Yeast Pol η Holds a Cis−Syn Thymine Dimer Loosely in the Active Site during Elongation Opposite the 3′-T of the Dimer, but Tightly Opposite the 5′-T[†]

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ABSTRACT: Polymerase η is a member of the Y family of DNA polymerases which is able to bypass thymine dimers efficiently and in a relatively error-free manner. To elucidate the mechanism of dimer bypass, the efficiency of dAMP and pyrene nucleotide insertion opposite the thymine dimer and its N3-methyl derivatives was determined. Pol η inserts pyrene nucleotide with greater efficiency than dAMP opposite the 3′-T of an undimerized or dimerized T and is an effective inhibitor of DNA synthesis by pol η. Substitution of the N3H of the 3′-T of an undimerized T or a dimerized T with a methyl group has little effect on the insertion efficiency of pyrene nucleotide but greatly inhibits the insertion of dAMP. Together, these results suggest that the error-free insertion of dAMP opposite the 3′-T of the cis−syn thymine dimer happens by way of a loosely held dimer in the active site which can be displaced from the active site by pyrene nucleotide. In contrast, pol η cannot insert pyrene nucleotide opposite the 5′-T of the dimer, whereas it can insert dAMP with efficiency comparable to that opposite the 3′-T. The inability to insert pyrene nucleotide opposite the 5′-T of the dimer is consistent with the idea that while the polymerase binds loosely to a templating nucleotide, it binds tightly to the nucleotide to its 3′-side. Overall, the results show a marked difference from similar studies on pol I family polymerases, and suggest mechanisms by which this Y family polymerase can process damaged DNA efficiently.

Cis-syn cyclobutane pyrimidine dimers (CPD's)[1] are the major photoproducts of DNA produced by the UV component of sunlight (*1, 2*). If left unrepaired, these photoproducts can block DNA replication. In response, living systems have evolved a number of different pathways to bypass DNA photoproducts (*3, 4*). In *Saccharomyces cerevisiae*, two major pathways have been discovered, one of which is catalyzed by pol ζ, which forms by the association of Rev3 and Rev7 proteins (*5*). Pol ζ is capable of limited translesion synthesis over a cis−syn thymine dimer in vitro and has been demonstrated to be involved in the error-prone lesion bypass of UV-induced damage by genetic studies. The alternative pathway is catalyzed by pol η, which is encoded by the Rad30 gene. Yeast pol η can efficiently and accurately bypass a cis−syn thymine dimer by incorporating two A's across from the two T's of the dimer (*6, 7*). Recently, pre-steady-state kinetic studies have shown that pol η utilizes an induced fit mechanism to insert nucleotides opposite

undamaged DNA, much like the Klenow fragment or T7 DNA polymerase (*8*). Despite all these studies, the mechanism by which pol η selects A's for insertion opposite the cis−syn thymine dimer is not known.

Previously we have proposed models for the active sites of replicative and DNA damage bypass polymerases to account for the specificity of nucleotide insertion opposite dipyrimidine photoproducts (Figure 1) (*9*). Crystal structures of polymerases involved in the replication of DNA, such as T7 DNA polymerase, reveal that these enzymes have highly constrained active sites which cannot accommodate dipyrimidine photoproducts during insertion opposite the 3′-nucleotide. It was therefore proposed that insertion of a nucleotide opposite the 3′-T of a dipyrimidine photoproduct must take place via a transient abasic site-like intermediate and that the specificity of nucleotide insertion would be the same as for an abasic site (*10*). The existence of a transient abasic site-like intermediate was confirmed for T7 DNA polymerase by the preferred addition of pyrene nucleotide opposite the 3′-T of a number of different dipyrimidine photoproducts (*9*). Pyrene is a large flat aromatic molecule that cannot fit opposite a normal nucleotide, but can be preferentially inserted opposite abasic sites by both *E. coli* pol I and T7 DNA polymerase (*11*).

Unlike replicative polymerases, some DNA damage bypass polymerases appear to be able to accommodate dipyrimidine photoproducts in their active sites during elongation opposite the 3′-base (*9*). In support of this idea, A has been found to be preferentially incorporated opposite the 3′-T of a cis−

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[1] Abbreviations: cis−syn dimer, cis−syn cyclobutane pyrimidine dimer; dNMP, deoxynucleoside monophosphate; dPMP, pyrene deoxynucleoside monophosphate; dNTP, deoxynucleoside triphosphate; dPTP, pyrene deoxynucleoside triphosphate; pol η, polymerase eta.
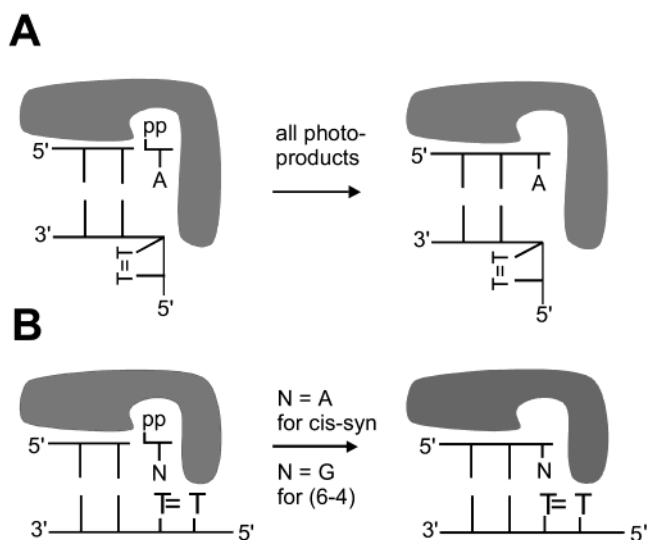
A

