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# Effect of *N*-2-Acetylaminofluorene and 2-Aminofluorene Adducts on DNA Binding and Synthesis by Yeast DNA Polymerase $\eta^{\dagger}$

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### **Abstract**

The well-studied aromatic amine carcinogen, N-2-acetylaminofluorene (AAF), forms adducts at the C8 position of guanine in DNA. Unlike replicative polymerases, Y-family polymerases have been shown to have the ability to bypass such bulky DNA lesions. To better understand the mechanism of translesion synthesis by the yeast DNA polymerase  $\eta$  (yPol $\eta$ ), a gel retardation technique was used to measure equilibrium dissociation constants of this polymerase to unmodified DNA or DNA containing dG-C8-AAF or the related deacylated dG-C8-AF adduct. These results show that the binding of yPol $\eta$  to the unmodified primer-template is substantially stronger in the presence of the next correct nucleotide than when no or an incorrect nucleotide is present. In addition, binding of yPol $\eta$  to either dG-C8-AAF or AF-modified templates is also stronger in the presence of dCTP. Finally, the yPol $\eta$  complex is destabilized if the primer extends to a position across from the adduct and stronger binding is not observed in the presence of the next correct nucleotide. Taken together, these data are consistent with the ability of yPol $\eta$  to undergo a conformational change to a closed ternary complex in the presence of the next correct nucleotide and on templates containing an AAF or AF adduct, but do not rule out other possible explanations.

Accurate replication of cellular DNA is necessary to maintain the integrity of the cell's genome during cell division. Normally DNA synthesis is carried out by a high-fidelity polymerase that has a strong preference for the insertion of the correct nucleotide across from the template base in the active site. This process has been shown to involve multiple steps, one of which is a conformational change from an open to closed complex that leads to a structure that can best accommodate a correct base pair (1). If the DNA contains a damaged base, nucleotide incorporation by a high-fidelity polymerase can be compromised, often leading to a slowing or stoppage of the replication process (2,3).

Most organisms have been found to contain specialized polymerases that are able to synthesize past the damage in DNA that has escaped nucleotide or base excision repair. Unlike replicative polymerases, these translesion synthesis  $(TLS^1)$  polymerases show a high degree of functional divergence in terms of both the lesion they can accommodate and the step insertion vs. extension in the bypass process they catalyze (4). Pol $\eta$  is an example of a translesion synthesis (TLS) polymerase that is part of the so called Y-family, whose main structural differences from replicative polymerases are a more open active site caused by stubby fingers and thumb domains and the presence of a polymerase-associated domain

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<sup>&</sup>lt;sup>1</sup>Abbreviations: AAF, *N*-2-acetylaminofluorene; AF, *N*-2-aminofluorene; dG-C8-AAF, N-(2'-deoxyguanosin-8-yl)-2-acetylaminofluorene; dG-C8-AF, *N*-(2'-deoxyguanosin-8-yl)-2-aminofluorene; PAGE, polyacrylamide gel electrophoresis; yPolη, yeast DNA polymerase η hPolη, human DNA polymerase η, TLS, translesion synthesis.

(PAD) region in the active site (4). Presumably, this more open structure allows the Y-family polymerases to accommodate the structural distortion induced by the presence of bulky DNA adducts. Pol $\eta$  is the only known TLS that can efficiently synthesize across from a cis-syn TT dimer (4) and both the yeast and human Pol $\eta$  can incorporate, although at reduced rates, nucleotides opposite other bulky adducts, such as dG-C8-AAF or cisplatin-GG lesions (5,6). A common feature for synthesis on these types of adducts is that once incorporation occurs across from the adduct position, Pol $\eta$  is unable to efficiently extend from these structures.

