioactive Labeling of Oligonucleotides. Oligonucleotides were 5′-phosphorylated using T4 polynucleotide kinase and [γ-^{32}P]ATP. Unreacted [γ-^{32}P]ATP was separated from the oligonucleotides by chromatography on a G-25 Sephadex spin column (Boehringer Mannheim) using the manufacturer's suggested protocol. The concentration of the ^{32}P-labeled DNA was determined by scintillation counting.

Primer−Template Formation. An RNA primer (R_{15} or R_{15}I) was annealed to the appropriate template as described previously and purified from a 20% nondenaturing polyacrylamide gel (*15*).

Photochemical Cross-Linking. Reaction mixtures (20 μL) typically contained 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 2 mM DTT, 0.1% Triton, 1 μM recombinant human primase, and 24 nM ^{32}P-labeled DNA template (3000 Ci/mmol). Samples were loaded into a 200 μL pipet tip and photolyzed through the large open end of the tip. Photolysis was performed at room temperature using a HeCd laser (Omnichrome, 40 mW output power at 325 nm). Reactions were stopped by adding 3× SDS loading buffer [187.5 mM Tris (pH 6.8), 6% SDS, 30% glycerol, 0.125 M DTT, and 0.03% (w/v) phenol red] and heating at 90 °C for 3−5 min. The

photochemically cross-linked protein−DNA samples were separated by 10% SDS−PAGE and analyzed via phosphorimaging (Molecular Dynamics).

Azidophenacyl Coupling to the Phosphorothioate Template and Photo-Cross-Linking by UV. Azidophenacyl bromide was coupled to the phosphorothioate containing template D_{43}-33AA using established procedures (*16*). The photoreactive substrate was resuspended in ultrapure water and analyzed by 15% denaturing polyacrylamide gel electrophoresis. Photo-cross-linking reaction mixtures were prepared as described above, placed in polystyrene tissue culture plates, and irradiated for 1.5 min using a UV Stratalinker (312 nm).

Immunoprecipitation of Cross-Linked Products. Immunoprecipitation experiments were performed on photolyzed reaction mixtures (typically 80 μL) using polyclonal rabbit antibodies against the p49 and p58 subunits (*8*). The precipitated products were then separated by 10% SDS−PAGE and analyzed via phosphorimaging (Molecular Dynamics).

K_D Determination. Reaction mixtures (20 μL) contained 50 mM Tris (pH 7.6), 10 mM $MgCl_2$, 2 mM DTT, 0.1% Triton, 5 nM ^{32}P-labeled DNA template (3000 Ci/mmol), and 0.2−2 μM protein. The reaction mixtures were photolyzed at room temperature for 5 min using the HeCd laser. Reactions were stopped, and the amount of protein−DNA cross-linking was determined as described above. The data were analyzed by Scatchard analysis as modified by Molnar et al. (*17*). This treatment accounts for the fact that only a fraction of the enzyme−DNA complex (i.e., the bound fraction in Scatchard analysis) is actually photo-cross-linked and detected after gel electrophoresis of the products. Plotting [cross-linked protein]/[free protein] versus [cross-linked protein] gives a straight line where the slope $= -K_D - 1$. Since the amount of protein is 40−400-fold greater than the amount of DNA in the assays, the amount of free protein can be approximated as total protein.

Data Simulation. Pre-steady-state kinetic data were analyzed using the program KaleidaGraph (Synergy Software, Reading, PA). Data were fit to the model in eq 1 where $k_1 \gg k_2$ by independently varying the starting amount of ES, k_1, and k_2.

$$\mathrm{ES} \xrightarrow{k_1} \mathrm{EP} \xrightarrow{k_2} \mathrm{ES} + \mathrm{P} \qquad (1)$$

RESULTS

Kinetics of Primer Synthesis by the p49−p58 Primase Complex. Previously, we showed that primer synthesis by the four-subunit calf thymus pol α−primase complex exhibits biphasic kinetics (*13*). In a rapid initial phase, each molecule of primase synthesizes one unit length primer, while further primer synthesis is negatively regulated until either pol α elongates the primer or the primer−template species dissociates from the enzyme. To determine if the absence of the p180 and p70 subunits affected the regulation of primase activity, we examined the kinetics of primer synthesis by the isolated p49−p58 human primase complex. Figure 1 shows that on $d(TC)_{30}$, the p49−p58 primase complex exhibits biphasic kinetics. We previously demonstrated that the simple two-step model depicted in eq 1 accurately describes the kinetics of primer synthesis by the pol α−primase complex (*13*). This model also describes the kinetics of primer synthesis by the p49−p58 primase complex using rate constants of 0.34 and 0.019 min^{-1} for the rapid and slow phases, respectively (Figure 1). Similar to the results with the pol α−primase complex, each molecule of primase synthesized approximately one unit length primer during the initial rapid phase of primer synthesis. Biphasic kinetics were likewise observed on the template D_{43}, indicating that this result is not unique to $d(TC)_{30}$ (data not shown). Stability appears to be critical for the newly generated primer−template species to negatively regulate further primase activity. With poly(dT) as the template, the rate of primer synthesis by the p49−p58 primase complex remained linear with time (Figure 1). Importantly, we previously showed that the pol α−primase comples also does not exhibit negative regulation of primase activity on poly(dT) due to the relative instability of the newly generated primer−template species (*13*). The similar negative regulation of primase activity observed with both the p48−p58 primase complex and the p49−p58−p70−p180 pol α−primase complex indicates that the p180 and p70 subunits are not essential for regulation.