B

FIGURE 1: Models for bypass of dipyrimidine photoproducts by two classes of DNA polymerases. (A) Replicative polymerases such as T7 DNA polymerase which bind to a bent template and exclude dipyrimidine photoproducts from the active site during insertion opposite the 3′-T. (B) DNA damage bypass polymerases such as *E. coli* pol V which presumably can bind linear templates and do not exclude the pyrimidine dimer from the active site.

syn dimer in *Escherichia coli* under SOS conditions, whereas G is incorporated opposite the 3′-T of a (6−4) photoproduct (*12−14*). The same selectivity of nucleotide incorporation has been observed to occur with Pol V, the enzyme that has been established to be involved in the bypass reaction (*15*). More significantly, it has been found that in *E. coli* under SOS conditions, G is incorporated opposite the 3′-C of the cis−syn dimer of TC, whereas A is incorporated opposite the 3′-T of the cis−syn dimer of TT (*16*).

In yeast and human cells, bypass of cis−syn dipyrimidine photoproducts is thought to be carried out by pol η. Unlike pol V, pol η appears to be only able to bypass cis−syn thymine dimer, and not other dipyrimidine photoproducts such as the (6−4) photoproduct (*17−19*), though like pol V it inserts a G opposite the 3′-T of the TT (6−4) product (*18, 19*). With respect to its limited bypass ability, pol η behaves more like T7 DNA polymerase, which bypasses the cis−syn dimer more easily than the other classes of photoproducts. To characterize the active site of pol η and to elucidate its bypass mechanism, we have investigated the nucleotide insertion selectivity of dPTP versus dATP opposite an undamaged TT site, its cis−syn cyclobutane dimer, and their N³-methyl derivatives. On the basis of these studies, we conclude that yeast pol η loosely holds a cis−syn thymine dimer during nucleotide insertion opposite the 3′-base, but holds it tightly during insertion opposite the 5′-base. The results suggest marked differences in active site structure and substrate interactions compared to previous results with high-fidelity enzymes of the Pol I family.

MATERIALS AND METHODS

Substrates and Enzymes. T4 polynucleotide kinase and [γ-³²P]-ATP were purchased from Amersham Pharmacia Biotech. Exo⁻ T7 DNA polymerase (Sequenase version 2.0) and exo⁻ Klenow fragment were purchased from USB. Undamaged ODNs and the abasic site analog-containing 50-mer were purchased from IDT. Deoxynucleotide triphos-

phates were purchased from Promega, and dPTP was prepared as previously described (*11, 20*). Yeast pol η with a His₆ tag on its N terminus was purified from yeast as previously described (*21*).

Dimer-Containing Substrates. The cis−syn thymine dimer-containing substrates were prepared via automated DNA synthesis using a cis−syn thymine dimer building block (*22, 23*). N3-methylated T-containing ODNs were prepared by automated synthesis with the β-cyanoethyldiisopropyl-phosphoramidite of N³-methyl thymidine that was prepared by a standard method (*24*) from 5′-dimethoxytrityl N³-methyl-thymidine (*25, 26*) which was prepared by methylation of thymidine followed by dimethoxytritylation. N3-methyl-thymidine is commercially available (ICN Pharmaceuticals, Inc. Costa Mesa, CA). The N3-methylated analogues of the cis−syn thymine dimer were prepared by triplet sensitized irradiation of d(GAGTATTATGAGA) containing N3-methylated thymidine at either or both the 5′-T and 3′-T, followed by reverse phase HPLC purification. The products were characterized by a recently developed enzyme coupled mass spectrometry assay for DNA photoproducts (*27*) and ligated into the 50-mer templates according to previously described methods (*28*).