N-2-acetylaminofluorene (AAF) is the most extensively studied example of the aromatic amine class of carcinogen and it has been extensively used as a model for studying carcinogenic mechanisms (7). Following AAF treatment of experimental animals, two major DNA adducts (C8-dG-AAF and C8-dG-AF) have been observed (Fig. 1), each linked to the pathway involved in the metabolic activation process (7). Most spectral, enzymatic, and theoretical studies suggest that the AF structure, which represents the major adduct observed in all tissue studied so far, produces much less distortion in the DNA helix than the closely related AAF adduct (8-12). Multidimensional NMR experiments show that the guanine bearing the C8-AAF adduct rotates from an anti to syn conformation in the double stranded DNA helix so that the fluorene ring becomes inserted into the helix (base displacement model, (13). This contrasts with the AF-adduct that can adopt interchangeable conformations: in the major structure the fluorene remains outside the helix (outside binding model) while the minor conformation has the fluorene ring stacked within the helix(14). The proportion of these conformations seems to be dependent on the sequence within which the adduct lies (15). The mutagenic consequences of each adduct are also quite distinct. While the dG-C8-AAF adduct results in mostly frameshift mutations in bacteria, the dG-C8-AF adduct produces predominately base substitution mutations (16).

In vitro studies using templates modified with either a dG-C8-AF or -AAF adduct have shown that the AF adduct is bypassed much more readily than the corresponding AAF adduct by a variety of replicative DNA polymerases (17,18). Two crystal structures of replicative polymerase complexed to AAF or AF-modified primer-templates provide insight into the these contrasting effects. Structures of the T7 DNA polymerase show that the AAF adduct retains a syn conformation and is positioned behind the O-helix causing this helix to block the nucleotide binding site (19). This structures has been shown to inhibit the conformational change to a closed ternary complex by both kinetic studies (18) and trypsin cleavage inhibition (20). The AF adduct does not appear to be associated with the O-helix but its precise position in the structure could not be resolved (19). However, there is evidence that the AF adduct did not inhibit the conformational change to a closed ternary complex (18,20). Similar structures with the Bacillus DNA polymerase I show that the dG-C8-AF adduct adopts an anti conformation with the adduct protruding into the major groove, a structure that allows normal base paring with dCTP (21). Recent molecular modeling and molecular dynamics simulations indicate that the AAF adduct can be accommodated in the TLS polymerase Dpo4 in an anti conformation that allows proper Watson-Crick base paring with and incoming dCTP (22).

Several studies have shown that both the human and yeast Poln can incorporate nucleotides opposite an dG-C8-AAF adduct, with dCTP being preferred (5,6). Neither enzyme is effective at extending from a dC:dG-C8-AAF primer terminus, although full extension could be obtained using a large excess of hPoln (5). In vivo studies in yeast have suggested that yPoln is responsible for both accurate and inaccurate bypass of a dG-C8-AAF lesion in a two step process that requires at least two TLS polymerases (23).

Pre-steady-state kinetic analysis that compared the yeast and human Polη showed substantial differences for the mechanism of nucleotide incorporation used by these two polymerases. Although the steady-state rates were similar, the nucleotide binding affinity was significantly less for the human enzyme while nucleotide incorporation rate was much faster (24). Prior studies had also suggested that yPolη undergoes a conformational change upon the binding of the next correct nucleotide (25) but that the induced fit was not able to discriminate between correct and incorrect nucleotide to the same extent as A family polymerases. The ability of the Y-family polymerases to undergo a conformational change is in some question because the crystal structure of the Dbh polymerase, another Y-family polymerase, shows the little fingers domain to already be in a closed orientation, similar to the closed structures observed for A and B-family polymerases (26).

In this paper we have determined the apparent dissociation constants for the interaction of yPolη with unmodified and AAF- or AF-modified primer-templates in the presence or absence of dNTPs. On unmodified DNA, the binding of yPolη is strengthened in the presence of the next correct nucleotide and reduced in the presence of an incorrect nucleotide. In addition, yPolη binds more tightly to an dG-AAF or AF-modified template in the presence of dCTP. These results parallel prior studies with Klenow fragment that have been used to measure the nucleotide-induced conformational change (18,20). Finally, the yPolη-DNA complex is destabilized if the primer extends to a position across from the adduct and stronger binding is not observed in the presence of the next correct nucleotide.

