The p58 Subunit of Human DNA Primase Is Important for Primer Initiation, Elongation, and Counting†

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ABSTRACT: The p58 subunit of human DNA primase contains a region, M288−K344, that is homologous to part of the 8 kDa domain of DNA polymerase *β*. Since regions of a protein that are highly conserved evolutionarily often play important catalytic functions, we examined the effects of mutating this region of the p58 subunit on primase activity. Deleting M288−L313 of the p58 subunit results in a protein that binds to the primase p49 subunit but cannot support primer synthesis on any template when assays only contain Mg^{2+} as the divalent metal. Including Mn^{2+}, a metal that stimulates initiation of primer synthesis, in the assays now allows the enzyme to synthesize primers at a rate only moderately lower than that of the wild-type enzyme on templates consisting solely of deoxycytidylates. While the enzyme is active under these conditions, it has lost the ability to synthesize primers of defined length (i.e., count). Alanine scanning mutagenesis of charged residues in this region revealed three amino acids, R302, R306, and K314, that play important roles in both primer initiation and translocation. Conversion of these residues to alanine interfered with initiation and significantly decreased the processivity of primase. Together, these studies indicate that this "pol *β*-like" region of p58 is important for three distinct aspects of primer synthesis:; initiation, translocation, and counting. The implications of these results with respect to the biological role of the p58 subunit and the mechanism of primer synthesis are discussed.

DNA primase is a DNA-dependent RNA polymerase that is essential for DNA replication (*1*). During leading and lagging strand synthesis, primase synthesizes an RNA primer de novo on single-stranded DNA onto which DNA polymerase α (pol α)[1] can polymerize deoxynucleotides. Additionally, primase is involved in the checkpoint pathway coupling DNA replication to repair (*2*−*5*). Yeast containing mutations in primase fail to elicit a G1/S block in response to DNA damaging agents, and *Xenopus* extracts require primase activity for activation of the DNA replication checkpoint.

Primase and pol α copurify as a four-protein complex, with subunits of approximately 49, 58, 70, and 180 kDa (*6*−*8*). The p180 subunit contains pol α catalytic activity, and the p49 subunit contains the catalytic core of primase (*9*, *10*). The p70 subunit may be important for p180 production and nuclear localization as well as tethering the pol α−primase complex to the replication fork (*11*, *12*). To date, the precise function(s) of the p58 subunit remain(s) unclear. Previous studies showed that p58 stabilizes the p49 subunit, increases the rate of primer synthesis, and can bind ssDNA as well as the partially duplex RNA·DNA product formed after primer synthesis (*9*, *13*, *14*). In combination with immunoprecipitation experiments indicating that p58 is positioned between p49 and p180 (*15*), these observations suggest that p58 plays a role in transferring the primase-synthesized RNA primer from primase to pol α during initiation of new DNA strands.

The kinetic mechanism of primase consists of three discrete processes: initiation, extension, and termination (*16*, *17*). Initiation involves primase binding ssDNA and two NTPs to form a quaternary complex, followed by dinucleotide synthesis. Primase rapidly extends the dinucleotide to a total length of 7−10 ribonucleotides (i.e., "unit length") during the extension process. Once primase synthesizes a unit length primer, further primase activity is negatively regulated until the primer is transferred intramolecularly to the pol α active site and dNTPs are polymerized (termination). Under typical in vitro conditions, the initiation step of dinucleotide synthesis is rate-limiting in primer synthesis.

Primase frequently synthesizes a dinucleotide or trinucleotide and then allows this product to dissociate into solution (*16*). When this occurs, primase rapidly reinitiates primer synthesis in order to try to synthesize a unit length primer. Since this reinitiation process is substantially faster than the rate of the original initiation event, conditions that prevent primase from synthesizing a unit length primer increase the rate of primer synthesis as measured in terms of total initiation events.

A second unique feature of primase is its ability to "count" (*18*−*20*). Regardless of the template sequence, unit length primers always appear to be ca. 7−10 nucleotides long. Additionally, primase will further elongate a unit length primer into longer products. Importantly, these longer products (primer multimers) result from primase again polymerizing defined numbers of nucleotides onto the original unit length primers. Thus, for example, eukaryotic

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[1] Abbreviations: pol, DNA polymerase; Tris-HCl, tris(hydroxymethyl)aminomethane, hydrochloride salt.

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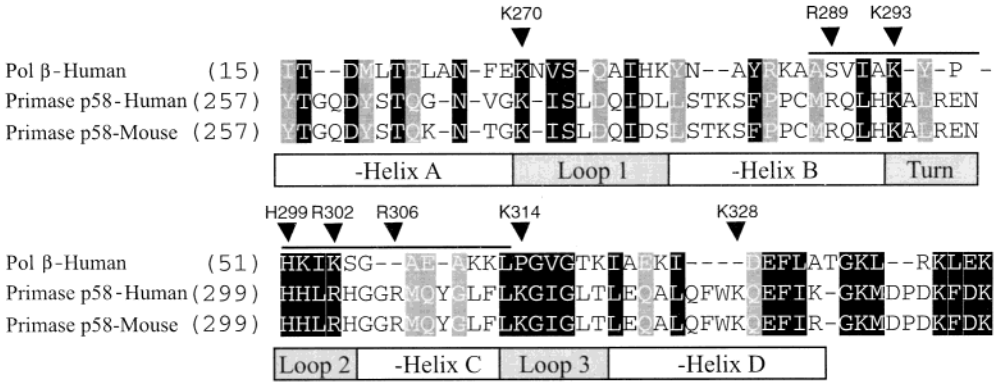


FIGURE 1: Sequence homology between the 8 kDa domain of pol β and part of the 58 kDa subunit of primase. The sequence of the 8 kDa domain of human pol β was aligned against the sequences of the human and mouse p58 primase subunits using CLUSTALW as previously described (*21*). Residues highly conserved between primase and pol β are highlighted in black, and conserved residues are highlighted in gray. Positively charged residues in the p58 subunit that were mutated to alanine are designated with a triangle. A line marks the sequence removed from the p58 deletion mutant. Secondary structural elements determined from the NMR and crystal structures of pol β are shown below the amino acid sequences (Swiss Protein Database, SW: DPOB-RAT). 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) 544186, 1346793, and 631665.

primase synthesizes primer multimers of ca. 20, 30, 40, etc. nucleotides long.

A combination of sequence homologies and site-directed mutagenesis has been used to identify important residues in eukaryotic primase (*21*). Eukaryotic primase, archaebacterial primase, DNA polymerase β, and other members of the class X nucleotidyl transferase family form a larger superfamily and share significant homology in their catalytic cores. Between the catalytic p49 subunit of primase and the dNTP-polymerizing 31 kDa domain of pol β, this homology consists of two sets of four residues that include the three aspartates that form the metal binding center of pol β along with an arginine (R304) that performs similar roles during catalysis by each enzyme. No high-resolution structural data on eukaryotic primase exist that could provide insights into the mechanism of primase, although the structure of an archaebacterial primase was recently solved to 2.3 Å (*22*).

