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Interactions of calf thymus DNA polymerase α with primer/templates

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ABSTRACT

The interactions of calf thymus DNA polymerase α (pol α) with primer/templates were examined. Simply changing the primer from DNA to RNA had little effect on primer/template binding or dNTP polymerization (K_m, V_{max} and processivity). Surprisingly, however, adding a 5′-triphosphate to the primer greatly changed its interactions with pol α (binding, V_{max} and K_m and processivity). While changing the primer from DNA to RNA greatly altered the ability of pol α to discriminate against nucleotide analogs, it did not compromise the ability of pol α to discriminate against non-cognate dNTPs. Thus the nature of the primer appears to affect 'sugar fidelity', without altering 'base fidelity'. DNase protection assays showed that pol α strongly protected 9 nt of the primer strand, 13 nt of the duplex template strand and 14 nt of the single-stranded template from hydrolysis by DNase I and weakly protected several bases outside this core region. This large DNA binding domain may account for the ability of a 5′-triphosphate on RNA primers to alter the catalytic properties of pol α.

INTRODUCTION

DNA polymerase α–primase (pol α–primase) is essential for initiation of new DNA strands during eukaryotic chromosomal replication (1). The tightly associated four subunit complex possesses two activities, DNA primase and DNA polymerase (2–4). On single-stranded DNA the primase activity synthesizes *de novo* a short oligoribonucleotide primer which the pol α activity subsequently elongates.

Pol α may be unique among the nuclear DNA polymerases in that it likely elongates both RNA and DNA primers, even though RNA:DNA and DNA:DNA duplexes have different structures (1,5). Differential interactions of pol α with RNA and DNA primers may be particularly important for modulating the effects of nucleotide analogs on pol α activity. For example, changing the primer from DNA to RNA greatly alters polymerization of ddNTPs and araNTPs by pol α (6).

Most previous studies examining the interactions of pol α–primase with substrates have used DNA primers. During DNA replication, however, pol α elongates primase-synthesized primers that will consist of RNA and contain a 5′-triphosphate. In fact, there is no evidence that pol α ever binds to and then elongates pre-existing DNA primers *in vivo*. Thus to understand the mechanism of pol α and how pol α interacts with nucleotide analogs, RNA primers containing a 5′-triphosphate are likely much more physiologically relevant than DNA primers.

In order to understand how the nature of the primer can affect the interactions of pol α with nucleotide analogs one must first understand how pol α interacts with the primer. Presently, however, conflicting data exist regarding the ability of pol α to recognize RNA and DNA primers and no data exist on the role of a 5′-triphosphate on the primer. Studies on pol α–primase from various sources using homopolymer primer/templates such as poly(dT)/oligo(rA) and poly(dT)/oligo(dA) as substrates indicated that pol α preferred to elongate RNA primers over DNA primers (7–10). In contrast, studies comparing 'hooked' primer/templates [$(dA)_{100}/(dT)_{25}(U)_2$ versus $(dA)_{100}/(dT)_{25}$] yielded similar K_m and V_{max} values for both RNA and DNA 3′-termini (11). However, interpretation of these studies is complicated, because DNA containing regions of oligo(dT)/oligo(dA) forms an unusual bent structure (12) and the hybridization conditions of the DNA affect the structure of the resulting duplex (13).

In order to overcome these potential problems we examined the ability of pol α to use synthetic primer/templates of defined sequence as substrates. Importantly, the primer/templates were of mixed base composition and their sequence remained constant as the primer composition was varied between RNA and DNA. Whereas changing the primer from DNA to RNA had only small effects on the ability of pol α to elongate these primers, a 5′-triphosphate on the RNA primer significantly altered catalysis. Nuclease protection assays were then used to define the regions of the primer/template with which pol α interacts.

MATERIALS AND METHODS

Materials

Unless noted otherwise calf thymus pol α–primase and all reagents were as previously described (14,15). DNA and RNA containing a 5′-hydroxyl were synthesized by Oligos, Etc. RNA primers containing a 5′-triphosphate were synthesized using T7 RNA polymerase (16). Products were purified using non-denaturing gel electrophoresis (20% acrylamide) and detected by UV shadowing. Bands containing product were excised from the gel and subjected

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to three freeze/thaw cycles in H_2O to elute the product RNA. The supernatant fluids were lyophilized to dryness and the RNA was resuspended in 10 mM Tris, pH 7.5. Primer/templates were annealed and quantified as previously described (15) and the concentrations are given in terms of 3′-termini.