# **MATERIALS AND METHODS**

#### **Materials**

Yeast DNA polymerase  $\eta$  was provided by Dr. Aneel Aggarwal of Mount Sinai School of Medicine. It was prepared as described (27). T4 DNA ligase, T4 DNA kinase, terminal deoxynucleotidyl transferase, deoxyribonucleotide-5'-triphosphate (dNTPs) and dideoxyribonucleotide-5'-triphosphate (ddNTPs) were purchased from USB Corporation. All unmodified oligonucleotides were obtained from Operon Technologies, Inc. [ $\gamma$ -32P] ATP, 7000Ci/mmol, was purchased from MP-Biomedical, LLC.

### Construction and Purification of the AAF and AF-Modified Oligonucleotides

The unmodified oligonucleotides were purified by 20% denaturing PAGE and isolated by the crush and soak method as described(28). Site-specifically modified 12-mer (GTGATG (C8-AAF) ATAAGT) was synthesized by solid phase synthesis (29) and purified by HPLC as described (30). The 28-mer oligonucleotides containing the AAF at G6 was synthesized by ligating 12-mer with AAF to a 5'-phosphorylated 16-mer, using a 21-mer template as a scaffold. The 28-mer product containing the AAF was separated from the 21-mer scaffold using a 20% PAGE in the presence of 8M urea. The AAF-modified 28-mer was recovered from the gel by the crush and soak method and desalted using centrifugal filter devices, YM-3 (Centricon). A portion of AAF-28mer was deacetylated as described (31). The modified oligonucleotides were characterized by HPLC analysis and primer extension studies.

### Synthesis of Dideoxynucleotide Terminated Primer

The dideoxy terminated 22-mer was obtained by incubating the 21-mer (4  $\mu M)$  with terminal deoxynucleotidyl transferase (70 units) in the presence of ddTTP (350  $\mu M)$  in the reaction buffer at pH 7.2 containing, 100 mM sodium cacodylate, 2 mM CoCl<sub>2</sub> and 0.1 mM 2-mercaptoethanol, incubated for 6 hrs. The resulting dideoxy-terminated 22-mer was purified by 20% denaturing polyacrylamide gel electrophoresis in the presence of 8M urea and isolated by the crush and soak method.

### **Gel Retardation Assay**

The equilibrium dissociation constant (K<sub>d</sub>) for the polymerase-primer-template was carried out by a method similar to that described by Astatke et al (32) and Dzantiev et al (18). The DNA binding reactions were performed in reaction buffer containing 40 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 60 mM KCl, 2.5 µg/10uL bovine serum albumin, and 10% glycerol. The binding was carried out at 25°C for 20 min in a 10 μL reaction containing 2nM labeled dideoxy terminated primer-templates with increasing amounts of yPolη (0-400 nM) and (if present) 0.4 mM dNTP. The reaction mixtures were loaded onto a native 7% polyacrylamide gel pre-equilibrated with 0.04 M Tris-borate, pH 8.8, and the gel run at 4°C for 30 min at 250 mV. Gels were fixed with 7% acetic acid, dried, and scanned using a phosphorimager. The amount of complex formed at equilibrium was estimated as the difference in the band intensities of free primer-template and the intensity of this band without the polymerase addition. To obtain the apparent dissociation constant  $(K_D)$ , the fraction of the DNA bound to the protein was plotted against the initial protein concentrations, and the data were analyzed using the Ultrafit (Biosoft, Cambridge, U.K.) fitting to the equation for single-site ligand binding ([DNA] $_{bound}$ /[DNA] $_{o}$ =[KF] $_{o}$ /(KD+  $[KF]_0$ ). The values for the  $K_D$  were obtained from the resulting fit of this equation (18). Each experiment was repeated at least three times.

