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Bypass and termination at apurinic sites during replication of single-stranded DNA *in vitro*: A model for apurinic site mutagenesis

(SOS mutagenesis/carcinogenesis/DNA polymerase III/error-prone repair)

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ABSTRACT **Mutations produced in *Escherichia coli* by apurinic sites are believed to arise via SOS-assisted translesion replication. Analysis of replication products synthesized on depurinated single-stranded DNA by DNA polymerase III holoenzyme revealed that apurinic sites frequently blocked *in vitro* replication. Bypass frequency of an apurinic site was estimated to be 10–15%. Direct evidence for replicative bypass was obtained in a complete single-stranded → replicative form replication system containing DNA polymerase III holoenzyme, single-stranded DNA binding protein, DNA polymerase I, and DNA ligase, by demonstrating the sensitivity of fully replicated products to the apurinic endonuclease activity of *E. coli* exonuclease III. Termination at apurinic sites, like termination at pyrimidine photodimers, involved dissociation of the polymerase from the blocked termini, followed by initiations at available primer templates. When no regular primer templates were available, the polymerase underwent repeated cycles of dissociation and rebinding at the blocked termini and, while bound, carried out multiple polymerization–excision reactions opposite the apurinic sites, leading to turnover of dNTPs into dNMPs. From the *in vitro* turnover rates, we could predict with striking accuracy the specificity of apurinic site mutagenesis, as determined *in vivo* in depurinated single-stranded DNA from an M13–*lac* hybrid phage. This finding is consistent with the view that DNA polymerase III holoenzyme carries out the mutagenic "misinsertion" step during apurinic site mutagenesis *in vivo* and that the specificity of the process is determined primarily by the polymerase. SOS-induced proteins such as UmuD/C might act as processivity-like factors to stabilize the polymerase–DNA complex, thus increasing the efficiency of the next stage of past-lesion polymerization required to complete the bypass reaction.**

Apurinic (AP) and apyrimidinic sites are common lesions in DNA and are believed to be important intermediates in chemical carcinogenesis by a variety of chemical agents (1, 2). Depurination is the most frequent spontaneous alteration in DNA under physiological conditions and occurs *in vitro* at the rate of 3×10^{-11} per nucleotide per sec (3). Extrapolation to the *in vivo* situation would suggest that 0.5 purine is lost from an *Escherichia coli* cell per generation, and as many as 10,000 purines are lost in each mammalian cell per day (3). Depyrimidination is much slower and occurs at rates 100 times lower than depurination (4). Exposure of cells to a variety of carcinogens leads to the formation of modified bases, some of which are converted to AP sites either because of enhanced spontaneous release (e.g., 7-methylguanine) (5) or by specific DNA glycosylases (e.g., 3-methyladenine) as part of the DNA repair processes (6). Specific DNA glycosylases form AP sites also by removing uracils or hypoxanthines misincorporated during replication or produced via deamination of cytosines or adenines, respectively (6).

In *E. coli*, AP sites are highly mutagenic, but only when the SOS system is induced (7, 8). The current hypothesis is that mutations are formed by SOS-assisted replication through AP sites, similar to the mechanism proposed for UV mutagenesis and other SOS-dependent mutagens. According to this suggestion the polymerase is usually unable to replicate through blocking lesions but can do so after its interaction with SOS-induced proteins. This hypothesis predicts that the identity of the nucleotides inserted opposite AP sites plays a central role in determining the specificity of the mutational spectra of AP sites (1, 9, 10). In support of this proposition it was found that DNA polymerase I of *E. coli* as well as avian myeloblastosis virus reverse transcriptase and DNA polymerase α insert dAMP preferentially opposite AP sites *in vitro*, a reaction that is potentially mutagenic when it occurs *in vivo* (11–14).

DNA polymerase III, the multisubunit polymerase primarily responsible for replicating the *E. coli* chromosome (15), is likely to be the polymerase that encounters AP sites and other lesions during replication. Moreover, a variety of genetic evidence indicated that it is directly involved in SOS mutagenesis (16–18). As part of our effort to elucidate the molecular mechanism of SOS mutagenesis, we undertook a detailed biochemical analysis of *in vitro* replication of damaged DNA substrates with DNA polymerase III (pol III) holoenzyme. We used the single-stranded → replicative form (RF) reaction (15) in which single-stranded DNA (ssDNA) is primed with a synthetic oligodeoxynucleotide and replicated with pol III holoenzyme in the presence of ssDNA binding protein to form the nicked duplex product. This system, used previously to study the replication of UV-irradiated ssDNA (19–21), was used to investigate the replication of depurinated M13 ssDNA.

