Pyrene Nucleotide as a Mechanistic Probe: Evidence for a Transient Abasic Site-like Intermediate in the Bypass of Dipyrimidine Photoproducts by T7 DNA Polymerase[†]

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ABSTRACT: We recently proposed a mechanism for why dAMP is primarily inserted opposite both T's of photoproducts of TT sites by T7 DNA polymerase [Smith, C. A., Baeten, J., and Taylor, J.-S. (1998) *J. Biol. Chem., 273*, 21933-21940] that was based on analysis of a recent crystal structure of a complex of this enzyme with a template, a primer, and a dideoxynucleotide. We proposed that indiscriminate insertion of dAMP opposite the 3′-T of each photoproducts takes place via a transient abasic site-like intermediate, with the photoproduct outside the active site, whereas insertion of dAMP opposite the 5′-T takes place with the photoproduct inside the active site. To obtain further support for this mechanism, we have investigated the selectivity of dNMP and pyrene nucleotide (dPMP) insertion opposite each T of the cis,syn, trans,syn-I, trans,syn-II, (6−4), and Dewar photoproducts of TT and opposite a tetrahydrofuran abasic site analogue by the exonuclease-deficient T7 DNA polymerase, Sequenase Version 2.0. Selectivity was determined by a direct competition assay that makes use of a stacked gel to resolve the various extension products. Pyrene nucleotide was chosen for investigation because it has been previously shown to be selectively inserted opposite abasic sites and was therefore expected to probe whether the photoproducts were inside the active site during a particular insertion step. In accord with the proposed mechanism, dPMP was inserted in preference to dAMP opposite the 3′-T of all the photoproducts with the exception of the trans,syn-I product, whereas dAMP was inserted in preference to dPMP opposite the 5′-T of all the photoproducts. In addition to supporting the proposed mechanism, these results suggest that pyrene nucleotide may be a useful probe for investigating the mechanism of DNA damage bypass by polymerases and for characterizing their active sites.

Most targeted mutations induced by UV[1] light can be ascribed to mutagenic insertion of nucleotides opposite DNA photoproducts by DNA polymerases (*1*). Studies with uniquely photodamaged substrates have revealed that the mutagenicity of trans-lesion synthesis bypass depends on the structure of both the photoproduct and the polymerase, but the mechanistic basis for these differences is poorly understood (*2*). UV light produces a number of DNA photoproducts, some of which are unstable and are converted to other products by the further action of light or by other spontaneous reactions (Figure 1). The major classes of photoproducts resulting from direct absorption of UV light are the cis,syn cyclobutane dimers and (6−4) products (*3*). The trans,syn cyclobutane dimers are formed with much lower frequency than the cis,syn dimers, and the (6−4) products are not stable in sunlight and are converted to their Dewar valence isomers
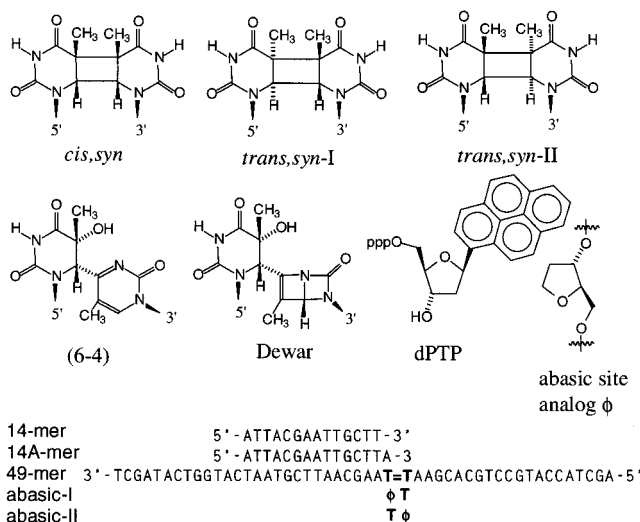

FIGURE 1: Structures of DNA photoproducts and substrates used in this study.

by 320 nm light (Figure 1) (*4*). There has been an attempt to classify these DNA photoproducts as being either instructive (misinstructive) or non-instructive by comparing the spectrum of mutations that they cause with that caused by an abasic site, the prototypical non-instructional lesion (*5*).

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[1] Abbreviations: dNTP, deoxynucleotide triphosphate; dPTP, pyrene deoxynucleotide triphosphate; DTT, dithiothreitol; exo⁻, 3′-5′ exonuclease deficient; UV, ultraviolet.
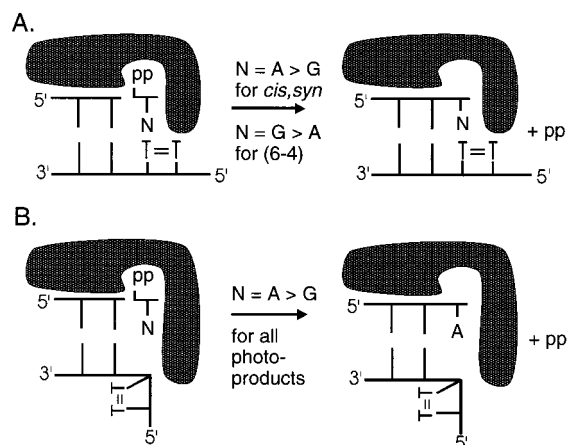
A.