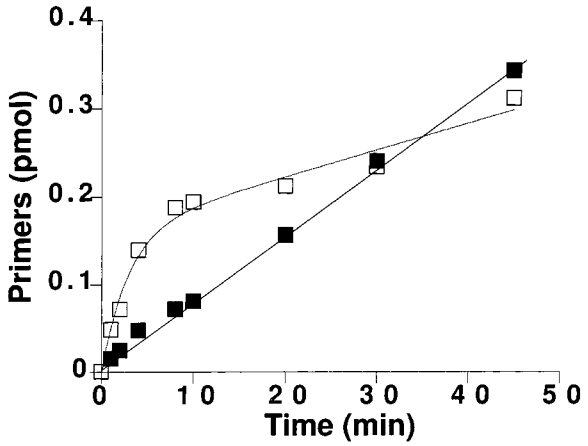

FIGURE 1: Time course of primase activity on $d(TC)_{30}$ and poly(dT). Reaction mixtures contained either 100 nM primase, 1.7 μM $d(TC)_{30}$, 100 μM [α-^{32}P]GTP, and 100 μM [α-^{32}P]ATP (□) or 100 nM primase, 100 μM poly(dT) (total nucleotide), and 100 μM [α-^{32}P]ATP (■). The data from the experiment using $d(TC)_{30}$ were fit to eq 1 using values of 0.34 min^{-1} for k_1 and 0.019 min^{-1} for k_2 and 105 nM ES ($\chi^2 = 0.001$). Attempts to fit the data to a one-step model gave accurate fits to the initial rapid phase, but could not account for the second phase of the time course (i.e., time points after 10 min).

The abilities of dNTPs and a synthetic DNA primer−template species (DNA_G) to modulate regulation were examined. With the pol α−primase complex, negative regulation of further primase activity can be alleviated by including either dNTPs to allow pol α to elongate the primase-synthesized primer or a synthetic primer−template species that binds to pol α [i.e., DNA_G (*18*)]. With the p49−p58 primase complex, however, addition of either 5 μM dNTPs or 0.5 μM DNA_G did not affect the biphasic kinetics of primer synthesis (data not shown), consistent with these agents influencing negative regulation via their interactions with pol α. Thus, while negative regulation of primer synthesis is an inherent property of primase and does not require either the p70 or p180 subunit of the pol α−primase complex, interactions of primase with the p180 and/or p70 subunits can modulate regulation.

Table 2: Steady-State Kinetic Parameters of Primer Synthesis on DNA Templates Containing 5-IdU

	D_{43}	D_{43}-33I	D_{43}-11I
K_m (μM)	0.11 ± 0.02	0.14 ± 0.04	0.18 ± 0.04
V_{max} [pmol h^{-1} (μg of enzyme^{-1})]	0.5 ± 0.2	0.74 ± 0.2	1.22 ± 0.3

The observation that negative regulation of further primase activity after synthesis of a unit length primer requires the formation of a stable primer−template species indicates that the newly generated primer−template species remains bound to the p49−p58 primase complex during negative regulation (*13*). Since the p49 subunit has previously been shown to bind DNA (*8*) while the p58 subunit contains the equivalent of a pol β domain that is known to bind DNA (*19*), it was unclear as to where the DNA would reside. To address this issue as well as to provide insights into the role of the p58 subunit during catalysis, we incorporated photoreactive nucleotide analogues into DNA and examined their cross-linking to primase.

Photo-Cross-Linking of DNA Primase to Photoreactive DNA. To examine the location of the template both prior to and after primer synthesis, we synthesized a series of templates containing photoreactive 5-iododeoxyuridine (5-IdU) at various positions (Table 1). These templates contain a pyrimidine rich region that supports primer synthesis in the presence of just ATP and GTP, followed by a purine rich region where primase does not readily synthesize primers (*20*, *21*). Initially, we ascertained that 5-IdU does not significantly affect the interactions of primase with the template. The data in Table 2 show that for both the normal template, D_{43}, and two templates containing a single 5-IdU, D_{43}-33I and D_{43}-11I, primase exhibits similar steady-state kinetic parameters. Additionally, analysis of the products by gel electrophoresis showed that the length of the products generated on each template was similar (data not shown). Thus, the 5-IdU substitution does not significantly alter enzyme−DNA interactions.