Primer Extension Assays. Primers were 5′-end labeled with [γ-³²P]-ATP by T4 polynucleotide kinase and annealed to 1.5 molar excess templates by heating at 95 °C for 5 min and cooling to 25 °C over 2 h. For standing and running start bypass reactions, all four dNTPs (125 μM ea) were incubated with 10 nM primer/template and 15 ng pol η in 10 μL of 40 mM Tris·HCl (pH 7.5), 6 mM MgCl₂, 5 mM DTT and 0.1 mg/mL BSA. After 30 min at 37 °C, the reaction was quenched by an equal volume of 95% forma-mide quench buffer with 4 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue. In direct competition assays, 10 nM primer/template was incubated with 1.5 ng pol η and 20 μM of a mixture of dATP and dPTP and quenched after 5 min incubation at RT. Reaction products from a direct competition assay were separated on a 20% two-phase polyacrylamide gel as previously described (*9*). The selectiv-ity for insertion opposite the 3′-T was then obtained as the slope of a linear least-squares fit of a plot of the ratio of products resulting from initial elongation of the 14-mer primer with dAMP and dPTP as a function of the ratio of [dPTP] to [dATP]:

$$S = \frac{(V_{max}/K_M)_A}{(V_{max}/K_M)_P} = \frac{[14A + 14AA + 14AP...]}{[14P + 14PA + 14PP...]} \cdot \frac{[dPTP]}{[dATP]}$$

Single nucleotide insertion reactions were carried out as previously described (*29*) with 10 nM primer/template for 5 min at RT in the presence of 0−500 μM dATP or dPTP and 1.5 ng pol η in total volume of 10 μL. The reaction mixtures were electrophoresed on a 15% polyacrylamide gel contain-ing 7 M urea and then exposed to a Kodak 35 × 43 cm phosphorimager screen. The screen was scanned by a model 425 Molecular Dynamics Phosphorimager or a BioRad Personal FX phosphorimager, and radioactivity in the bands was quantified by volume integration with ImageQuant software version 3.3 (Molecular Dynamics) or Quantity One software (BioRad). Plots of the initial insertion velocity versus dNTP concentration were fitted to a Michaelis Menten equation by a nonlinear least-squares method using Kalaida-
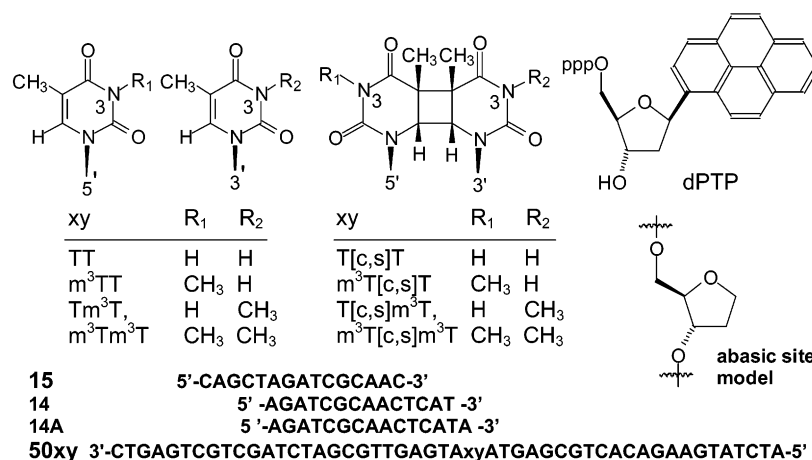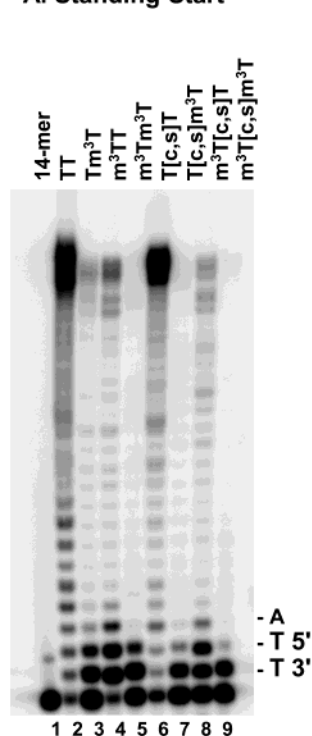
FIGURE 2: Substrates used in this study.

Graph software. Selectivity was calculated as the ratio of insertion efficiency (V_{max}/K_M) of dATP and dPTP.

RESULTS

Experimental Design. To probe the active site and cis−syn dimer bypass mechanism of yeast pol η, we have investigated the selectivity and efficiency of dAMP and pyrene deoxynucleoside monophosphate (dPMP) insertion opposite an undimerized and a dimerized TT site and their N3-methyl derivatives (Figure 2). Pyrene nucleotide was chosen to probe whether the dimer was in the active site during insertion because of its known preference for insertion opposite abasic sites (*11*) and abasic site-like intermediates in the bypass of DNA photoproducts (*9*) by pol I family polymerases. Whether or not pyrene nucleotide would behave in a similar fashion with pol η was unknown at the time. As an alternative probe of whether a thymine dimer is within the active site during an insertion step, we also decided to investigate the selectivity and efficiency of nucleotide insertion opposite N3-methylated derivatives of undimerized and dimerized TT sites. If a T is within the active site, insertion of A should be greatly inhibited by methylation of the N3 position, whereas it should have no effect if the T is outside the active site.