## **Primer Extension assay**

The  $^{32}\text{P-labeled}$  22mer primer was annealed to unmodified (3 nM) or modified template (40 nM) in a 4 to 6 ratio in reaction buffer (40 mM Tris-HCl, pH 8.0, 5 mM MgCl<sub>2</sub>, 10 mM dithiothreitol, 60 mM KCl, 0.025 mg/mL bovine serum albumin, and 10% glycerol). A mixture of dNTPs (100  $\mu\text{M}$  final concentration) and yPol $_{100}$  (600 fmol) in 35  $\mu\text{I}$  of reaction buffer was added to 15  $\mu\text{I}$  of the annealed primer template mixture and the reaction incubated at 25° C. Aliquots (5  $\mu\text{I}$ ) were removed at specific time intervals and added to 25  $\mu\text{I}$  of gel loading buffer containing 90% formamide, 0.5 mg/ml bromophenol blue and xylene cyanol, and 10 mM EDTA to halt the reaction. A portion (15  $\mu\text{I}$ ) of this mixture was electrophoresed on to the 20% denaturing PAGE and scanned using a phosphorimager.

#### Single Nucleotide Extension assay

To 7  $\mu$ l of annealing mixture prepared as described for the primer extension assay and containing either unmodified or modified primer templates was added 3  $\mu$ l of a solution of dNTP (100  $\mu$ M final concentration) and yPol $\eta$  (120 fmoles) and the mixture incubated at 25°C for 10 minutes. A portion (5  $\mu$ l) of the reaction mix was removed after 10 min and added to 25  $\mu$ l of gel loading buffer containing 90% formamide, 0.5 mg/ml bromophenol blue and xylene cyanol and 10 mM EDTA to halt the reaction. A portion (15  $\mu$ l) of this mixture was electrophoresed on a 20% denaturing PAGE and the gel scanned using a phosphorimager.

# **RESULTS**

#### **DNA Synthesis on AAF and AF Modified Template**

It is well established that replicative polymerases are strongly blocked by an dG-C8-AAF adduct but can much more easily bypass an dG-C8-AF adduct (33). Prior studies using yPolη has shown that incorporation can occur across from the AAF modified G, but that extension from this substrate is inhibited (6). Figure 2 compares synthesis by yPolη on templates containing either a dG-C8-AF or AAF adduct. As has been observed with polA-family polymerases (17,18), synthesis occurs past the AF adduct giving the same full length product that is observed for the unmodified template (Fig. 2). Nucleotide incorporation on an identical template modified with an AAF adduct occurred across from the AAF-modified

guanine, but no extension was observed past the adduct (Fig. 2). When synthesis was carried out in the presence of a single nucleotide, the preferred base that was incorporated opposite either a dG-C8-AAF or AF adduct is cytosine (Fig. 3) and no incorporation of any other nucleotide is observed across from the adduct for either the AAF or AF-modified template.

# Determination of Equilibrium Dissociation Constants (KD)

Prior studies have shown that DNA polymerase I (Klenow fragment) binds more tightly to a DNA primer-template in the presence of the next correct nucleotide (18,32) and more weakly in the presence of an incorrect nucleotide (18). This tighter binding is consistent structural studies that show that the presence of the next correct nucleotide results in the movement of the fingers region towards the active site to form a closed ternary complex required for proper alignment of the incoming nucleotide with the template base (34). Using similar studies, the apparent dissociation constants were determined for the binding of yPoln to DNA. A dideoxy-terminated, <sup>32</sup>P-labeled primer was annealed to the template in the presence or absence of a dNTP (Fig. 4A) and these mixtures were incubated with increasing amounts of yPoln. These mixtures were then immediately loaded and electrophoresed on a non-denaturing polyacrylamide gel producing a band that was the result of the formation of the polymerase-DNA complex (Fig. 4B). The ratio of the band intensities of the free primertemplate to that of the band in the absence of polymerase was used to estimate the amount of complex formed at equilibrium (Fig. 4C). This procedure allows us to estimate the amount of complex formed in solution prior to loading on the gel and to neglect the slight amount of dissociation of DNA-protein complexes that occurs in the gel. The data obtained this way were then fitted to an equation for single-site ligand binding and apparent K<sub>D</sub> values were calculated (Table 1). The experiments in the presence of dNTPs were carried out with primers that were terminated with a 2',3'-dideoxynucleotide so that nucleotide incorporation could not occur. A comparison of binding with primers containing a 3'-OH and a dideoxyterminated primer in the absence of nucleotide gave comparable K<sub>D</sub>s suggesting that the 3'OH did not effect the K<sub>D</sub>s measured in the presence of dNTPs (data not shown).