There is also a striking sequence similarity between the p58 primase subunit and the 8 kDa domain of pol β [Figure 1 (*21*)]. In fact, the homology between p58 and the 8 kDa domain is substantially greater than the homology between the two active sites involved in phosphodiester bond formation. Since regions of a protein that are highly conserved evolutionarily often play important catalytic functions, we hypothesized that this "pol β-like" sequence in p58 performs a major role in primase activity. Here we report the construction and analysis of eight single-site mutations and one deletion mutation in the pol β-like sequence of p58. These studies revealed that mutating this region of p58 inhibits both initiation and elongation of primers, largely due to an impaired ability of the enzyme to translocate along the template. Additionally, this region of p58 appears important for the counting ability of primase. The biological and mechanistic implications of these results are discussed.

EXPERIMENTAL PROCEDURES

Materials

Human recombinant primase p49 and p58 subunits were coexpressed in *Escherichia coli* JM105 transformed cells and purified using Ni-NTA (Qiagen) chromatography as previously described (*14*). Synthetic oligonucleotides of defined sequence were obtained from Oligos, Etc. The concentrations of ssDNAs were determined spectrally and are expressed in terms of total nucleotides. Radiolabeled NTPs and dNTPs were purchased from New England Nuclear. All other reagents were of the highest purity available.

Methods

Site-Directed Mutagenesis. Single-site and deletion mutants of the primase p58 subunit were engineered using a pACYC184-Hp58 plasmid generously provided by Dr. Bill Copeland (NIEHS, Research Triangle Park, NC) and the Chameleon dsDNA site-directed mutagenesis kit (Stratagene). To construct the large deletion mutant, pACYC184-Hp58 was incubated with *Sph*I to remove residues M288−N348. (The upstream *Sph*I site was endogenous to pACYC184-Hp58, and the downstream site was engineered by site-directed mutagenesis.) The construction of the small deletion mutant was similar except that the downstream *Sph*I site was engineered nearer the endogenous *Sph*I site in order to remove residues M288−L313. The digested pACYC184-Hp58 plasmids were then ligated overnight at 16 °C and transformed into competent JM105 cells. Plasmids from positive colonies were amplified and purified with Qiagen mini-preps and sequenced at the DNA sequencing facility of the University of Colorado to confirm the presence of the correct mutations.

Enzyme Assays. Primase assays (10 mL) were performed as previously described and typically contained 60 μM single-stranded DNA template, 1 μM primase, 200 μM [α-^{32}P]NTPs, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.05 mg/mL bovine serum albumin (*16*, *21*). Reactions were initiated by the addition of enzyme and incubated at 37 °C for 0.5−60 min. After the reactions were quenched by adding 2.5 volumes of gel loading buffer (90% formamide), products were separated by denaturing polyacrylamide gel electrophoresis (18−20% polyacrylamide, 8 M urea) and analyzed via phosphorimagery (Molecular Dynamics).

Assays to measure primase-catalyzed extension of exogenously added primer-templates (10 μL) contained 50 μM RNA primed-DNA template, 1 μM primase, 200 μM [α-^{32}P]-NTPs, 50 mM Tris-HCl, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 0.05 mg/mL bovine serum albumin. Reactions were initiated by the addition of enzyme and incubated at 37 °C for 0.5−60 min. Products were separated and quantified as described above.

Base Hydrolysis of Primers. Primase assays were performed as described above. NaOH was added to a final concentration of 0.08 M, and the samples were heated at 90 °C for 10 min and quenched with 2.5 volumes of gel loading buffer.

RESULTS

Effects of Deleting Part of the Pol β-like Region of p58. To determine the importance of the pol β-like motif in p58, a deletion mutant was constructed that lacks a large segment of this region [M288−L313[2] (Figure 1)]. We chose to delete this segment because the two conjoined amino acids (C287 and K314) correspond to two residues in the homologous 8 kDa domain of pol β (A43 and P63) that are close together (*23*, *24*). The deletion mutant was overexpressed and purified in complex with wild-type p49 (p49/DMp58) in *E. coli* using Ni-NTA chromatography. Copurification of both the p49 and DMp58 subunits, as evidenced by their presence on a stained sodium dodecyl sulfate−polyacrylamide gel, indicated that the deletion did not grossly alter p49/p58 binding since only the DMp58 subunit contained a His tag.

To initially assess how the deletion mutation affected primase activity, p49/DMp58 and wild-type primase were incubated with $(dC)_{40}$ and 200 μM [α-^{32}P]GTP. As expected, in assays containing the wild-type enzyme and only Mg^{2+} as the divalent cation, the majority of products were full-length primers, ≥7 nucleotides long. In contrast, p49/DMp58 synthesized no detectable products under these conditions (Figure 2). Since adding Mn^{2+} to assays containing Mg^{2+} stimulates primase activity via an effect on the p58 subunit (*25*), the ability of Mn^{2+} to enhance p49/DMp58 activity was measured. Including 100 μM Mn^{2+} dramatically stimulated p49/DMp58 activity such that the level of primase activity was now only moderately less than that of wild-type primase [42 ± 7% of wild type (Figure 2)]. We tested the hypothesis that the products synthesized by p49/DMp58 resulted solely from activity of the p49 subunit by measuring the ability of the isolated p49 subunit to synthesize products on $(dC)_{40}$ in assays containing 100 μM Mn^{2+} (Figure 2). Only small amounts of products were synthesized, <1% of the amount synthesized by p49/DMp58. Thus, high rates of primer synthesis by p49/DMp58 requires the presence of both subunits.

Primer synthesis by p49/DMp58 was tested on a large panel of templates in assays containing both Mg^{2+} and Mn^{2+}. p49/DMp58 synthesized no detectable products on poly(dT) and $d(TC)_{30}$, while on $d(CCT)_{20}$, $d(TTCC)_{15}$, $d(C_3R)$, $(dC)_{20}$, and $(dC)_{30}$, the level of p49/DMp58 activity was 0.3%, 0.6%, 3.7%, 2.7%, and 26% that of wild-type activity, respectively. Since previous studies demonstrated that both Mn^{2+} and cytosine-rich templates enhance primer initiation (*21*, *25*), these results suggest that p49/DMp58 initiates primer synthesis much less efficiently than the wild-type enzyme.

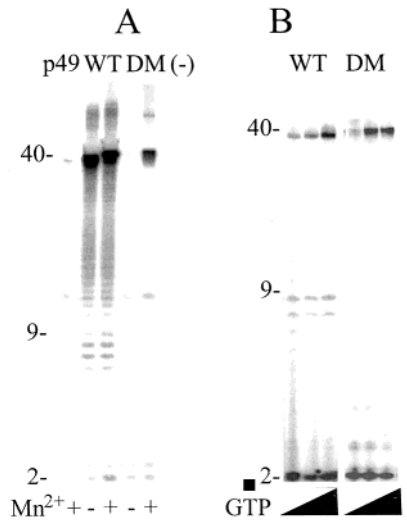


FIGURE 2: Effect of the p58 deletion on primase activity. (A) The activity of the isolated p49 subunit (1 μM) and p49/p58 primase complexes (1 μM) containing either wild-type primase (WT) or the deletion mutant p49/DMp58 (DM) was measured in assays containing 60 μM $(dC)_{40}$ and 200 μM [α-^{32}P]GTP as described under Experimental Procedures. The presence or absence of 100 μM $MnCl_2$ in the assays is noted in the figure. A control reaction was conducted under identical conditions that lacked enzyme (−). Products were separated on a 20% denaturing polyacrylamide gel and quantified via phosphorimagery. Product length is noted to the left of the image. (B) Primase assays contained either wild-type or p49/DMp58 primase were performed as in (A), except the [α-^{32}P]-GTP was replaced with either 20, 50, or 100 μM [γ-^{32}P]GTP. The apparent large differences in the relative amounts of dinucleotide and longer products between panel A and panel B result from the assays in panel A employing [α-^{32}P]GTP while those in panel B employed [γ-^{32}P]GTP. Whereas in panel A the dinucleotide and long products will contain 2 and approximately 40 ^{32}P-labeled nucleotides, respectively, in panel B both dinucleotide and long products will contain just 1 ^{32}P-labeled nucleotide because only the 5′-terminal nucleotide of the primer retains its γ-phosphate.