Ability of Pol η to Bypass N-Methyl Thymine and Thymine Dimer-Containing Substrates. The ability of yeast pol η to synthesize past the various DNA modifications used in this study was first investigated under standing-start (immediately before the dimer, panel A) and running-start (four nucleotides before the dimer) conditions in the presence of all dNTPs (Figure 3). This was done to see whether there is any difference in nucleotide insertion and bypass efficiency between a polymerase that is prebound to the dimer site and one that is actively replicating when it encounters the dimer site. No significant difference in the termination and bypass product distribution is seen under the two conditions, though significant amounts of primer extension product terminating opposite the A to the 3′-side of the 3′-methylated substrates can be seen in the running start reactions (lanes 3, 5, 7, and 9) which are not readily apparent in the standing start reactions. In addition to the well-known ability of pol η to bypass of a cis−syn thymine dimer, pol η can also be seen to synthesize past a single N3-methylated thymine or thymine dimer, though the amounts of full-length bypass product
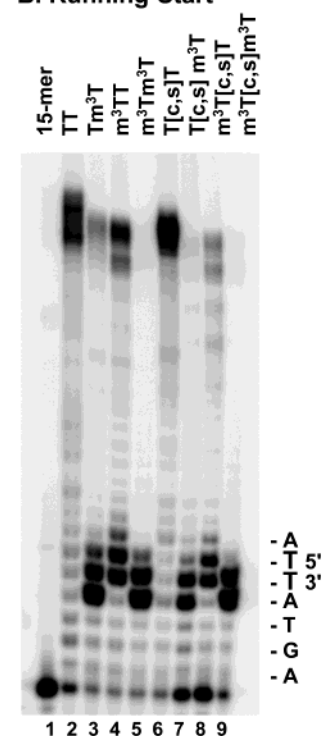

FIGURE 3: Standing start and running start DNA synthesis opposite templates containing unmethylated and N3-methylated TT sites and cis−syn TT dimer sites by yeast pol η. Reactions were conducted with 20 nM pol η, 125 μM each dNTP, and 10 nM 14-mer or 15-mer primer/50-mer template for 30 min at 37 °C.

depended on the site of N3-methylation. For substrates containing two N3-methyl thymines, there was almost no full-length bypass products, and only primer extension up to the adenine to 5′-side of the thymine dimer is observed.

Selectivity of Nucleotide Insertion by a Direct Competition Assay. To determine directly the selectivity of dPMP insertion relative to dAMP, we made use of a competition assay in which the extension products were quantified as a function of varying ratios of dATP to dPTP (Figure 4, Table 1) (*9*). The advantage of this assay is that selectivity can be directly determined without the need for independent measurements of K_M and V_{max} for each competing nucleotide. The competition assay shows that pyrene nucleotide is preferentially inserted by yeast pol η opposite the 3′-T of all the substrates used in this study, including the undimerized

Table 1: Michaelis Menten Parameters and Selectivities of Nucleotide Insertion Opposite the 3'-T of the Unmethylated and Methylated TT and cis−syn TT Dimer Sites (T[c,s]T)

template (5'-3')	dNTP	V_{max} (nM/min)	K_m (μM)	V_{max}/K_m	relative efficiency	F^a	F^b
T<u>T</u>	dATP	0.082 ± 0.007	2.0 ± 0.6	0.042 ± 0.013	1.0	4 ± 2	74 ± 3
	dPTP	0.14 ± 0.01	0.8 ± 0.4	0.17 ± 0.07	4.0		
Tm³<u>T</u>	dATP	0.035 ± 0.005	340 ± 90	0.0001 ± 0.00003	0.0024	1270 ± 630	5190 ± 330
	dPTP	0.064 ± 0.006	0.5 ± 0.2	0.13 ± 0.05	3.1		
m³T<u>T</u>	dATP	0.12 ± 0.02	2.8 ± 1.5	0.043 ± 0.024	1.0	3.3 ± 2.5	168 ± 6
	dPTP	0.14 ± 0.02	1.0 ± 0.50	0.14 ± 0.073	3.3		
T[c,s]<u>T</u>	dATP	0.034 ± 0.005	11 ± 4	0.003 ± 0.001	0.07	32 ± 19	166 ± 10
	dPTP	0.12 ± 0.01	1.2 ± 0.6	0.10 ± 0.05	2.3		
T[c,s]m³<u>T</u>	dATP	0.010 ± 0.001	120 ± 40	0.00009 ± 0.00003	0.0021	860 ± 410	8110 ± 330
	dPTP	0.12 ± 0.01	1.7 ± 0.6	0.08 ± 0.03	1.8		
m³T[c,s]<u>T</u>	dATP	0.059 ± 0.005	3.7 ± 1.8	0.016 ± 0.008	0.4	12 ± 7	21 ± 0.5
	dPTP	0.090 ± 0.006	0.48 ± 0.16	0.19 ± 0.06	4.5		
Ta<u>b</u>	dPTP	0.23 ± 0.02	0.48 ± 0.17	0.48 ± 0.17	11	-	-

[a] Selectivity $F = (V_{max}/K_m)_{dPTP}/(V_{max}/K_m)_{dATP}$. [b] Selectivity F from competition experiment.