Photolysis of primase and 5′-^{32}P-labeled D_{43}-33I at 325 nm generates two radiolabeled bands that migrate with apparent molecular masses of approximately 70−90 kDa (Figure 2).[2] The amount of cross-linked products increases and then saturates with increasing irradiation time, giving a maximal cross-linking yield of 2−3%. To ensure that the observed products were due to protein−DNA cross-linking, we performed a series of controls. Photolysis of D_{43}-33I alone, photolysis of enzyme alone, and heating the enzyme to 90 °C prior to adding D_{43}-33I and irradiating the sample

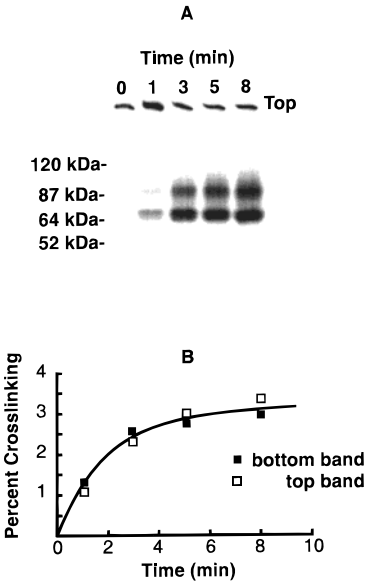

FIGURE 2: Photo-cross-linking of D_{43}-33I to primase at 325 nm as a function of irradiation time. Reactions were performed and analyzed as described in Experimental Procedures. Panel A shows a phosphorimage of the products generated with increasing photolysis times. The positions of molecular mass markers are indicated. Panel B shows the percentage of DNA present in either the faster (□) or slower (■) migrating species.

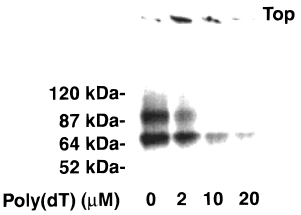

FIGURE 3: Poly(dT) inhibits cross-linking of primase to D_{43}-33I. Reaction mixtures contained 24 nM 5′-[^{32}P]D_{43}-33I and 1 μM primase. Lanes 1−4 contained 0, 2, 10, and 20 μM poly(dT) (total nucleotide), respectively. Reactions were photolyzed at room temperature for 5 min. The positions of molecular mass markers are indicated.

all resulted in no cross-linked products (data not shown). Finally, poly(dT), a good primase substrate, was added to the reaction mixtures to block binding of D_{43}-33I. Figure 3 shows that as the concentration of poly(dT) increased, the amount of cross-linked products decreased. Together, these data demonstrate that the observed products are due to specific protein−DNA cross-linking.

To identify the primase subunit(s) with which the DNA reacted, the isolated p49 and p58 subunits were individually cross-linked to D_{43}-33I. Figure 4 shows that the products generated upon photolysis of p58 with D_{43}-33I comigrated with those generated from the p49−p58 complex. Isolated p49 also reacted with D_{43}-33I upon photolysis; however, the resulting product migrated substantially faster than that obtained with the p49−p58 complex. Thus, only the p58 subunit of the p49−p58 primase complex reacted with D_{43}-33I upon photolysis. As a further test of these results, we generated the p49−p58 complex from the purified p49 and p58 subunits, a process that results in the active p49−p58 complex (*8*). Photolysis of this enzyme with D_{43}-33I generated the same two products, indicating that only p58 reacted with D_{43}-33I (data not shown).

[2] Cross-linking experiments were also attempted using D_{43} containing 5-BrdU at the same positions as the 5-IdU and irradiation at 308 nm. Like the results with 5-IdU, two cross-linked species with an apparent mass of 70−90 kDa were generated after irradiation for 30 s. However, these labeled products disappeared upon irradiation of the samples for an additional 5 min, while a high-molecular mass product that did not enter the separating gel appeared (data not shown). Control experiments showed that irradiation of the p49−p58 complex at 308 nm in the absence of DNA converted the two primase subunits into a high-molecular mass species that did not enter the separating gel, presumably due to the generation of protein−protein cross-links. These data suggest that there is substantial interaction between individual primase complexes in solution. To avoid this protein−protein cross-linking, all further experiments were performed using 5-IdU and irradiation at 325 nm.

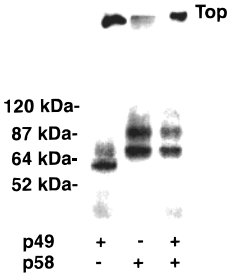

FIGURE 4: Photo-cross-linking of p49, p58, and the p49−p58 primase complex to D_{43}-33I. Reaction mixtures contained 1 μM protein and 24 nM 5′-[^{32}P]D_{43}-33I. Lanes 1−3 contained p49, p58, and the p49−p58 complex, respectively. Reactions were photolyzed at room temperature for 5 min and analyzed as described in Experimental Procedures. The positions of molecular mass markers are indicated.

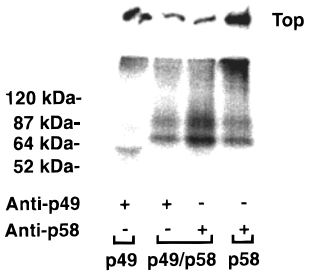

FIGURE 5: Immunoprecipitation of the cross-linked products. Proteins were cross-linked to 5′-[^{32}P]D_{43}-33I, immunoprecipitated with polyclonal antibodies specific for either p49 or p58, and analyzed as described in Experimental Procedures. The positions of molecular mass markers are indicated.