Curiously, the p49/DMp58 and wild-type primases synthesized products of very different size distribution on $(dC)_{40}$. Whereas the wild-type enzyme generated a small amount of dinucleotide, significant amounts of unit length primers 7−10 nucleotides long, and primer−multimers ca. 40 nucleotides long, p49/DMp58 only generated dinucleotide and products ca. 40 nucleotides long. To ensure that the 40 nucleotide long products were, in fact, bona fide RNA primers, two controls were performed. First, treatment of the products synthesized by p49/DMp58 with base resulted in their complete destruction, demonstrating that they did consist of RNA (data not shown). Second, we examined primer synthesis in assays containing [γ-^{32}P]GTP and $(dC)_{40}$ (Figure 2, panel B). If the products were true primers, labeled products should be generated starting with [γ-^{32}P]GTP since the 5′-terminal nucleotide retains its γ-phosphate. In contrast, if the products resulted from polymerization of NTPs directly onto either the template or a small contaminating oligonucleotide present in the reactions, no labeled products should be observed. As shown in Figure 2, p49/DMp58 synthesizes radiolabeled products in assays containing [γ-^{32}P]-

[2] A larger deletion mutant lacking the sequence (M288−N348) was also engineered. However, this form of p58 was not successfully expressed in JM105 cells, either in the presence or in the absence of the p49 subunit. Interestingly, the corresponding residues in pol β, A43 and D92, are located quite far apart from each other.

Table 1: DNA Templates Used[a]

$d(TC)_{30}$	$^{(5')}$TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
$d(CCT)_{20}$	$^{(5')}$CCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCTCCT
$d(C_3R)$	$^{(5')}$CCCTACGATGCCCTACGATGCCCTACGATGCCCTACGATG
$d(TTCC)_{15}$	$^{(5')}$TTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCCTTCC
$d(TCTA)_{15}$	$^{(5')}$TCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTATCTA
$d(TCTG)_{15}$	$^{(5')}$TCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTGTCTG
$d(TCTCTA)_{10}$	$^{(5')}$ATCTCTATCTCTATCTCTATCTCTATCTCTATCTCTATCTCTATCTCTATCTCT
$d(TCTCTG)_{10}$	$^{(5')}$GTCTCTGTCTCTGTCTCTGTCTCTGTCTCTGTCTCTGTCTCTGTCTCTGTCTCT
$d((TC)_5A)_5$	$^{(5')}$TCTCTATCTCTCTCTCATCTCTCTCTCATCTCTCTCTCATCTCTCTCTCATCTCTCTCTC
$d((TC)_5G)_5$	$^{(5')}$TCTCTGTCTCTCTCTCGTCTCTCTCTCGTCTCTCTCTCGTCTCTCTCTCGTCTCTCTCTC
$d(TCA)_{20}$	$^{(5')}$TCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCATCA
RNA8/43mer[a]	$^{(5')}$UUUGAGAG $^{(3')}$AAACTCTCTCTCTCTCTGGGGGTAAAATGGGGCAAAATGGGGC

[a] In RNA8/43mer, the primer is RNA while the template is DNA.

GTP. Together, these two controls indicate that the long products are true RNA primers.

The lack of unit length primers suggested that deleting part of p58 interfered with the ability of primase to count. Primer synthesis on $(dC)_{40}$ was examined in assays containing only 100 μM GTP, conditions that result in the accumulation of large amounts of primers 7−10 nucleotides long relative to primer multimers. As shown in Figure 2, this again resulted in the accumulation of no detectable unit length primers by p49/DMp58. Likewise, further decreasing the concentration of GTP to either 50 or 20 μM still resulted in the accumulation of no unit length products by p49/DMp58. In contrast, wild-type primase synthesized large amounts of unit length primers under these conditions (Figure 2). These results were not limited to just $(dC)_{40}$, since on the other templates where p49/DMp58 primase showed measurable activity, $(dC)_{30}$, $(dC)_{20}$, and $d(C_3R)$, the enzyme synthesized only dinucleotide and very long products (data not shown). Thus, deleting part of the pol β-like region of p58 appears to interfere with the ability of primase to count.

Construction and Evaluation of Single-Site Mutants. To further analyze the pol β-like sequence in p58, eight conserved, positively charged amino acid residues (K270, R289, K293, H299, R302, R306, K314, and K328, Figure 1) were individually mutated to alanine and expressed alone in JM105 cells. Each p58 mutant was combined with wild-type p49 to form the p49/p58 complex, and activity was measured in assays containing $(dC)_{40}$ and 200 μM [α-^{32}P]-GTP. Primase complexes bearing the K270A, R289A, K293A, H299A, and R302A mutations had activity similar to that of the wild-type enzyme (85%, 104%, 102%, 88%, and 115% of wild-type activity, respectively) while complexes bearing the R306A, K314A, and K328A mutations exhibited reduced activity (57%, 29%, and 64% of the wild-type enzyme, respectively). We then focused on the six amino acids nearest the three residues that most severely affected primase activity in order to better understand the role(s) of these residues in primase activity. p58 proteins containing either K293A, H299A, R302A, R306A, K314A, or K328A were coexpressed with wild-type p49 and purified using Ni-NTA chromatography. Similar to the deletion mutant, each single-site mutant was successfully purified in complex with p49, indicating that these mutations did not grossly alter the interaction of p58 with p49.