B.

FIGURE 2: Proposed models for two types of DNA polymerase active sites. (A) Polymerases typified by *E. coli* polymerase V have less constrained active sites that can accommodate dipyrimidine photoproducts during insertion opposite the 3′-T of the photoproduct. These polymerases must be able to accommodate a linear template. (B) Polymerases typified by T7 DNA polymerase have highly constrained active sites that force dipyrimidine photoproducts out of the active site during insertion past the 3′-T of the photoproduct. Template primers bound by these polymerases show a characteristic bend of the template following the active site that results from exclusion of the nucleotide to the 5′-side of the templating nucleotide.

As we will show, this type of classification depends not only on the photoproduct but also on the polymerase and the mechanism by which it bypasses the photoproduct.

Mutagenesis studies in *Escherichia coli* under SOS conditions with site-specific products have suggested that cis,syn and, to a slightly less extent, the trans,syn dimers of TT behave like instructive lesions because dAMP's are inserted opposite these dimers with a much higher frequency than observed for abasic sites (*5−7*). The cis,syn cyclobutane dimer of TC also appears to behave like an instructive lesion as dGMP is almost exclusively inserted opposite the 3′-C, whereas dAMP is inserted opposite the 5′-T (*8*). Because the structures of the cis,syn dimers of TC and TT only differ in the base pairing properties of the 3′-base, it appears that base pairing must be responsible for the observed specificity of nucleotide insertion opposite the 3′-base. Whereas dAMP's are primarily inserted opposite the cis,syn dimer of TT, dGMP is primarily inserted opposite the 3′-T of the (6−4) product, and dAMP is inserted opposite the 5′-T (*7, 9*). The Dewar photoproduct of TT is also highly mutagenic, but nucleotides are inserted opposite the 3′-T of this product with lower specificity than opposite the (6−4) product (*7, 9*). The (6−4) photoproduct of TC behaves much like the (6−4) product of TT (*10*). Most recently, the selectivity of nucleotide insertion observed to occur opposite the cis,syn and (6−4) products of TT in *E. coli* under SOS have been reproduced in vitro by *E. coli* pol V (*11*). All the data are consistent with a bypass mechanism in which the 3′-T of the photoproducts is able to direct the insertion of a nucleotide and as such must lie within the active site of the polymerase (Figure 2A).

In contrast to what is observed for *E. coli* under SOS, and presumably accounted for by the pol V enzyme, dAMP appears to be primarily inserted opposite the 3′- and 5′-T's of both cis,syn dimers and (6−4) products of TT by exo⁻ T7 DNA polymerase (*12*), *E. coli* pol III (*11*), yeast (*13,*

14), and human cell extracts (*15, 16*). These results suggest that nucleotides are inserted opposite the 3′-T of these photoproducts by a different mechanism than takes place with *E. coli* pol V. Recently, we proposed that dAMP is selectively inserted opposite the 3′-T of dipyrimidine photoproducts by exo⁻ T7 DNA polymerase by a mechanism involving a transient abasic site-like intermediate, in which the photoproduct is outside of the active site (Figure 2B) (*12*). This mechanism was based on a crystal structure of a complex between a template primer and an exo⁻ T7 DNA polymerase in the presence of a ddNTP, which shows that the O-helix excludes nucleotides to the 5′-side of the templating nucleotide from the active site which creates a bend in the template (*17*). Because the 5′-T of a dipyrimidine photoproduct is covalently attached to its 3′-T, the entire photoproduct must be forced out of the active site by the O-helix during insertion opposite the 3′-T. As a result, an abasic-like site would be created, opposite which dAMP would be inserted if T7 DNA polymerase behaved like most other polymerases do when encountering an abasic site. Following the addition of dAMP, we argued that these photoproducts could be accommodated into the active site and that dAMP would be introduced opposite their 5′-T's by way of a base-pair-like structure.

We now report further experiments that support the key elements of the proposed mechanism for bypass of photoproducts by T7 DNA polymerase: (i) that dAMP is selectively introduced opposite abasic analogues by T7 polymerase relative to other nucleotides of DNA, (ii) that insertion opposite that 3′-T of a dipyrimidine photoproduct takes place with the photoproduct outside the active site, and (iii) that insertion opposite the 5′-T of a dipyrimidine photoproduct takes place with the photoproduct inside the active site. To probe whether a photoproduct is inside or outside the active site during an insertion step, we have made use of the recently demonstrated preference of T7 DNA polymerase for incorporating pyrene nucleotide opposite abasic sites (*18*).

