Arg304 of Human DNA Primase Is a Key Contributor to Catalysis and NTP Binding: Primase and the Family X Polymerases Share Significant Sequence Homology†

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ABSTRACT: Comparison of the amino acid sequences of eucaryotic DNA primase and the family X polymerases indicates that primase shares significant sequence homology with this family. With the use of DNA polymerase β (pol β) as a paradigm for family X polymerases, these homologies include both the catalytic core domain/subunit of each enzyme (31 kDa domain of pol β and p49 subunit of primase) as well as the accessory domain/subunit (8 kDa domain of pol β and p58 subunit of primase). To further explore these homologies as well as provide insights into the mechanism of primase, we generated three mutants (R304K, R304Q, and R304A) of the p49 subunit at an arginine that is highly conserved between primase and the eukaryotic family X polymerases. These mutations significantly decreased the rate of primer synthesis, due primarily to a decreased rate of initiation, and the extent of impairment correlated with the severity of the mutation (A > Q > K). R304 also contributes to efficient utilization of the NTP that will become the 5′-terminus of the new primer, and these effects are at least partially mediated through interactions with the phosphates of this NTP. The implications of these results with respect to the structure and biological role of primase, as well as its relationship to the family X polymerases, are discussed.

Eucaryotic DNA primase is a two subunit RNA polymerase essential for DNA replication (*3*−*6*). Primase initiates the synthesis of each Okazaki fragment on the lagging strand and, presumably, the leading strand by synthesizing a RNA primer that is then elongated by DNA polymerase α (pol α).[1] In addition, it is likely that primase helps couple DNA replication and repair. Yeast-containing mutations in primase are unable to delay entry into S phase and slow DNA synthesis upon treatment with DNA-damaging agents (*7*, *8*).

Primase typically consists of 2 subunits of mass 49 and 58 kDa. The p49 subunit contains phosphodiester bond formation activity, and mutagenesis studies have identified several Asp and Glu residues essential for activity (*1*). Relatively little is known about the role of the p58 subunit during primer synthesis (*9*). It lacks detectable phosphodiester bond formation activity, although it greatly stabilizes the p49 subunit. In the 4 subunit pol α-primase complex, an additional role of p58 is to tether p49 to the 180 kDa pol α subunit (*10*). Presently, there is little structural information on primase that might provide further insights into either the function(s) of each subunit or the relationship of primase to other nucleotide polymerases.

Functional aspects of primase in vitro have been well-studied and have helped define a minimal kinetic model (*10*−*12*). The catalytic cycle of primase consists of three segments: initiation (dinucleotide formation), elongation (converting the dinucleotide into a primer typically 7−10 nucleotides long), and termination (movement of the primer to the pol α active site and reactivation of primase for another round of primer synthesis). Under in vitro assay conditions, the initiation step typically limits the overall rate of primer synthesis.

We have found sequence homologies between primase and DNA polymerase β, as well as the rest of the family X polymerases. These include residues in the catalytic cores of pol β and the p49 subunit of primase, as well as homology between the 8 kDa domain of pol β and the p58 subunit of primase. Mutagenesis of a highly conserved arginine (R304) in the p49 subunit of human primase revealed that it plays a key role in both catalysis and NTP binding. The biological, mechanistic, and structural implications of these results are discussed.

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[1] Abbreviations: A. aeol., *Aquifex aeolicus*; A. fulg., *Archaeoglobus fulgidus*; A. thal., *Arabidopsis thaliana*; ASFV, African swine fever virus-strain BA71V; Axolotl, *Ambystoma mexicanum*; B. subt., *Bacillus subtilis*; C. eleg., *Caenorhabditis elegans*; D. mela., *Drosophila melanogaster*; dsDNA, double-stranded DNA; DTT, dithiothreitol; hprimase, human primase; M. ento., *Melanoplus sanguinipes entomopxvirus*; M. jann, *Methanococcus jannaschii*; M. ther., *Methanobacterium thermoautotrophicum*; Mit., Mitochondrion of *Crithidia fasciculata*; P. falc., *Plasmodium falciparum*; P. hor., *Pyrococcus horikoshii*; Pol α, DNA polymerase α; Pol β, DNA polymerase β; S. cere., *Saccharomyces cerevisiae*; S. pomb., *Schizosaccharomyces pombe*; ssDNA, single-stranded DNA; t.a. B−O, *thermophilic archaeon "Bonch-Osmolovskaya"*; T. aqua., *Thermus aquaticus*; TDT, terminal deoxynucleotidyltransferase; Tris, tris(hydroxylmethyl)aminomethane (HCl salt); WT, wild-type; X. laev, *Xenopus laevis*.

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Table 1: DNA Templates Used

$d(ACT)_{20}$	$^{(5')}$ACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACT
$d(ACC)_{20}$	$^{(5')}$ACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACC
$d(TC)_{30}$	$^{(5')}$TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
$(dC)_{40}$	$^{(5')}$CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC

MATERIALS AND METHODS

Materials

Unless noted, all materials and methods were as described previously (*12*, *13*). Both the p58 and the p49 subunits of hprimase were overexpressed in an *Escherichia coli* JM105-(DE3) strain deficient in Exonuclease I and purified via Ni−NTA (Qiagen) chromatography as described previously (*10*), except 1 mM $MgCl_2$ and 0.1 mM $MnCl_2$ were added to the lysate solution (*14*). Synthetic oligonucleotides of defined sequence were from Integrated DNA Technologies, Inc., and Oligos Etc., Inc., and their sequences are listed in Table 1. Poly(dT) and poly(dC) were from Sigma. The concentrations of ssDNAs were determined spectrally and are expressed in terms of total nucleotide. Extinction coefficients for ssDNA were determined as described in ref *15*. All other reagents were of the highest purity available.

Methods

Site-Directed Mutagenesis. Site-directed mutants of the p49 subunit of hprimase were developed using a pQE9-p49 plasmid provided by Dr. Bill Copeland (NIEHS, Research Triangle Park, NC) and the Chameleon dsDNA Site Directed Mutagenesis kit (Stratagene). Plasmids from positive colonies were amplified and purified via Qiagen Midi-Preps, and sequenced at the DNA sequencing facility of the University of Colorado to confirm the presence of the correct mutation.

Assembly of the p49/p58 hPrimase Complex. The p49 and p58 subunits were expressed and purified separately. To generate the p49/p58 primase complex, we combined the purified subunits in a buffer of 50% glycerol, 50 mM potassium phosphate, pH 7.5, 20 mM KCl, and 10 mM DTT to give a final concentration of 7.5 μM p49 and 15 μM p58. All primase concentrations are given in terms of the p49 subunit.

