Abasic Template Lesions Are Strong Chain Terminators for DNA Primase but Not for DNA Polymerase α during the Synthesis of New DNA Strands†

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ABSTRACT: The effects of abasic lesions on both primase activity and DNA polymerase α- (pol α) catalyzed elongation of primase-synthesized primers were examined. Abasic lesions were strong chain terminators during primer synthesis by primase. However, extension of primase-synthesized primers by pol α resulted in 60−93% bypass of abasic lesions. Sequencing of bypass products generated during this primase-coupled pol α activity showed that dAMP was preferentially incorporated opposite the abasic lesion, indicating that pol α was responsible for bypass. In contrast, previous analyses of pol α-catalyzed elongation of exogenously supplied DNA primer−templates showed that abasic lesions strongly terminated DNA synthesis. Thus, elongation of primase-synthesized primers by pol α−primase is fundamentally different than elongation of exogenously added primer−templates with respect to interaction with abasic lesions. Furthermore, this high level of abasic lesion bypass during primase-coupled pol α activity provides an additional mechanism for how translesional synthesis may occur in vivo, an event hypothesized to be mutagenic.

Abasic lesions result from the spontaneous hydrolysis of the N-glycosidic bond connecting the base (purine or pyrimidine) to the sugar phosphate backbone of DNA and are one of the most frequent forms of DNA damage in mammalian cells (*1*). It is estimated that 10^4 depurination and 10^2 depyrimidination events occur spontaneously in a eukaryotic cell per day (*2, 3*). Hydrolysis of the glycosidic bond is enhanced by several known carcinogens through addition of bulky adducts to the base (*4*). Additionally, abasic sites are generated as a normal intermediate in the base excision repair pathway through the action of various cellular N-glycosylases (e.g., uracil glycosylase) (*3*). While most apurinic and apyrimidinic sites are properly repaired by base-excision repair, it is hypothesized that a few lesions persist and are replicated, resulting in a variety of mutations including deletions and base substitutions (*4*−*6*).

Although it is clear from studies in whole cells that the DNA replication machinery can polymerize dNTPs past abasic sites in plasmid DNA (*7*−*9*), the mechanism of bypass is not well understood. This could be accomplished by any of the six nuclear eukaryotic polymerases (pol α, *β*, *δ*, *ϵ*, *ζ*, and *η*).[1] Pol α is required for initiation of new DNA strands, pol *β* is involved in DNA repair, pol *δ* and pol *ϵ* are essential for replicative DNA synthesis (for a review see ref *10*), and the roles of pol *ζ* and *η* are unclear since they were only recently identified (*11*−*13*). In vitro studies to date show that both pol *β* and pol *δ* (in complex with proliferating cell nuclear antigen) can replicate through abasic lesions (*14, 15*). In contrast, reports on pol α show that polymerization terminates either at or near abasic sites (*16*−*18*). However, these pol α studies may not accurately reflect replication in vivo since exogenously added DNA primer−templates were used. In whole cells, primers used for the initiation of new DNA strands are provided by primase, an RNA polymerase that forms a tight complex with pol α (*19*−*21*). On single-stranded DNA, primase synthesizes a RNA primer that is transferred intramolecularly to the pol α active site and dNTPs are polymerized [i.e., primase-coupled pol α activity (*22*)].

We previously found that during primase-coupled pol α activity, pol α interacts very differently with nucleoside analogues (acyclovir triphosphate and araATP) as compared to pol α-catalyzed extension of exogenously added primer−templates (*23, 24*). For example, while polymerization of araATP caused strong chain termination during elongation of exogenously added primer−templates, incorporation of araATP by pol α did not cause chain termination during primase-coupled pol α activity (*24*). Importantly, the primase-coupled pol α findings correspond to observations from whole cells showing that araATP is internally incorporated into DNA (*25, 26*).

Given the inherently different properties of pol α when it elongates primase-synthesized primers versus exogenously added primers, it seemed likely that the interaction of pol α with abasic lesions during primase-coupled pol α activity would be vastly different as compared to pol α activity on

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[1] Abbreviations: araATP, 9-(*β*-D-arabinofuranosyl)adenosine triphosphate; ddNTP, 2′,3′-dideoxynucleoside triphosphate; pol, DNA polymerase; pol α−primase, DNA polymerase α−primase; Tris-HCl, tris(hydroxymethyl)aminomethane, hydrochlorine salt.

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exogenously added primer−templates. Here we report that, during primase-coupled pol α activity, abasic lesions do not lead to strong chain termination. The mechanism of bypass likely involves pol α activity, since abasic lesions were strong inhibitors for primase. The biological implications of these results are discussed.