Somewhat surprisingly, photolysis of D_{43}-33I and either primase or p58 resulted in two radiolabeled species. To provide evidence that both cross-linked species resulted from reaction of DNA with p58, we immunoprecipitated the cross-linked complexes using polyclonal rabbit antibodies directed against either p58 or p49 and then analyzed the precipitated proteins (Figure 5). If one of the products observed in cross-linking reactions resulted from high-efficiency cross-linking to a contaminating protein present at low levels, one would not expect it to be immunoprecipitated by these antibodies. The antibody against p58 precipitated both radiolabeled products generated in cross-linking reactions including either p58 or the p49−p58 complex. Likewise, the antibody against p49 also immunoprecipitated both products generated in reactions including the p49−p58 complex, consistent with p49 and p58 forming a very stable complex. Thus, both species generated during photolysis likely result from cross-linking of p58 with DNA. Additionally, replacing the full-length p58 with truncated forms lacking the N- or C-terminal halves of the protein results in the disappearance of the two species generated with full-length p58 and the appearance of a species whose apparent mass correlates with the truncated forms of p58 (see below).

We explored the possibility that changing the location of the 5-IdU would affect either the cross-linking yield or the subunit with which cross-linking occurred. Positioning the 5-IdU either in the pyrimidine rich, primer synthesis region (D_{43}-33I and D_{43}-39I) or in the purine rich region where primase does not readily initiate synthesis (D_{43}-11I and D_{43}-2I) resulted in only the p58 subunit reacting with the DNA upon photolysis, and the same two bands were always generated (data not shown). Additionally, the cross-linking

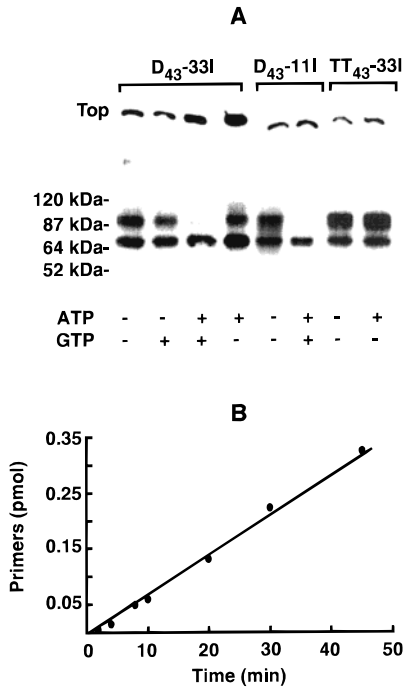

FIGURE 6: Effects of adding NTPs on cross-linking of DNA to primase. (A) Reaction mixtures contained 1 μM primase, either 5′-[^{32}P]D_{43}-33I, 5′-[^{32}P]D_{43}-11I, or 5′-[^{32}P]TT_{43}-33I at 24 nM, and the indicated NTP(s) at a concentration of 200 μM. The positions of molecular mass markers are indicated. (B) The time course of primer synthesis on TT_{43}-33I was measured in assays containing 3.5 μM TT_{43}-33I, 200 μM [α-^{32}P]ATP, and 100 nM primase.

yield was similar with all four templates (1.3% with D_{43}-2I, 2.2% with D_{43}-11I, 3% with D_{43}-33I, and 2.3% with D_{43}-39I).

Effect of NTPs and Primer Synthesis on Cross-Linking. To synthesize a primer, primase first binds DNA followed by two NTPs to generate a primase−DNA−NTP−NTP quaternary complex poised for catalysis. Due to the template sequence of D_{43} in the primer synthesis region (TCTC...), we can add either only ATP or GTP to generate a stable primase−DNA−NTP ternary complex that cannot synthesize a primer. However, formation of this complex by mixing enzyme, D_{43}-33I, and either ATP or GTP (200 μM) had no effect on cross-linking (Figure 6).

The effects of primer synthesis on cross-linking were examined by adding both ATP and GTP to the cross-linking reaction mixtures, conditions that allow primase to synthesize a primer in the pyrimidine rich region of the template. Primer synthesis on D_{43} begins near the 3′-end of the pyrimidine rich region and continues for 20−25 nucleotides (L. Rubsam and R. Kuchta, unpublished data). Thus, primer synthesis on D_{43}-33I will result in a RNA primer−template species where the photoactive IdU ends up in a RNA−DNA duplex, while primer synthesis on D_{43}-11I will result in a RNA primer−template species where the photoactive IdU will remain in single-stranded DNA. Cross-linking experiments with both templates revealed that after primer synthesis, the slower migrating species disappeared, while the faster migrating species remained (Figure 6). Once again, however, no cross-linking to p49 was observed in any of these experiments.