The presence of the next correct nucleotide, dCTP, resulted in a stabilization of the complex (Table 1), a result similar to that observed for KF when it undergoes a conformational change to a closed ternary complex. No enhancement of binding was observed in the presence of any incorrect nucleotide and dGTP caused a substantial destabilization of the polymerase primer-template complex. A G:dGTP mismatch in the active site also caused a substantial destabilization of the KF primer-template complex, as did a dG:dATP mismatch (18).

# Binding of yPoIn with AAF- and AF-modified Templates in the Presence or Absence of dNTPs

Several lines of evidence indicates that KF is unable to undergo a conformational change when an AAF adduct is positioned in the active site. These include the fact that no enhancement of binding occurs in the presence of the next correct nucleotide (18), the retention of a trypsin cleavage site that is lost when KF undergoes a conformational change to a closed ternary complex (20), and the crystal structure of the homologous T7 DNA polymerase showing that the AAF adduct induces a structural change that interferes with nucleotide binding (19). The effect on binding by yPoln to a primer template containing an dG-C8-AAF adduct positioned in the active site was measured using the same methods described for the unmodified template (Fig. 5). The apparent K<sub>D</sub> for binding to the AAF template was within the limits of error of that determined for the unmodified template (Table 1). When dCTP was added there was approximately a two fold decrease in K<sub>D</sub>, consistent with the possibility that a conformational change to a closed complex may have occurred. As was observed on unmodified primer-templates, there was also a measurable

drop in  $K_D$  when dTTP or dATP was present and an increase in  $K_D$  when dGTP was present. Similar results were observed when the template contained a dG-C8-AF adduct in the active site (Table 1).

The fact that extension is inhibited when the primer terminus extends to a position across from a dG-C8-AAF adduct (Fig. 2, (6)) prompted us to measure the  $K_D$  for yPol $\eta$  binding in the presence and absence of nucleotide on such a primer template (Fig. 6). In the absence of nucleotide, we observed that the  $K_D$  was increased from 127±30 (Table 1) when the primer extended to the position preceding the adduct to 226±39 when it extended across from the adduct. The  $K_D$  in the presence of the next correct nucleotide (dATP) was 291±61, which is within the limits of error for binding in the absence of dNTP. These data suggest that binding by yPol $\eta$  to what is likely a distorted primer terminus is inhibited and more significantly, that this structure is likely to be unable to undergo a conformational change to a closed complex in the presence of the next correct nucleotide.

# **DISCUSSION**

The molecular mechanism that DNA polymerases use to accurately incorporate nucleotides during DNA synthesis has been intensively studied over the last four decades. This process is intriguing because a replicative polymerase must simultaneously enforce the high fidelity of each incorporation while still allowing each of the possible base pair combinations to be accommodated in the active site. Structural studies have revealed a significant conformational change occurs upon the binding of the correct nucleotide to the polymeraseprimer template complex (34). Examination of kinetic data from a variety of polymerases has shown that a non-covalent step precedes the chemistry step that is often, but not always, rate-limiting (35). Recent studies from several labs have used fluorescence-based assays to measure the conformational change from an open binary to a closed ternary complex and shown that this step is rapid compared with the rate of nucleotide incorporation and is therefore not likely to be the rate-limiting step for the incorporation of the correct nucleotide(36-38). Moreover, studies by Joyce, et al (36) and Rothwell and Waksman (38) present evidence that dNTP binding to the template base occurs in an open conformation, thus proceeding the fingers-closing conformational change. Using single-molecule, ensemble-averaged stopped-flow, and continuous-flow methods Luo, et. al (37) showed that the fingers closing is triggered by the binding of the correct dNTP and that there was no equilibrium between the open and closed conformation in the absence of dNTP.