Methods

Unless noted otherwise all assays were performed under initial velocity conditions at 37°C and contained 50 mM Tris, pH 7.5, and 5 mM $MgCl_2$.

Pol α activity. Polymerase assays typically contained 1 μM primer/template, 2–10 μM [α-^{32}P]dNTPs and 0.66–6.6 nM pol α. Reactions were quenched with either 1.5 vol. 50 mM EDTA or 1–2.5 vol. gel loading buffer (90% formamide). Products were quantified using either a DE81 filter binding assay (15) or polyacrylamide gel electrophoresis followed by phosphorimagery (Molecular Dynamics). In experiments to determine V_{max} and K_m as a function of DNA concentration the dNTP concentration was held constant at 10 μM. Assays to measure the V_{max} and K_m for polymerization of multiple dNTPs contained 0.5 μM primer/template. Assays to determine V_{max} and K_m for insertion of a single dNTP contained 1 μM primer/template.

Processivity assays contained 1 μM primer/template and 10 μM each dNTP ([α-^{32}P]dTTP) (17). For these measurements to be valid pol α must always rebind to the starting DNA after dissociating from a product. This was confirmed in control experiments that showed that the size distribution of products did not change with increasing amounts of product synthesized, indicating that after dissociating from a DNA product pol α always bound to unelongated primer/template.

K_d measurements. Pol α (17–22 nM) was incubated with varying concentrations of primer/template for 2 min at 25°C. This mixture (4–5 μl) was then diluted into a 'trap' solution (5–10 μl, 25°C) containing an [α-^{32}P]dNTP (1.2 μM [α-^{32}P]dTTP for the 26mer templates, [α-^{32}P]dGTP for the 60mer templates) and heparin (0.5 mg/ml), final concentrations. Any enzyme not bound to primer/template was bound by heparin, while enzyme bound to primer/template can incorporate one dNTP onto the primer. Reactions were quenched with gel loading buffer after 30 s, a time sufficient for pol α to elongate any primer/template to which it was bound, and the products analyzed by gel electrophoresis and phosphorimagery. From the amount of [^{32}P]dNMP incorporated into products the K_d can be determined (18).

In the case of TPR9/26 and TPR9/60 (Table 1) the trap solution contained RNase H (5 U) and 2.4 μM dNTPs ([α-^{32}P]dGTP). This allows polymerization of multiple dNTPs onto the primer and the products were quantified by gel electrophoresis followed by phosphorimagery. The RNase H destroys the RNA primers of any primer/templates free in solution. Control experiments were performed as described previously and showed that both traps prevented free pol α from binding to primer/template (15).

DNA protection assays. To determine the length of primer protected 4 nM enzyme was incubated with 50 nM 12/34mer, 10 μM [α-^{32}P]dGTP and 10 μM aphidicolin. Pol α converts the 12/34mer into a 3′-[^{32}P]13/34mer, which remains tightly bound to pol α via formation of a pol α·3′-[^{32}P]13/34mer·aphidicolin complex (15). DNase I (0.05–0.2 mg/ml) was added and the products analyzed by gel electrophoresis and phosphorimagery after 2–30 min. A 3′-[^{32}P]21/34mer (Table 1) was similarly examined. Pol α was incubated with 18/34 mer, [α-^{32}P]dATP and aphidicolin prior to addition of DNase.

Table 1. Synthetic primer/templates used[a]

Primer/template	Sequence
9/26mer	GCGCCGAAA-3′ TTTCGCGGCTTTAGTCCCCAGTCCCC
9/60mer	GGAGGGAGG-3′ TCGTCCTCCCTCCCATCCTCCTCCATCTTTCTTTACTTCTTCTTATCTCTCTGCTGCT
9/5′-[^{32}P]34mer[b]	GCGCCGAAA-3′ AGGTATAGTGTACGCGGCTTTGTGTAATAGTAA**G**
12/34mer[b]	TCCATATCACAT-3′ AGGTATAGTGTACGCGGCTTTGTGTAATAGTAA**G**
12/3′-[^{32}P]35mer[b]	TCCATATCACAT-3′ **A**AGGTATAGTGTACGCGGCTTTGTGTAATAGTAAG
3′-[^{32}P]13/34mer[b]	TCCATATCACAT**G**-3′ AGGTATAGTGTACGCGGCTTTGTGTAATAGTAAG
18/34mer	TCCATATCACATGCGCCG-3′ AGGTATAGTGTACGCGGCTTTGTGTAATAGTAAG
3′-[^{32}P]21/34mer[b]	TCCATATCACATGCGCCGAA**A**-3′ AGGTATAGTGTACGCGGCTTTGTGTAATAGTAAG
21/5′-[^{32}P]34mer[b]	TCCATATCACATGCGCCGAAA-3′ AGGTATAGTGTACGCGGCTTTGTGTAATAGTAA**G**

[a]The nomenclature used is length of primer strand/length of template strand. D9/26, D9/60 and D9/5′-[^{32}P]34mer have DNA primers, R9/26 has an RNA primer containing a 5′-hydroxyl and TPR9/26, TPR9/60 and TPR9/5′-[^{32}P]34mer have RNA primers containing a 5′-triphosphate. The templates are always DNA.
[b]Bold, underlined text shows the position of the ^{32}P label in those DNAs used for protection studies.