We further explored the effects of primer synthesis on cross-linking using TT_{43}-33I, a template where the pyrimidine

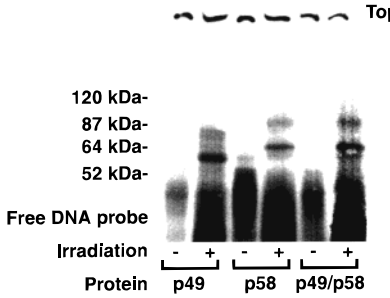

FIGURE 7: UV photo-cross-linking of p49, p58, and the p49−p58 primase complex to an aryl azide-containing template, D_{43}-33AA. Reaction mixtures contained 1 μM protein and 24 nM 5′-[^{32}P]D_{43}-33AA. The protein and the absence or presence of irradiation (1.5 min) are as noted. The positions of the free DNA probe and molecular mass markers are indicated.

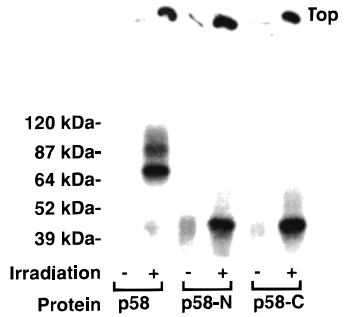

FIGURE 8: Both the N- and C-terminal halves of p58 contain DNA binding domains. Reaction mixtures contained 24 nM 5′-[^{32}P]D_{43}-33I and either p49−p58, p49−p58-N, or p49−p58-C at 1 μM. The protein and the absence or presence of irradiation (5 min) are as noted. The positions of molecular mass markers are indicated.

rich primer synthesis region lacks any deoxycytidylates (Table 1). As expected from the lack of G·C base pairs in the product primer−template species (vide infra and ref *13*), primer synthesis on this template does not result in negative regulation of further primer synthesis after formation of a unit length primer (Figure 6). Prior to primer synthesis, photo-cross-linking of TT_{43}-33I to primase gave the two bands observed with the other templates. Unlike the results with D_{43}-11I and D_{43}-33I, however, adding ATP to the reaction mixtures to allow primer synthesis on TT_{43}-33I did not affect the relative amounts of the fast and slow migrating species.

Lack of Cross-Linking to p49 Is Not Due to either the Amino Acid Specificity of 5-IdU or p58 Binding All of the DNA. Since p49 contains the primase active site and can clearly bind DNA (vide infra and ref *8*), we were extremely surprised by the complete lack of DNA−p49 cross-linking. Therefore, we tested if the lack of cross-linking was due to either the amino acid specificity of photoexcited 5-IdU or the presence of separate DNA binding sites on p49 and p58 such that under our experimental conditions, we had only saturated the p58 binding site.

To test a photoactivatable cross-linking agent that generates an intermediate with broader reactivity upon photolysis, we incorporated an aryl azide into the template. Photolysis of an aryl azide generates a highly reactive nitrene that can react with most amino acid residues (*22*). The azidophenacyl group was coupled to the sulfur of the phosphorothioate-containing template D_{43}-33AA, such that the cross-linking reagent was now located in the sugar phosphate backbone of the DNA. As with the templates containing 5-IdU, photolysis of D_{43}-33AA and the p49−p58 complex only resulted in cross-linking to p58, and two products were again generated (Figure 7). Isolated p49 and p58 also reacted with D_{43}-33AA upon photolysis and gave products similar to those obtained with the 5-IdU-containing templates (Figure 7).

To exclude the possibility that the p58 subunit was binding all of the DNA present in the reaction mixtures, cross-linking was examined in reaction mixtures containing a 4-fold molar excess of DNA (0.4 μM D_{43}-33I and 0.1 μM p49−p58). Under these conditions, there will be a large amount of free DNA available for binding to p49 even if p58 binds a D_{43}-33I. Furthermore, this DNA concentration is 3-fold higher than the K_M for D_{43}-33I; hence, most of the enzyme should be bound to DNA. Once again, the D_{43}-33I only reacted with the p58 subunit upon photolysis and gave the same two products observed at lower D_{43}-33I concentrations (data not shown).

The N- and C-Terminal Halves of p58 Both Contain DNA Binding Domains. To begin to locate the DNA binding domains on p58, cross-linking of two truncated forms of p58 to D_{43}-33I was examined. In p58-N, only the N-terminal half of p58 remains (amino acids 1−270), while in p58-C, only the C-terminal half remains [amino acids 270−509 (*12*)]. p49 forms stable complexes with both p58-N and p58-C, although neither the p58-N−p49 nor the p58-C−p49 complex supports primer synthesis (*12*). Figure 8 shows that both p49−p58-N and p49−p58-C cross-link to the labeled D_{43}-33I to a similar extent, and the relative mobility of the cross-linked products corresponds to the truncated forms of p58. Additionally, the two cross-linked species formed when using intact p58 both disappear. Thus, DNA binding by p58 involves residues located in both the N- and C-terminal halves of the protein.