The activity of the coexpressed mutant p49/p58 complexes was assessed on three templates, poly(dT), $d(TC)_{30}$, and $(dC)_{40}$, where the initiating dinucleotide synthesized would vary in stability from two A·T base pairs [poly(dT)] to two G·C base pairs [$(dC)_{40}$]. On poly(dT), both R306A and K314A exhibited greatly reduced rates of initiation, 92% less than wild type, and synthesized only trace amounts of products greater than two nucleotides long (Table 2 and Figure 3). Under similar conditions, the K293A, H299A, R302A, and K328A proteins exhibited minimal changes relative to the wild-type enzyme. In contrast to the decreased rate of initiation on poly(dT), the rate of initiation by the R302A, R306A, and K314A proteins increased 3−4-fold relative to wild type on the template $d(TC)_{30}$ (Table 2). Primer initiations likewise increased on the template $(dC)_{40}$, albeit to a lesser extent. As discussed later, this increased initiation rate results from an accumulation of dinucleotide. The mutations K293A, H299A, and K328 had no effect on primase activity using either $d(TC)_{30}$ or $(dC)_{40}$.[3]

To provide further evidence that the mutations R306A and K314A interfere with initiation on poly(dT), we added 100 μM Mn^{2+}, a metal that enhances initiation (*25*), to the assays. Initiation events increased by 3-fold for both proteins; however, the rate of initiation remained much less than for the wild-type protein. Interestingly, these mutant proteins still

Table 2: Primase Activity on Poly(dT), $d(TC)_{30}$, and $(dC)_{40}$

template/protein	total products[a]	dinucleotide (% total product)
poly(dT)		
wild type	1	61 ± 5
R296A	1.0 ± 0.10	55 ± 2
H299A	0.97 ± 0.04	56 ± 1
R302A	1.1 ± 0.2	50 ± 5
R306A	0.08 ± 0.03	90 ± 2
K314A	0.08 ± 0.06	84 ± 9
K328A	0.8 ± 0.1	55 ± 2
$d(TC)_{30}$		
wild type	1	66 ± 2
K293A	1.1 ± 0.3	59 ± 8
H299A	1.4 ± 0.3	64 ± 3
R302A	3.1 ± 0.7	81 ± 3
R306A	3.8 ± 0.6	88 ± 2
K314A	3.1 ± 0.3	87 ± 1
K328A	1.7 ± 0.4	63 ± 4
$(dC)_{40}$		
wild type	1	12 ± 4
K293A	0.98 ± 0.06	10 ± 7
H299A	1.0 ± 0.1	8 ± 1
R302A	1.4 ± 0.2	23 ± 1
R306A	1.0 ± 0.1	24 ± 1
K314A	1.20 ± 0.08	36 ± 1
K328A	1.0 ± 0.01	12.3 ± 0.1

[a] Assays were performed as described in Figure 2. The total moles of product synthesized for the wild-type enzyme on each template was normalized to 1.

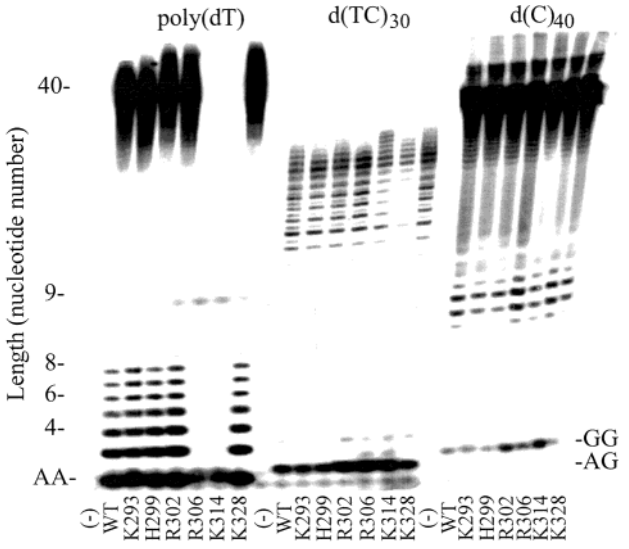


FIGURE 3: Effect of mutating individual amino acids in p58 on primase activity. Activity was measured in assays containing 1 μM primase complexes [either wild type (WT) or the designated p58 mutant], 60 μM template [poly(dT), $d(TC)_{30}$, or $(dC)_{40}$], and 200 μM [α-^{32}P]NTPs, as described under Experimental Procedures. Control reactions for each template were conducted under identical conditions that lacked enzyme (−). Products were separated on a 20% denaturing polyacrylamide gel and visualized via phosphorimagery.

did not synthesize any products longer than two nucleotides long (data not shown). Together with the data from p49/DMp58, these effects of mutating R306 and K314 provide strong evidence that the pol β-like sequence in p58 is important for initiation.

In addition to affecting the amount of total product synthesized, the mutations R302A, R306A, and K314A also altered the size distribution of the products as compared to the wild-type enzyme (Figure 3). As indicated above, the R306A and K314A mutants almost exclusively synthesized the dinucleotide on poly(dT). On the template $d(TC)_{30}$, the R302A, R306A, and K314A mutants synthesized 3.1-, 3.7-, and 2.9-fold, respectively, more dinucleotide than the wild-type enzyme and increased the trinucleotide product by 1.9−2.1-fold relative to the wild-type enzyme. The wild-type, R306A, and K314A primase complexes synthesized similar amounts of products >3 nucleotides long, while K314A synthesized slightly fewer long products on $d(TC)_{30}$ (Figure 3). Similarly, on $(dC)_{40}$ the three mutant proteins generated 3.1−4.2-fold more dinucleotide than the wild-type enzyme, while each protein synthesized similar levels of products longer than a dinucleotide (trinucleotide levels were below the limit of detection). This accumulation of short products indicates that, in addition to inhibiting initiation of primer synthesis, these mutations interfere with primer elongation.

Because previous studies indicated that $MnCl_2$ can enhance primer extension (*25*), we tested the effect of $MnCl_2$ on the products synthesized by the mutant enzymes. However, adding $MnCl_2$ did not alter the relative amounts of dinucleotide and longer products formed by the R302A, R306A, or K314A proteins (data not shown).

Effect of Mutating p58 on Extension of the Dinucleotide. Conversion of the dinucleotide to the trinucleotide reflects a competition between dissociation of the dinucleotide from the enzyme and polymerization of the next correct NTP (*26*). Thus, accumulation of dinucleotide could reflect either increased rates of dinucleotide dissociation or decreased rates of any step during polymerization of the next correct NTP and with which dissociation competes. Minimally, NTP polymerization requires at least three processes, a translocation event to the next template base to be read, binding of the next correct NTP, and polymerization of the next correct NTP. Since we cannot directly measure the rate of dinucleotide dissociation, we measured the competition between dissociation and binding and polymerization of the next correct NTP.

Wild-type and mutant primase were incubated with the template $d(TCTA)_{15}$, [γ-^{32}P]ATP, and GTP. Previous studies indicate that primase greatly prefers to incorporate GTP as the second nucleotide of the primer (*16*); hence primase can synthesize the dinucleotide pppApG and the trinucleotide pppApGpA, but synthesis of longer products will be minimized due to the absence of UTP. Thus, we can measure dinucleotide extension without the complication of subsequent polymerization reactions. Additionally, using [γ-^{32}P]-ATP will result in only those products containing a 5′-terminal ATP being radioactive, thereby eliminating any complications due to initiation opposite the template deoxycytidylate (i.e., pppGpA synthesis).[4] In assays containing 200 μM ATP and GTP, the R302A, R306A, and K314A mutants were significantly impaired in their ability to elongate the dinucleotide. Dinucleotide elongation decreased by 23%, 82%, and 93%, respectively, for each of the mutants relative to wild-type primase (Figure 4), consistent with the results described earlier on poly(dT), $d(TC)_{30}$, and $(dC)_{40}$. Then, the

[3] The slightly greater effects of mutating K328 when p58 is individually expressed and then combined with p49 versus when the two subunits are coexpressed may indicate that this residue is important for the stability of p58 and/or binding to p49. Coexpression in *E. coli* may mask these effects due to the presence of cellular chaperones that could catalyze complex formation.