Table 2. Kinetic parameters for binding and elongation of RNA and DNA primers by pol α[a]

Parameter	D9/26	R9/26	TPR9/26	D9/60	TPR9/60
$K_{d\ (DNA)}$ (μM)	1.0 ± 0.3	0.30 ± 0.05	0.45 ± 0.03	0.10 ± 0.01	0.070 ± 0.008
$K_{m\ (DNA)}$ (μM)	0.70 ± 0.03	1.1 ± 0.1	0.30 ± 0.11	0.50 ± 0.12	0.075 ± 0.016
V_{max} (pmol/μg/min)	530 ± 15	720 ± 70	130 ± 13	1900 ± 100	220 ± 30
V_{max}/K_m (pmol/μg/min/μM)	750 ± 30	650 ± 60	430 ± 90	3800 ± 900	2900 ± 600
$K_{m\ (dNTPs)}$ (μM)	5.0 ± 2.0	3.0 ± 0.4	1.9 ± 0.3	2.0 ± 0.2	2.6 ± 0.7

[a]The values and standard errors were calculated using the program Enzfitter© (Biosoft, Ferguson, MO).

Template protection was examined by 5′-^{32}P-labeling the 34mer template and annealing it to the 18mer primer. The resulting 18/5′-[^{32}P]34mer was incubated with 4 nM pol α, 50 μM dATP and 10 μM aphidicolin to generate a pol α·21/5′-[^{32}P]34mer·aphidicolin complex. DNase I treatment was performed as described above. Template protection in the opposite direction was measured using the 12/3′-[^{32}P]35mer. The 34mer was 3′-^{32}P-labeled using 3′-[α-^{32}P]dATP and terminal transferase. Differences in template protection when the primer was either RNA or DNA were measured similarly, except digestion was with micrococcal nuclease.

RESULTS

To better understand how pol α interacts with RNA and DNA primers we examined the kinetic and thermodynamic parameters for polymerization on synthetic primer/templates of mixed base composition (Table 1). Unlike homopolymeric DNA, the primer could only anneal to the template at one location and result in one structure. A primer length of 9 nt was chosen because RNA primers attached to Okazaki fragments *in vivo* are typically 8–10 nt long (19,20).

The primers consisted of either DNA displaying a 5′-hydroxyl (D9/26 and D9/60), RNA displaying a 5′-hydroxyl (R9/26) or RNA displaying a 5′-triphosphate (TPR9/26 and TPR9/60) (Table 1). Triphosphate-containing primers were examined because primers generated *in vivo* by DNA primase retain the triphosphate group of the initiating nucleotide (21). Importantly, the primer and template sequences remained constant among the three 26mer primer/templates and the two 60mer primer/templates. Thus any difference in the ability of pol α to bind or elongate the different primer/templates can be attributed to primer composition, rather than sequence effects.

As shown in Table 2, changing the primer from DNA to RNA had only small effects on the K_m of the primer/template and a 5′-triphosphate slightly decreased this value. To explicitly determine how changing the primer from DNA to RNA affects binding, K_d values of the various primer/templates were measured. Changing the primer from DNA to RNA (with or without a 5′-triphosphate) had little effect on binding, whereas increasing the length of the template resulted in a slightly lower K_d.

Even though the K_d values for the different primers were similar, the nature of the primer does affect the pol α·primer/template complex. K_d values were measured via trapping experiments. For DNA and RNA primers containing a 5′-hydroxyl a pol α·primer/template complex was diluted into the next correct dNTP (^{32}P-labeled) and heparin. Any primer/template bound to pol α was elongated by 1 nt, while the heparin served to 'trap' any free pol α and prevent it from binding additional primer/template molecules. Curiously, this approach failed with the two RNA primers that contained a 5′-triphosphate; no elongation of the primer was observed when heparin was present. Replacing the heparin trap with an RNase H trap overcame this problem and allowed measurement of the K_d for triphosphate-containing primer/templates.