Primer-Template Cross-Linking to Primase Subunits. Since primase must interact with both single-stranded DNA and a primer−template species during its catalytic cycle, we examined the cross-linking of primer−template species to primase. Initially, we examined primer−template species where the template contained the IdU. Either D_{43}-33I or D_{43}-11I was 5′-^{32}P-labeled and annealed to an RNA primer (R_{15}) so it would mimic the product of primase activity. With the RNA primer−DNA template R_{15}−D_{43}-11I, the IdU is located in the single-stranded template region, while in the case of R_{15}−D_{43}-33I, the IdU is in the double-stranded region. Each primer−template species was mixed with either p49−p58, p49, or p58, and subjected to photolysis. Figure 9 shows that R_{15}−D_{43}-11I reacts with isolated p58, isolated p49, and the p58 subunit in the p49−p58 complex. The extent of cross-linking to p58 and the p49−p58 complex was similar to that obtained with the D_{43}-11I template, although the cross-linking yield of p49 with R_{15}−D_{43}-11I was 5-fold lower than with D_{43}-11I. In contrast, R_{15}−D_{43}-33I gave minimal cross-linking to p49, p58, and the p49−p58 complex (data not shown). Compared to that of single-stranded D_{43}-33I, the cross-linking yield decreased by >98, 94, and 92%, respectively.

To further explore primer−template binding to primase, we incorporated 5-iodouracil into an RNA primer (R_{15}I, Table 1). The single-stranded RNA reacted with p49, p58, and the p49−p58 complex upon photolysis (Figure 9), indicating that both p49 and p58 can bind single-stranded

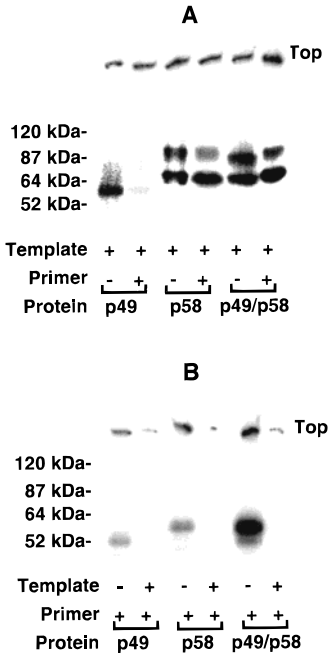

FIGURE 9: Comparison of template and primer−template cross-linking to either p49, p58, or the p49−p58 primase complex. Reaction mixtures contained 1 μM protein and were photolyzed at room temperature for 5 min and analyzed as described in Experimental Procedures. (A) As noted in the figure, reaction mixtures contained either 5′-[^{32}P]D_{43}-11I or R_{15}−5′-[^{32}P]D_{43}-11I at 20 nM. (B) As noted in the figure, reaction mixtures contained either 5′-[^{32}P]R_{15}I or 5′-[^{32}P]R_{15}I−D_{43}a at 20 nM. The positions of molecular mass markers are indicated.

RNA. However, upon conversion of the single-stranded RNA into the primer−template R_{15}I−D_{43}a, the cross-linking yield decreased by >95% with each protein. Thus, primer−template species bind to and can be cross-linked to both p49 and p58, although efficient cross-linking only occurs when the photoactive 5-iodouridine is present in the single-stranded template region.

Calculation of DNA−Protein Binding Affinities. The K_D for binding of D_{43}-33I to p49, p58, and the p49−p58 complex was measured by DNA−protein cross-linking. Apparent K_D values for binding to the isolated p49 subunit, isolated p58 subunit, and the p49−p58 complex were 0.97 ± 0.07, 0.20 ± 0.02, and 0.44 ± 0.1 μM, respectively. This slightly greater affinity for p58 as compared to that for p49 for DNA is consistent with the DNA primarily binding to the p58 subunit of the p49−p58 complex.

DISCUSSION

We have found that negative regulation of further primase activity after synthesis of a unit length primer is an inherent property of the p49−p58 primase complex and does not require the p180 and p70 subunits of the pol α−primase complex. Cross-linking studies using two different chemistries indicate that both prior to and after primer synthesis, DNA exclusively reacts with the p58 subunit. Both single-stranded DNA and partially duplex RNA primer−DNA templates bound to p58. These data suggest a model for regulation of primase activity whereby binding of the newly generated primer−template species to p58 helps mediate negative regulation. Additionally, the ability of p58 to bind primer−template species in conjunction with its location in the pol α−primase complex suggests that p58 is involved in the transfer of newly generated primer−template species from the primase active site to the pol α active site.