MATERIALS AND METHODS

Enzymes and Substrates. T4 polynucleotide kinase and [γ-³²P]ATP were purchased from Amersham Corp. (Arlington Heights, IL). Exo⁻ T7 DNA polymerase (Sequenase version 2.0)(*19*) was purchased from either Amersham Corp. or USB. The abasic site models containing 49-mers (abasic-I and abasic-II) were purchased from IDT Inc. (Coraville, IA) and purified by PAGE. Dimers containing 49-mers were prepared as previously described (*20*). Pyrene nucleotide triphosphate was prepared as previously described (*18, 21*). Radioactive bands on the electrophoresis gels were detected with a phosphorimager screen (35 × 43 cm) and a model 425 Molecular Dynamics PhosphorImager and quantified by volume integration with ImageQuant software version 3.3.

Gel Electrophoresis. Reaction products were separated on a two-phase gel comprised of a 20%, 1:19 cross-linked, native polyacrylamide gel in the lower portion of a 400 mm × 300 mm × 0.4 mm gel and a 20%, 1:19 cross-linked 7 M urea denaturing polyacrylamide gel in the top 50 mm of the gel (*22*).

Primer-Extension Reactions with dNTP's. The primers were 5′-end-labeled with [γ-³²P]ATP and T4 polynucleotide

kinase and annealed to 3.3 molar excess template. Extension reactions were carried out with exo⁻ T7 DNA polymerase, 50 nM primer/template, and 500 μM total dNTP's in 6 μL of 40 mM Tris-HCl (pH 7.5), 5 mM MgCl$_2$, 50 mM NaCl, and 5 mM DTT at 37 °C for 1 h. For the cis,syn, trans,syn-I, and abasic analog-containing templates, 5 units (660 nM) of enzyme was used, and for the trans,syn-II, (6−4), and Dewar photoproduct-containing substrates, 13 units (1.8 μM) of enzyme was used. In competition experiments between dATP and dPTP, primer templates were prepared by annealing a 2.5 molar excess of 5′-end-labeled primer to template. Extension reactions opposite the 3′-T were carried out with 3.25 (or 6.5) units of enzyme and 100 μM (or 200 μM) total dNTP concentration in 10 μL of 40 mM Tris-HCl (pH 7.5), MgCl$_2$ (20 mM), NaCl (50 mM), and 5 mM DTT. Extension reactions opposite the 5′-T were carried out under identical conditions except that Mg^{2+} was replaced with 0.6 mM Mn^{2+} to improve the efficiency of extension. Extensions were carried out at 37 °C for 30 min for photoproduct-containing templates or 10 min for abasic site-containing templates. Selectivity factors were calculated from the slope of the line resulting from linear least-squares fit of a plot of the ratio of the sum of the volume integrals corresponding to products from initial insertion by X and Y, versus the ratio of the concentrations of the competing nucleotides X and Y:

$$\frac{(I_{PX} + I_{PXX} + I_{PXY} + ...)}{(I_{PY} + I_{PYY} + I_{PYX} + ...)} = \frac{(k_{cat}/K_M)_X\ [X]}{(k_{cat}/K_M)_Y\ [Y]} \quad (1)$$

RESULTS

Determination of Nucleotide Insertion Selectivity. To obtain experimental support for the mechanism that we had proposed, we needed to compare the selectivity of nucleotide insertion opposite abasic sites and photoproducts. Most often, selectivity is calculated by taking the ratio of the specificity constants (k_{cat}/K_M) or efficiencies (V_{max}/K_M) for each substrate, which is often a time-consuming procedure and subject to propagation of errors. The selectivity can also be more directly determined by examining the products of the reaction in the presence of competing substrates by making use of the following equation (*23*):

$$\frac{v_X}{v_Y} = \frac{(k_{cat}/K_M)_X\ [X]}{(k_{cat}/K_M)_Y\ [Y]} \quad (2)$$

where v_X is the velocity of the reaction for substrate X, and v_Y is the velocity of the reaction for substrate Y. In this case of primer extension opposite the first site, the ratio of the nucleotide insertion velocities, v_X/v_Y, is equivalent to the ratio of the sum of the integrated gel band intensities I of all the products arising from the initial insertion of nucleotides X and Y opposite the first site:

$$\frac{v_X}{v_Y} = \frac{(I_{PX} + I_{PXX} + I_{PXY} + ...)}{(I_{PY} + I_{PYY} + I_{PYX} + ...)} \quad (3)$$

where PX represents the product of primer extension by X. The selectivity is thus equal to the slope of the line obtained from plotting the ratio of the sum of the integrated band intensities versus [X]/[Y]. A direct competition assay has been previously used to verify the fidelity of nucleotide insertion determined from k_{cat} and K_M (*24*).