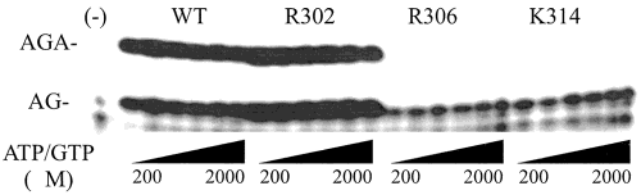


FIGURE 4: Effect of the p58 mutations R302A, R306A, and K314A on extension of the dinucleotide generated during initiation of primer synthesis. Activity was measured in assays containing 1 μM primase complexes [either wild type (WT) or the designated p58 mutant], 60 μM d(TCTA)$_{15}$, and 200, 400, 800, 1000, 1500, and 2000 μM [γ-^{32}P]ATP and GTP as described under Experimental Procedures. Control reactions for each template were conducted under identical conditions that lacked enzyme (−). Products were separated on a 20% denaturing polyacrylamide gel and quantified via phosphorimagery.

Table 3: Steady-State Kinetic Parameters for the NTP When Using RNA8/43mer as Template[a]

protein	$K_{m,ATP}$ (μM)	$V_{max,ATP}$ [pmol of ATP/ (pmol of enzyme·h)]	$V_{max,ATP}/K_{m,ATP}$
wild type	192	76	0.40
R302A	106	106	1.00
R306A	74	72	0.97
K314A	78	38	0.49
DMp58	88	33	0.38

[a] Assays for addition of a nucleotide onto a preexisting RNA primer were performed as described under Experimental Procedures.

effect of increasing the NTP concentration on the competition between dissociation of the dinucleotide from the enzyme and polymerization of the next correct NTP was measured. If the mutations either increased the rate of dinucleotide dissociation relative to NTP binding/polymerization or decreased the rate of NTP binding/polymerization, increasing the NTP concentration should have decreased dinucleotide accumulation. However, Figure 4 shows that increasing the concentration of GTP and ATP up to 2 mM did not enhance the fraction of dinucleotide elongated for any of the mutants tested. Thus, dinucleotide accumulation did not result from a decreased rate of binding/polymerization of the next correct NTP relative to dinucleotide dissociation.

Mutating p58 Does Not Inhibit Elongation of Exogenously Supplied RNA Primers. To directly test the possibility that mutations in p58 affected phosphodiester bond formation, we measured the effects of the mutations on primase-catalyzed NTP polymerization onto a synthetic RNA primer-template. Primase was incubated with RNA8/43mer (Table 1) in the presence of only [α-^{32}P]ATP, conditions where primase can polymerize a single NTP onto the primer (*15*). All of the single-site mutants as well as the deletion mutant polymerized ATP onto the RNA primer with steady-state kinetic parameters either comparable to or slightly greater than wild type (Table 3). Thus, the effects of these mutations on primer synthesis are likely not due to effects on phosphodiester bond formation and are consistent with previous studies showing that large portions of p58 can be deleted without affecting phosphodiester bond formation by p49 (*13*).

[4] Similar experiments containing [α-^{32}P]GTP instead of [γ-^{32}P]ATP were conducted to label both pppApG and pppGpA dinucleotide products. Wild-type primase produced similar amounts of dinucleotide in assays containing either [α-^{32}P]GTP or [γ-^{32}P]ATP, and the mutant primases showed similar reductions in dinucleotide elongation regardless of whether [α-^{32}P]- or [γ-^{32}P]ATP was employed (data not shown). Thus, these mutations do not affect the preference of primase for using GTP as the second nucleotide of the primer.

Table 4: Steady-State Kinetic Parameters for the NTPs When Using d(TC)$_{30}$ as Template[a]

protein	K_m^{NTP} (μM)	V_{max}^{NTP} [pmol of primer/ (pmol of enzyme·h)]	V_{max}^{NTP}/K_m^{NTP}
	Total Products		
wild type	152	26	0.2
R302A	167	35	0.2
R304A	865	72	0.1
K314A	2500	116	0.05
	Unit Length Products[b]		
wild type	69	4	0.06
R302A	67	2	0.03
R304A	461	2	0.004
K314A	604	1	0.002

[a] Primase assays were performed as described under Experimental Procedures with 60 μM DNA. [b] Unit length products represent primers ≥8 nucleotides long.

Steady-State Kinetic Parameters of the p58 Mutants. To further evaluate the effects of the mutations R302A, R306A, and K314A on NTP polymerization, steady-state kinetic parameters were measured on d(TC)$_{30}$. The DNA concentration used for these studies, 60 μM, is substantially above its K_m for each enzyme (wild type, 10 μM; R302A, 6 μM; R306A, 6 μM; K314A, 9 μM). Kinetic parameters were measured in terms of both total products synthesized and products synthesized that pol α can elongate (i.e., unit length primers). With both measurements, the K_m for NTPs increased substantially (Table 4). Interestingly, the V_{max} for the mutant enzymes could both increase (total products synthesized) and decrease (unit length primers synthesized), depending upon which products were analyzed. This increased V_{max} when measuring total products results almost entirely from the large accumulation of dinucleotide upon mutation of p58. In fact, the dinucleotide is the predominate product synthesized by all of the enzymes when measured on a molar basis, as shown by the much larger V_{max} obtained when measuring total product.

Mutating p58 Dramatically Affects Dinucleotide Synthesis in the Absence of a Cognate NTP Even If That NTP Is Not Involved in Dinucleotide Synthesis. Surprisingly, the R306A and K314A proteins exhibited substantially reduced dinucleotide synthesis on the template d(TCTA)$_{15}$ as compared to the wild-type enzyme (75−80% less, Figure 4), while the R302A protein synthesized only slightly more dinucleotide than the wild-type enzyme (20% more, Figure 4). In contrast, the mutant proteins generated much more dinucleotide on the templates d(TC)$_{30}$ and (dC)$_{40}$ (Figure 3 and Table 2). We considered two potential explanations: (i) the presence of template purines inhibited dinucleotide synthesis by the mutant enzymes, and (ii) the lack of a required NTP inhibited dinucleotide synthesis. To demonstrate that the dramatically decreased dinucleotide synthesis was not due to the presence of template purines, dinucleotide synthesis was measured on the template d(TTCC)$_{13}$. In the presence of only GTP, the R306A and K314A proteins generated 80% and 66% less dinucleotide than the wild-type enzyme, respectively (Table 5).

Table 5: Comparison of Dinucleotide Synthesis on Various Templates in the Presence of All Required NTPs with Dinucleotide Synthesis in the Absence of One Required NTP[a]

template/enzyme	all required NTPs[b]	absence of one required NTP	missing NTP
$d(ATCT)_{15}$			
wild type	1	1	UTP
R306A	2.6	0.2	UTP
K314A	1.4	0.3	UTP
$d(TTCC)_{15}$			
wild type	1	1	GTP
R306A	1.3	0.1	GTP
K314A	1.9	0.05	GTP
$d(TTCC)_{15}$			
wild type	1	1	ATP
R306A	1.3	0.2	ATP
K314A	1.9	0.3	ATP
$d(TCA)_{20}$			
wild type	1	1	UTP
R306A	1.9	0.3	UTP
K314A	1.5	0.3	UTP
$d(TCA)_{20}$			
wild type	1	1	ATP
R306A	1.9	0.04	ATP
K314A	1.5	0.01	ATP
$d(TCA)_{20}$			
wild type	1	ND[c]	GTP
R306A	1.9	ND	GTP
K314A	1.5	ND	GTP

[a] Assays were performed as described under Experimental Procedures with 60 μM DNA and 200 μM NTPs. [b] Dinucleotide product synthesized for wild-type enzyme on each template was normalized to 1.[c] No product detected.