MATERIALS AND METHODS

Materials. *E. coli* DNA pol III holoenzyme and ssDNA binding protein were purified as described (22–24). *E. coli* DNA polymerase I, T4 DNA ligase, exonuclease III, and T4 polynucleotide kinase were purchased from Pharmacia P. L. Biochemicals. ssDNA from phages M13 *Gori1* ssDNA and M13 *wt* was purified as described (25). The primer for M13 *Gori1* and M13 *wt* ssDNAs was 5′ GAAACCATCGATAGC 3′ (5′ end at position 2538 on M13 *Gori1* DNA map). It was synthesized by the chemical services unit of the Weizmann Institute by phosphoramidate coupling.

Bovine serum albumin was obtained from Miles and nucleotides were obtained from Sigma. Polyethylenimine-

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Abbreviations: ssDNA, single-stranded DNA; pol III, DNA polymerase III; AP, apurinic; RF, replicative form.

cellulose plates were purchased from Merck and radiolabeled [α-^{32}P]dNTPs at 400 Ci/mmol (1 Ci = 37 GBq) were obtained from The Radiochemical Center, Amersham. Buffer R contained 20 mM Tris·HCl (pH 7.5), bovine serum albumin (80 μg/ml), 5 mM dithiothreitol, 4% (vol/vol) glycerol, 8 mM $MgCl_2$, 40 mM NaCl, and 0.1 mM EDTA.

Depurination of ssDNA. Depurination of ssDNA was carried out by heating the DNA (11 μg) in a buffer containing 0.01 M sodium citrate and 0.1 M KCl (pH 5.0) (40 μl) at 70°C for various periods of time (7). The depurinated DNAs were neutralized by adding 10 μl of 0.2 M Tris·HCl (pH 7.8), and the samples were diluted in 0.1 M Tris·HCl/1 mM EDTA, pH 7.5, to a concentration of 100 ng/μl for M13 *Goril* or 75 ng/μl for M13 *wt*, each representing 35 fmol of DNA circles per μl. Heat/acid treatment for 15, 30, and 45 min produced an average of 1.0, 1.9, and 2.5 AP sites per M13 *Goril* ssDNA molecule, respectively.

Hydrolysis of apurinic sites was carried out by incubation of the depurinated DNA in 0.1 M NaOH at 37°C for 1 hr (26). Samples were then neutralized by adding 1.3 vol of 0.5 M Mops KOH (pH 6.6).

Replication of Oligonucleotide-Primed ssDNA (ss → RF Reaction). Replication was performed as described (20) except that depurinated M13 *Goril* or M13 *wt* ssDNA was used instead of UV-irradiated DNA. DNA synthesis was determined by acid precipitation and product distribution was analyzed by agarose gel electrophoresis as described (20).

Bypass of AP Sites During Replication *in Vitro*. Bypass of AP sites was tested by determining the sensitivity of fully replicated depurinated DNA to the AP endonuclease activity of *E. coli* exonuclease III (27). Fully replicated covalently closed DNA molecules containing AP sites (as a result of bypass) should be converted by exonuclease III into the circular nicked form.

Replication was carried out under standard conditions except that 0.2 μg of DNA polymerase I and 5.6 units of T4 DNA ligase were added to convert the nicked RF II obtained with pol III holoenzyme into the covalently closed form of DNA. After replication, 0.1 vol of 3 M sodium acetate (pH 5.5) and 2 vol of ethanol were added and the DNA was precipitated for 30 min in dry ice. The DNA was then spun down and the pellet was resuspended in 20 mM $CaCl_2$/50 mM Tris·HCl, pH 7.8. (In the presence of Ca^{2+}, exonuclease III has AP endonuclease activity but no exonuclease activity.) The extent of DNA synthesis was determined at this stage by acid precipitation of 1/10th of the DNA solution.

The DNA samples were boiled for 5 min and immediately cooled on ice. This denatures all partially replicated molecules, but it leaves fully replicated covalently closed DNA molecules intact. The samples were treated with 50 units of exonuclease III for 5 min on ice, after which the reaction was stopped by adding 20 mM EDTA/1% $NaDodSO_4$/5% (vol/vol) glycerol/0.02% bromophenol blue, and the mixture was loaded onto a neutral agarose gel. After electrophoresis, the gels were dried and autoradiographed as described (20).