Although the precise role of the conformational change in the fidelity of nucleotide incorporation remains unclear, the fact that it is an integral part of the mechanism of every high fidelity polymerase studied suggests that this step plays a key role in the accurate selection of the correct nucleotide. Because Y-family polymerases typically have reduced fidelity, the question of whether these polymerases use a different mechanism is a reasonable one. Prakash and coworkers (25) provided the first evidence that yPoln uses an induced fit mechanism in which a conformational change step proceeds the chemistry step. However, a comparison of crystal structures of several Y-family polymerases suggests that the fingers domain is in the closed structure prior to nucleotide binding (26). These observations, coupled with the need for a more open active site that can accommodate bulky distortions to the DNA argue against the conformational change step that is such an integral part of the mechanism used by high-fidelity replicative polymerases. Other kinetic studies using the Dbh polymerase were unable to observe whether nucleotide selection used an induced fit mechanism(28), while similar studies suggested that an induced fit conformational change occurs with Dpo4 polymerase (39). More recent structural (40) and molecular dynamic (41) studies suggest that rather than a large-scale subdomain

conformational change, more subtle changes occur that involve the little fingers and thumb domains.

Prior studies in our lab have attempted to measure directly the conformational change that occurs when a polymerase binds the next correct nucleotide in the active site. Using a tryptic digestion assay we found a diagnostic cleavage site in Klenow fragment that is protected if the polymerase is in a closed ternary complex, bound to the primer-template and the next correct nucleotide (42). We have also measured the apparent equilibrium  $K_Ds$  in the presence and absence of dNTPs and shown that the binding strength of Klenow fragment to a primer-template increases in the presence of the next correct nucleotide, consistent with what is expected when a closed ternary complex forms (18). In addition, we have shown using tryptic digestion studies (20) and X-ray crystallography (19) that a dG-C8-AAF adduct inhibits both the conformational change and the nucleotide-induced increase in binding strength (18), providing further support that the  $K_D$  measurements can be used as an indication of the formation of a closed ternary complex.

In the present study, we have measured nucleotide incorporation and template binding of yPol $\eta$  using unmodified and AAF or AF-modified primer-templates. We find that this polymerase is able to bypass the dG-C8-AF adduct, similar to what is observed with replicative polymerases (17,18). As was shown previously (6) using the AAF-modified template, we find that exclusive incorporation of cytosine can occur opposite the dG-C8-AAF adduct but that extension from this structure is not observed. Studies with a DNA polymerase I mutant gave similar results on AAF-modified primer templates (43). In this mutant, the tyrosine at the base of the O-helix is replaced by a serine, resulting in a more open active site. This mutation allows greatly enhanced incorporation opposite the adduct but reduced incorporation from this extended primer-template. Similarly, it has also been shown that extension is inhibited using hPol $\eta$  on B[a]P modified templates and that the level of inhibition depended on both the stereochemistry of the adduct and sequence context across from and past the lesion (44).

As we have shown with both the Klenow fragment (18) and T7 DNA polymerase (Ullah and Romano, unpublished results), yPol $\eta$  binds more strongly to a primer template in the presence of the correct dNTP. However, unlike what is observed with these replicative polymerases (18) (Ullah and Romano, unpublished results) where we do not observe a dNTP-induced decrease in  $K_D$  if an AAF adduct is positioned in the active site, we find that yPol $\eta$  binds more tightly to an AAF modified template in the presence of dCTP. Similar results are observed for templates containing the less distorting AF adduct. In addition, the presence of each of the incorrect nucleotides has a similar effect on binding to an AAF-modified template as is observed for an unmodified template (Table 1). Based on the crystallographic, binding, kinetic, and protease digestion experiments obtained with Klenow fragment and its homologs (18,42,45) these results are consistent with the ability of yPol $\eta$  to undergo a conformational change to a closed ternary complex in the presence of the next correct nucleotide on both an unmodified or AAF-modified template. However, it is also possible that this enhanced binding could represent a more subtle change in structure, such as that predicted by structural (40) and molecular dynamic (41) studies.