To determine if reduced dinucleotide synthesis by the R306A and K314A proteins reflected the absence of a required NTP, we first examined the effects of varying the frequency with which primase will encounter a template base for which it lacks the cognate NTP. As described above, on those templates where enhanced dinucleotide synthesis occurred [$d(TC)_{30}$ and $(dC)_{40}$], assays contained all of the required NTPs, whereas on those templates where diminished dinucleotide synthesis occurred [$d(TCTA)_{13}$ and $d(TTCC)_{13}$], assays lacked a required NTP. If decreased dinucleotide synthesis resulted from primase encountering a template base for which it lacks the cognate NTP, decreasing the frequency with which it encounters these bases should increase dinucleotide synthesis. Dinucleotide levels were measured on a series of templates containing varying lengths of TC repeats interrupted by either deoxyadenylate or deoxyguanylate with just ATP and GTP included in the reaction. These templates were designed to contain either 15 [$d(TCTA)_{15}$ and $d(TCTG)_{15}$], 10 [$d(TCTCTA)_{10}$ and $d(TCTCTG)_{10}$], 5 [$d((TC)_5A)_5$ and $d((TC)_5G)_5$], or 0 [$d(TC)_{30}$] purine residues for which primase lacks the cognate NTP (Table 1). As shown in Figure 5, the amount of dinucleotide synthesized by all three mutants increased as the number of template-purines decreased when assays contained only ATP and GTP. On a template containing only five purines, $d((TC)_5A)_5$, dinucleotide levels were increased by 14- and 8-fold for the R306A and K314A mutants, respectively, as compared to a template containing 15 purines, $d(TCTA)_{15}$. The R302A mutant showed a much smaller increase in dinucleotide production (2.5-fold), analogous to the much smaller effect of this mutation on dinucleotide elongation (Figure 4). Thus, increasing the frequency with which the mutant primase encounters a template base for which it lacks the complementary NTP strongly inhibits primer synthesis by the mutant primases but has no effect on the wild-type enzyme.

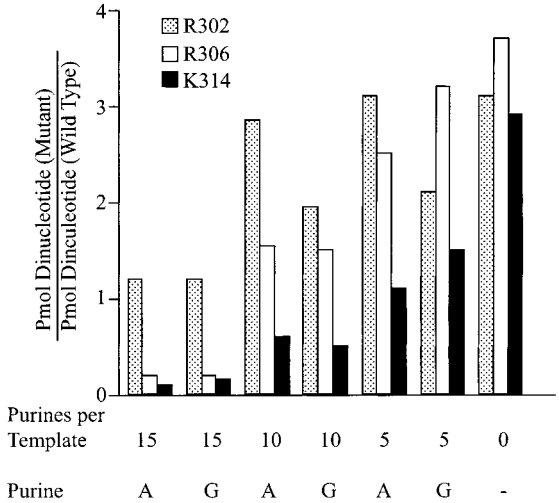


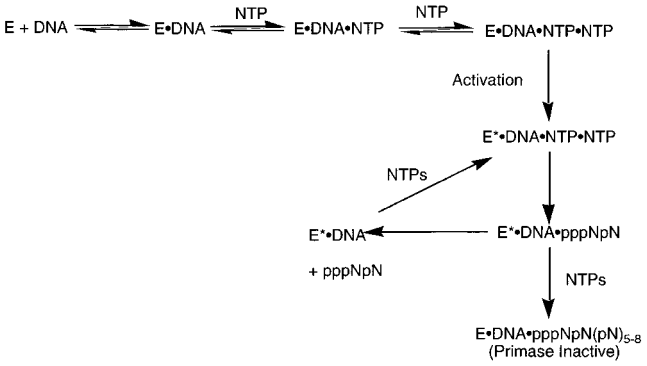
FIGURE 5: Effect of changing the frequency with which primase will encounter a template base for which it lacks a cognate NTP. Activity was measured in assays containing 1 μM primase complexes (either wild type or the designated p58 mutant), 60 μM template, 200 μM [α-^{32}P]ATP and GTP, 5 mM $MgCl_2$, 0.05 mg/mL BSA, 1 mM DTT, and 50 mM Tris, pH 7.9, for 1 h. The templates contained either 15 [$d(TCTA)_{15}$ and $d(TCTG)_{15}$], 10 [$d(TCTCTA)_{10}$ and $d(TCTCTG)_{10}$], 5 [$d((TC)_5A)_5$ and $d((TC)_5G)_5$], or 0 [$d(TC)_{30}$] purine residues [deoxyadenylate (A) or deoxyguanylate (G)]. Products were separated on a 20% denaturing polyacrylamide gel and quantified via phosphorimagery.

To provide further evidence that the reduced levels of dinucleotide synthesis by the mutant enzymes resulted from the lack of a required cognate NTP, we measured dinucleotide synthesis on three templates [$d(TCTA)_{15}$, $d(TTCC)_{15}$ and $d(TCA)_{20}$] both in the presence of all required NTPs and in the absence of a required NTP. In assays containing $d(TCTA)_{15}$ and all required NTPs, the R306A and K314A proteins synthesized 2.6- and 1.4-fold more dinucleotide than the wild-type enzyme, respectively (Table 5). When the assays lacked UTP, however, they synthesized 80% and 70% less dinucleotide than the wild-type enzyme, respectively. Similar results were observed on the templates $d(TTCC)_{15}$ and $d(TCA)_{20}$ upon omission of a required NTP (Table 5). When assays containing the template $d(TCA)_{20}$ lacked GTP, neither wild-type nor mutant primase synthesized detectable amounts of products. Under these conditions, initiation of primer synthesis would require that UTP serve as the 5′-terminal nucleotide. The lack of primer synthesis by the wild-type enzyme is consistent with previous studies showing that primase exhibits a tremendous preference for using a purine NTP as the 5′-terminal nucleotide (*16*, *27*, *28*). Importantly, the lack of dinucleotide synthesis by the mutant primases in the absence of GTP indicates that mutating p58 does not affect this preference.

DISCUSSION

The studies described here show that the pol β-like region of the p58 subunit plays an important role in primer synthesis. Alignment of the 8 kDa domain of pol β with part of the primase p58 subunit showed significant homology (Figure 1). Mutagenesis of this region in p58 revealed that it is critical for both initiation and elongation reactions during primer

Scheme 1: Mechanism of DNA Primase[a]



[a] E* represents an activated form of primase.

synthesis, although it does not appear to play a role in phosphodiester bond formation. Three basic amino acids, R302, R306, and K314, appear to be especially important.