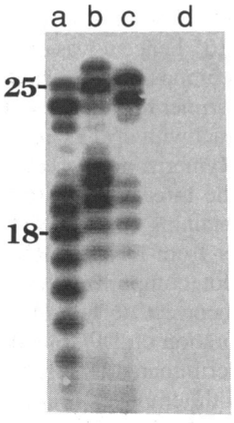

Figure 1. Effects of primer composition on the processivity of pol α. Processivity was measured as described under Materials and Methods using either D9/26 (lane a), R9/26 (lane b) or TPR9/26 (lane c) as substrate. Lane d lacked enzyme. The lengths of DNA standards of known length are marked on the left side of the figure. It should be noted that products containing an RNA primer (± triphosphate) migrate slightly slower than products that are all DNA (6).

Further evidence that a 5′-triphosphate alters the properties of the pol α·primer/template complex comes from processivity measurements. Figure 1 shows the products generated on D9/26, R9/26 and TPR9/26. Since the products synthesized on R9/26 and TPR9/26 contain RNA, they migrate slightly slower than those from D9/26 (6). When the substrate was R9/26 or D9/26 only 18 and 21%, respectively, of the products were elongated to within 3 nt of the end of the template. In contrast, 48% of the products had been elongated to within 3 nt of the end of the template when the primer contained a 5′-triphosphate.

In both cases examined the V_{max} for the triphosphate-containing primer/templates was significantly slower than for the corresponding DNA-primed template (Table 2). However, if one considers the selectivity parameter, V_{max}/K_m, there was virtually no difference in the ability of pol α to use DNA or RNA (± triphosphate) primers; the lower V_{max} for RNA primers containing a 5′-triphosphate was largely compensated for by a lower K_m.

Both V_{max} and V_{max}/K_m for the longer primer/templates were significantly higher than for the shorter substrates. In combination with the data showing that pol α binds the 60mer primer/templates more tightly than the 26mer primer/templates, these results suggest that the 9/26mers are too short to optimally interact with pol α. As will be shown later in the DNA protection studies, pol α indeed interacts with more DNA than just a 9/26mer.

Changing the primer from DNA to RNA has small effects on the rate of dNTP misincorporation

Previous studies have shown that changing the primer from DNA to RNA can dramatically alter the ability of pol α to discriminate against dNTP analogs (6), hence it was of interest to determine if changing the primer composition affected misincorporation of dNTPs. Changing the primer from DNA to RNA did not alter the ability of pol α to interact with dNTPs, as assessed by the K_m for dNTPs (Table 2). Polymerization of the correct (dTTP) and incorrect dNTPs on the three 9/26 primer/templates was then measured in assays containing 10 μM dNTPs. As shown in Table 3, changing the primer from DNA to RNA (with or without a 5′-triphosphate) did not compromise the ability of pol α to discriminate against incorrect dNTPs. A more detailed kinetic analysis of misincorporation on D9/26 and R9/26 demonstrated that pol α primarily discriminated against non-cognate dNTPs via an increased K_m on both templates (Table 4). These results are consistent with previous studies on other DNA primer/templates showing that pol α discriminates against non-cognate dNTPs largely via K_m discrimination (22) and indicate that the nature of the primer does not greatly alter the mechanism of discrimination.

Table 3. Changing the primer from DNA to RNA does not greatly affect misincorporation of dNTPs[a]

	D9/26	R9/26	TPR9/26
dTTP	1	1	1
dCTP	5×10^{-3}[b]	1×10^{-2}[b]	$<1.0 \times 10^{-3}$
dATP	2×10^{-2}[b]	$<1.0 \times 10^{-3}$	1.0×10^{-3}
dGTP	$<3.0 \times 10^{-4}$	$<1.0 \times 10^{-3}$	$<1.0 \times 10^{-3}$

[a]The first template base to be replicated is adenine and all rates are relative to the rate of dTTP incorporation, which was normalized to 1.0. Assays contained 1 μM primer/template and a single [α-^{32}P]dNTP (10 μM). Remaining [α-^{32}P]dNTPs were separated from any polymerization products by gel electrophoresis and the amount of product due to incorporation of an [α-^{32}P]dNTP onto the primer was calculated by phosphorimagery.
[b]Values were calculated from the data in Table 4.