To be able to use the latter method to determine nucleotide insertion selectivity, we needed to resolve the products of primer extension reactions that have the same length but differ only in sequence by one or more nucleotides. This could be achieved for 14-mer and 15-mer primers with a two-phase gel system developed by Shibutani that consists of a short denaturing section on the top 20% of the gel and a longer native section on bottom 80% of the gel (*22*). The denaturing section serves to dissociate the primer from the template, and the native portion of the gel serves to separate oligodeoxynucleotide differing in both sequence and length. In the two-phase gel used herein, the relative mobilities of 14X-mers differing only in the 3′-terminal nucleotide were in the order X = dC > dA > dT > dG as determined by authentic samples. This is different from what was reported in the original study using different primers, in which the order was X = dC > dT > dA > dG (*22*). Under the conditions of this gel system, primers extended by pyrene nucleotide migrate more slowly than those extended by dG and are only slightly more mobile than the same primers extended by two dA's.

Selectivity of Nucleotide Insertion Opposite the Photoproducts and Abasic Sites. In our previously proposed mechanism, we had proposed that dAMP's are introduced opposite the 3′-T of all the photoproducts via a transient abasic site-like intermediate. If true, we expected that the selectivity for insertion of dAMP opposite the 3′-T of all the photoproducts should be similar to that for insertion opposite an abasic site. Abasic sites that result from loss of a base by glycosidic bond hydrolysis are labile and are prone to isomerization, ring opening, and strand cleavage via β-elimination reactions. As a result, we chose to use a stable tetrahydrofuran analogue ϕ instead (Figure 1) (*25, 26*). To probe the selectivity of nucleotide insertion opposite the 3′-T of the photoproducts or opposite ϕ in place of the 3′-T, primer-extension reactions were carried out with the 14-mer primer in the presence of competing pairs of dNTP's. From experiments with competing pairs of nucleotides, it was clear that dATP was the best substrate and that dGTP was the only other nucleotide that could compete effectively with dATP. To determine the precise selectivity for dATP relative to dGTP, primer-extension reactions were carried out with mixtures ranging in concentration from 100:0 to 0:100 dATP: dGTP (Figure 3). As can be seen in the lanes where a 50/50 ratio of dNTP's is used, there is a visible preference for the insertion of dAMP in preference to dGMP. When the selectivity was calculated as described in the previous section, it was found to vary from 10.6 to 46.8 for insertion opposite the 3′-T of the photoproducts and was 47.5 for insertion opposite the abasic site analogue ϕ at the same site (Table 1). Similar sets of experiments were also carried out for extension of 14A-mer opposite the 5′-T of the photoproducts and opposite the abasic site analogue ϕ at the same site (Figure 4). For these sites, the selectivity was much higher (>100) except for the trans,syn-I dimer, which showed a selectivity of 25.5. For the trans,syn-II dimer, no direct extension by dGMP was observed, instead a band appeared that was consistent with replacement of the terminal dAMP with dGMP, presumably via exonucleolytic or pyrophosphorolytic removal of the dAMP.
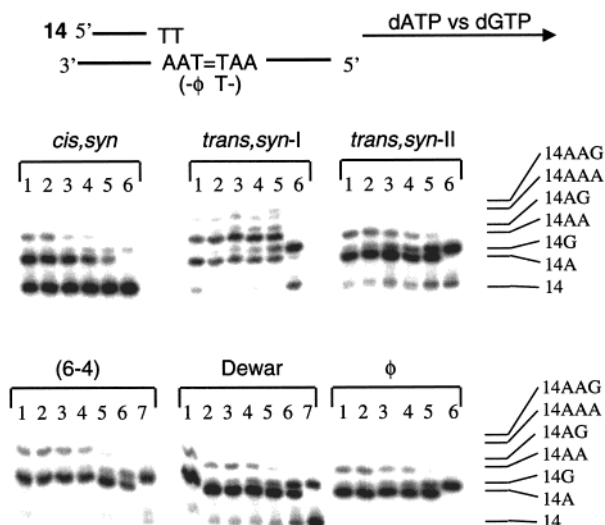
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FIGURE 3: Nucleotide insertion selectivity opposite the 3′-T's of dipyrimidine photoproducts and the corresponding abasic site analogue. Autoradiograms of 20% polyacrylamide gels of the products of 14-mer extension by exo⁻ T7 DNA polymerase for 1 h. For experiments with cis,syn, trans,syn-I, and trans,syn-II photoproducts and the abasic site analogue, lanes 1−6 correspond to dATP:dGTP ratios of 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100, respectively. For experiments with the (6−4) and Dewar photoproducts, lanes 1−7 correspond to ratios of 100:0, 90:10, 70:30, 50:50, 30:70, 10:90, and 0:100, respectively.