Recycling of Pol III Holoenzyme. Pol III holoenzyme was recycled as described (20) except that depurinated M13 *wt* ssDNA was used instead of UV-irradiated DNA.

Turnover Assay. The assay for turnover of a given dNTP into its corresponding dNMP by the 3′ → 5′ exonucleolytic activity of the polymerase was performed as described (21) except that depurinated M13 *wt* ssDNA was used instead of UV-irradiated DNA.

RESULTS

Replication of Depurinated ssDNA by Pol III Holoenzyme. When depurinated M13 *wt* ssDNA was replicated with pol III holoenzyme in the presence of ssDNA binding protein there was a decrease in nucleotide incorporation as a function of depurination time, indicating that elongation frequently stops at AP sites. The extent of DNA synthesis decreased to 55%, 39%, and 29% for DNA samples pretreated by heat/acid for 15, 30, and 45 min, respectively, as compared to untreated DNA. Assuming a random distribution of replication blocks in the depurinated DNA substrates, the average number of blocks can be determined from the distribution of replication products fractionated in alkaline or neutral agarose gels by electrophoresis based on Poisson distribution (19, 20). We found that pretreatment of the depurinated ssDNA with alkali, which breaks AP sites thus rendering them complete blocks to replication, yielded upon synthesis a slightly higher fraction of shorter termination products, indicating that AP sites were not complete blocks to replication and were bypassed to some extent (data not shown). Based on the fraction of full length in these densitometric traces, we estimated that AP sites were bypassed at a frequency of 10–15% in this *in vitro* system. However, because of the low extent of bypass, direct proof for bypass is needed.

Bypass of AP Site During *in Vitro* Replication. Replicative bypass will form a fraction of full-length products that contain AP sites and are thus expected to be sensitive to AP endonucleases, which specifically cleave depurinated double-stranded DNA at AP sites. The AP endonuclease we used was exonuclease III of *E. coli*, which has multiple enzymatic activities but in the presence of Ca^{2+} functions only as an AP endonuclease (27).

As shown in Fig. 1, replication of depurinated ssDNA yielded full-length covalently closed DNA as well as termination products. Treatment of the DNA samples with the AP endonuclease converted a significant part of the supercoiled replication products synthesized on depurinated DNA into the nicked form. The control undamaged DNA gave rise to products that were not cleaved by the AP endonuclease. This provides direct evidence for the presence of AP sites in fully replicated molecules.

DNA Pol III Holoenzyme Recycles After Replication of Depurinated DNA. Although bypass of AP sites can occur, most encounters of the polymerase with AP sites still lead to termination, indicating that AP sites are major obstacles to replication.

Similar to termination of replication at pyrimidine photodimers (20), termination at AP sites involved dissociation of pol III holoenzyme from DNA followed by fresh initiations at

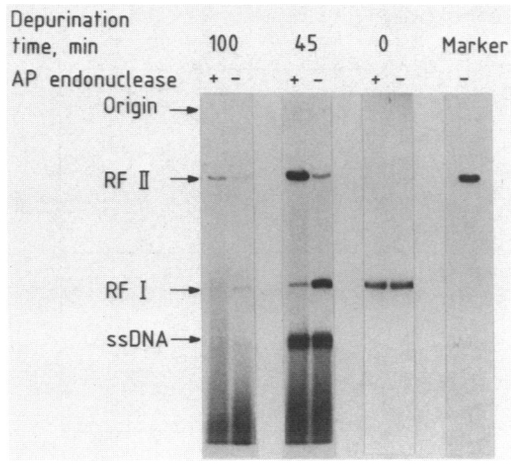

FIG. 1. Bypass of AP sites during *in vitro* replication. Depurinated M13 *wt* was replicated with DNA pol III holoenzyme ssDNA binding protein, DNA polymerase I, and T4 ligase, and fully replicated covalently closed molecules were probed for the presence of AP sites with exonuclease III. Autoradiogram of DNA products fractionated by neutral agarose gel electrophoresis.

available primer templates. This was demonstrated by the ability of the polymerase to carry out multiple cycles of replication in a reaction mixture in which depurinated primed DNA was present in excess. As shown in Fig. 2, when either untreated or depurinated DNAs were used as templates there was an initial fast phase of DNA synthesis, which corresponded to the completion of the first cycle of replication followed by a phase with a slower rate of DNA synthesis. This slower phase involved dissociation of pol III holoenzyme from the replicated molecules followed by reinitiation and elongation of unreplicated ones (20, 28). We have previously pointed out that termination at lesions occurred at structures that are basically primer template junctions, to which the polymerase usually binds with high affinity (21). It is thus remarkable that the presence of a lesion such as an AP site or a pyrimidine photodimer at the primer template junction signals termination and causes dissociation of the polymerase from the primer template.