DNA protection studies

The binding studies described above suggested that pol α interacts with more DNA than just a 9/26mer. Consistent with this idea, previous footprinting studies showed that pol α interacts with a substantial region of DNA (23,24), although the precise amount was not well defined. To better define the amount of DNA with which pol α interacts we used DNase protection studies. Unlike previous footprinting studies that utilized 'single-hit' methods, we employed conditions that resulted in extensive degradation of the DNA by DNase in order to define 'highly protected' and 'weakly protected' regions of the DNA. This approach required that we first stabilize the pol α·DNA binary complex and was accomplished by including aphidicolin in the experiments. Aphidicolin appears to bind in the dNTP binding site of pol α and stabilize DNA binding by converting the pol α·DNA binary complex into a pol α·DNA·aphidicolin ternary complex (14).

Figure 3 shows that pol α strongly protected 9 nt of the 3′-[^{32}P]13/34mer (Table 1) from digestion by DNase. Interestingly, there was also some protection of the entire primer strand (13 nt). This suggests that pol α interacts strongly with ~9 nt of the primer, but can interact weakly with additional bases. Similarly, pol α strongly protected 9 nt of a 3′-[^{32}P]21/34mer primer/template (data not shown), showing that a change in the length of the duplex region does not affect the amount of primer DNA protected.

To characterize the interaction of pol α with the template strand we utilized the 21/5′-[^{32}P]34mer (Table 1). Digestion with DNase I resulted in strong protection of a 25 nt product (Fig. 2) and weaker protection of up to 31 nt of DNA. Since the 5′ radiolabel was retained, these data indicate that pol α strongly protects the entire single-stranded region of this template (13 nt) and 12 nt of the template strand in the duplex primer/template region and may also interact less well with an additional 6 nt of the template. Thus pol α protects the double-stranded primer/template region asymmetrically, with strong template protection extending 3 nt beyond the primer region that was strongly protected.

We utilized a 12/3′-[^{32}P]35mer (Table 1) to determine the full length of single-stranded template protected by pol α (Fig. 2). As shown by the experiments described above, the 3′-[^{32}P] label will be protected from DNase I digestion. The primary product observed after DNase digestion was 27 nt long, although smaller amounts of products up to 34 nt were also observed. These data indicate that pol α strongly protects 14 nt of the single-stranded template from digestion by DNase and also interacts more weakly with at least seven additional bases.

Table 4. V_{max} and K_m values for incorporation of correct and incorrect nucleotides onto RNA and DNA primers

	D9/26			R9/26		
	V_{max}[a]	K_m (μM)	V_{max}/K_m	V_{max}[a]	K_m (μM)	V_{max}/K_m
dTTP	1.0	0.33	3	1.0	0.38	2.6
dATP	0.71	360	2×10^{-3}	<0.01[b]	–	–
dCTP	4.4	6000	7.3×10^{-4}	5.0	5000	1.2×10^{-3}

[a]V_{max} values for incorporation of the correct nucleotide, dTTP, were normalized to 1 and other V_{max} values are given relative to this value.
[b]There was no detectable dATP incorporation at any concentration. This value is an upper limit on the rate of misincorporation with 1 mM dATP.

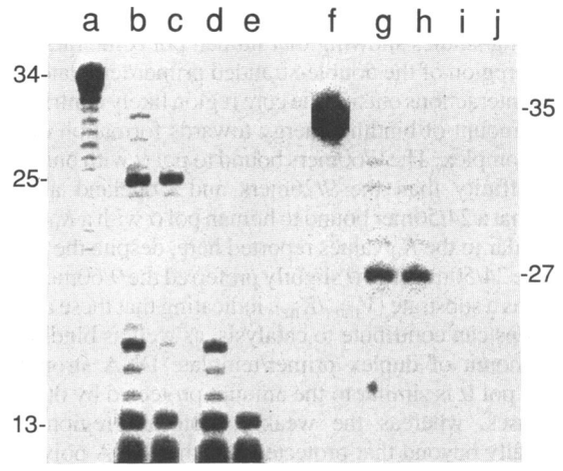

Figure 2. Pol α strongly protects 13 nt of the template strand in the duplex primer/template region. Lane a shows the 21/5′-[^{32}P]34mer prior to treatment with DNase. In lanes b and c the 21/5′-[^{32}P]34mer was treated with DNase I for 5 and 10 min in the presence of pol α and aphidicolin. Lanes d and e were identical to lanes b and c, except the pol α was heat inactivated. Lanes f–j are identical to lanes a–e, except that the labeled DNA was the 12/3′-[^{32}P]35mer and DNase digestions were for 20 and 30 min. DNA lengths are noted.