Primase Activity Assays. Reactions (10 μL) typically contained 50 mM Tris, pH 7.9, 50−75 μM ssDNA template (total nucleotide), 100−200 nM primase, 0.05 mg mL^{-1} bovine serum albumin, 1 mM DTT, 100−200 μM [α-^{32}P]-NTPs, and 5 mM $MgCl_2$. Reactions were initiated by adding enzyme and incubated at 37 °C for 30 min. After quenching the assays by adding 2.5 volumes of gel loading buffer (90% formamide), we separated the products by denaturing polyacrylamide gel electrophoresis (18% polyacrylamide, 8 M urea) and analyzed them using a Molecular Dynamics Phosphorimager. The intensity of product bands was determined individually via area integration and converted to picomoles of product (5′-ends). Background values were determined from identical reactions that lacked enzyme.

The relative V_{max}/K_M for using GTP versus either guanosine or GMP was determined in primase assays containing 20−40 μM [α-^{32}P]GTP, 53 μM $(dC)_{40}$ and varying amounts of either guanosine or GMP. The molar amount of each length product was determined and then used to calculate the frequency with which primase used GMP/guanosine for initiation versus using GTP. The relative V_{max}/K_M for using GTP versus either guanosine or GMP was computed as described previously (*16*). Kinetic parameters were obtained by analyzing the data using the program Enzfitter (Biosoft).

Initiation Assays. Reactions (10 μL) typically contained 50 mM Tris, pH 7.9, 70 μM $d(ACT)_{20}$, 100−200 nM primase, 0.05 mg mL^{-1} bovine serum albumin, 1 mM DTT, ATP, [α-^{32}P]GTP, and 5 mM $MgCl_2$. Reactions were initiated by adding enzyme and incubated at 37 °C for 30 min. Assays were heat inactivated at 65 °C for 10 min, and 1.5 μL of 0.2 M Tris, pH 8, and 0.1 M $MgCl_2$ plus 1 unit of shrimp alkaline phosphatase were added. (One unit hydrolyzes 1 μmol of *p*-nitrophenyl phosphate per minute at pH 9.6 and 37 °C.) Reactions were incubated at 37 °C for 30 min and then quenched with 30 μL of gel loading buffer. Products were analyzed as described above. Since only the cognate NTPs required for pppApG synthesis were present, products consisted of only dinucleotides.

Isolation and Analysis of the pGpG Product. A 10 μL primase assay containing 1.5 μM wild-type primase, 53 μM $(dC)_{40}$, 40 μM [α-^{32}P]GTP, and 0.31 mM GMP was incubated at 37 °C for 30 min, then quenched by adding 10 μL of gel loading buffer and heating to 80 °C for 5 min. Products were separated by gel electrophoresis (20% acrylamide, 8 M urea), and the pGpG dinucleotide was located by autoradiography and eluted from the gel into 2 mL of H_2O as described previously (*16*). The product was dried in vacuo and resuspended in 0.5 mL of H_2O. In a 40 μL reaction, 0.054 pmol (4000 cpm) of pGpG was incubated with 2 units of shrimp alkaline phosphatase in 20 mM Tris, pH 8, and 10 mM $MgCl_2$ for 30 min at 37 °C. The reaction was heated to 70 °C for 10 min to inactivate the phosphatase, at which point half of the reaction was dried in vacuo and then resuspended in gel loading buffer. To the other half of the reaction, 2 μL of 10 units μL^{-1} polynucleotide kinase, 1 μL of 2 mM ATP, and 2 μL of 0.5 M Tris, pH 7.5, and 50 mM $MgCl_2$ were added. After 30 min at 37 °C, the reaction was heated to 70 °C for 5 min, dried in vacuo, and resuspended in gel loading buffer. The products were analyzed by denaturing polyacrylamide gel electrophoresis followed by analysis on a Molecular Dynamics Phosphoimager.

Sequence Analysis. Protein sequences were obtained from the NCBI database (http://www.ncbi.nlm.nih.gov/). For alignment of the small subunit of primase to the Pol X family, conserved blocks were first identified for the group of primases and Pol X family independently using Block Maker (*17*). Then, conserved blocks of the Pol X family were aligned against the entire sequence of the group of primases using CLUSTALW from the European Molecular Biology Laboratory (*18*). Finally, the regions of family X polymerases and primases that had been identified as being homologous were aligned using CLUSTALW. The 8 kD domains of human, rat, and *Xenopus laevis* pol β (amino acids 2−92)