Two lines of evidence indicate that mutations in the pol β-like region of p58 destabilize the primase·DNA·NTP·NTP quaternary complex required for initiation, thereby inhibiting this process. On poly(dT), p49/DMp58, p49/R306Ap58, and p49/K314Ap58 all exhibited significantly reduced rates of initiation. In contrast, these three proteins initiated synthesis on $(dC)_{40}$ at rates either comparable to or faster than the wild-type enzyme under appropriate experimental conditions. On poly(dT), the enzyme must stabilize two A·T base pairs in order to initiate primer synthesis, while on $(dC)_{40}$, the enzyme must stabilize two G·C base pairs. In light of the much greater stability of a G·C base pair than an A·T base pair, mutations that destabilize the primase·DNA·NTP·NTP quaternary complex would be expected to have a much more severe effect on initiation with the template poly(dT). Indeed, the results from these studies are analogous to previous work showing that mutating R304 in the p49 subunit impacts synthesis on poly(dT) much more severely than synthesis on $(dC)_{40}$ (*21*). This arginine interacts with the 5′-triphosphate of the NTP that will become the 5′-terminal nucleotide of the primer, and mutation of this residue specifically inhibits primer initiation. Second, we observed that adding small amounts of $MnCl_2$ to assays was required for p49/DMp58 to show activity and increased the rate of primer initiation by the R306A and K314A proteins on a poly(dT) template. Previously, we found both that Mn^{2+} stimulates initiation and that the p58 subunit likely contains at least one Mn^{2+} binding site (*25*).

Even though the mutations in p58 could inhibit primer initiation, the total number of initiations often increased in a template-dependent fashion [e.g., $d(TC)_{30}$]. This increase results from an unusual aspect of the primase mechanism. As shown in Scheme 1, synthesis of the dinucleotide is very slow and is the rate-limiting step for primer synthesis (*16*). While the precise nature of the rate-limiting process has not been delineated, it results in primase entering an "activated" state. If the activated primase now synthesizes a unit length (or longer) primer, primase becomes "deactivated" and must go through this slow, rate-limiting step in order to synthesize another primer. However, the activated primase will also very frequently generate a dinucleotide or trinucleotide and allow this product to dissociate into solution. In contrast to what occurs after synthesis of a unit length primer, synthesis of these abortive products does not result in deactivation of primase, and primase remains bound to the template. Since it is still in the activated state, primase will initiate synthesis of a new primer at a rate much faster than that of the original initiation event. Mutating either R302, R306, or K314 resulted in enzymes that aborted primer synthesis much more frequently than the wild-type enzyme after dinucleotide synthesis. These enzymes remain in the activated state after dinucleotide synthesis and, therefore, generate many more total products than the wild-type enzyme as measured by total initiation events.

In addition to being important for initiation, this region of p58 appears to be involved in primer elongation. Several experiments showed that mutating p58 substantially decreased the processivity of primase, especially with respect to elongation of the pppNpN dinucleotide, indicating that the rate of dinucleotide dissociation increased relative to the rate of polymerization of the next correct NTP. Since increasing the NTP concentration could not eliminate these effects, the increased dinucleotide dissociation was not a consequence of inefficient binding of the next correct NTP. If decreased processivity had resulted from impaired binding of the next required NTP, then increasing the NTP concentration should have restored the processivity of the mutant primases to that of the wild-type enzyme, but this was not observed. Likewise, the mutations in p58 did not appear to affect phosphodiester bond formation, as evidenced by the lack of effect of these mutations on the elongation of an exogenously added RNA primer-template. Thus, these mutations in p58 must result in an increased rate of primer dissociation relative to NTP polymerization by altering a process that does not involve either NTP binding or polymerization. Potential candidates for the altered process include a decreased affinity for the newly generated RNA primer and decreased translocation along the DNA to the next template base to be replicated after NTP polymerization. As described in greater detail below, we believe that mutating this region inhibits translocation along the DNA.

While mutating p58 increased the amount of dinucleotide synthesized when assays contained NTPs required for synthesizing all potential dinucleotides, omission of a potentially required NTP dramatically reduced the rate of dinucleotide synthesis. This occurred regardless of the template base for which the enzyme lacked the cognate NTP, indicating that the reduced levels of dinucleotide did not result from altered interactions with a specific base. Rather, these data suggest that (i) after primase generates a dinucleotide and this dinucleotide dissociates into solution, the enzyme must translocate along the template by at least one nucleotide in order to initiate synthesis of another primer and (ii) mutating either R302, R306, or K314 in the p58 subunit interferes with translocation, with mutation of either R306 or K314 having especially severe effects. With the wild-type enzyme, omitting a NTP required for dinucleotide synthesis is not problematic since the enzyme apparently can rapidly translocate to another site on the template where it synthesizes a dinucleotide using the supplied NTPs. However, if mutating p58 interferes with translocation, omitting a NTP required for synthesis of the next dinucleotide is potentially problematic. Since under these conditions primase would have to translocate along the DNA to a site for which it had all required NTPs, the mutations should interfere with reinitiation, the observed result. Furthermore, this model

predicts that decreasing the frequency with which primase requires a NTP that is not available should decrease the effects on dinucleotide synthesis. The data in Figure 5 show that, indeed, reducing the frequency with which the mutant primases encounter a template base for which they lack the cognate NTP decreases the effect of the p58 mutations on the rate of primer initiation. Additionally and as noted earlier, the effects of the p58 mutations on dinucleotide elongation are also consistent with the mutations inhibiting translocation along the template. Together, these two sets of data indicate that this region of p58 is important for translocation of primase along the template as well as initiation of primer synthesis.

Just as mutating individual amino acids in this region significantly inhibited primer synthesis, deleting a large portion of this region also greatly diminished primer synthesis. Deleting M288 through L313 impaired primer synthesis to the extent that no synthesis was observed on any template in assays containing only Mg^{2+}. Also, including 100 μM Mn^{2+} in the assays resulted in relatively high levels of primase assay on $(dC)_{40}$ and $(dC)_{30}$ but only very low or undetectable activity on a variety of other templates. The enhancement of activity by Mn^{2+} along with much greater activity on templates consisting of only deoxycytidylate is consistent with this region playing an important role in initiation of primer synthesis. Curiously, and in contrast to the results obtained upon mutation of the individual residues R302, R306, and K314, deleting this region did not result in enhanced accumulation of dinucleotide (Figure 2). This surprising result suggests that while this region of the p58 subunit is involved in translocation, it may not be essential for translocation. The point mutations may result in structural changes that actively interfere with translocation, while deletion of this region does not interfere with translocation. Alternatively, this region of p58 may contain both positive and negative regulators of processivity, and the amino acids that were individually mutated may all normally enhance processivity.

The pol β-like region of p58 also appears to play a critical role in the normal counting mechanism of primase. On those templates where p49/DMp58 exhibited activity, the enzyme synthesized and released into solution only dinucleotide and very long products. In contrast, wild-type primase synthesized and released significant amounts of unit length primers 7−10 nucleotides long. Thus, deleting part of p58 impairs termination of further primer synthesis after generation of a unit length primer.