EXPERIMENTAL PROCEDURES

Materials

Unless noted, all materials and methods were as described previously (*22*). The recombinant human p180/p70/p58/p49 pol α−primase and the p180/p70 pol α complexes were purified by use of a baculovirus/insect cell expression system as previously described with minor modifications (*27*, *28*). Briefly, Hi5 insect cells were infected for 48 h with baculovirus expression vectors containing the cDNAs for the desired subunits of the pol α−primase complex (multiplicity of infection = 10). The cells were then harvested by centrifugation, lysed, and loaded onto a p180 antibody column. Subsequently, the column was washed in 50 mM Tris-HCl, pH 8.6, 150 mM KCl, and 1 mM 2-mercaptoethanol. The enzyme complex was eluted with 10 mM triethylamine, pH 10.8, 1 M NaCl, and 10% glycerol and dialyzed overnight into 50 mM Tris-HCl, pH 8.6, 5 mM 2-mercaptoethanol, 1 mM EDTA, and 50% glycerol.

The 43-mer templates containing a single abasic site were synthesized as previously described (*29*). The control 43-mer template, RNA primers, and DNA primers were purchased from Oligos, Etc. The concentrations of single-stranded DNAs were determined spectrally and are expressed in terms of the total nucleotides (*30*). For sequencing experiments, the thermosequenase radiolabeled terminator cycle sequencing kit was purchased from Amersham Pharmacia Biotech. All other reagents were of the highest purity available.

Methods

Enzyme Assays. Unless noted, all assays contained 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. 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% polyacrylamide, 8 M urea) and analyzed via phosphorimagery (Molecular Dynamics).

Primase assays (10 μL) contained 60 μM single-stranded DNA template, 100 nM pol α−primase, and 200 μM [α-^{32}P]-NTPs. Primase-coupled pol α assays (10 μL) contained 60 μM single-stranded DNA template, 100 nM pol α−primase, 200 μM NTPs, and 10 μM [α-^{32}P]dNTPs. Assays to measure pol α-catalyzed elongation of exogenously added primer−templates (10 μL) contained 1−10 μM primer−template, 100 nM pol α−primase, and 10 μM [α-^{32}P]dNTPs. To control for the relatively low processivity of pol α−primase, the frequency of abasic lesion bypass was normalized for the frequency with which pol α paused at that position on the normal template, D_{43}. This was accomplished by dividing the measured frequency of bypass by the frequency with which pol α polymerized nucleotides past that position on D_{43}.

Table 1: Synthetic DNAs of Defined Sequence Used in These Studies

Templates [a]		
	D_{43}	AAACTCTCTCTCTCTCTGGGGGTAAAATGGGGCAAAATGGGGC-5′
	D_{43}-7	AAACTC**x**CTCTCTCTCTGGGGGTAAAATGGGGCAAAATGGGGC-5′
	D_{43}-14	AAACTCTCTCTCT**x**TCTGGGGGTAAAATGGGGCAAAATGGGGC-5′
	D_{43}-19	AAACTCTCTCTCTCTCTG**x**GGGTAAAATGGGGCAAAATGGGGC-5′
	D_{43}-7,14	AAACTC**x**CTCTCT**x**TCTGGGGGTAAAATGGGGCAAAATGGGGC-5′
Primers [b]		
	D_{11}	TTTGAGAGAGA-3′
	D_{15}	TTTGAGAGAGAGAGA-3′
	R_{11}	UUUGAGAGAGA-3′
	R_{15}	UUUGAGAGAGAGAGA-3′
	Sequencing Primer	GTAAAATGGGGCAAAAT-5′

[a] Abasic sites in the template are noted by a **x**. [b] D_{11} and D_{15} are DNA, while R_{11} and R_{15} are RNA. The relative orientation and position of the primers and templates are arranged to facilitate viewing how they will hybridize to each other.

Purification of Individual Bypass Products from Coupled Assays. Reactions (10−30 μL) containing 60 μM single-stranded DNA template, 100 nM pol α−primase, 200−500 μM NTPs, and 10 μM [α-^{32}P]dNTPs were incubated for 1 h at 37 °C. After the products were separated by denaturing gel electrophoresis, individual product bands were excised from the gel. DNA was extracted from the gel by adding 1 mL of H_2O to each product, passing it through a 1 mL syringe 5−10 times, and subjecting it to three freeze−thaw cycles. Samples were then rotated overnight at 4 °C, filtered to remove gel material, and concentrated in vacuo. Finally, DNA was purified by isopropyl alcohol precipitation.

Base Hydrolysis. Purified individual products were treated with 100 mM NaOH for 10 min at 90 °C. After addition of 2.5 volumes of gel loading buffer, products were analyzed by denaturing gel electrophoresis and phosphorimagery.