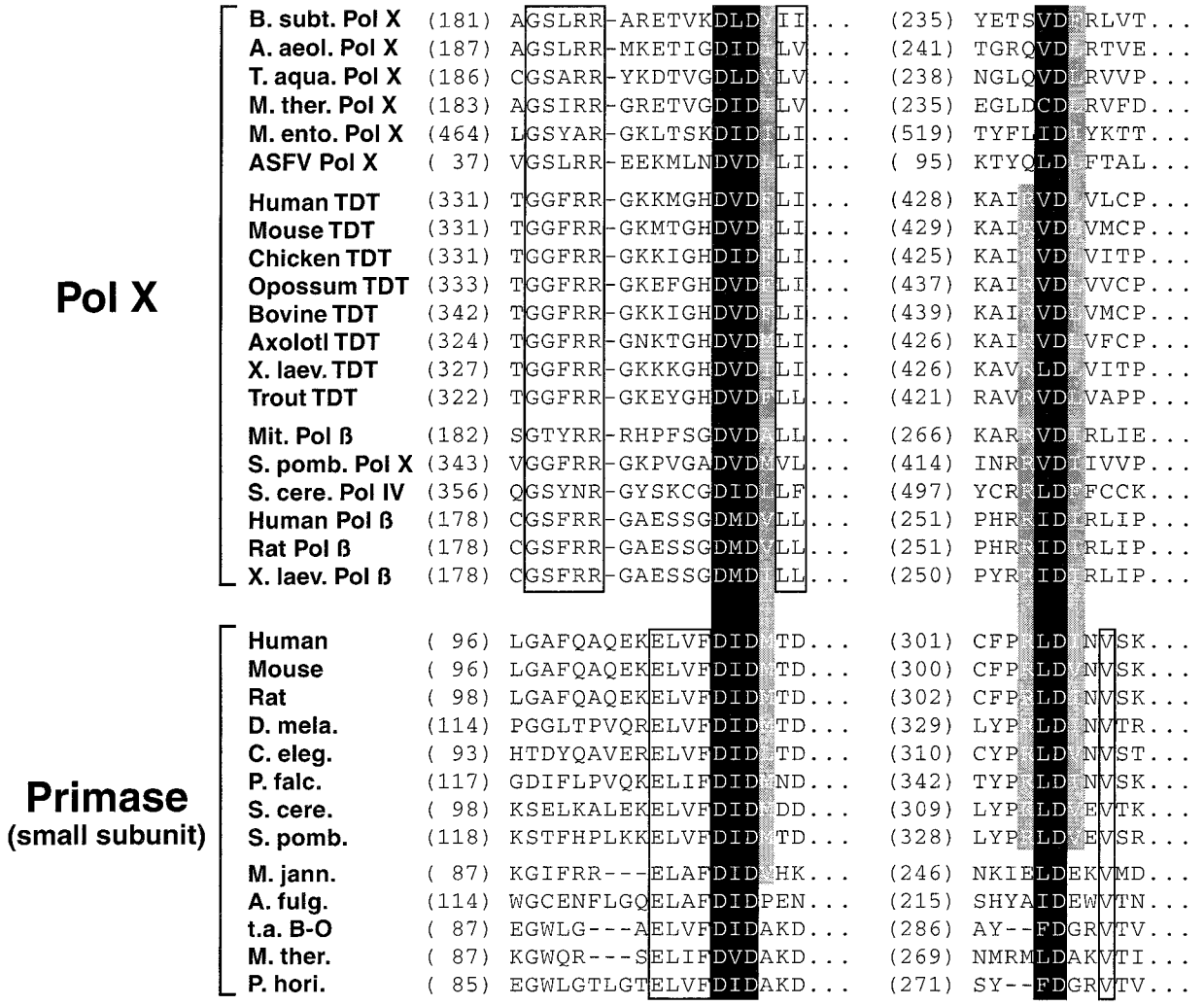

FIGURE 1: Multiple amino acid sequence alignment between the primase small subunit and the tri-acidic regions of the family X polymerases. Positions highly conserved between the family X polymerases and the primases are highlighted in black. Positions that are generally hydrophobic between the family X polymerases and the p49 subunits are highlighted in gray. Positions that are highly conserved only within the family X polymerases and the primases, respectively, are enclosed by a box. Numbers in parentheses to the left of the sequence indicate the amino acid position relative to the N-terminus of the respective polymerase. The accession number for each sequence is (top to bottom) the following: 2635324, 2983818, 1526547, 2621626, 4049700, 1171878, 2144439, 1174628, 1362717, 3024713, 135561, 2724141, 2117942, 1354475, 1362543, 2130375, 140461, 544186, 1055330, 2661842, 1346792, 423418, 3676248, 2498803, 3877820, 1430925, 730382, 3183125, 3915957, 2648218, 3342819, 2621663, and 3256581.

were aligned against the entire sequence of the primase large subunits listed using CLUSTALW.

RESULTS

Primary Sequence Homology between DNA Primase and DNA Polymerase β. Even though DNA pol β and DNA primase catalyze different reactions, they share a number of functional and structural similarities. Most strikingly, the primase p49 subunit shares important sequence similarity to the pol β 31 kDa domain (Figure 1), while the primase p58 subunit contains a primary structure analogous to the pol β 8 kDa domain (see below). Structurally, pol β consists of two domains, a 31 kDa domain that contains deoxynucleotide polymerization activity, and an 8 Da domain that binds both ssDNA and dsDNA, enhances the processivity of dNTP polymerization, and contains deoxyribose-5′-phosphate lyase activity (*19*). Primase is similar in that it consists of two subunits, a small subunit (~49 kDa) that contains nucleotide polymerization activity, and a large subunit (~58 kDa) that helps stabilize the 49 kDa subunit and can bind single-stranded DNA (Unpublished data, B. Arezi and R. D. Kuchta). Functionally, both enzymes interact with Mn^{2+}, and this interaction stimulates some polymerization reactions (Unpublished data, B. Kirk and R. Kuchta, and refs *20*, *21*).

Between polymerases of the same family, higher order structure is usually maintained, while primary sequence homology is less conserved (*22*−*24*). In fact, about the only significant amino acid sequence of high conservation across all nucleotide polymerases is a motif of either 2 or 3 acidic amino acids that coordinates the binding of a divalent cation-(s) involved in phosphodiester bond formation (*25*, *26*). Therefore, we attempted to align the 3 essential aspartate residues, Asp190, 192, and 256, from the active site of the mammalian pol β with the small subunit of primase.[2] Consistent with previous work (*27*), Asp190 and Asp192 of human pol β align with 2 aspartates in conserved region IV of the small primase subunit (p49) (Figure 1 (for simplicity,

[2] As described by Copeland and Tan (*1*), the absolutely conserved acidic residues of p49 do not clearly align with the catalytic aspartates in motifs A, B, and C of DNA polymerases.

we will discuss the residues using the human pol β numbering system)). Furthermore, we have found that Asp256 of pol β aligns with an aspartate in conserved region V of the primase small subunit (*28*). Importantly, a series of elegant mutagenesis studies by Copeland and co-workers have shown that these 3 aspartate residues in mouse primase are critical for activity (*1*), consistent with their forming the catalytic core. In addition, the regions around the primase tri-aspartates are similar to the highly conserved regions of the family X polymerases (Figure 1). Intriguingly, Arg254 is highly conserved between the eukaryotic primases and family X polymerases. Structural studies of human pol β showed that this is only arginine or lysine in the 31 kDa domain making contact with the phosphate backbone of the primer strand (*25*, *29*). This residue can also make an interaction with one of the catalytic aspartates (Asp256) and plays a role in catalysis (*30*).

To both further test the hypothesis that primase and pol β are closely related functionally as well as to provide insights into an important catalytic residue, we generated 3 mutants at R304 of the p49 subunit of hprimase: R304K, R304Q, and R304A. This residue was chosen since R304 of hprimase aligns with R254 of pol β, a residue that has already been shown to greatly affect both substrate binding and catalysis (*30*). The chosen amino acid substitutions differ in both charge and structure, thereby providing a diverse set of mutants to better understand the role of R304 during primase-catalyzed primer synthesis.

Mutating R304 Does Not Affect Either Expression of p49 or Association of p49 with p58. Both wild-type as well as the 3 mutant forms of p49 were overexpressed in an *E. coli* expression system. The yield of purified p49 from 1 L of cells was similar for all 4 proteins (wild-type = 0.50 mg, R304K = 0.65 mg, R304Q = 0.39 mg, and R304A = 0.32 mg), indicating that mutation of R304 does not significantly affect either the expression or purification properties of p49.

Proper association of the p58 subunit with the p49 subunit is required for high levels of activity in wild-type primase. To confirm that mutating R304 does not prevent binding to p58, we determined the effect of increasing amounts of p58 on both wild-type and mutant p49 activity (Figure 2). In each case, the addition of p58 to primase assays using $(dC)_{40}$ as a template greatly increased primase activity. For wild-type p49 we observed a 38-fold increase in activity at the optimal p58 concentration (500 nM). For the mutants, we detected no activity in the absence of p58, but all three mutants were activated upon addition of the accessory subunit. Importantly, both the wild-type and all three mutants demonstrated maximum activity with a ~5-fold molar excess of p58 over p49, and the shapes of the titration curves were similar. This result indicates that mutations at R304 do not significantly alter the affinity of p58 for p49.