FIGURE 4: Direct competition assay between dAMP and dPMP insertion opposite the 3'-T of unmethylated and methylated TT and cis−syn TT dimer sites. Reactions were conducted with 2 nM pol η and 10 nM 14-mer primer/50-mer template at room temperature for 5 min with 20 μM total dPTP and dATP in the following ratios for lanes 1−10 of: 0:100, 1:99, 5:95, 10:90, 20:80, 30:70, 40:60, 50:50, 75:25, and 100:0.
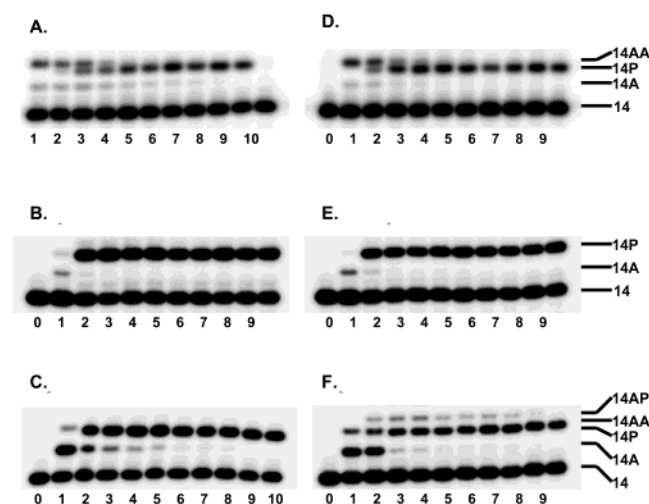

FIGURE 5: Direct competition assay between dAMP and dPMP insertion opposite the 5'-T of unmethylated and methylated TT and cis−syn TT dimer sites. Reactions were carried out with 2 nM pol η, 10 nM 14A-mer primer/50-mer template for 10 min at RT. Lanes 1−5 correspond to ratios of 100:0, 70:30, 50:50, 30:70, and 0:100, respectively, at a total concentration of 100 μM for the unmethylated dimer and 500 μM for the 5'-methylated dimer.

FIGURE 6: Effect of dPTP on synthesis by pol η and Klenow fragment opposite an undamaged template. Reactions were conducted with 5 nM pol η, 2.5 units of KF, 10 nM 14-mer primer/50-mer primer, 100 μM each dNTP, at RT for 15 min.

sites. This can be easily appreciated by examining lane 8 of each panel in Figure 4 in which both nucleotides are present in equal concentration, but the exclusive or major bands result from insertion of pyrene nucleotide opposite the 3'-base of the dimer. At higher ratios of dATP:dPTP, dAMP can be seen to be inserted in competition with dPMP allowing selectivities of dPMP:dAMP insertion opposite an unmethylated 3'-T (panels A, C, D, and F of Figure 4) to be calculated to be in the range of 21−168 (Table 1). Methylation of the N3 position of the 3'-T, however, caused a more than 70-fold increase in the dPMP:dAMP insertion selectivity opposite the undimerized T's and about 50-fold increase for the dimerized T's. In contrast to what was observed opposite the 3'-T of the dimer, only dAMP could be inserted opposite the 5'-T of the dimer (Figure 5), and neither dAMP nor dPMP could be inserted opposite a 5'-methylated dimer. Synthesis by pol η opposite a normal template was also found to be greatly inhibited by low concentrations of pyrene nucleotide in comparison to the exonuclease-deficient Klenow fragment (Figure 6), which is known not to selectively insert pyrene nucleotide opposite normal bases as efficiently as abasic sites. In the presence of dPTP, termination products
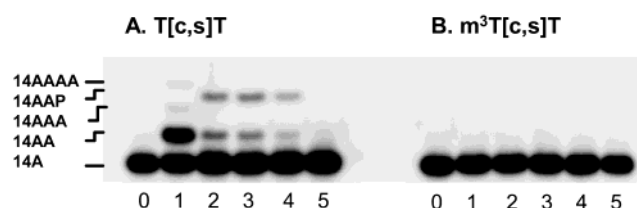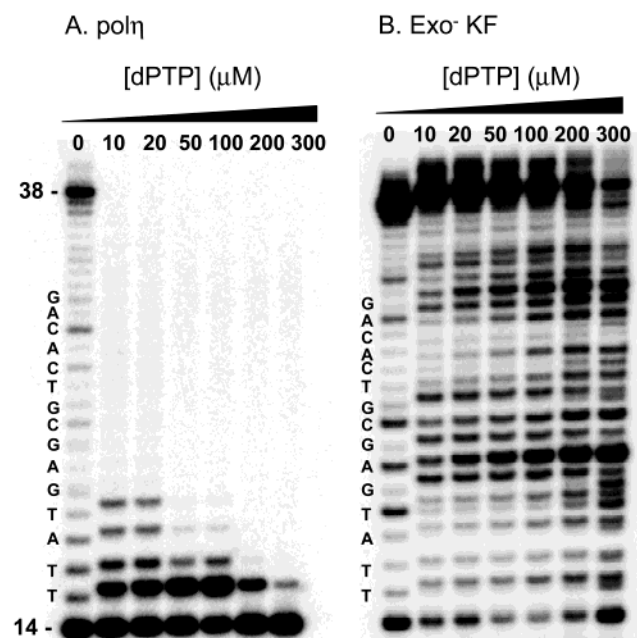
with altered mobility relative to those formed in its absence are observed with both pol η and Klenow fragment that are consistent with the insertion of dPMP. Exactly, how dPTP is inhibiting primer extension is currently under study.