Sequencing Bypass Products. Purified individual products were sequenced with the thermosequenase cycle sequencing kit according to the manufacturer's instructions (Amersham). Briefly, 5−25 fmol of purified individual products were incubated with 0.5 pmol of ssDNA primer (5′-TAAAACGG-GGTAAAATG), dITP nucleotide mix, [α-^{33}P]ddNTPs, reaction buffer, and thermosequenase. Samples were then amplified by linear PCR for 60−90 cycles with the following conditions: 95 °C, 30 s; 40 °C, 30 s; and 60 °C, 1 min.

RESULTS

To investigate the interaction of the pol α−primase complex with abasic sites, we examined a series of synthetic 43-mer templates containing model abasic (tetrahydrofuran) lesions (Table 1). On these templates, primase can synthesize primers on the pyrimidine-rich 3′ end in the presence of ATP and GTP, and pol α can elongate the primers via dNTP polymerization. Two templates, D_{43}-7 and D_{43}-14, have single lesions positioned within the pyrimidine-rich 3′-end to determine the effect of abasic sites on primase-catalyzed polymerization of NTPs and on polymerization of dNTPs immediately after the switch to pol α activity. The third template, D_{43}-7,14, contains two lesions in this region and allows us to determine the effects of an abasic site under conditions where primase must interact with the lesion in order to synthesize a primer. The fourth template, D_{43}-19, contains a single lesion downstream in the purine-rich section

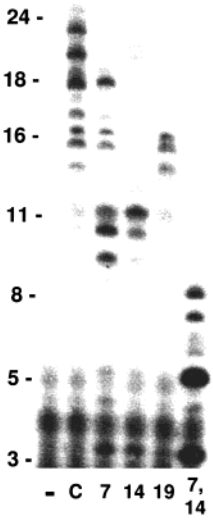

FIGURE 1: Effects of abasic lesions on primase activity. Assays contained pol α−primase, 200 μM [α-^{32}P]ATP/GTP, and 60 μM D_{43} (lane C), D_{43}-7 (lane 7), D_{43}-14 (lane 14), D_{43}-19 (lane 19), or D_{43}-7,14 (lane 7,14). They were incubated at 37 °C for 1 h and analyzed as described under Experimental Procedures. The assay in lane (−) lacked pol α−primase. The lengths of oligo(A) products generated in primase assays on poly(dT) are shown on the left side.

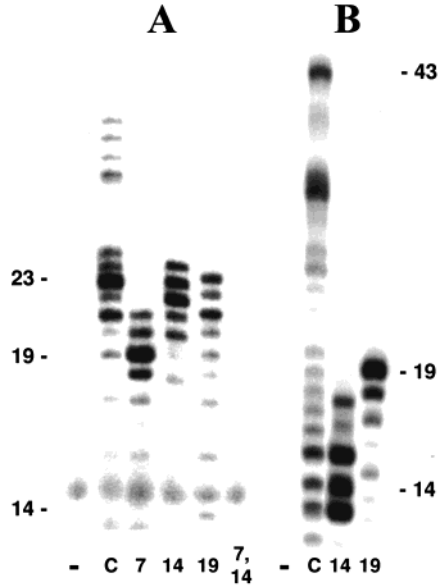

FIGURE 2: Effects of abasic lesions on primase-coupled pol α activity and pol α activity. (A) Primase-coupled pol α assays were performed as described under Experimental Procedures and contained pol α−primase, 200 μM ATP/GTP, 10 μM [α-^{32}P]dATP/dGTP/dCTP, and 60 μM D_{43} (lane C), D_{43}-7 (lane 7), D_{43}-14 (lane 14), D_{43}-19 (lane 19), or D_{43}-7,14 (lane 7,14). (B) Pol α assays contained 10 μM [α-^{32}P]dATP/dGTP/dCTP/TTP, and 1−10 μM D_{11}/D_{43} (lane C), D_{11}/D_{43}-14 (lane 14), or D_{11}/D_{43}-19 (lane 19), each primed with a 11 nucleotide long DNA oligonucleotide. In both panels, assays in lane (−) lacked pol α−primase. All assays were incubated at 37 °C for 1 h. The mobility of 5′-[^{32}P]-labeled DNA standards is shown on the left side. It should be noted that products containing a RNA primer (±triphosphate) migrate slightly slower than products that are all DNA (*41*).

to evaluate the effect of abasic lesions on pol α-catalyzed polymerization of dNTPs.