Photolysis of reaction mixtures containing the p49−p58 primase complex and DNA containing either 5-IdU or an aryl azide resulted in cross-linking to only the p58 subunit under a wide variety of experimental conditions (prior to and after primer synthesis, presence or absence of NTPs, etc.). The result is most surprising since p49 (i) contains the primase active site, (ii) can catalyze polymerization of ATP onto oligo(A)−poly(dT) in the absence of p58 (*8*), (iii) can catalyze complete primer synthesis in the absence of p58 if purified in the presence of Mn^{2+} or Mg^{2+} (*11*), and (iv) binds single-stranded DNA and reacts with DNA containing either 5-IdU or an aryl azide when p58 is not present. This lack of reactivity suggests that in the context of the complex, DNA primarily binds to p58. During primer synthesis, the DNA may move transiently into the catalytic site in p49, or alternatively, p58 may continuously bind the DNA and "present" it to the active site in p49. Consistent with these possibilities, p49 purified in the absence of Mn^{2+} or Mg^{2+} absolutely requires p58 to catalyze primer synthesis on single-stranded DNA, and even when p49 is purified with Mn^{2+} of Mg^{2+}, adding p58 greatly increases the extent of primer synthesis (*11*). Isolated p58 also binds DNA 5-fold more tightly than isolated p49, in accord with DNA primarily binding to the p58 subunit of the p49−p58 complex. However, we cannot exclude the alternative possibility that the DNA binding domain in p49 becomes extremely nonreactive in the context of the p49−p58 complex such that it does not react with either photoactivated 5-IdU or a nitrene.

The ability of primase to slide along single-stranded DNA likely accounts for the results showing that regardless of where the IdU was located in D_{43}, the resulting DNAs cross-linked to p58 with similar efficiencies and gave similar products. Previously, we used kinetic methods to show that primase can slide along single-stranded DNA (*13*), and formation of a stable E−DNA complex only occurs after binding two NTPs (*14*). D_{43} consists of two distinct regions, a pyrimidine rich region that primase prefers for initiating primer synthesis and a purine rich region. The ability of primase to slide along the DNA would allow primase to react with IdU residues located in either the pyrimidine or purine rich regions of the DNA.

The DNA binding domain of p58 includes residues in both the N- and C-terminal halves of the protein (amino acids 1−270 and 270−509, respectively), as evidenced by cross-linking of DNA to p58-N and p58-C. This could indicate either an extended DNA binding domain located in the center of p58 such that part of it remains intact in each truncated form of p58 or the presence of two (or more) noncontiguous domains with at least one domain in each half of the protein. Interestingly, while each half of the protein binds DNA and binds to p49, neither form of p58 supports primase activity by p49 (*12*). Thus, the ability of p58 to support primase activity requires more than p58 just having the ability to bind DNA.

One potential DNA binding site in p58 includes the region from residues 288−342, a region that is highly homologous to the 8 kDa domain of DNA polymerase β (*19*). Pol β consists of two domains, a 31 kDa domain that contains the deoxynucleotide polymerase activity (*23*) and an 8 kDa

domain that enhances the processivity of pol β during gap filling synthesis and contains deoxyribose 5′-phosphate lyase activity (*24*, *25*). Importantly, the effects on processivity probably result from the ability of the 8 kDa domain to bind both single-stranded and double-stranded DNA (*26*). Given the significant homology between p58 and part of this 8 kDa domain, it would not be surprising if this region binds DNA. Experiments for testing this hypothesis are in progress.

The presence of DNA binding sites in both the C- and N-terminal halves of p58 may also account for the two bands observed during cross-linking of DNA with either p58 or the p49−p58 complex. Cross-linking at two different sites might result in altered conformations of p58 during SDS−PAGE and/or affect binding of SDS to the p58, either of which could affect the electrophoretic mobility of the p58−DNA conjugate. Three sets of data indicate that both species result from cross-linking to p58, as opposed to one of the cross-linked species arising from high-efficiency cross-linking to a contaminating protein present at low levels. (i) Immunoprecipitation of primase using antibodies against either p49 or p58 precipitates both species generated during cross-linking of the p49−p58 complex. (ii) Both species disappear when full-length p58 is replaced with either p58-N or p58-C. (iii) The formation of the two cross-linked species requires the presence of p58 in the purified material. If one of the cross-linked species was due to a contaminating protein, one would have expected it to be present in the p49 reaction mixtures since p49, p58, and the p49−p58 complex were purified using identical procedures. Furthermore, the generation of multiple cross-linked species with quite different electrophoretic mobilities has been observed with other proteins. For example, cross-linking of the *lac* promoter to bacterial RNA polymerase generated three products with altered mobilities, all of which resulted from cross-linking between DNA and the σ subunit (*27*).

Primer synthesis can significantly alter the interaction of DNA with p58. After primer synthesis on D_{43}, most of the slower migrating cross-linked species disappears while the amount of faster migrating cross-linked species remains constant. This occurred both when the IdU was in the pyrimidine rich region of the template where primase synthesizes primers (D_{43}-33I) and when the IdU was in the purine rich region near the 5′-end of the template where primase does not synthesize primers (D_{43}-11I). In contrast, photolysis of R_{15}−D_{43}-33I resulted in virtually no detectable cross-linked products, while photolysis of R_{15}−D_{43}-11I produced both the slower and faster migrating species in approximately equal amounts. First, these results provide another illustration of the pol α−primase complex interacting with exogenously added primer−template species very differently than with primase-generated primer−template species. As described in greater detail below, kinetic studies have shown that the nature of the primer−template species (exogenously added vs primase-generated) dramatically affects the interactions of the pol α−primase complex with both nucleotide analogues and template lesions. Second, these data suggest that after primer synthesis, the newly generated primer−template species either does not interact with the site on p58 that gives rise to the slower migrating species or interacts with this site very differently compared to an exogenously added primer−template species. R_{15}−D_{43}-11I and the products of primer synthesis on D_{43}-11I should have similar structures since both will be RNA−DNA duplexes and cover the pyrimidine rich region of D_{43}-11I. Nonetheless, the slower migrating product largely disappears after primase-catalyzed primer synthesis on D_{43}-11I but is still formed in cross-linking reaction mixtures containing R_{15}−D_{43}-11I. Finally, since cross-linking to the site that gives rise to the faster migrating species still occurs after primer synthesis, DNA must remain bound to this site after primer synthesis.