Efficiency of dPMP and dAMP Insertion Opposite the Undimerized and Cis−Syn Dimer of TT and their N-Methyl

Table 2: Michaelis Menten Parameters and Efficiencies of dAMP Insertion Opposite the 5′-$\underline{T}$ of Normal, Methylated, and cis−syn Dimer-Containing TT Site

template (5′-3′)	dNTP	V_{max} (nM/min)	K_m (μM)	V_{max}/K_m	relative efficiency
TT	dATP	0.20 ± 0.01	20 ± 3	0.010 ± 0.002	1
$\underline{T}$T	dPTP	0.29 ± 0.02	1.1 ± 0.3	0.25 ± 0.08	25
$\underline{T}$m³T	dATP	0.07 ± 0.02	70 ± 30	0.001 ± 0.0005	0.1
m³$\underline{T}$T	dATP	0.016 ± 0.001	65 ± 15	0.0002 ± 0.00006	0.02
$\underline{T}$[c,s]T	dATP	0.08 ± 0.02	20 ± 12	0.0038 ± 0.002	0.4
$\underline{T}$[c,s]m³T	dATP	0.006 ± 0.002	250 ± 200	0.000024 ± 0.00002	0.0024
m³$\underline{T}$[c,s]T	dATP	0.011 ± 0.005	430 ± 450	0.00003 ± 0.00003	0.003
$\underline{T}$[c,s]T[a]	dATP	2.4 ± 0.2	14 ± 4	0.17 ± 0.05	1
m³$\underline{T}$[c,s]T[a]	dATP	2.2 ± 0.3	900 ± 300	0.0024 ± 0.001	0.014

[a] Higher concentration of enzyme.

Derivatives, and an Abasic Model Site. To gain insight into the origin of the preference for dPMP insertion relative to dAMP opposite the undimerized and dimerized TT sites, we determined V_{max} and K_M for dAMP and dPMP insertion (Tables 1 and 2). The results show that the efficiency of dPMP insertion opposite the 3′-T varies only 2-fold for all substrates, regardless of dimerization or methylation state, and is from 2.5- to 6-fold lower than that for insertion opposite an abasic site model. Insertion of dAMP opposite the unmethylated 3′-Ts of undimerized TT sites is only 2−4-fold less efficient than that for dPMP but is 12−32-fold less efficient opposite the dimerized TT sites. Methylation of the 3′-T caused a dramatic reduction in efficiency of dAMP insertion of about 300-fold for the undimerized T and about 30-fold for the dimerized one. The large drop in efficiency of dAMP insertion on going from a normal T to a methylated T which cannot base pair is similar to the 2000-fold difference in efficiency of dAMP insertion reported for going from a T to an abasic site model (tetrahydrofuran analog) (30). It is also interesting to note that the selectivities calculated from the single nucleotide insertion assays opposite the 3′-T are much lower than those calculated from the direct competition assays. The origin of this difference is not understood at the moment and is under study, but may be due to an inability to accurately quantify closely spaced bands in the gel. It may be that pyrene nucleotide is functioning as some sort of noncompetitive inhibitor of dAMP insertion, which could explain in part the inhibitory effect of dPTP on primer extension opposite undamaged templates (Figure 6).

In contrast to what was observed for insertion opposite the 3′-T site of the dimer, insertion of dPMP is completely inhibited opposite both the unmethylated and methylated 5′-T of the dimer even at 500 μM (Figure 5) and higher concentrations of dPTP. On the other hand, the efficiency of dAMP insertion opposite the 5′-T of the dimer is about the same as that opposite the 3′-T of the dimer but is dramatically reduced (>100-fold) by methylation of the 5′-T of the dimer (Table 2). For the undimerized site, the efficiency of dAMP insertion opposite the 5′-T is about 10-fold less than that opposite the 3′-T, and methylation of the 5′-T also greatly inhibits the insertion of dAMP by about 50-fold. Methylation of the 3′-T of both the undimerized and dimerized TT sites also inhibits insertion of dAMP opposite the 5′-T, but the effect appears to be much less for the undimerized T than for the dimerized T (10-fold vs 150-fold). Presumably, the 3′-T of the dimer is better able to communicate distorting effects resulting from methylation than the 3′-T compared to an undimerized site by virtue of its covalent linkage to the 5′-T.