Table 1. Selectivity of Nucleotide Insertion Opposite Dipyrimidine Photoproducts and Abasic Sites by Exo⁻ T7 DNA Polymerase by Linear Regression Analysis According to Eq 1

	dAMP vs dGMP		dPMP vs dAMP	
	3′-T	5′-T	3′-T	5′-T
cis,syn	46.8 ± 3.4	>100[a]	15.1 ± 0.8[b]	< 0.01[d]
trans,syn-I	38.4 ± 1.4	24.5 ± 1.6	<1[c]	<0.10
trans,syn-II	14.9 ± 0.6	>100[a]	21.4 ± 0.8[b]	<0.01[d]
(6−4)	11.7 ± 0.6	>100[a]	16.9 ± 0.8[b]	<0.01[d]
Dewar	10.6 ± 0.7	>100[a]	24.9 ± 0.5[b]	<0.01[d]
abasic model	47.5 ± 2.3	105.7 ± 8.6	57 ± 7[b]	390 ± 35[b]

[a] No incorporation of dGMP was observed opposite the 5′-T of the photoproducts. [b] Selectivity determined for ratios of dPTP:dATP from 100:0 to 60:40 due to deviations from expected behavior for some substrates at a 50:50 ratio. [c] The plot of the data did not have a zero intercept as expected for eq 1. [d] No incorporation of dPMP was observed opposite the 5′-T of the photoproducts in the competition experiment.

Selectivity of Pyrene Nucleotide Insertion Opposite Photoproducts. In our previously proposed mechanism, we had proposed that insertion of dAMP opposite the 3′-T of the photoproducts occurred with the photoproduct outside of the active site and opposite the 5′-T with the photoproducts inside the active site. To probe whether the photoproduct was inside or outside the active site during each of these steps, we made use of the recently discovered preference for the insertion of pyrene nucleotide opposite abasic sites and analogues by KF and T7 DNA polymerase (*18*). If the photoproduct is outside the active site during an insertion step, we expected that dPMP would be preferentially inserted over dAMP, whereas if it was inside the active site we expected that dAMP would be preferentially inserted over dPMP. Primer insertion opposite the 3′-T and corresponding abasic site model were carried out with the 14-mer primer and mixtures ranging from 50:50 to 100:0 dATP:dPTP (Figure 5). With the exception of the trans,syn-I isomer, dPMP was prefer-
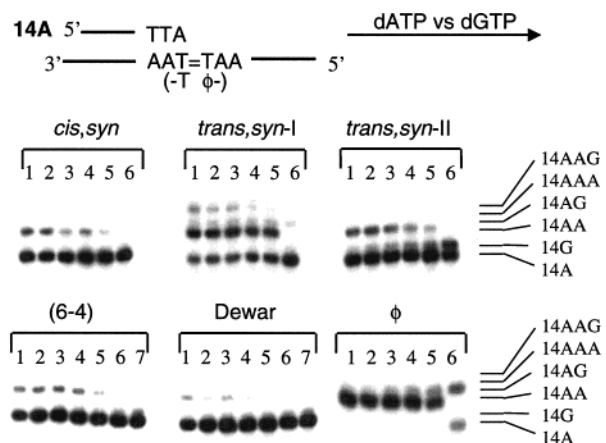

FIGURE 4: Nucleotide insertion selectivity opposite the 5′-T's of the dipyrimidine photoproducts and the corresponding abasic site analogue. Autoradiograms of 20% polyacrylamide gels of the products of DNA 14A-mer extension by exo⁻ T7 DNA polymerase for 1 h. For experiments with cis,syn, trans,syn-I, and trans,syn-II photoproducts, lanes 1−6 represent dATP:dGTP ratios of 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100, respectively, at a total concentration of 100 μM. For experiments with the (6−4) and Dewar photoproducts, lanes 1−7 correspond to ratios of 100:0, 90:10, 70:30, 50:50, 30:70, 10:90, and 0:100, respectively.
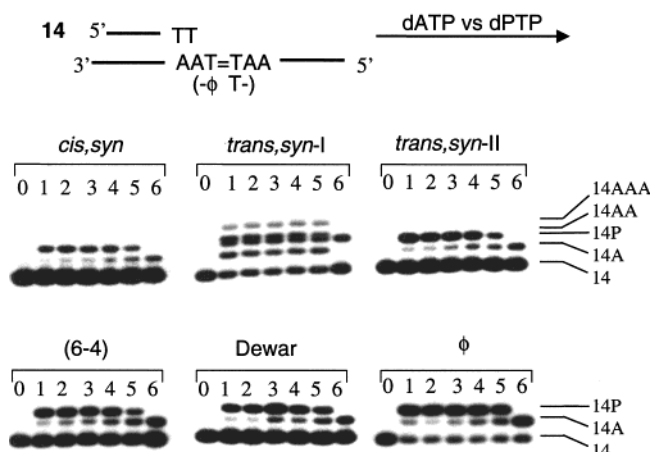
FIGURE 5: Selectivity of A vs P insertion opposite the 3′-T of photoproducts and the corresponding abasic site analogue. For experiments with cis,syn, trans,syn-II, (6−4), and Dewar photoproducts and the abasic site analogue, lanes 1−6 correspond to dPTP:dATP ratios of 50:50, 40:60, 30:70, 20:80, 10:90, and 0:100, respectively. For experiments with the trans-syn-I dimer, lanes 1−6 correspond to ratios of 50:50, 60:40, 70:30, 80:20, 90:10, and 100:0 respectively, at a total concentration of 100 μM.

entially inserted by greater than 12:1 relative to dAMP (Table 1). Primer insertion opposite the 5′-T of the photoproducts and corresponding abasic site analogue were carried out in a similar fashion with the 14A-mer primer, except that we replaced Mg²⁺ with Mn²⁺ (*27*) to increase the efficiency of extension opposite the photoproducts (Figure 6). The result, however, was the same as that observed with Mg²⁺. In this case, dAMP was preferentially inserted opposite the 5′-T of all the photoproducts, whereas dPMP was preferentially inserted opposite the abasic site analogue.