Analysis of Primer Synthesis on Abasic Templates. We first examined how abasic lesions affect primase activity by incubating pol α−primase with each of the four abasic templates and [α-^{32}]ATP/GTP (each at 200 μM). As shown in Figure 1, primase activity on D_{43} (lane C) resulted in greater than 99% full-length primers (≥7 nucleotides long), primarily consisting of primer dimers and primer trimers (ca. 16 and 24 ribonucleotides long, respectively). The formation of large amounts of primer dimers and primer trimers occurs when dNTPs are absent (*31*). Under these conditions primase extends off a newly synthesized primer by the distance of another primer (7−10 nucleotides), resulting in products that are multiples of approximately 8 nucleotides. Figure 1 also demonstrates the extraordinary infidelity of primase (*32*). These reactions, containing only GTP and ATP, generated products up to 24 nucleotides long, while the longest TC region in the 43-mer template is 14 nucleotides long.

Primase was also able to synthesize primers on each template containing a single abasic lesion (D_{43}-7, D_{43}-14, and D_{43}-19). The amount of product <5 nucleotides long increased substantially on each template relative to D_{43}; however, 70−80% of total product formed still consisted of full-length primers. While primase could synthesize primers on abasic templates, it appeared that this was accomplished through polymerization of NTPs either upstream or downstream of the abasic lesion but not by insertion of an NTP opposite the abasic lesion. The longest primers synthesized on D_{43}-14 and D_{43}-19 were only 11 and 16 nucleotides long, respectively, whereas primers up to 24 nucleotides long were synthesized on D_{43} (Figure 1). The correlation between the extent to which primers became shorter and the position of the lesion relative to the 3′-end of the pyrimidine-rich region strongly suggests that primer synthesis terminates at or near the abasic lesion. On D_{43}-7, primers were approximately 3−4 nucleotides shorter as compared to the normal 43-mer, indicating that primase avoids the abasic lesion by initiating primer synthesis 3−4 nucleotides downstream relative to initiation on D_{43}. Confirmation of this hypothesis came from the results on D_{43}-7,14, where synthesis of a primer >6 nucleotides long would require polymerization of an NTP opposite one of the abasic sites. In assays containing D_{43}-7,14, only 8% of total product was >6 nucleotides long, although large amounts of smaller products accumulated. Thus, abasic lesions appear to strongly inhibit primer synthesis by causing chain termination.

Abasic Lesions Are Not Chain Terminators during Primase-Coupled Pol α Activity. The effect of abasic lesions on primase-coupled pol α activity was measured by including substrates for both primase (ATP/GTP) and pol α (dNTPs). These assays only included [α-^{32}P]dATP, dGTP, and dCTP (each at 10 μM) so that polymerization would terminate at the first template dAMP (nucleotide 23), resulting in a relatively sharp band at this position and facilitating analysis of products.[2] As shown in Figure 2A, assays containing D_{43} (lane C) showed an accumulation of products ranging from 19 to 23 nucleotides long, indicating that this is a good substrate for primase-coupled pol α activity. When the template was D_{43}-7, products were 3−4 nucleotides shorter than observed with the normal 43-mer. This observation provides further support for the hypothesis that primase

[2] Including dTTP did not appear to affect the extent of abasic lesion bypass, although it greatly complicated calculation of the percent bypass (data not shown). Due to the relatively low processivity of pol α, many products of slightly different length were generated when dTTP was included in the assay. Thus, dTTP was omitted from all further assays.

Table 2: Effect of Abasic Lesions on Steady-State Kinetic Parameters for Primase-Coupled Pol α Activity

	K_m (template) (μM)	V_{max} [pmol of dNTP h^{-1} (μg of enzyme)$^{-1}$]
D_{43}	17 ± 3	3.8 ± 0.8
D_{43}-7	43 ± 14	4.2 ± 1.1
D_{43}-14	29 ± 0.1	2.3 ± 0.2
D_{43}-19	21 ± 10	3.4 ± 1.5

initiates primer synthesis downstream of the abasic lesion on D_{43}-7, thereby resulting in shorter products. For D_{43}-7,-14, coupled activity was below the limit of detection. Since pol α does not readily elongate primase-synthesized primers <7 nucleotides long (*31*), these results are consistent with the primase assays showing that the two lesions in this template potently inhibited synthesis of mature primers (Figure 1). Most importantly, analysis of D_{43}-14 and D_{43}-19 showed substantial bypass of abasic lesions (93% ± 5% and 60% ± 3% bypass, respectively). In fact, for both of these templates, the distribution of products was similar to assays containing D_{43} where products greater than 14 and 19 nucleotides long comprised 97% ± 4% and 86% ± 5% of total product, respectively. Additionally, the abasic sites in D_{43}-14 and D_{43}-19 did not substantially alter the steady-state kinetic parameters for primase-coupled pol α activity (Table 2). Together, these results show that pol α−primase can readily bypass abasic lesions during primase-coupled pol α activity.