Interestingly, primer synthesis on TT_{43}-33I did not alter the cross-linking pattern. Unlike D_{43}-11I and D_{43}-33I, primer synthesis on TT_{43}-33I does not show negative regulation of further primer synthesis after generation of a unit length primer, likely due to the relative instability of oligo(rA)−oligo(dT) duplexes (*13*). Thus, a decreased level of formation of the slower migrating cross-linked species may require that primer synthesis result in a stable primer−template species that can mediate negative regulation.

The p58 subunit of primase probably helps transfer the newly synthesized primer−template species from the primase active site into the pol α active site. Immunoprecipitation studies indicate that p58 tethers the catalytic p49 subunit of primase to the p180 subunit of pol α (*8*), consistent with p58 mediating the intramolecular movement of the newly generated primer−template species between the primase and pol α active sites. Importantly, our cross-linking studies indicate that p58 binds RNA primer−template species, a capacity that would seem to be essential for p58 to mediate primer transfer.

Primer−template species only reacted with p58 when the IdU was located in the single-stranded region of the template. The lack of cross-linking when the IdU was located in the duplex region could indicate either that this region of the primer−template species does not interact with p58 or, alternatively, that the restricted mobility and/or different location of the IdU blocked cross-linking. In an RNA−DNA duplex, the iodine will likely reside within the major groove, as does the 5-methyl group of thymidine (*28*), thereby restricting its ability to react with amino acids to only those that are very near the major groove. In contrast, the IdU will not be subject to these constraints when present in single-stranded DNA and, therefore, has an enhanced ability to react with the protein.

Binding of RNA primer−template species by p58 may also be important for negative regulation of primase activity after synthesis of a unit length primer. The rate of primer synthesis remained linear with time when the template was poly(dT), indicating that negative regulation did not occur after generation of a unit length primer (Figure 1). We previously observed a similar phenomenon with the pol α−primase complex (*13*), and this lack of negative regulation was due to the relative instability of oligo(A)−oligo(dT) compared to primer−template species that contained G·C base pairs. Thus, negative regulation of the p49−p58 complex appears to require stable association of the primer and template strands such that the newly synthesized primer−template species remains bound to primase. In the absence of dNTP polymerization by pol α, alleviation of this negative regulation requires that the newly generated primer−template species dissociate from the primase complex. The results showing that primer−template species only cross-linked to the p58 subunit of the p49−p58 complex and that they reacted much more efficiently with isolated p58 than with

p49 suggest that after primer synthesis, the newly generated primer−template species resides on p58. This implies that reactivation of primase in the absence of dNTP polymerization reflects the rate at which the primer−template species dissociates from the p58 subunit.

Interactions between newly generated primer−template species and p58 can also help explain why pol α exhibits fundamentally different properties when elongating primase synthesized primers as compared to when it elongates exogenously added primer−template species. For example, whereas arabinofuranosyl nucleoside triphosphates are extremely strong chain terminators when pol α elongates an exogenously added primer−template species, these compounds are not chain terminators when pol α elongates a primase-synthesized primer (*29*). Likewise, pol α strongly discriminates against acyclovir triphosphate polymerization when using an exogenously added primer−template species, but not when elongating a primase-synthesized primer (*30*). Finally, abasic template lesions are strong chain terminators during elongation of exogenously added primer−template species, but not during elongation of primase-synthesized primer−template species (*31*). At least part of this enhanced bypass was probably due to the presence of the p49−p58 primase complex. The ability of pol α to bypass abasic lesions on exogenously added primer−template species increased 1.5−7-fold when the p180 pol α subunit was bound to the p49−p58 primase complex as compared to when p49−p58 was absent (*31*). With an exogenously added primer−template species, the substrate may bind primarily to pol α. However, when pol α elongates a primase-synthesized primer−template species, the primer is likely first bound by p58 before entering the pol α active site. If the primase-synthesized primer−template species remains bound to p58 during the initial stages of dNTP polymerization by pol α, this might slightly alter the conformation of the primer−template species in the active site and thereby affect interactions of pol α with both nucleoside analogues and template lesions. Likewise, interactions of the primer−template species with the p58 subunit may have also caused the increased level of bypass of abasic sites during elongation of exogenously added primer−template species that occurred when pol α was bound to the p49−p58 complex. Experiments for further testing this hypothesis are in progress.

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