DISCUSSION

In support of our proposed mechanism for how T7 polymerase incorporates dAMP opposite both T's of dipyrimidine photoproducts, in which the photoproducts are
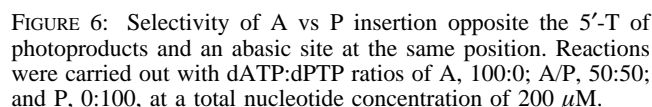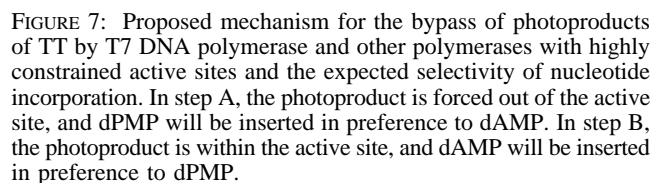
FIGURE 6: Selectivity of A vs P insertion opposite the 5′-T of photoproducts and an abasic site at the same position. Reactions were carried out with dATP:dPTP ratios of A, 100:0; A/P, 50:50; and P, 0:100, at a total nucleotide concentration of 200 μM.

FIGURE 7: Proposed mechanism for the bypass of photoproducts of TT by T7 DNA polymerase and other polymerases with highly constrained active sites and the expected selectivity of nucleotide incorporation. In step A, the photoproduct is forced out of the active site, and dPMP will be inserted in preference to dAMP. In step B, the photoproduct is within the active site, and dAMP will be inserted in preference to dPMP.

outside the active site during insertion opposite their 3′-T's (Figure 7A) but inside during insertion opposite their 5′-T's (Figure 7B), we have shown the following: (i) that dAMP is preferentially inserted relative to all other nucleotides of DNA opposite abasic site analogues; (ii) that dPMP is preferentially inserted relative to dAMP opposite abasic site models and the 3′-T of dipyrimidine photoproducts with only one exception; and (iii) that dAMP is preferentially inserted relative to dPMP opposite the 5′-T of all the dipyrimidine photoproducts. Below we now discuss these results in more detail and their implications.

Nucleotide Insertion Selectivity Opposite Abasic Site Models. We had previously proposed that dAMP is preferentially inserted opposite the 3′-T of the photoproducts over the other three nucleotides of DNA because of the intermediacy of a transient abasic-like site. In support of this proposal, we have found that T7 DNA polymerase, like many other polymerases, does indeed obey what has been termed the "A rule" (*28*) and preferentially incorporates dAMP opposite the tetrahydrofuran abasic site analogue φ in competition with dCMP, dGMP, and dTMP. What is most interesting, however, is that the observed selectivities of 48 and 106 for inserting dAMP relative to dGMP opposite φ in

the position of the 3′-T or the 5′-T by T7 DNA polymerase are about 10-fold higher than what has been reported for other polymerases. Selectivities of 5.0 (*29*), 6.4 (*30*), and 10 (*18*) have been reported for Klenow fragment (KF) in different sequence contexts, 5.0 for HIV RT, and from 5.7 to 11.1 for *Drosophila* pol α in different sequence contexts (*31*). Calf thymus polymerase δ has also been found to prefer to insert dAMP opposite the tetrahydrofuran abasic site model (*32*). The large difference in selectivity observed for T7 DNA polymerase relative to the other polymerases is not likely to be due to the particular sequence context of the abasic site analogues, as judged from competition experiments that were also carried out with exonuclease deficient KF. With this enzyme, selectivity values of 12.1 and 5.1 were observed for the abasic site models in the position of the 3′-T and 5′-T, respectively, which are similar in value to those reported for other sequence contexts (data not shown).

T7 DNA polymerase has also been shown in this and a previous study (*18*) to follow the "P-rule", in which pyrene nucleotide, dPMP, is preferentially inserted relative to all four nucleotides of DNA opposite abasic sites and analogues. The precise selectivity, however, appears to be sequence context dependent, being quite high for insertion opposite the abasic site model φ in the position of the 5′-T but about 7-fold lower for that in the position of the 3′-T. The selectivity of 390 observed for dPMP insertion relative to dAMP opposite the abasic site analogue at the 5′-T position by T7 DNA polymerase is quite similar to that of 264 observed for insertion by KF opposite the same abasic site analogue in a different sequence context (*18*). It is possible that the diminished selectivity observed opposite the 3′-site is due to insertion of dAMP via a competitive slippage mechanism of the type observed for polymerase β (*33*). In this mechanism, the nucleotide following the abasic site, which in this case is a T, serves to template the addition of the nucleotide. It is also possible that sequence context affects the selectivity in some other way, such as π-stacking. In support of this notion, the selectivity of dPMP addition relative to dAMP opposite the 3′-T of the photoproducts, which cannot undergo the same type of slippage as an abasic site, is also fairly low and similar to that for the abasic site model at this same site. Recently, it has been found that 5′-pyrene nucleotide shows a preference for stacking upon nucleotides in the order dA > dG ≥ T = C (*34*).