To control for the possibility that the high level of bypass observed during primase-coupled pol α activity was specific to the DNA sequence of these templates, pol α assays were also conducted on the 43-mer templates. A DNA primer (D_{11}) was annealed to D_{43}, D_{43}-14, and D_{43}-19, and the resulting primer−templates were incubated with pol α−primase and [α-^{32}P]dNTPs (see Table 1 for sequences). As shown in Figure 2B, assays containing D_{11}/D_{43}-generated products ranging in size up to 43 nucleotides long, as expected. In contrast, analysis of assays containing either D_{11}/D_{43}-14 or D_{11}/D_{43}-19 revealed strong termination at or near the abasic site in each template (23% and 4% bypass on D_{11}/D_{43}-14 and D_{11}/D_{43}-19, respectively). Furthermore, the majority of bypass on D_{11}/D_{43}-14 (80−90%) resulted in products that extended just 1−3 nucleotides past the abasic site. In contrast, almost all of the bypass products in primase-coupled pol α assays containing D_{43}-14 extended 6−10 nucleotides past the lesion (Figure 2). Thus, the facile bypass of abasic lesions observed during primase-coupled pol α activity is not a consequence of the DNA sequence.

Bypass of abasic lesions during coupled activity likely does not involve pol α dissociating from and then rebinding the DNA. After just 5 min, 50% bypass was observed on both D_{43}-14 and D_{43}-19 and increased by no more than 1.8-fold over 60 min (Figure 3). In contrast, significant bypass of abasic lesions during pol α-catalyzed elongation of exogenously added DNA primer−templates did require long incubations: on D_{11}/D_{43}-14, bypass increased from 8% to 31% as time increased from 5 to 60 min, and on D_{11}/D_{43}-19, bypass increased from 3% to 7%.[3]

Parameters Involved in Abasic Lesion Bypass. To better understand why pol α−primase readily bypassed abasic

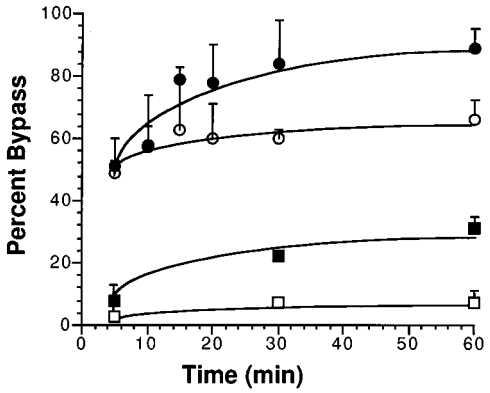

FIGURE 3: Time course of abasic lesion bypass. Bypass of abasic lesions during both primase-coupled pol α activity on D_{43}-14 (●) and D_{43}-19 (○) and during pol α-catalyzed elongation of an exogenously added primer−template on D_{11}/D_{43}-14 (■) and D_{11}/D_{43}-19 (□) was measured as described under Experimental Procedures.

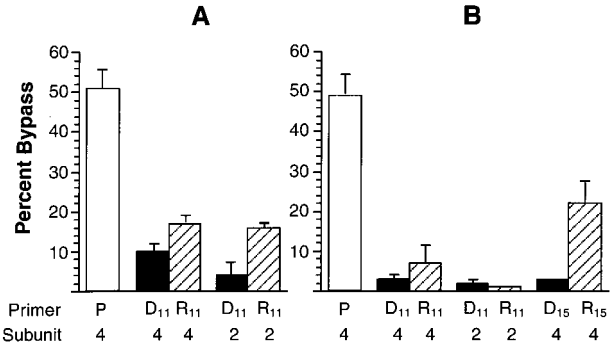

FIGURE 4: Parameters involved in bypass of abasic lesions. On D_{43}-14 (panel A) and D_{43}-19 (panel B), the frequency with which primase-coupled pol α bypassed an abasic lesion (open bars) was compared to bypass during elongation of an exogenously added primer template where the primer was either DNA (solid bars) or RNA (hatched bars). The primer length and enzyme complex used (four-subunit pol α−primase or two-subunit p180/p70) are noted in the figure. A primer provided by primase is denoted by P.

lesions during primase-coupled pol α activity but not during elongation of an exogenously added primer−template, we evaluated the following three parameters that could affect activity.

(1) Changing the Primer from DNA to RNA. Changing the primer from DNA to RNA significantly enhanced the ability of pol α to bypass abasic lesions (Figure 4). With an 11-nucleotide long primer, bypass was enhanced by 1.7-fold on R_{11}/D_{43}-14 and 2.3-fold on R_{11}/D_{43}-19 when compared to D_{11}/D_{43}-14 and D_{11}/D_{43}-19, respectively.