dNTPs Turn Over into dNMPs During Termination. Fractionation by polyethylenimine-cellulose TLC of replication mixtures in which a radiolabeled dNTP was included revealed its conversion to the radiolabeled dNMP (Fig. 3). These originated from radiolabeled dNMPs, which were first incorporated into the DNA by the polymerase and excised out by its 3′ → 5′ proofreading exonuclease activity (21, 29, 30). No free dNMPs were detected unless replication took place. We followed in parallel DNA synthesis and release of free dNMPs during replication of untreated DNA and depurinated DNA. As shown in Fig. 3, DNA synthesis was rapid and reached its maximal value within 1–2 min. The monophosphates, however, continued to accumulate at termination—i.e., when no further DNA synthesis took place. This indicates that during termination, the polymerase, which underwent repeated cycles of dissociation and reinitiation, carried out polymerization–excision reactions while being bound to the DNA. The rates of monophosphate accumulation during termination on depurinated DNA were 4.10, 0.95, 0.48, and 1.29 pmol of nucleotide per pmol of DNA per sec

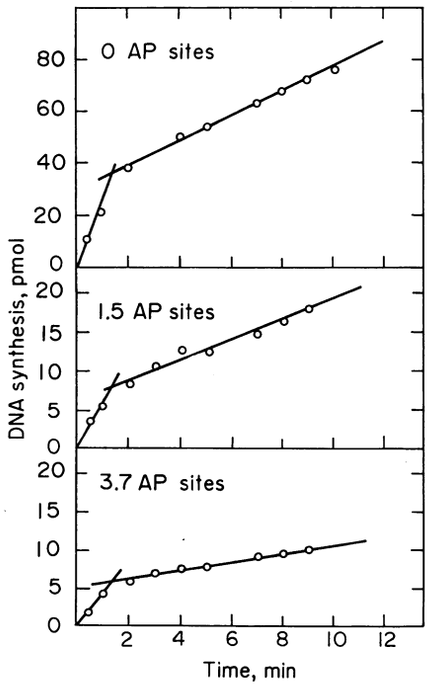

FIG. 2. Recycling of DNA pol III holoenzyme during replication of depurinated DNA. DNA synthesis in a reaction mixture with a limiting amount of pol III holoenzyme was determined as described (20) except that depurinated M13 *wt* ssDNA was used instead of UV-irradiated DNA.

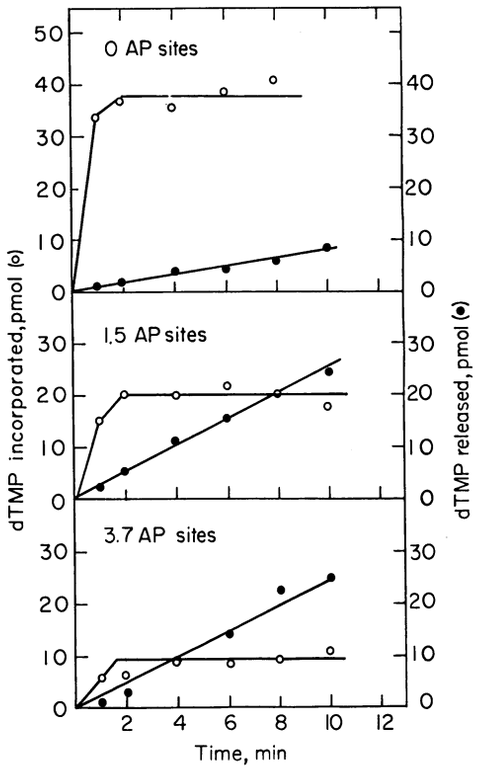

FIG. 3. Turnover of dTTP into dTMP during replication of untreated or depurinated ssDNA. Reaction conditions and the assay were as described (21), except that depurinated ssDNA was used instead of UV-irradiated DNA. The reaction mixture was fractionated on polyethylenimine-cellulose TLC plates from which the extent of incorporation of [α-^{32}P]dTMP into DNA and the released free [α-^{32}P]dTMP were determined simultaneously.

for dATP, dGTP, dCTP, and dTTP, respectively. They were 2.6- to 4.0-fold higher than on untreated DNA, for which the values were 1.03, 0.28, 0.15, and 0.50, respectively. The turnover rates were similar for UV-irradiated (21) and depurinated DNA, and in both cases dATP turned over preferentially. Taking into account the different chemical structures of pyrimidine photodimers and AP sites, and the different sequence contexts in which they were formed (runs of pyrimidines for the photodimers vs. purine-containing sequences for the AP sites), the similar turnover rates provide further evidence for polymerization–excision directly opposite the lesion (21).