Nucleotide Insertion Specificity Opposite the 3′-T of the Dipyrimidine Photoproducts. According to our proposed model, insertion opposite the 3′-T of the photoproducts occurs via a transient abasic site-like intermediate with the photoproduct outside of the active site. If true, we expected that T7 DNA polymerase would show a similar preference for inserting dAMP relative to dGMP opposite the 3′-T of all the photoproducts as was observed for the abasic site model (A rule). While the preference was the same, we were surprised to find that the precise selectivity for inserting dAMP relative to dGMP opposite the 3′-T of the photoproducts depended on the structure of the photoproduct and varied 4.4-fold. The selectivity was highest and most similar to that for the abasic site model for the cis,syn dimer and lowest for the Dewar photoproduct. These differences might be the result of the way that each photoproduct disrupts the active

site of the polymerase when it is forced out, perhaps by altering the conformation of the primer terminus and/or the protein.

Our proposed mechanism also requires that T7 DNA polymerase would show a similar preference for inserting pyrene nucleotide (dPMP) relative to dAMP opposite the 3′-T of the photoproducts as was observed for the abasic site model (P rule). With the exception of the trans,syn-I dimer, the preference for insertion opposite the 3′-T of the photoproducts was similar but less in magnitude to that of 57 for the abasic site analogue, and the precise selectivity varied from 15 to 25. The selectivity for dPMP insertion was highest and most similar to that of the abasic site analogue for the Dewar product and lowest for the cis,syn dimer. The variation of the selectivity is different from that observed for the insertion of dAMP relative to dGMP and suggests that distortions induced by the photoproducts might have different effects on different incoming nucleotides. In contrast, insertion opposite the trans,syn-I dimer appeared to have the opposite selectivity, although the precise selectivity could not be calculated because a plot of the data according to eq 1 resulted in an intercept that was significantly different from the expected value of zero. The unusual behavior of this substrate might be due in part to the *syn*-glycosyl conformation of the 5′-T of this photoproduct, whereas all the other photoproducts studied have 5′-T's in the anti conformation. The syn conformation might enable the trans,syn-I photoproduct to occasionally fit into the active site during nucleotide insertion opposite the 3′-T, which would prevent the insertion of pyrene nucleotide. The preference for incorporating dAMP opposite the 3′-T of the trans,syn-I dimer in this case is also consistent with its structure, in that the 3′-T is similar in structure and glycosyl bond orientation to a normal thymine.

Nucleotide Insertion Specificity Opposite the 5′-T of the Dipyrimidine Photoproducts. According to our proposed model, insertion opposite the 5′-T of the photoproducts occurs with the photoproducts inside the active site, so that the 5′-T could influence the selection of the nucleotide to be inserted. If true, we expected that T7 DNA polymerase would prefer to incorporate dAMP relative to dGMP opposite the 5′-T of all the photoproducts with the exception of the trans,syn-I dimer, because in principle the 5′-T of these products are all similar in structure and glycosyl bond orientation to a normal thymidine. The trans,syn-I dimer, however, has the 5′-T locked in a syn glycosyl conformation and would not necessarily be expected to direct the insertion of dAMP via a standard Watson−Crick A−T-like base pair. In support of this proposition, the selectivity for incorporating dAMP relative to dGMP was greater than 100 for all the photoproducts except for the trans,syn-I isomer, which showed a much lower selectivity of 24.5.

Our proposed mechanism also requires that T7 DNA polymerase would show a much greater preference for incorporating dAMP relative to dPMP opposite the 5′-T of the photoproducts, as pyrene should not be able to fit opposite a photoproduct that is in the active site. This appears to be the case for all the photoproducts as judged by the competition assay in the presence of equimolar dATP and dPTP (Figure 6). Incorporation of dPMP was very inefficient opposite the 5′-T's of the photoproducts, even in the absence of competing dATP, except for the trans,syn-I dimer. The

more facile incorporation of dPMP opposite the trans,syn-I dimer might be due to the unusual syn glycosyl conformation of the 5′-T, which might make it possible to accommodate pyrene nucleotide in the active site in the presence of the dimer.