Mutation of R304 Decreases Primase Activity in a Template-Dependent Fashion. Assays of p49/p58 primase complexes were performed with wild-type and the three R304 mutants of p49 on poly(dT), $d(TC)_{30}$, and $(dC)_{40}$ (Figure 3 and Table 2). On all three templates, the mutants displayed a significant decrease in total primers synthesized. For assays performed with poly(dT), only the R304K mutant displayed detectable activity (4.7% that of wild-type enzyme). No full-length products were detected with this enzyme, and only small amounts of dinucleotide were formed. However, when

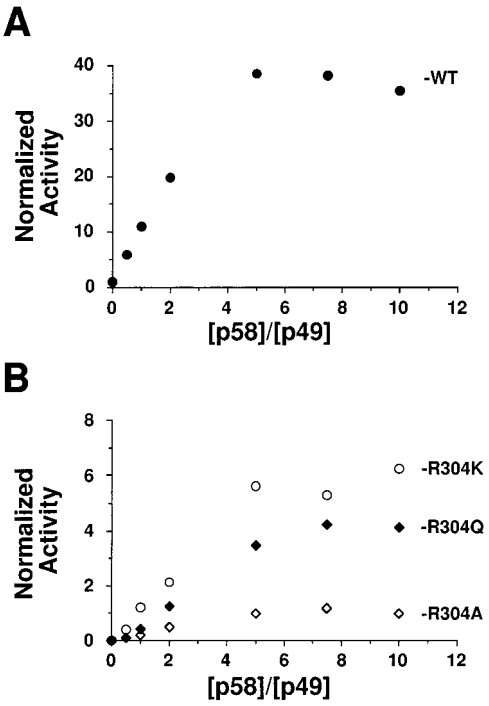

FIGURE 2: Effects of adding p58 to wild-type and mutant p49. Assays contained 100 nM p49, 200 μM [α-^{32}P]GTP, 60 μM $(dC)_{40}$, and the indicated concentrations of p58. The amount of full-length product was quantified as described under Experimental Procedures. The activity of wild-type p49 in the absence of p58 was defined as 1.0, and the amount of activity measured in the other assays was normalized to this value.

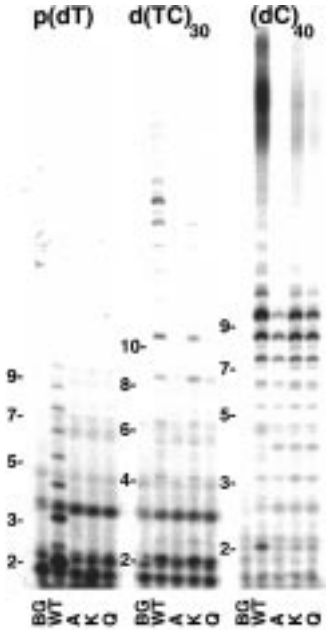

FIGURE 3: Effects of mutating R304 on primase activity depend on the template used. Assays contained 60 μM ssDNA, 200 μM cognate [α-^{32}P]NTPs, and either 100 nM wild-type or 200 nM mutant primase. Products were separated on an 18% denaturing polyacrylamide gel and quantified as described under Experimental Procedures. Product length is noted to the left of each set of assays.

$(dC)_{40}$ was used as the template, all three mutants displayed activity. Activity of the mutants correlated with the polarity of the substituted residue, with R304K displaying the greatest activity (29% of wild-type) and R304A displaying the lowest activity (8.1% of wild-type). Similar results were obtained

Table 2: Activity on Different Templates

template	total products[a]	full-length products[b]	full-length/total
poly(dT)			
wild-type	1	0.02	0.02
R304K	0.05	nd[c]	0
R304Q	nd	nd	
R304A	nd	nd	
$d(TC)_{30}$			
wild-type	1	0.23	0.23
R304K	0.43	0.086	0.20
R304Q	0.29	0.026	0.09
R304A	0.25	0.02	0.08
$(dC)_{40}$			
wild-type	1	0.80	0.80
R304K	0.29	0.23	0.81
R304Q	0.17	0.13	0.76
R304A	0.08	0.042	0.53

[a] The total moles of product synthesized for wild-type enzyme on each template was normalized to 1. [b] "Full-length products" is defined as those products at least 7 nucleotides long. [c] nd = none detected

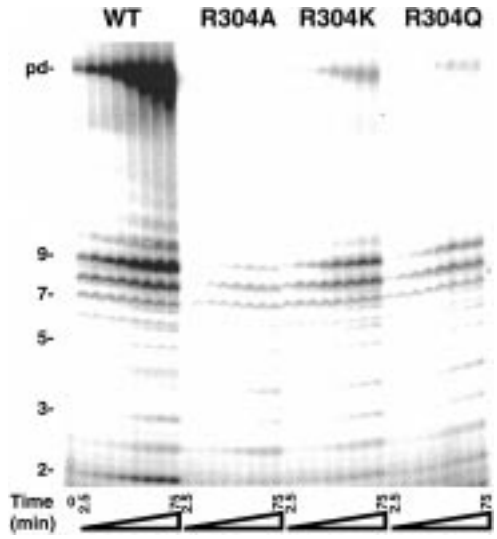

FIGURE 4: Time course of primer synthesis. Assays contained 200 μM [α-^{32}P]GTP and 60 μM $(dC)_{40}$. Aliquots were quenched in gel loading buffer at 2.5, 5, 7.5, 15, 30, 45, 60, and 75 min. Product length is noted on the left side of the image. "pd" refers to primer-dimers, products that are due to primase elongating an already synthesized primer by another 8−10 nucleotides.

with $d(TC)_{30}$ as the template, but the decrease in activity of the mutants was less severe (Table 2). To exclude the possibility that the greater activity on $d(TC)_{30}$ and $(dC)_{40}$ was due to the shorter length of these templates, we also measured activity on poly(dC), a 2000−4000 nucleotide long template similar to poly(dT). The activity on this template was virtually identical to that on $(dC)_{40}$, indicating that template length was not modulating activity (data not shown). These results demonstrate both that R304 is important for primer synthesis, as well as that the severity of the effects greatly depends on the template used for analysis.

The decreased rates of primer synthesis largely reflect changes in the rate of dinucleotide synthesis. First, the lower rate of primer synthesis for the R304 mutants was not due to enhanced thermal denaturation at 37 °C, since the time course of primer synthesis remained linear for at least 45 min (Figure 4). More importantly, there was no detectable accumulation of short primers 2−6 nucleotides long at early time points that were later converted into full-length products (i.e., single-turnover conditions (*12*)). Previous studies have shown that the formation of the dinucleotide is completely rate-limiting with wild-type enzyme (*12*), and elongation of the dinucleotide is too fast to measure directly. If elongation had become rate-limiting, one would have expected rapid accumulation of the dinucleotide followed by elongation into longer products. This was not observed, indicating that dinucleotide formation remains rate-limiting for the mutant enzymes.