The turnover data would argue that insertion of nucleotides opposite AP sites is relatively easy. All nucleotides can be inserted with a slight preference for dAMP. A more pronounced preference for insertion of dAMP opposite AP sites was found for DNA polymerase I of *E. coli*, avian myeloblastosis virus reverse transcriptase, and for DNA polymerase α from *Drosophila melanogaster* and calf thymus (11–14).

Turnover Data Accurately Predict the Mutagenic Specificity of AP Sites. The turnover rates are composed primarily of the rates of polymerization and excision. During elongation on undamaged templates, polymerization is much faster than excision, and thus the rates of turnover reflect primarily the slow step of excision (31). We have argued that during termination, when turnover occurs opposite a lesion, the rate of polymerization is reduced because of the difficulty in copying a damaged and distorted site, whereas the rate of excision is increased because of the mismatched nature of the incorporated nucleotides (21). We further argued that the net result is that polymerization becomes slower than excision and thus the turnover rates opposite lesions primarily represent polymerization. In support of this suggestion, we have

found that purified RecA protein, which inhibited the exonuclease activity of the DNA polymerase III holoenzyme in the presence of ssDNA binding protein (31, 32) but not its polymerization activity (H. Shwartz and Z.L., unpublished data), had no effect on turnover during termination on UV-irradiated DNA, suggesting that polymerization is the slower step. This predicts that at any given time the nascent DNA chain is likely to be terminated 1 nucleotide prior to the lesion, as indeed was found with a variety of polymerases when termination sites were mapped (e.g., see refs. 12 and 33).

During termination on depurinated DNA, nearly all the molecules (>90%) are terminated at AP sites. Thus, turnover rates of the various dNTPs during termination represent their rates of polymerization opposite AP sites. Can they predict the distribution of mutations produced by AP sites *in vivo*?

The distribution of mutations produced in the *lacZ'* segment of depurinated ssDNA from phage M13mp2 has been determined by direct sequence analysis (34). Table 1 summarizes the *in vivo* data along with our prediction for mutation distributions, assuming that they were formed by incorporation of nucleotides opposite AP sites with relative rates identical to the *in vitro* turnover rates. As shown in Table 1, the *in vitro* turnover data predicted the *in vivo* mutagenic specificity for all base substitutions with striking accuracy.

Is this a coincidence? Will DNA polymerase I, for example, yield the same prediction? Accurate turnover data opposite AP sites are difficult to obtain with DNA polymerase I because of its relatively low processivity and the variety of side reactions that it catalyzes (e.g., strand displacement, strand switching, etc.). Still, based on data obtained by Sagher and Strauss (12) for turnover on depyrimidinated DNA with the large fragment of DNA polymerase I, the prediction is not as accurate as with pol III holoenzyme. The large deviation is for G → A transitions where the predicted value is 1/4th that found (7% vs. 29%).

DISCUSSION

Complete replicative bypass of AP sites involves at least two potentially problematic polymerization steps. The insertion of a nucleotide opposite the lesion (opposite-lesion polymerization; olp step) and the next polymerization step in which the first nucleotide past the lesion is inserted (past-lesion polymerization; plp step; Fig. 4). As evident from the presence of AP sites in fully replicated molecules, these two steps can be carried out during replication *in vitro* unassisted by any SOS-induced proteins, albeit at a low extent. Why do most attempts by the polymerase to bypass AP sites fail, leading to termination of elongation?