(2) Changing the Distance between the Primer 3′-Terminus and the Lesion. We measured the ability of pol α to bypass lesions on D_{11}/D_{43}-19, R_{11}/D_{43}-19, D_{15}/D_{43}-19, and R_{15}/D_{43}-19. When the primer was DNA, decreasing the distance between the primer 3′-terminus and the lesion did not enhance bypass (compare D_{11}/D_{43}-19 and D_{15}/D_{43}-19, Figure 4). In contrast, when the primer was RNA, decreasing the

[3] These experiments are complicated by the extremely low turnover number of primase relative to pol α. The rate of nucleotide polymerization by pol α is typically 50−100-fold greater than the rate of nucleotide polymerization by primase, and the $t_{1/2}$ for a single turnover of primase is typically 3−6 min. Hence, we cannot exclude the possibility that the primer−template undergoes one or a few dissociation and rebinding events in the first 5 min (*22*).

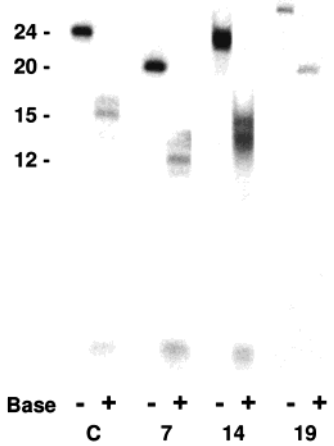

FIGURE 5: Base hydrolysis of products from primase-coupled pol α assays. Individual products isolated from coupled assays containing either D_{43} (lanes C), D_{43}-7 (lanes 7), D_{43}-14 (lanes 14), or D_{43}-19 (lanes 19) were analyzed either directly or after treatment with base as described under Experimental Procedures. The mobility of 5′-[^{32}P]-labeled DNA standards is shown on the left side.

distance between the primer 3′-terminus and the lesion increased bypass 3.1-fold (compare R_{11}/D_{43}-19 and R_{15}/D_{43}-19, Figure 4). In fact, the extent of bypass on R_{15}/D_{43}-19 (22%) was similar to the level observed on R_{11}/D_{43}-14 (17%), two substrates where the distance between the primer 3′-terminus and the lesion is similar.

(3) Role of the p49/p58 Primase Subunits. The ability of the four-subunit pol α−primase complex (p180/p70/p58/p49) and a two-subunit pol α complex (p180/p70) to bypass abasic lesions was compared. The lack of p49/p58 reduced the ability of pol α to bypass lesions by from 1.5-fold on D_{11}/D_{43}-19 to 7-fold on R_{11}/D_{43}-19 (Figure 4). Together, these studies show that the nature of the primer (DNA versus RNA), the distance of the primer 3′-terminus from the lesion, and the presence of the p49/p58 primase subunits all influence the ability of pol α to polymerize dNTPs past an abasic site when exogenously added primer−templates were used.

Identification of the Nucleotide Inserted opposite the Abasic Lesion. To further understand the mechanism of bypass during primase-coupled pol α activity, the identity of the nucleotide inserted opposite the abasic site was determined. One potential, albeit unlikely, mechanism for bypass during coupled activity is that primase polymerizes an NTP opposite the abasic lesion. To directly test this hypothesis, individual products from primase-coupled pol α assays were gel purified and treated with base to hydrolyze ribonucleotides. If the only ribonucleotides present in the products were from primase-synthesized primers, each product should be shortened by ca. 8 nucleotides. However, if an NTP was polymerized during bypass of the abasic site, base hydrolysis would result in much smaller products. In the case of D_{43}-19, if an NMP had been incorporated opposite the abasic site during bypass, base treatment should have shortened the product by approximately 15 nucleotides.[4] As shown in Figure 5, products obtained from assays containing the normal 43-mer or one of the abasic templates were

[4] This calculation assumes that primer synthesis begins near the 5′ end of the pyrimidine rich region, as seems likely on the basis of the data in Figures 1 and 2.