DISCUSSION

Pol η is the only eukaryotic DNA polymerase discovered to date that can efficiently bypass a cis−syn thymine dimer in a nonmutagenic manner, and thus it is of great interest to determine the structure and mechanism by which it can do so. Two general active site and associated bypass mechanisms can be envisioned to explain the preferential insertion of dAMP over other nucleotides opposite the 3′- and 5′-T's of a thymine dimer (Figure 1) (31). The first type of active site is one that is highly constrained and which cannot accommodate a thymine dimer during insertion opposite the 3′-T. In this case, nucleotide insertion would have to occur via a transient abasic site-like intermediate, in which the preference for dAMP incorporation comes from an inherent selectivity for inserting A opposite a template devoid of a base. The second type of active site is one that is much less constrained and can accommodate a thymine dimer during insertion opposite the 3′-T. In this case the selectivity for incorporation of dAMP occurs by thymine-directed insertion of dAMP. The latter mechanism would appear to be possible for pol η based on a model created by fitting a computer generated cis−syn thymine dimer-containing template primer to the crystal structure of the catalytic core of yeast pol η (32). In that model, the active site of pol η is large enough to accommodate the cis−syn dimer during nucleotide insertion opposite the 3′-T of the dimer.

Evidence For a Loosely Held Templating Nucleotide. Initially, we expected that we could differentiate between the two possible bypass mechanisms by the selectivity of dPMP insertion relative to dAMP insertion, as we had done for T7 DNA polymerase (9). In the case of T7 polymerase, dPMP is preferentially inserted relative to dAMP opposite abasic sites, but not opposite an unmodified T. Because dPMP is also preferentially inserted opposite the 3′-T of dipyrimidine photoproducts, but not opposite the 5′-T, we argued that the dimer is outside of the active site during insertion opposite the 3′-T, but inside during insertion opposite the 5′-T. Surprisingly, we find that pol η preferentially inserts dPMP relative to dAMP opposite the 3′-T of both an undimerized and a dimerized TT site, as well as their N3-methylated derivatives (Figure 4, Table 1) and normal DNA bases (Figure 6). In order for this to occur, the 3′-T must have either been forced out of its normal templating position or been prevented from entering the
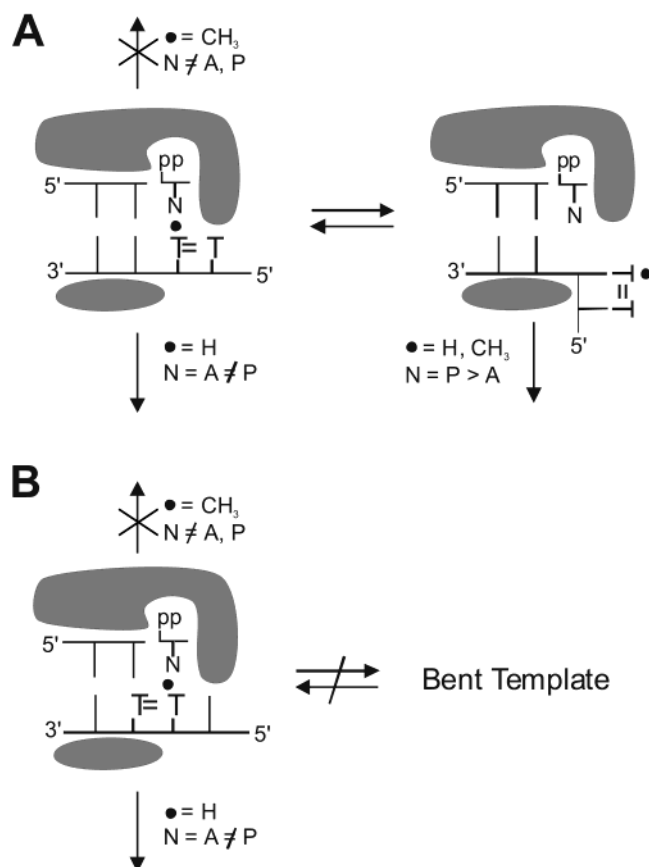
FIGURE 7: Proposed mechanism to account for the observed nucleotide insertion preferences and efficiencies opposite the 3′-T and the 5′-T of unmethylated and methylated cis−syn dimers by pol eta. (A) Insertion opposite the 3′-T of the dimer can take place with the dimer either inside or outside of the active site, depending on whether it can fit opposite the incoming nucleotide. Insertion of dAMP opposite the 3′-T takes place efficiently with the dimer inside the active site directing the insertion, whereas the preferential insertion of the bulkier dPMP must take place with the dimer outside the active site. When the N3 of the 3′-T is methylated (indicated by a dot), insertion of dAMP takes places much less efficiently and presumably takes place with the dimer outside the active site. (B) Insertion opposite the 5′-T of the dimer can only take place with the dimer inside the active site because the 3′-T of the dimer, to which the 5′-T is covalently fused, is held tightly by the polymerase. In this case only dAMP can be inserted efficiently because dPMP cannot fit opposite the 5′-T that is fixed within the active site. Likewise, methylation of the 5′-T dramatically inhibits insertion of dAMP because the dAMP cannot fit opposite the methylated 5′-T in the active site.