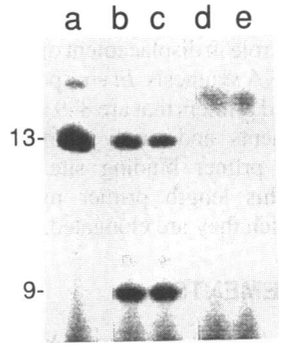

Figure 3. Pol α strongly protects 9 nt of the primer from DNase I digestion. Lane a shows the 3′-[^{32}P]13/34mer prior to treatment with DNase. In lanes b and c the 3′-[^{32}P]13/34mer was treated with DNase I for 5 and 10 min in the presence of pol α and aphidicolin as described under Materials and Methods. Lanes d and e were identical to lanes b and c, except that the pol α was heat inactivated (5 min at 65°C). DNA lengths are noted.

Changing the primer from DNA to RNA causes only small changes in the protection pattern

Changing the primer from DNA to RNA alters the kinetic properties of a primer/template, thus it seemed possible that the nature of the primer could affect the protection pattern. D9/34mer and TPR9/34mer were 5′-^{32}P-labeled on the template strand and the amount of nuclease protection determined. The studies described above demonstrated that pol α will protect the entire single-stranded template region in front of the 3′-end of the primer, hence these experiments detected changes in binding of the template region near the 5′-end of the primer. As shown in Figure 4, there were only small differences in the protection pattern between the two primer/templates.

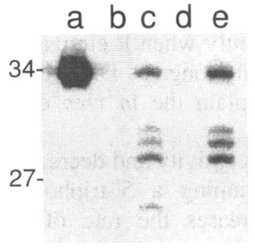

Figure 4. Changing the primer from a triphosphate-containing RNA to DNA has only small effects on the protection pattern. Lane a shows the 5′-[^{32}P]34mer prior to digestion with micrococcal nuclease. Lanes b and c show the digestion pattern of D9/5′-[^{32}P]34mer after a 1 min incubation with nuclease, aphidicolin and either heat-inactivated or active pol α, respectively. Lanes d and e are identical to lanes b and c, except that the primer/template was TPR9/5′-[^{32}P]34mer and digestion was for 2 min.

DISCUSSION

The ability of pol α to bind and polymerize dNTPs onto RNA (± 5′-triphosphate) and DNA primers was compared. For a given length template the specificity constant (V_{max}/K_m) remained relatively constant, regardless of whether the primer was RNA or DNA. Importantly, these results were obtained on two primer/templates of different sequence, suggesting that pol α will not generally differentiate between elongation of RNA and DNA primers. This may be important *in vivo*, since pol α must interact with both RNA and DNA primers.

The ability of pol α to bind and elongate RNA and DNA primers with similar efficiency is quite impressive, since DNA:DNA duplexes and RNA:DNA duplexes adopt very different structures in solution (1,5). This may indicate that the DNA binding site of pol α is quite flexible, such that it can productively interact with both the B-helix of a DNA:DNA duplex and the apparent non-A-, non-B-type helix of an RNA:DNA duplex. Alternatively, pol α might bind only a single helical conformation and would thus need to change the conformation of one of the substrates during binding.

Even though pol α binds RNA and DNA primers with similar affinity, it is clear that the interactions are not identical. Changing the primer from DNA to RNA greatly affects the ability of pol α to interact with certain nucleotide analogs (6). A 5′-triphosphate decreased V_{max} for dNTP polymerization, increased the processivity of pol α and altered the properties of the pol α·primer/template complex as assessed by the trapping experiments using heparin. While the protection studies indicate that the 5′-triphosphate will likely interact with pol α–primase, it is still somewhat surprising that a 5′-triphosphate can alter dNTP polymerization, since it is so far removed from the 3′-terminus of the primer. Alternatively, these differences might reflect interactions of the triphosphate-containing primers with DNA primase. RNA primers containing a 5′-triphosphate will readily move between the primase and pol α active sites via an intramolecular mechanism (14) and these primers interact with primase much more efficiently than RNA primers that lack a 5′-triphosphate (25).

Since primase-synthesized primers are both RNA and contain a 5′-triphosphate, the data described above predict that the properties of pol α during DNA replication will be very different to during elongation of the more widely studied DNA primers.

Indeed, we have already found that pol α interacts with nucleotide analogs very differently when it elongates primase-synthesized primers than when it elongates DNA primers and these differences may help explain the *in vivo* effects of these analogs (26,27).