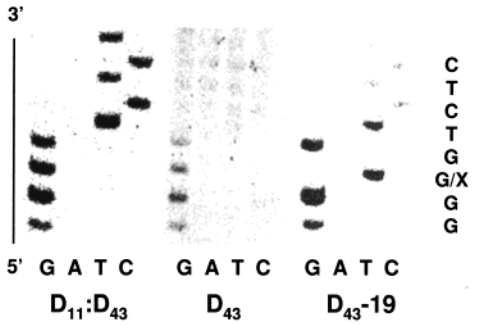

FIGURE 6: Sequencing of products from coupled assays. Individual products from pol α assays containing D_{11}/D_{43} or from primase-coupled pol α assays containing either D_{43} or D_{43}-19 were purified and sequenced as described under Experimental Procedures. The anticipated sequence of the template strand is shown on the right side of the figure (X = abasic site). The actual sequence of the products will be the complement of this sequence.

shortened by about 8 nucleotides. Therefore, it is clear that all ribonucleotides originated from the primer and bypass involves dNTP polymerization opposite the abasic site. During primase-coupled pol α activity on D_{43}-14, the transition region between RNA primer synthesis and DNA synthesis will occur very close to the abasic site. Thus, the observation that base hydrolysis reduces the length of bypass products by about 8 nucleotides strongly suggests, but does not prove, that a deoxynucleotide was incorporated opposite the abasic site on D_{43}-14. Finally, these data show that the abasic lesions do not affect what length primers are elongated by pol α during coupled activity.

The identity of the dNMP inserted opposite the abasic site was determined by sequencing individual gel-purified products from primase-coupled pol α assays by linear PCR. A control experiment showed that the sequence of product generated during pol α-catalyzed elongation of the normal substrate, D_{11}/D_{43}, was the expected 5′-GAGAGAGAGAGA-GACCCC (Figure 6). While the products from a primase-coupled pol α assay on D_{43} could not be sequenced as far, probably due to the RNA primer, the first four nucleotides were clearly identified as 5′-CCCC. Finally, sequencing of several bypass products from coupled assays containing D_{43}-19 (template sequence 3′...TGXGG..., where X is the abasic lesion) each gave the sequence 5′-CACC, showing that dATP had been inserted opposite the abasic lesion.

DISCUSSION

We have found that pol α-catalyzed extension of primase-synthesized primers results in substantial bypass of model abasic lesions. In contrast, as shown here and in previous reports, abasic lesions caused strong chain termination during pol α-catalyzed elongation of exogenously supplied DNA primer−templates (*16*−*18*). Furthermore, the small level of bypass measured during pol α extension of exogenous primers was a slow reaction compared to bypass during primase-coupled pol α activity and consisted of products only 1−4 nucleotides past the abasic site. Importantly, these results provide an additional mechanism by which the DNA replication machinery could polymerize deoxynucleotides past abasic lesions in whole cells.

Two sets of data indicate that polymerization of the nucleotide opposite the abasic lesion during primase-coupled pol α activity was catalyzed by pol α. First, a dNMP was

polymerized opposite the abasic site, and primase prefers to polymerize NTPs rather than dNTPs (*33*). Second, abasic lesions result in strong chain termination during primer synthesis by primase.

These studies show that pol α interacts with template lesions in a fundamentally different manner when the primer is supplied via primase activity than when the primer−template is added exogenously, analogous to what we previously observed with nucleoside analogues. For example, comparison of primase-coupled pol α activity versus pol α-catalyzed elongation of exogenously added primer−templates showed that the antiviral compound acyclovir triphosphate inhibits pol α−primase more potently during coupled activity (*23*), and polymerization of araNTPs does not result in chain termination during coupled activity (*24*). Additionally, pol α−primase will readily incorporate the cognate NTP in the absence of a required dNTP during primase-coupled pol α activity, whereas this situation results in chain termination during elongation of an exogenously added primer−template (*24, 34*). In combination with the lack of evidence for pol α ever using the equivalent of an exogenously added primer−template in vivo, these results indicate that it is important to examine primase-coupled pol α activity when the potential effects of a DNA lesion or nucleoside analogue on pol α activity are considered.

At least four factors may mediate bypass of abasic lesions: the composition of the primer, the distance between the primer 3′-terminus and the lesion, the presence of the primase p49/p58 subunits, and the temporal coupling of primase and pol α activity. Changing the primer from DNA to RNA and decreasing the distance from the primer 3′-hydroxyl to the lesion both increased the ability of pol α to bypass abasic lesions when an exogenously added RNA primer−template was elongated and can account for a large amount of the bypass observed during coupled activity. These results suggest that the unique non-A, non-B helical structure of a RNA/DNA duplex can strongly influence pol α activity (*35*−*37*). We previously observed a similar effect of RNA primers on the ability of pol α to discriminate against ddNTPs (*35*−*37*). Changing the primer from DNA to RNA greatly diminished the ability of pol α to discriminate against polymerization of a ddNTP, and the magnitude of this effect decreased rapidly upon increasing the distance between the 3′-end of the RNA primer and the site where the ddNTP was polymerized. Also, the presence of the primase subunits (p49/p58) enhanced the ability of pol α to bypass abasic lesions during elongation of exogenously added primer−templates. One potential mechanism is that binding of primase to pol α mediates a conformational change in pol α that alters its catalytic activity. Alternatively, these changes could be mediated by interactions of the primer−template with the p58 subunit of primase. Consistent with this idea, we have found that RNA primer−templates bind to p58 (*38*), and Copeland and Wang (*33*) showed that p58 binds directly to the p180 pol α catalytic subunit. Finally, because RNA primers, primer-lesion distance, and the p49/p58 subunits can only partially account for the higher level of bypass observed during primase-coupled pol α activity, it appears that an as yet undefined factor intrinsic to the coordinated activities of primase and pol α is important for bypass.