Instructional vs Non-Instructional Damage. There have been many attempts in the past to classify DNA damage as either non-instructional or instructional (misinstructional) to account for the specificity of nucleotide insertion opposite the damage site (*5, 35−37*). Whether or not DNA damage has the possibility of directing the insertion of a nucleotide, however, depends on whether it is present in the active site during the insertion step. Now that it is apparent that bypass of dipyrimidine photoproducts can occur by at least two distinct classes of mechanisms, it would seem important to reconsider the instructional properties of photoproducts within the context of the bypass mechanism and polymerase involved. In one class of bypass mechanism, typified by T7 DNA polymerase, we have established that the photoproduct is outside the active site during insertion opposite the 3′-pyrimidine (Figure 7A) and cannot directly influence the selection of the nucleotide to be inserted. In the subsequent insertion step opposite the 5′-pyrimidine, however, we have shown that the photoproduct is in the active site (Figure 7B) and can influence the selection of the nucleotide to be inserted. For this type of bypass mechanism, the 3′-pyrimidine can be considered to be non-instructional, and the 5′-T can be considered to be instructional. It has been argued, however, that no type of lesion can be truly non-instructional, as it may still influence the selection of nucleotides by other, less direct effects (*28*). In this regard, we found that the precise selectivity for nucleotide insertion opposite the 3′-T of the photoproducts does depend on their structure, even though all the evidence points to their being outside the active site.

In another class of bypass mechanism, typified by *E. coli* pol V, there is much evidence to suggest that the photoproduct is in the active site during insertion opposite the 3′-pyrimidine (Figure 2A), because the photoproduct is able to influence the selection of the nucleotide to be inserted (see discussion in the introduction). At the moment, there is no evidence to suggest that the photoproduct is not in the active site during the insertion step opposite the 5′-T and cannot also influence the selection of the nucleotide to be inserted. For this type of bypass mechanism, both pyrimidines of a dipyrimidine photoproduct can be considered to be instructional. Thus, it would appear that one cannot broadly classify a photoproduct or any other type of damage as being either instructional or non-instructional, and one must take into account the bypass mechanism involved. One could imagine that other types of bulky DNA damage might also show dual behavior that is similar to dipyrimidine photoproducts, such as cisplatin and psoralen monoadducts. Cisplatin adducts of GG sites are similar in structure to dipyrimidine photoproducts in that these two adjacent bases are covalently linked together and thus might not be able to fit into an active site typified by T7 DNA polymerase during insertion opposite the first of these bases. A psoralen monoadduct of T might also not be expected to fit because of the large steric hindrance caused by the attachment of the psoralen to the 5,6 double bond of the T via a cyclobutane ring.

Implications for the Active Sites of Replicative and Damage Bypass Polymerases. There are now a number of crystal structures of complexes of primer templates with polymerases involved in replication and short patch repair, such as T7 DNA polymerase (*17*), pol β (*38*), Taq (*39*), and *Bacillus stearothermophilus* pol I (*40*). All these polymerases create a sterically demanding active site for nucleotide addition by flanking one side of the incipient base pair with a protein wall that excludes the next nucleotide in the template from the active site. This protein wall forces the two template bases apart and causes a characteristic bend in the template. It makes sense that such an active site would have evolved for such polymerases, whose major role is the high fidelity replication of DNA. When DNA damage is encountered that cannot fit into the active site, the rate of synthesis decreases dramatically, as found for the rate of photoproduct bypass by T7 polymerase (*12*) and *E. coli* polymerase III (*11*). This would cause DNA synthesis to terminate and minimize the chance of mutagenesis that might result from trans-lesion synthesis. On the other hand, the role of damage bypass polymerases, typified by *E. coli* pol V, is to be able to synthesize past DNA damage, and as a result one would expect that their active sites would have evolved to accommodate a wide variety of damage. Unlike *E. coli* pol V, which can synthesize past both cis,syn and (6−4) photoproducts (*11*), the related DNA damage bypass polymerases yeast pol η (*41*), yeast pol ζ (*42*), and human polymerase η (*43*) have only been found to synthesize past cis,syn photoproducts. Although it cannot bypass a (6−4) photoproduct, human pol η has recently been found to be able to bypass cisplatin adducts, which can also be classified as sterically demanding or bulky types of damage (*44*).

Conclusion. We have demonstrated that bypass of a number of dipyrimidine photoproducts by the replicative polymerase of T7 phage occurs by way of a transient abasic site-like intermediate during insertion opposite the 3′-pyrimidine, but not the 5′-pyrimidine, which results from an inability to accommodate the photoproduct in the active site. This type of bypass mechanism is distinctly different from that of the DNA damage bypass polymerase V of *E. coli*, which appears to be able to accommodate the photoproducts in the active site during insertion opposite both pyrimidines. We were able to demonstrate the involvement of a transient abasic site-like intermediate in the bypass of the photoproducts by T7 DNA polymerase by making use of its preference for inserting pyrene nucleotide opposite abasic sites (P rule). As a result, we expect that pyrene nucleotide should be generally useful for probing the mechanism of DNA damage bypass by other polymerases that also follow the P rule. Furthermore, by examining the preference for pyrene nucleotide insertion opposite a cis,-syn thymine dimer, it should be possible to determine whether the template in the active sites of such polymerases is linear or bent.

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