The increased processivity and decreased V_{max} observed with RNA primers containing a 5′-triphosphate suggests that a 5′-triphosphate decreases the rate of pol α·primer/template dissociation. Since processivity is defined by a competition between the rates of primer/template dissociation and polymerization of the next correct dNTP (28), the increased processivity could be due to either a faster rate of polymerization or a slower rate of dissociation. Pol α is somewhat processive during elongation of a DNA primer, thus dNTP polymerization must be faster than DNA dissociation (i.e. DNA dissociation is more rate limiting than dNTP polymerization). Therefore, increasing the processivity by increasing the polymerization rate should either have no effect or slightly increase V_{max}, but this was not observed. In contrast, increasing the processivity by decreasing the rate of dissociation should decrease V_{max}, the observed result.

Neither primer composition nor template length had any significant effect on K_m for dNTP incorporation (Table 2). Likewise, changing the primer from DNA to RNA had little effect on the ability of pol α to discriminate against non-cognate dNTPs and discrimination primarily occurred at the level of K_m. The higher V_{max} values for dCTP misincorporation may reflect a change in the rate limiting step. As described above, polymerization of a cognate dNTP must be faster than DNA dissociation. During misincorporation, however, the rate of product dissociation may increase, such that polymerization is now the rate limiting step. Faster dissociation rates would correspond to faster cycling of free enzyme to additional primer/template molecules and thus increase the measured V_{max}.

It may be important that the nature of the primer does not greatly affect discrimination, since pol α must elongate primers consisting of both RNA and DNA near the 3′-terminus *in vivo*, particularly during replication of the lagging strand. Consistent with these results, there appear to be only small differences in the fidelity of DNA replication on the leading and lagging strands (29,30). Nonetheless, these results are somewhat surprising, since changing the primer from RNA to DNA greatly alters the ability of pol α to interact with sugar-modified nucleotide analogs (6). For example, changing the primer from DNA to RNA decreased the ability of pol α to discriminate against ddNTPs by 85-fold. These data, albeit limited, suggest that the nature of the primer can greatly affect 'sugar fidelity', but not 'base fidelity'.

The protection studies indicate that pol α strongly protects 9 nt of the primer and 12 nt of the template in the duplex primer/template region, as well as 13 nt of single-stranded template region, from digestion by DNase. [These studies employed the four subunit pol α–primase complex, thus we cannot exclude the possibility that some of the protection from DNase digestion involved interactions of the DNA with subunits other than the pol α catalytic subunit. However, the pol α catalytic subunit alone is reported to interact with an extended region of the duplex primer/template (23).] It should be noted, however, that the actual amount of DNA that pol α strongly protects could be slightly less than these values, since steric interference may preclude DNase from hydrolyzing DNA very near the surface of pol α. Pol α also interacts with additional DNA outside the highly protected core region, although these interactions appear to be weaker. This is consistent with previous 'single hit' DNase footprinting studies showing that human pol α interacts with an extended region of the double-stranded primer/template (23,24).

These interactions outside the core region likely contribute only a small amount of binding energy towards formation of the pol α·DNA complex. The 9/60mers bound to pol α with only slightly greater affinity than the 9/26mers and Copeland and Wang showed that a 24/50mer bound to human pol α with a K_d of 43 nM (23), similar to the K_d values reported here, despite the increased size of the 24/50mer. Pol α slightly preferred the 9/60mer over the 9/26mer as a substrate (V_{max}/K_m), indicating that these additional interactions can contribute to catalysis, as well as binding.

The amount of duplex primer/template DNA strongly protected by pol α is similar to the amount protected by other DNA polymerases, whereas the weakly protected region extends substantially beyond that protected by other DNA polymerases. DNA protection studies showed that Klenow fragment binds to ~8 base pairs of the primer/template (31), whereas T4 DNA polymerase strongly protects ~14 base pairs of the primer/template (32). X-ray crystallography studies of DNA polymerase β, a much smaller polymerase, suggest that only ~5 base pairs of DNA interact with the enzyme (33). It is unknown how much single-stranded template DNA these latter enzymes interact with.

The interaction with an extended region of the single-stranded template and strong protection of 9 nt of the primer strand may be relevant to the *in vivo* functions of pol α during DNA replication. The large amount of single-strand template protected ahead of the primer might play a role in displacement of DNA binding proteins in order to allow DNA synthesis. *In vivo* pol α routinely elongates primase-synthesized primers that are 8–9 nt long during initiation of Okazaki fragments and rarely elongates shorter primers (19,20). That the primer binding site is ideally suited for interacting with this length primer may well enhance the efficiency with which they are elongated.