Effects of Abasic Lesions on Primase. Interestingly, abasic lesions caused strong chain termination of primase activity. This was surprising since primase is perhaps the most inaccurate nucleotide polymerizing enzyme known, with error frequencies as high as 1 in 30 (*32*). This infidelity of primase is further emphasized in the primase assays on D_{43} that contained only ATP and GTP. In these assays, primase-synthesized products up to 24 nucleotides long, while the oligo(dTdC) region is only 14 nucleotides long. Conceivably, this misincorporation of multiple consecutive nucleotides could be due to a primer-slippage mechanism whereby primase remains bound to the oligo(dTdC) region and adds extra adenylate and guanylate residues to the primer. However, we previously found that, during misincorporation on other templates, primer slippage was not involved (*32*). The remarkable ability of primase to misincorporate nucleotides while also showing extreme sensitivity to the absence of a template base suggests that interactions with the base are of paramount importance for catalysis, as opposed to interactions with a correct base pair.

The "A Rule". During primase-coupled pol α activity, the enzyme complex only incorporated dATP opposite the abasic lesion, similar to previous reports on purified DNA polymerases isolated from prokaryotic and eukaryotic organisms (*6, 11, 29, 39, 40*). While this specificity for dATP, coined the "A rule", is obeyed in most purified polymerase systems and in bacterial cells, it is not observed in mammalian cells. Monkey COS7 cells and human lymphoblastoid cells preferentially incorporated dGTP (*7, 8*), while NIH3T3 cells preferentially incorporated dTTP opposite abasic sites (*9*). There are several potential explanations for the discrepancy between these in vitro and in vivo results. First, it is possible that other proteins associated with pol α−primase could affect the catalytic properties and dNTP specificity of pol α. Indeed, we have already found that one protein associated with pol α, primase, alters the ability of pol α to bypass abasic lesions. Alternatively, the rapidly incorporated deoxyadenylate may not represent the final base incorporated opposite the abasic lesion once replication is completed. The cellular postreplicative repair machinery might interpret the deoxyadenylate/abasic site "base pair" as a mismatch and attempt to repair the newly synthesized DNA by excising the deoxyadenylate. If this repair involved pol β, an enzyme that does not follow the A rule (*14*), incorporation opposite the abasic site could involve any of the four dNTPs.

Biological Implications. It has been hypothesized for over 10 years that replication past abasic sites results in mutagenesis (*4, 5*). Presumably this occurs when an abasic lesion escapes the many DNA repair processes of the cell and becomes a substrate for DNA polymerases. While in vivo studies clearly demonstrate that bypass of abasic lesions in DNA plasmids leads to mutagenesis, the molecular mechanism is not well understood. Only recently was it reported that pol β and pol δ can bypass abasic lesions in vitro (*14, 15*). The rapid bypass of abasic lesions during initiation of new strands of DNA by pol α−primase provides another mechanism by which abasic sites could be mutagenic in mammalian cells.

The observation that primase is sensitive to abasic lesions may also be important for understanding how generating large amounts of these lesions, as occurs with some anticancer agents (e.g., alkylating agents) and γ irradiation, elicits cytotoxicity (*4*). In a normal cell, it would be expected that primase rarely encounters an abasic site, since this lesion is

a relatively uncommon event. However, in a rapidly growing cancer cell treated with irradiation or drug, the interaction of primase with abasic sites would increase due to the elevation in DNA lesions. We hypothesize that this event would potently block initiation of DNA synthesis based on the strong primase inhibition observed here in vitro.

Finally, pol α may encounter a large number of different and potentially mutagenic lesions during initiation of new strands of DNA. For those lesions that have been examined, they have typically only been characterized by using an exogenously added DNA primer−template. Given the fundamentally different manner in which pol α−primase interacts with abasic sites during coupled activity as compared to when an exogenously supplied primer−template is used, the effects of these other lesions on primase-coupled pol α activity clearly merits investigation. This information could provide insights into the effects of these lesions on the integrity of DNA replication as well as the mechanism of pol α−primase.

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