templating site by the large pyrene group, suggesting that the templating base is loosely held and is not required for efficient dPMP insertion (Figure 7). Indeed, dPMP is inserted with the highest efficiency opposite an abasic site, for which there is no base to force out or prevent from entering. In the absence of dPTP, however, the 3′-T must be able to occupy the normal templating position because pol η faithfully copies undamaged templates, though with a much higher error frequency than replicative polymerases (*33*).

The higher efficiency of dPMP insertion relative to dAMP appears to be largely due to a higher V_{max} and lower K_M (Table 1). The lower K_M might be due to a low K_d for dPTP binding to the active site. Because pyrene is highly hydrophobic and does not have H-bonding ability, a low K_d suggests that it is interacting primarily by a hydrophobic and/ or stacking interaction. This would also explain why dPMP

insertion is favored over dAMP opposite abasic sites by pol η, as well as exonuclease deficient Klenow fragment and T7 DNA polymerases (*11*). In support of this idea, dangling end thermal denaturation studies have shown that the free energy of π stacking by pyrene on the end of duplex DNA terminating in a C·G base pair is −1.7 kcal/mol, which is 0.7 kcal more favorable than that for A (*34*). This, together with the observation that the flanking base can influence the efficiency of nucleotide insertion opposite an abasic site (*35*), suggests that π stacking of dPMP with the primer terminus in pol η is at least in part responsible for its high insertion efficiency opposite modified and unmodified template bases. It would appear that pyrene nucleotide must also be able to drive the conformational change that has been proposed to precede phosphodiester bond formation without forming a base pair (*8*). Perhaps the larger pyrene can drive the conformational change due to its hydrophobicity and its ability to mimic a base pair due to its large size.

Evidence That Both T's of the Cis−Syn Dimer Direct Insertion of dAMP. Because dPMP is preferentially inserted opposite the 3′-T of unmodified or modified TT sites, it cannot be used to determine whether the dimer is inside or outside the active site during insertion of dAMP opposite the 3′-T. The location of the dimer can be determined, however, by use of N3-methyl derivatives of the 3′-T. If the dimer is outside the active site and does not play any role in directing the insertion of A opposite the 3′-T, then methylation of N3 of the 3′-T should not have any effect on the efficiency of dAMP insertion. If, on the other hand, the 3′-T of the dimer must be inside the active site to direct the insertion of A, methylation of the N3 of the 3′-T would be expected to greatly decrease the efficiency for dAMP insertion (Figure 7). In support of the latter pathway, the efficiency of dAMP insertion opposite 3′-T of the dimer drops by a factor of about 33 upon methylation (Table 1). In the case of insertion opposite the 5′-T of the dimer, evidence for the dimer being in the active site is even more compelling. First, dAMP is found to be efficiently inserted opposite the 5′-T of the dimer, whereas under the same conditions, the insertion of dPMP cannot be detected. Second, the efficiency of dAMP insertion is reduced about 100-fold upon methylation of the N3 of the 5′-T (Table 2).

Evidence that the Nucleotide to the 3′-Side of the Templating Nucleotide Is Tightly Held by Pol η. The selectivity and efficiency of nucleotide insertion opposite the 5-T′ of the dimer also tells us something about how tightly the nucleotide to the 3′-side of the templating base is held by the polymerase. Because cis−syn dimer formation fuses the 5′-T of the dimer to the 3′-T, the flexibility of the 5′-T is governed by the flexibility of the 3′-T and vice versa. We already know that when the templating base is a nondimerized T or the 3′-T of a cis−syn dimer, it must be loosely held by virtue of the fact that dPMP is efficiently inserted. The inability to insert dPMP, but not dAMP, opposite the 5′-T of the dimer therefore indicates that the 3′-T is being rigidly held and prevents the 5′-T of the dimer from being displaced from the templating position (Figure 7).

CONCLUSION

All the evidence obtained supports a mechanism for the error free bypass of a thymine dimer by pol η in which both

T's of the dimer direct the insertion of dAMP. This is markedly different from pol I family enzymes, which insert dAMP opposite the 3′-T of the dimer via a transient abasic site-like intermediate. The evidence also suggests that, with the exception of the 5′-T of the dimer, templating bases are held loosely, which would explain the lower fidelity of this polymerase compared to replicative polymerases and its unusual preference for inserting pyrene nucleotide in preference to dAMP opposite both modified and unmodified T's. The 5′-T of the dimer, on the other hand, appears to be held tightly when in the templating position, which suggests that the 3′-T to which it is covalently linked is being held tightly by the enzyme.

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