While elongation of the dinucleotide remains rapid upon mutation of R304, the efficiency with which it is converted into a full-length primer $\geq$7 nucleotides long decreases (Figures 3 and 4). We examined in detail the size distribution of the products synthesized with each form of primase in assays containing 200 μM NTPs. All three mutant enzymes synthesized full-length products on the template $d(TC)_{30}$, but the ratio of (full-length products)/(total products) was significantly lower than that for wild-type enzyme, and this ratio also decreased as the polarity of the substituted amino acid decreased (Table 2). For wild-type enzyme, 23% of the total products were full-length products, while for R304K, Q and A, this fraction decreased by 13%, 61%, and 66%, respectively. Similar, albeit smaller, effects were observed when $(dC)_{40}$ served as the template: mutating R304 to K, Q, and A decreased the ratio of (full-length products)/(total products) by 0%, 5%, and 33%, respectively. Since processivity is defined by a competition of elongation versus dissociation of a short product, these results indicate that mutating R304 slightly reduces the net rate constant for elongation relative to the net rate constant for dissociation during the catalytic cycle (*30*).[3]

While R304 affects all polymerization reactions, this residue is not essential for a unique feature of primase catalysis: the ability to "count" and thereby synthesize primers of specific lengths (*12*). Even with the most severe mutant, R304A, the size distribution of products 2−10 nucleotides long is remarkably similar to wild-type enzyme: relatively large amounts of dinucleotide and full-length products 7−10 nucleotides long are formed, with much smaller amounts of products 3−6 nucleotides long (Figure 3).

R304 Is Involved in both Catalysis and Interactions with NTPs. The dramatically greater effects of mutating R304 on activity using a poly(dT) template as compared to the effects with the other 2 templates suggested that R304 might be involved in stabilizing interactions with the substrate(s). To initiate synthesis on poly(dT), the enzyme must stabilize two ATP−thymidylate base pairs, while with the other two templates, formation of the quaternary E−NTP−NTP−DNA complex involves at least one GTP−deoxycytidylate base-pair. Because of the lower stability of A−T base pairs, enzyme−substrate interactions may be more important for the formation of the quaternary complex required for initiation when on poly(dT). In pol β, R254 interacts with

[3] Changes in processivity observed upon mutation of R304 are not easily interpretable in terms of individual steps during catalysis. In pol β, the equivalent Arg helps bind the primer−template, is important for catalysis, and, presumably, must translocate to the next phosphodiester bond after each dNTP polymerization event. Since changes in any of these processes could alter processivity and mutation of R304 will likely affect these processes in primase, it is not possible to define why mutating R304 affects processivity.

Table 3: Effect of Temperature on Primase Activity

		(Activity at 37 °C)/(Activity at 25 °C)		
template	wild-type	R304K	R304Q	R304A
Poly(dT)	1.54	0.53	nd[a]	nd
$d(TC)_{30}$	1.64	0.90	0.77	0.69
$(dC)_{40}$	2.8	2.7	2.0	1.8

[a] nd = no activity detected at either 25 or 37 °C.

Table 4: Steady-State Kinetic Parameters for Primase Activity When Using $(dC)_{40}$ as Template

varied substrate	wild-type	R304K	R304Q	R304A
	K_M (μM)[a]			
GTP	270 ± 60	460 ± 75	560 ± 140	1500 ± 460
$(dC)_{40}$	12 ± 3.0	12 ± 3.0	8.2 ± 1.5	7.5 ± 1.9
	V_{max}[a] (pmol primers) (h μg of primase)			
GTP	235 ± 14	25 ± 2	18 ± 2	15 ± 3
$(dC)_{40}$	32 ± 2	5.0 ± 0.2	2.9 ± 0.1	1.1 ± 0.1

[a] Assays contained 200 μM [α-^{32}P]GTP when $(dC)_{40}$ was the varied substrate and 90 μM $(dC)_{40}$ when GTP was the varied substrate. Kinetic parameters with $(dC)_{40}$ as the varied substrate were not performed using saturating [α-^{32}P]GTP because the resulting low specific activity precludes accurate measurement of these values.

the terminal phosphodiester bond of the primer strand, thereby stabilizing primer−template binding (*25*, *30*). If R304 in primase serves an analogous function during initiation of primer synthesis, it would likely interact with and stabilize the binding of one of the initiating NTPs.

To provide initial evidence that R304 helps stabilize the binding of substrates, we performed primase assays at both 25 and 37 °C on the templates poly(dT), $d(TC)_{30}$, and $(dC)_{40}$. For wild-type primase, we observed a typical increase in activity as the temperature was increased (Table 3). Conversely, we observed the opposite result for the mutants on poly(dT) and $d(TC)_{30}$: activity decreased with increasing temperature and the magnitude of this effect correlates inversely with the severity of the mutation (A > Q > K). While both mutant and wild-type enzyme show normal temperature dependencies on $(dC)_{40}$, the mutant enzymes show a smaller increase in activity at 37 °C than wild-type enzyme, analogous to the results on poly(dT) and $d(TC)_{30}$. These data are consistent with the hypothesis that R304 serves to stabilize binding of the substrates. As described above, the rate of primer synthesis remains constant with time at 37 °C, thereby excluding the possibility that the enhanced primase activity at 25 °C is due to thermal denaturation of the mutant forms of primase at 37 °C.

To directly demonstrate the involvement of R304 in substrate interactions, we measured the K_M and V_{max} for both NTPs and ssDNA of wild-type primase and the R304 mutants using $(dC)_{40}$ as the template. Table 4 shows that, while mutating R304 does not significantly alter the K_M for ssDNA, it markedly increases the K_M for GTP, and the magnitude of the increase corresponds to the severity of the mutation (A > Q > K). These data suggest that R304 makes important interactions with only the NTP substrates, and not with the DNA template. In addition, the greatly decreased V_{max} shows directly that R304 is important for dinucleotide formation since, as described above, this step is rate-limiting for both wild-type and mutant enzymes.

R304 of Primase Interacts with the 5′-Terminal NTP. During dinucleotide formation, primase must bind and polymerize 2 NTPs. To determine if R304 interacts with the NTP that becomes the 5′-terminal nucleotide or with the NTP that becomes the second nucleotide of the primer, we examined dinucleotide synthesis on the template $d(ACT)_{20}$ in assays containing only ATP and GTP. Under these conditions, primase only synthesizes 5′-pppApG dinucleotide (*12*). Table 5 shows that, whereas there was little change in the K_M of GTP upon mutation of R304, the K_M of ATP increased significantly. Thus, R304 appears to primarily interact with the NTP that will become the 5′-terminus of the newly generated primer (ATP).

Table 5: R304 Primarily Affects the K_M for the NTP that Will Become the 5′-Terminal Nucleotide of the Primer[a]

	K_M (μM)		
	wild-type	R304K	R304Q
ATP	160 ± 11	710 ± 130	870 ± 70
GTP	330 ± 12	350 ± 50	750 ± 210[b]

[a] For the measurement of the K_M for ATP, assays contained $d(ACT)_{20}$, 200 μM [α-^{32}P]GTP, and varying concentrations of ATP. For the measurement of the K_M for GTP, assays contained $d(ACT)_{20}$, 200 μM ATP, and varying concentrations of [α-^{32}P]GTP. Dinucleotide formation was analyzed using alkaline phosphatase as described under Experimental Procedures. [b] This value is subject to very high uncertainty due to the small amount of product formed, a consequence of the low activity of R304Q primase in combination with the specific activity of the [α-^{32}P]GTP decreasing at higher concentrations.