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REFERENCES

1 Kornberg,A. and Baker,T.A. (1992) *DNA Replication*. W.H.Freeman and Co., New York, NY.
2 Hu,S., Wang,T.S. and Korn,D. (1984) *J. Biol. Chem.*, **259**, 2602–2609.
3 Kaguni,L.S., Rossignol,J.M., Conaway,R.C., Banks,G.R. and Lehman,I.R. (1983) *J. Biol. Chem.*, **258**, 9037–9039.
4 Tseng,B.Y. and Ahlem,C.N. (1983) *J. Biol. Chem.*, **258**, 9845–9849.
5 Salazar,M., Fedoroff,O.Y., Miller,J.M., Ribeiro,N.S. and Reid,B.R. (1993) *Biochemistry*, **32**, 4207–4215.
6 Kuchta,R.D., Ilsley,D., Kravig,K.D., Schubert,S. and Harris,B. (1992) *Biochemistry*, **31**, 4720–4728.
7 Weiser,T., Gassmann,M., Thommes,P., Ferrari,E., Hafkemeyer,P. and Hubscher,U. (1991) *J. Biol. Chem.*, **266**, 10420–10428.
8 Hohn,K.-T. and Grosse,F. (1987) *Biochemistry*, **26**, 2870–2878.
9 Pritchard,C.G., Weaver,D.T., Baril,E.F. and DePamphilis, M. (1983) *J. Biol. Chem.*, **258**, 9810–9819.
10 Detera,S.D., Becerra,S.P., Swack,J.A. and Wilson,S.H. (1981) *J. Biol. Chem.*, **256**, 6933–6943.

11 Fisher,P.A. and Korn,D. (1981) *Biochemistry*, **20,** 4570–4578.
12 Koo,H., Wu,H. and Crothers,D.M. (1986) *Nature*, **320,** 501–506.
13 Mesner,L.D. and Hockensmith,J.W. (1991) *Proc. Natl. Acad. Sci. USA*, **89,** 2521–2525.
14 Sheaff,R., Ilsley,D. and Kuchta,R. (1991) *Biochemistry*, **30,** 8590–8597.
15 Sheaff,R.J. and Kuchta,R.D. (1993) *Biochemistry*, **32,** 3027–2254.
16 Milligan,J.F., Groebe,D.R., Witherell,G.W. and Uhlenbeck,O.C. (1987) *Nucleic Acids Res.*, **15,** 8783–8798.
17 Cheng,C.-H. and Kuchta,R.D. (1993) *Biochemistry*, **32,** 8568–8574.
18 Sheaff,R.J., Kuchta,R.D. and Ilsley,D. (1994) *Biochemistry*, **33,** 2247–2254.
19 Hay,R.T., Hendrickson,E.A. and DePamphilis,M.L. (1984) *J. Mol. Biol.*, **175,** 131–157.
20 Kitani,T., Yoca,K. and Okazaki,T. (1984) *Mol. Cell. Biol.*, **4,** 1591–1596.
21 Grosse,F. and Krauss,G. (1985) *J. Biol. Chem.*, **260,** 1881–1888.
22 Boosalis,M.S., Petruska,J. and Goodman,M.F. (1987) *J. Biol. Chem.*, **262,** 14689–14696.
23 Copeland,W.C. and Wang,T.S.-F. (1993) *J. Biol. Chem.*, **268,** 11028–11040.
24 Tsurimoto,T. and Stillman,B. (1991) *J. Biol. Chem.*, **266,** 1950–1960.
25 Podust,V.N., Vladimirova,O.V., Manakova,E.N. and Lavrik,O.I. (1992) *Eur. J. Biochem.*, **206,** 7–13.
26 Ilsley,D.D., Lee,S.-H., Miller,W.H. and Kuchta,R.D. (1995) *Biochemistry*, **34,** 2504–2510.
27 Thompson,H.C. and Kuchta,R.D. (1995) *Biochemistry*, in press.
28 Bryant,F.R., Johnson,K.A. and Benkovic,S.J. (1983) *Biochemistry*, **22,** 3537–3546.
29 Roberts,J.D., Thomas,D.C. and Kunkel,T.A. (1993) *Biochemistry*, **32,** 4083–4089.
30 Thomas,D.C., Nguyen,D.C., Peigorsch,W.W. and Kunkel,T.A. (1993) *Biochemistry*, **32,** 4083–4089.
31 Joyce,C.M. and Steitz,T.A. (1987) *Trends Biochem. Sci.*, **12,** 288–292.
32 Munn,M.M. and Alberts,B.M. (1991) *J. Biol. Chem.*, **266,** 20034–20044.
33 Pelletier,H., Sawaya,M.R., Kumar,A., Wilson,S.H. and Kraut,J. (1994) *Science*, **264,** 1891–1903.