Finally, when similar binding experiments were carried out with an AAF-modified primer template in which the primer extended to the position across from the adduct, we found that there was a decrease in binding strength compared with an identical unmodified primer-template or the case where the primer ended before the adduct. Moreover, the presence of the next correct dNTP did not result in enhanced binding. These results are consistent with our model that the presence of an adduct across from the primer terminus leads to a distorted

primer template when this structure is located in the more open active site afforded by a bypass polymerase (43).

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Primers: (22-mers) 5'-GACGAAAACGACGGCCACTTAT<sub>H</sub>
5'-GACGAAAACGACGGCCACTTAT<sub>OH</sub>

Templates: (28-mers) 3'-CTGCTTTTGCTGCCGGTGAATAGTAGTG

3'-CTGCTTTTGCTGCCGGTGAATAGTAGTG

AAF

3'-CTGCTTTTGCTGCCGGTGAATAGTAGTG

**Figure 1.** Structure of the dG-AF and dG-AAF adducts and the sequences of the primers and templates used in this study.

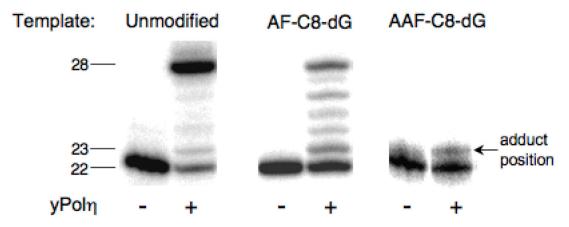
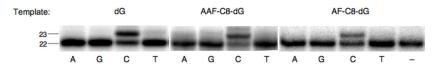
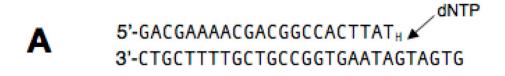
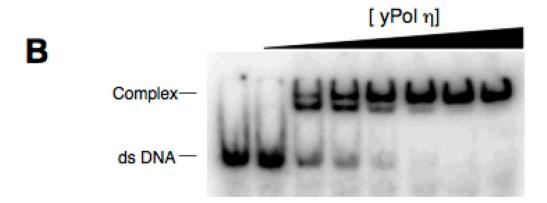


Figure 2. Primer extension reaction on AF- and AAF-modified templates. The 22-mer primer with a 3'-OH was annealed to either the unmodified 28-mer or the AF- or AAF-modified template. Primer extension was carried out using the yPol  $\eta$  and the reactions terminated after the period of time indicated under each lane as described in "Materials and Methods."



**Figure 3.**Single nucleotide primer extension directly across from the AAF or AF-dGuo adduct. Single nucleotide incorporations were measured in the presence of indicated dNTPs on either unmodified or AAF or AF-modified 28-mer templates as described in "Materials and Methods.".





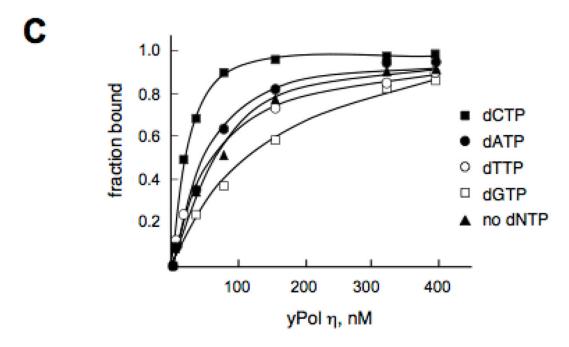
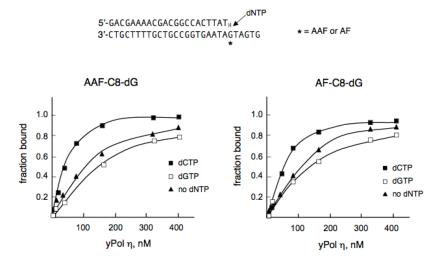