R304 Interacts with a Phosphate of the 5′-Terminal NTP. In pol β, R254 interacts with the phosphodiester bond linking the two nucleotides at the 3′-terminus of the primer. On the basis of the homology between R254 of pol β and R304 of primase as well as the positive charge of Arg, it seemed likely that R304 of primase would interact with the triphosphate of the 5′-terminal NTP. To test this hypothesis, we examined the ability of wild-type primase to use a NTP, NDP, NMP, and nucleoside as the 5′-terminal nucleotide(side) for primer synthesis, and then quantified the effects of mutating R304.

Dinucleotide formation was first analyzed in assays containing the template $d(ACT)_{20}$, [α-^{32}P]GTP, and either ATP, ADP, AMP, or adenosine. Products were treated with alkaline phosphatase to remove any 5′-phosphates and thereby convert the $(p)_n$ApG dinucleotides into ApG and enhance resolution of the product (*32*). Figure 5 shows that, while primase generates large amounts of dinucleotide when assays contain ATP, it synthesizes little dinucleotide when ADP or adenosine was present. Interestingly, when assays contained GTP and adenosine, the product observed migrates slightly slower than ApG and, in fact, comigrates with the GpG dinucleotide, suggesting that this product represents a trace amount of misincorporation of GTP during dinucleotide formation. In contrast to the apparent strong discrimination against using ADP and adenosine as the 5′-terminal nucleotide, primase uses AMP as the 5′-terminal nucleotide relatively well. In assays containing 1 mM AMP, the amount of dinucleotide synthesized with AMP was 12.5% of the amount synthesized with ATP. For the R304A mutant, ATP remained the preferred substrate, AMP allowed substantial activity, and ADP showed minimal activity, results that are similar to wild-type enzyme. Surprisingly, however, adenosine was now readily utilized by R304A. In assays containing 1 and 8 mM adenosine, the amount of dinucleotide synthesized was 8.3% and 83.1% of the amount synthesized with

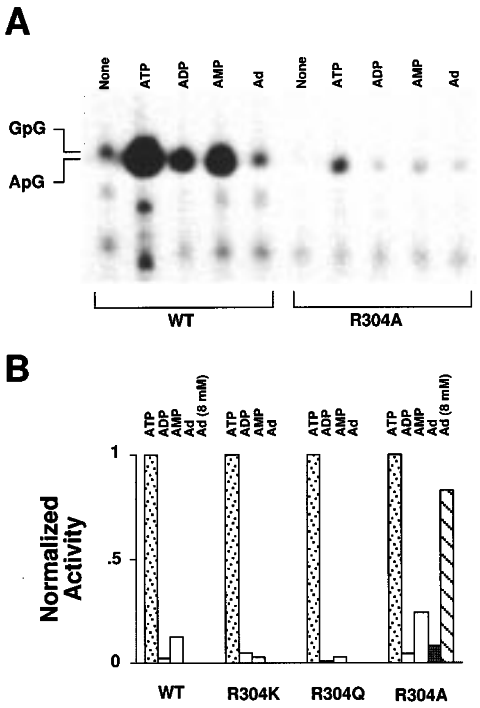

FIGURE 5: Utilization of ATP, ADP, AMP, and adenosine as the 5′-terminal nucleotide(side). (A) Assays contained 60 μM d(ACT)$_{20}$, 200 μM [α-^{32}P]GTP, and 1 mM of either ATP, ADP, AMP, or adenosine (Ad). Assays were heat inactivated and treated with shrimp alkaline phosphatase before being separated on an 18% denaturing polyacrylamide gel. The products for wild-type and R304A primase are shown. (B) The amount of ApG products synthesized in assays containing 1 mM ATP, 1 mM ADP, 1 mM AMP, and 1 or 8 mM adenosine was quantified as described under Experimental Procedures. For each enzyme, activity is normalized to that obtained with 1 mM ATP.

1 mM ATP. These results further demonstrate that R304 interacts with the nucleotide(side) that will become the 5′-terminus of the primer and suggest that this interaction involves the phosphates.

To directly demonstrate that R304 interacts with the phosphates of the NTP that becomes the primer 5′-terminus, we quantified the relative ability of primase to use a NTP, NMP, or nucleoside as the 5′-terminal nucleotide/side in competition assays on the template (dC)$_{40}$. Assays contained [α-^{32}P]GTP and increasing concentrations of the competitor species (GMP or guanosine), and the frequency with which primase uses either GTP or the competitor species as the 5′-terminal nucleotide/side measured.

Wild-type primase discriminated strongly against initiating primer synthesis using guanosine as the 5′-terminal nucleoside (Figure 6), consistent with the strong discrimination against using adenosine described above. At high concentrations of guanosine, small amounts of only a single new product that comigrated with GpG were detected. There were no detectable products that comigrated with the GpGpG trinucleotide generated by alkaline phosphatase treatment of primase reactions containing only GTP (Figure 6A), and no accumulation of new products that had electrophoretic mobilities different than the normal primers generated in assays containing only GTP (Figure 6B). Thus, initiation with guanosine only results in dinucleotide synthesis. Quantitative analysis of these data indicated that wild-type primase preferred to initiate primer synthesis with GTP rather than guanosine by ~450-fold (V_{max}/K_M, Table 6).

When GMP was titrated into the assays, a single new product was generated that migrated slightly slower than the normal dinucleotide (pppGpG, Figure 7). No other products with altered electrophoretic mobility were detected upon addition of GMP, indicating that initiation with GMP as the 5′-terminal nucleotide results in only the pGpG dinucleotide. As predicted by the results on d(ACT)$_{20}$, wild-type primase discriminated only moderately against GMP polymerization (25(±1)-fold). Several lines of evidence indicate that this new product is the dinucleotide pGpG. (1) The amount of product synthesized increases with increasing GMP concentrations. (2) It migrates slightly slower than the pppGpG dinucleotide, consistent with the reduced negative charge of pGpG. (3) Treatment of purified pGpG product with alkaline phosphatase resulted in a species that comigrated with GpG dinucleotide (Figure 7B). Upon inactivation of the alkaline phosphatase and further incubation with polynucleotide kinase and ATP to add a single 5′-phosphate, the resulting product comigrated with the starting pGpG dinucleotide.

Mutation of R304 significantly altered the relative ability of primase to use either GMP or guanosine as the 5′-terminal nucleotide/side, indicating that R304 likely interacts with a phosphate of the 5′-terminal nucleotide (Table 6). R304K and R304Q discriminated against GMP slightly more effectively than wild-type enzyme, while R304A showed similar discrimination as a wild-type enzyme. In contrast to the results with GMP, all 3 mutants showed significantly less discrimination between GTP and guanosine and increasing the severity of the mutation resulted in increased a loss of discrimination.