The effects of mutating the pol β-like region of p58, altered translocation and processivity, indicate that this region of p58 controls catalytic functions analogous to those regulated by the homologous 8 kDa domain of pol β. This domain of pol β consists of four helices packed in two antiparallel pairs and is important for binding DNA during pol β-mediated repair synthesis (*29*, *30*). Importantly, DNA binding by the 8 kDa domain regulates the processivity of pol β and its translocation along some DNA substrates. Pol β exhibits very low processivity during elongation of a DNA that contains only single-stranded template in front of the 3′-end of the primer. However, with a gapped DNA substrate containing only a short single-stranded template region in front of the primer followed by more duplex DNA, pol β has very high processivity (*31*, *32*). This high processivity likely results from the 8 kDa domain binding to the duplex DNA in front of the primer (*24*, *33*). Indeed, deletion of the 8 kDa domain results in a form of pol β with low processivity on the gapped DNA (*31*). In the absence of the 8 kDa domain, the enzyme dissociates from the template much more rapidly than it translocates to the next template base to be replicated and polymerizes the cognate dNTP. Thus, both the 8 kDa domain of pol β and the homologous region of the p58 subunit regulate the processivity of their respective enzymes.

Indeed, the most critical residues tested in the pol β-like sequence of p58, R302A, R306A, and K314A, are located in a region that corresponds to loop 2−α helix C−loop 3 in the 8 kDa domain of pol β. This region of pol β includes the sequence LPGVG, a highly conserved motif involved in non-sequence-specific DNA binding found in a wide variety of proteins including polymerases (*24*). Similarly, the analogous region in p58, LKGIG, appears to be important for both initiation of primer synthesis and translocation, two events which involve interactions with single-stranded DNA.

Previous studies showed that the p58 subunit of the p49/p58 complex interacts with the single-stranded DNA template bound to the complex prior to primer synthesis as well as the RNA·DNA duplex structure bound to the complex after primer synthesis (*14*). While the amino acids of p58 that interact with the DNA were not mapped, residues in both the N- and C-terminal halves of the protein interact with the nucleic acid. The pol β-like region of p58 is located near the middle of p58 in the C-terminal half and may well comprise one of the regions of p58 that interact with the nucleic acid.

In addition to translocation along the template, primase activity is associated with one additional translocation event, the movement of the newly generated primer-template from the primase active site into the pol α active site. As noted earlier, p58 is situated between the primase and pol α active sites and interacts with the newly generated primer-template. This raises the possibility that the pol β-like region of p58 will also mediate the movement of the primer-template from the primase active site to the pol α active site. Studies to test this hypothesis are currently underway.

REFERENCES

1. Kornberg, A., and Baker, T. (1992) *DNA Replication*, 2nd ed., W. H. Freeman, San Francisco.
2. Michael, W. M., Ott, R., Fanning, E., and Newport, J. (2000) *Science 289*, 2133−2137.
3. Foiani, M., Lucchinni, G., and Plevani, P. (1997) *Trends Biochem. Sci. 22*, 424−427.
4. Marini, F., Pellicioli, A., Paciotti, V., Lucchini, G., Plevani, P., Stern, D. F., and Foiani, M. (1997) *EMBO J. 16*, 639−650.
5. Longhese, M. P., Fraschini, R., Plevani, P., and Lucchini, G. (1996) *Mol. Cell. Biol. 16*, 3235−3244.
6. Goulian, M., and Heard, C. J. (1989) *J. Biol. Chem. 264*, 19407−19415.
7. Cotterill, S., Chui, G., and Lehman, I. R. (1987) *J. Biol. Chem. 262*, 16105−16108.
8. Lehman, I. R., and Kaguni, L. S. (1989) *J. Biol. Chem. 264*, 4265−4267.
9. Schneider, A., Smith, R. W., Kautz, A. R., Weisshart, K., Grosse, F., and Nasheuer, H. P. (1998) *J. Biol. Chem. 273*, 21608−21615.

10. Longhese, M. P., Jovine, L., Plevani, P., and Lucchini, G. (1993) *Genetics 133*, 183−191.
11. Collins, K. L., Russo, A. A. R., Tseng, B. Y., and Kelly, T. J. (1993) *EMBO J. 12*, 4555−4566.
12. Mizuno, T., Ito, N., Yokoi, M., Kobayashi, A., Tamai, K., Miyazawa, H., and Hanaoka, F. (1998) *Mol. Cell. Biol. 18*, 3552−3562.
13. Copeland, W. C. (1997) *Protein Expression Purif. 9*, 1−9.
14. Arezi, B., Kirk, B. W., Copeland, W. C., and Kuchta, R. D. (1999) *Biochemistry 38*, 12899−12907.
15. Copeland, W. C., and Wang, T. S. F. (1993) *J. Biol. Chem. 268*, 26179−26189.
16. Sheaff, R., and Kuchta, R. D. (1993) *Biochemistry 32*, 3027−3037.
17. Sheaff, R., Kuchta, R. D., and Ilsley, D. (1994) *Biochemistry 33*, 2247−2254.
18. Frick, D. N., and Richardson, C. C. (2001) *Annu. Rev. Biochem. 70*, 39−80.
19. Tseng, B. Y., and Ahlem, C. N. (1983) *J. Biol. Chem. 258*, 9845−9849.
20. Singh, H., Brooke, R. G., Pausch, M. H., Williams, G. T., Trainor, C., and Dumas, L. B. (1986) *J. Biol. Chem. 261*, 8564.
21. Kirk, B. W., and Kuchta, R. D. (1999) *Biochemistry 38*, 7727−7737.
22. Augustin, M. A., Huber, R., and Kaiser, J. T. (2001) *Nat. Struct. Biol. 8*, 57−61.
23. Pelletier, H., Sawaya, M. R., Kumar, A., Wilson, S. H., and Kraut, J. (1994) *Science 264*, 1891−1903.
24. Pelletier, H., Sawaya, M. R., Wolfe, W., Wilson, S. H., and Kraut, J. (1996) *Biochemistry 35*, 12742−12761.
25. Kirk, B. W., and Kuchta, R. D. (1999) *Biochemistry 38*, 10126−10134.
26. Kuchta, R. D., Reid, B., and Chang, L. M. S. (1990) *J. Biol. Chem. 265*, 16158−16165.
27. Grosse, F., and Krauss, G. (1988) *J. Biol. Chem. 260*, 1881−1888.
28. Yamaguchi, M., Hendrickson, E. A., and DePamphilis, M. L. (1985) *Mol. Cell. Biol. 5*, 1170−1183.
29. Kumar, A., Widen, S. G., Williams, K. R., Kedar, P., Karpel, R. L., and Wilson, S. H. (1990) *J. Biol. Chem. 265*, 2124−2131.
30. Liu, D., Prasad, R., Wilson, S. H., DeRose, E. F., and Mullen, G. P. (1996) *Biochemistry 35*, 6188−6200.
31. Singhal, R. K., and Wilson, S. H. (1993) *J. Biol. Chem. 268*, 15906−15911.
32. Chagovetz, A. M., Sweasy, J. B., and Preston, B. D. (1997) *J. Biol. Chem. 272*, 27501−27504.
33. Rajendran, S., Jezewska, M. J., and Bujalowski, W. (2001) *J. Mol. Biol. 308*, 477−500.

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