Figure 4. Determination of the equilibrium dissociation constants ( $K_D$ ) of the yPolη-DNA complex. A. Primer-template used to determine the  $K_D$ . B. A typical gel retardation assay used to determine the  $K_D$  of yPolη-DNA complexes.  $^{32}$ P-Labeled primer-templates were incubated with increasing amounts of yPolη and then loaded on a 7% nondenaturing polyacrylamide gel as described under "Materials and Methods." "dsDNA" is the position of the primer-template, "complex" is the position of the polymerase-DNA complex. C. Binding curves for the interaction of the yPolη with the unmodified primer-template in the presence of no nucleotide (▲), dCTP ( $\blacksquare$ ), dATP ( $\bullet$ ), dTTP ( $\circ$ ), or dGTP ( $\square$ ). The fraction of bound DNA determined as described under "Materials and Methods" was plotted versus the yPolη

concentration. All data point represent the average of at least three different experiments and each of the values shown in Table 1 was determined using this method.



**Figure 5.**Binding curves for the interaction of yPolη with AF or AAF-modified DNA in the presence or absence of dNTPs. The position of the adducts is indicated with an \* in the primer-template shown at the top. The fraction of bound AAF- or AF-modified DNA in the absence or presence of dNTPs was determined as described in the "Materials and Methods" and these values plotted against the concentration of yPolη. All data point represent the average of at least three different experiments and each of the values shown in Table 1 was determined using this method.

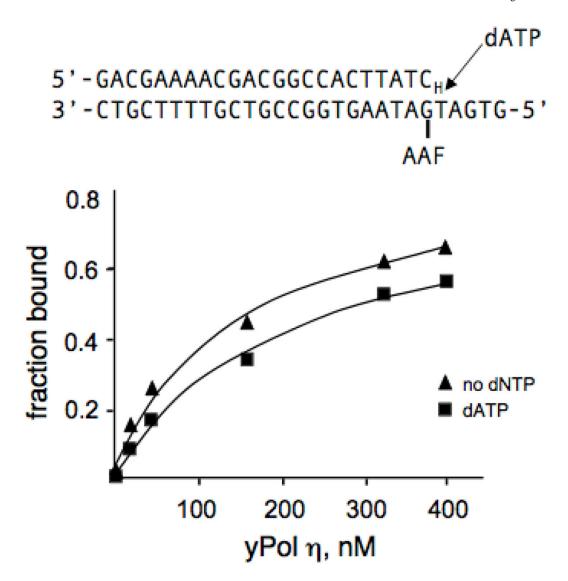


Figure 6.
Binding curves when the primer extends to the adduct site. The fraction of bound AAF-modified DNA in the absence or presence of dATP was determined as described in the "Materials and Methods" and these values plotted against the concentration of yPolη. All data point represent the average of at least three different experiments and each of the values shown in Table 1 was determined using this method.

# Table 1

Dissociation constants for the binding of yPoln to primer-templates in the presence or absence of dNTPs<sup>a</sup>.

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			$K_{D}$ $(nM)$		
Template		dCTP	dTTP	dATP	dGTP
Unmodified	99 ± 21	23 ± 5	$90 \pm 10$	73 ± 7	171 ± 12
AAF modified	$127\pm30$	$60 \pm 4$	$85\pm12$	$75\pm2$	$151\pm13$
AF modified	$147 \pm 2$	67 ± 3	87 ± 7	$92\pm12$	$179\pm28$

<sup>a</sup>The sequence of the 22 primer containing a dideoxy nucleotide at the 3' terminus and the templates used for this study are shown in Fig. 1. The equilibrium dissociation constant values were determined as described in "Material and Methods." Each experiment was repeated at least three times.

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