Two lines of evidence indicate that R304 interacts with and stabilizes the binding of the NTP that becomes the 5′-terminus of the primer through interactions with at least one of the phosphates. (1) Mutation of R304 reduces the ability of primase to discriminate between GTP and guanosine, and the only difference between GTP and guanosine is the 5′-phosphates. (2) The reduced ability to discriminate between GTP and guanosine results primarily from a decreased ability to use GTP, and only secondarily from changes in the ability to use guanosine. For the K, Q, and A mutants, the V_{max}/K_M for GTP on the (dC)$_{40}$ template decreases by factors of 15, 26, and 87, respectively. Likewise, mutating R304 to K, Q, and A reduces primase's preference for GTP over guanosine as the 5′-terminal nucleotide by factors of 5, 13, and 56. The similar reductions in the ability of each mutant to use GTP and discriminate against using guanosine are readily explained by R304 normally interacting with one of the phosphates on the NTP that will become the 5′-terminus of the primer. As the interaction with the phosphates becomes weaker, the ability of the enzyme to use a NTP should decrease but there should be little effect on utilization of a nucleoside.

DISCUSSION

Effects of Mutating R304 of hPrimase Are Analogous to Mutating R254 in Mammalian Pol β. The interaction of R304 with one of the phosphates of the 5′-terminal NTP is consistent with the role of the analogous R254 in pol β. With this enzyme, the Arg interacts with the 3′-terminal phos-

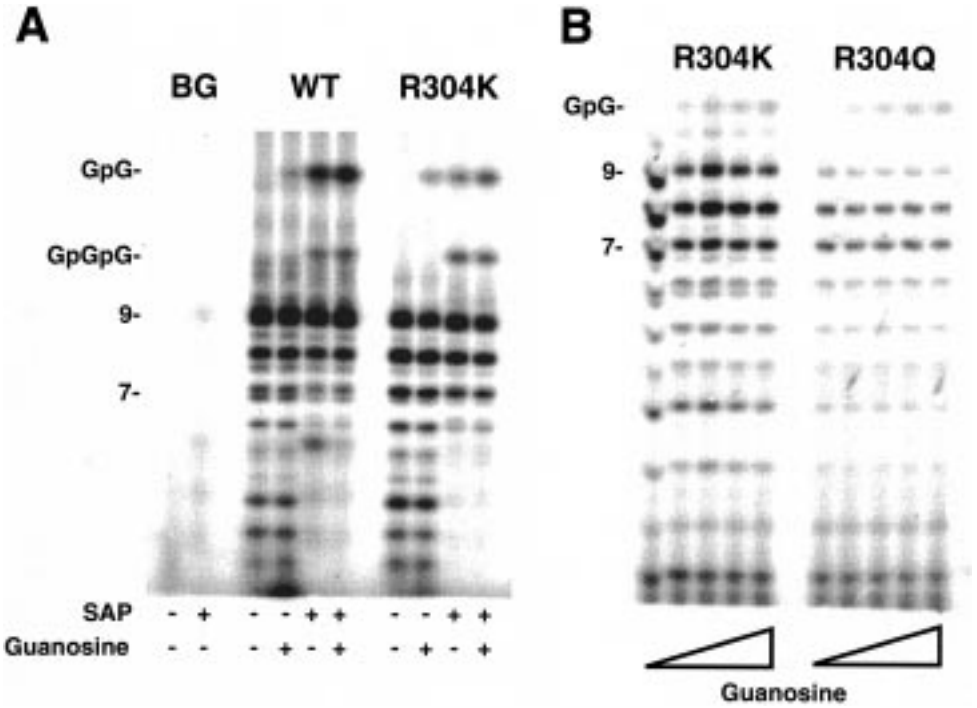

FIGURE 6: Utilization of guanosine as the 5′-terminal nucleoside. (A) The products from assays containing $(dC)_{40}$, 20 μM [α-^{32}P]GTP, 0 or 0.65 mM guanosine, and either wild-type or R304K primase were treated with shrimp alkaline phosphatase (SAP) as described under Experimental Procedures. The GpG and GpGpG products are noted to the left of the image. (B) The effects of titrating guanosine into assays containing either R304K or R304Q primase, 20 μM [α-^{32}P]GTP, $(dC)_{40}$, and either 0, 0.26, 0.39, 0.52, or 0.65 mM guanosine are shown. Guanosine concentration increases left to right. Product length is noted to the left of the image.

Table 6: R304 Interacts with One of the Phosphates of the NTP that Will Become the 5′-Terminal Nucleotide of the Primer

	wild-type	R304K	R304Q	R304A
$(V_{max}/K_M)_{GTP}/(V_{max}/K_M)_{GMP}$	25 ± 1	82 ± 11	60 ± 5	35 ± 10
$(V_{max}/K_M)_{GTP}/(V_{max}/K_M)_{guanosine}$	450[a]	93 ± 15	35 ± 7	8.0 ± 1.6

[a] Due to the relatively strong discrimination against guanosine in combination with its limited solubility, this value (450) is subject to substantial experimental error and represents the average of 2 experiments containing 20 μM GTP and either 0 or 0.65 mM guanosine.

phodiester bond of the primer strand and does not interact with the incoming dNTP (*25*). During initiation of primer synthesis by primase, the 5′-terminal NTP is the functional equivalent of the "primer strand". Mutagenesis studies of pol β showed that mutating R254 to Ala increases the K_M of the primer−template but has no effect on the K_M for the incoming dNTP (*30*), analogous to the increased K_M for the 5′-terminal NTP but little change for the second NTP during primer synthesis.

Identical to the results with R304 of primase, a large decrease in V_{max} was observed when the analogous R254 of pol β was mutated. From structural studies of pol β, R254 is a relatively mobile residue. In the free enzyme, it forms a salt bridge with the catalytic D256, while in the protein−DNA complex, it interacts with the 3′-terminal phosphodiester bond of the primer (*25*, *30*). Thus, potential roles of this Arg in catalysis for both pol β and primase include helping orient a catalytic aspartate, proper orientation of the primer 3′-OH, and mediation of a conformational change.