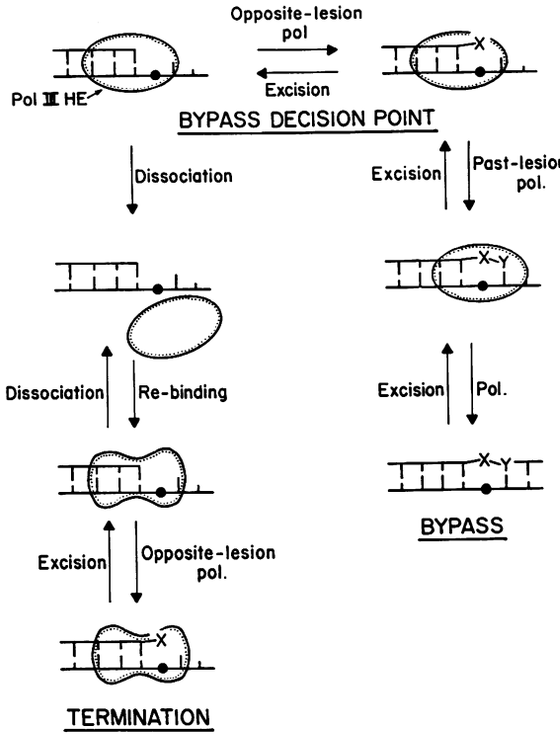

FIG. 4. A model for targeted mutagenesis of AP site via replicative bypass. The AP site is presented as a solid circle on the DNA, and other bases are designated by short vertical lines. X and Y, nucleotides; pol, polymerization; HE, holoenzyme. The polymerase is shown as an ellipse. Its altered shape at termination represents the formation of an unstable initiation-like complex. See *Discussion* for a detailed explanation.

As the polymerase encounters an AP site it pauses in a situation described as the bypass decision point (Fig. 4). Two reactions compete at this stage: (*i*) dissociation of the polymerase due to destabilization of its binding to the DNA caused by the lesion and (*ii*) the bypass reaction (Fig. 4). The turnover data indicated that the olp step is relatively easy, suggesting that it is the plp reaction that is slow. We suggest that under normal non-SOS conditions dissociation is faster than the plp reaction, yielding primarily terminations at AP sites. During termination the polymerase undergoes cycles of

Table 1. Prediction of AP site mutagenic specificity from turnover rates during termination of *in vitro* replication of depurinated DNA

Depurinated site	Inserted nucleotide	Turnover rate	Predicted misinsertion, %	Mutations in depurinated M13mp2	
				Predicted	Found
Adenine	dAMP	4.10	74	12	13
	dGMP	0.95	17	3	2
	dCMP	0.48	9	1	1
Guanine	dAMP	4.10	65	31	27
	dGMP	0.95	15	7	7
	dTMP	1.29	20	10	14

Turnover rates are in pmol of dNMP per pmol of DNA per sec. The turnover of dTTP was not included for the prediction of mutations at adenine AP sites since its insertion does not lead to a mutational event. The turnover of dCTP was excluded from the calculation for guanine AP sites for the same reason. Predicted percentage of the appropriate mutational events is based on the ratios of the turnover rates [e.g., % dAMP inserted opposite an adenine apurinic site: 4.10 × 100/(4.10 + 0.95 + 0.48) = 74%]. *In vivo* mutagenesis specificity was taken from Table 4 in ref. 34, in which 16 mutants at adenine putative AP sites and 48 mutants at guanine putative AP sites were listed. The predicted occurrences of the various mutants were obtained by multiplying the predicted misinsertion percent (column 4) by the total number of mutants analyzed (16 for adenine AP sites and 48 for guanine AP sites).

binding and dissociation from the lesion-blocked primer termini. Binding forms an unstable initiation-like complex, which does not allow elongation but permits repeated polymerization–excision reactions opposite the lesions (Fig. 4). This stage might represent the polymerase arrested at the "misincorporation" mutagenic step of SOS-dependent AP site mutagenesis as indicated by the ability to predict accurately *in vivo* mutagenic specificity of AP sites from the *in vitro* turnover activity of pol III holoenzyme during termination. The remarkable accuracy of the prediction suggests that the *in vitro* termination reaction faithfully represents the *in vivo* misincorporation mutagenic step, at least as far as specificity is concerned.

These findings are consistent with the view that DNA pol III holoenzyme is the enzyme that carries out the misincorporation step opposite the lesion and that the specificity of the process is determined primarily by the polymerase. Moreover, it suggests, in agreement with the two-step model for UV mutagenesis (35), that SOS-induced proteins are needed at a later stage to rescue the stalled polymerase and allow continuation of elongation. As we have previously suggested for SOS UV mutagenesis (20, 21), a possible mechanism would involve the clamping of the polymerase to the DNA by the UmuD/C proteins acting as processivity-like factors. This would increase the stability of the polymerase–DNA complex, thus increasing the efficiency of the next stage of past-lesion polymerization required to complete the bypass reaction. A similar role for the UmuD/C proteins in mutagenesis was recently proposed based on their sequence homology to phage T4 polymerase accessory proteins gp45, -44, and -62, which act as processivity factors (36). The verification of this mechanism must await the complete *in vitro* reconstitution of SOS-targeted mutagenesis.

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