R304 appears critical for dinucleotide synthesis, the rate-limiting step for both wild-type and mutant forms of primase. With the most severe mutation, R304A, V_{max} decreased by 15-fold. Interestingly, however, while mutating R304 clearly

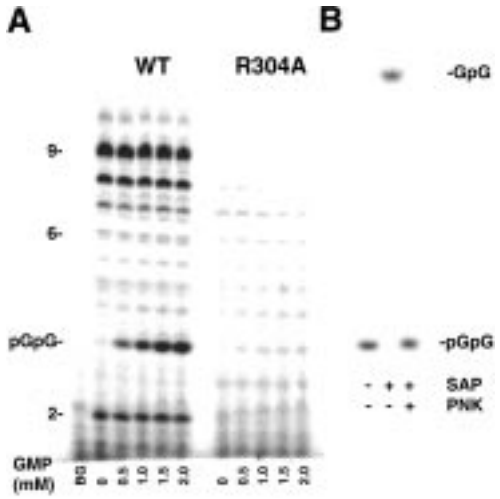

FIGURE 7: Utilization of GMP as the 5′-terminal nucleotide. (A) Primase assays contained $(dC)_{40}$, 40 μM [α-^{32}P]GTP, the indicated concentration of GMP, and either wild-type or R304A primase. Product lengths are noted to the left of the image. (B) The pGpG product was isolated as described under Experimental Procedures. As indicated under the image, pGpG was either (i) not treated; (ii) treated with shrimp alkaline phosphatase (SAP); or (iii) incubated with shrimp alkaline phosphatase, heated to 70 °C to inactivate the phosphatase, and then incubated with polynucleotide kinase and ATP. The electrophoretic mobility of the GpG product was obtained from a primase assay that contained 0.65 mM guanosine.

affects polymerization events following dinucleotide formation based on the decreased processivity, these effects are much smaller. Since processivity is defined by a competition between the net rate constants for polymerization (k_{pol}) and dissociation (k_{diss}), this result indicates that k_{pol} and k_{diss} are not greatly altered by mutation of R304 and further supports

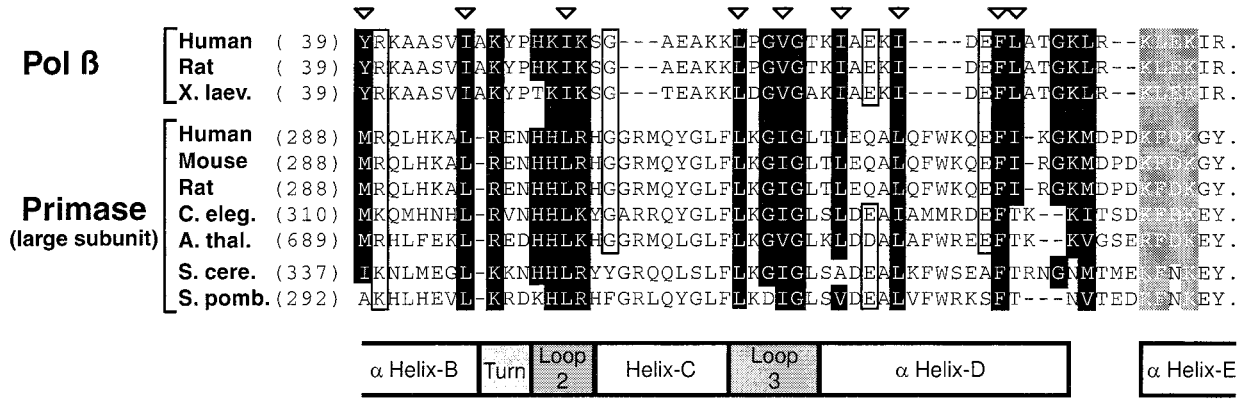

FIGURE 8: Amino acid sequence alignment between the 8 kD domain of pol β and the primase p58 subunit. Conserved amino acid positions in the hydrophobic core and loop/linker regions of pol β are highlighted in black, while a highly conserved block of residues in helix E is highlighted in gray. Highly conserved amino acids in other regions are enclosed by a box. Triangles above the sequences show hydrophobic core residues in pol β. Secondary structure elements determined from the NMR and crystal structures of pol β are below the amino acid sequences (Swiss Protein Database, SW: DPOB_RAT, ref *35*). Numbers in parentheses to the left of the sequence indicate the amino acid position relative to the N-terminus of the respective polymerase. The accession number for each sequence is (top to bottom) the following: 544186, 1055330, 2661842, 1346793, 631665, 3676246, 3183129, 2760323, 666094, and 3650405.

the idea that initiation and elongation are fundamentally different reactions (*12*).

The 8 kDa Domain of Pol β and the p58 Subunit of Primase Share Significant Sequence Homology. In addition to the catalytic 31 kDa domain, pol β contains an independently folding 8 kDa domain that has multiple activities important for DNA repair by pol β (*25*, *33*, *34*). Most surprisingly, we found significant homology between the 8 kDa domain of pol β with the middle of the large subunit of primase (Figure 8). The 8 kDa domain of pol β consists of 4 helices packed into two antiparallel pairs, helices A/B and helices C/D, and is attached to the 31 kDa domain via a flexible helical linker (helix E (*20*, *35*)). While there was minimal homology in the region from helix A through helix B, the region from Loop-2 through Helix E shows significant conservation between pol β and p58. In this region all of the buried hydrophobic residues are conserved, while loops 2 and 3 and parts of helices D and E are extremely similar. While the significance of such homology with respect to primase function is not clear, this striking homology indicates that similarities between primase and pol β extend beyond the catalytic subunit.

Relationship between the Family X Polymerases and DNA Primase. Sequence comparison between pol β and primase allowed us to identify R304 of the p49 subunit of hprimase as an important residue involved in both catalysis and binding of the NTP that becomes the 5′-terminus of the primer. In combination with previous studies identifying D109, D111, and D306 as being functionally important (*1*), 4 residues homologous with residues in pol β have now been identified. Thus, it seems likely that these enzymes will employ similar catalytic mechanisms.

Primase is likely part of a larger superfamily, the class-X nucleotidyl transferases, that includes the family X polymerases (Terminal deoxynucleotidyl transferase, pol β, and several other polymerases of unknown function, Figure 1) and several nucleotidyl transferases that transfer the nucleotide to non-nucleic acid receptors (*36*). For all of the family X polymerases, the 3 catalytic aspartates are arranged with the dual aspartate motif on the N-terminal side of the single aspartate motif. Primase also shares this arrangement of the 3 catalytic aspartates, and the amino acids neighboring these 3 aspartates are extremely similar between primase and the family X polymerases. Interestingly, while the Archeabacterial and eukaryotic primases share significant homology around the catalytic aspartates (Figure 1 and ref *37*), primases do not contain several sequences that are highly conserved among family X polymerases (Figure 1). Thus, the primases likely form their own family that is contained within the putative class-X nucleotidyl transferase superfamily. The family X polymerases and several nucleotidyl transferases were previously proposed to be related on the basis of sequence alignments, primarily in the region around the dual aspartate motif, and a low resolution structure of kanamycin nucleotidyl transferase (*36*). Additionally, the dual aspartate motif in kanamycin nucleotidyl transferase is on the N-terminal side of the single carboxylic acid motif. Thus, this putative class-X nucleotidyl transferase superfamily contains enzymes involved in very diverse processes, including RNA polymerization, template-dependent and template-independent DNA polymerization, antibiotic detoxification, and gene regulation. Further evidence that all of these enzymes do, in fact, form a superfamily will require further structural analysis.[4]

[4] Interestingly, the C family of DNA polymerases may actually be a subset of the class-X nucleotidyl transferase superfamily. Recent studies by McHenry and co-workers showed that for *E. coli* DNA polymerase III, the catalytic dual aspartate motif is located N-terminal of the single aspartate motif (*2*), and the sequence of these two motifs in DNA polymerase III (DFDV and KFDF, respectively) is very similar to those of the class-X nucleotidyl transferases.

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