Mechanism for *N*-Acetyl-2-aminofluorene-Induced Frameshift Mutagenesis by *Escherichia coli* DNA Polymerase I (Klenow Fragment)†

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ABSTRACT: *N*-Acetyl-2-aminofluorene (AAF) is a chemical carcinogen that reacts with guanines at the C8 position in DNA to form a structure that interferes with DNA replication. In bacteria, the *Nar*I restriction enzyme recognition sequence ($G_1G_2CG_3CC$) is a very strong mutational hot spot when an AAF adduct is positioned at G_3 of this sequence, causing predominantly a −2 frameshift GC dinucleotide deletion mutation. In this study, templates were constructed that contained an AAF adduct at this position, and primers of different lengths were prepared such that the primer ended one nucleotide before or opposite or one nucleotide after the adduct site. Primer extension and gel shift binding assays were used to study the mechanism of bypass by the *Escherichia coli* DNA polymerase I (Klenow fragment) in the presence of these templates. Primer extension in the presence of all four dNTPs produced a fully extended product using the unmodified template, while with the AAF-modified template synthesis initially stalled at the adduct site and subsequent synthesis resulted in a product that contained the GC dinucleotide deletion. Extension product and gel shift binding analyses were consistent with the formation of a two-nucleotide bulge structure upstream of the active site of the polymerase after a nucleotide is incorporated across from the adduct. These data support a model in which the AAF adduct in the *Nar*I sequence specifically induces a structure upstream of the polymerase active site that leads to the GC frameshift mutation and that it is this structure that allows synthesis past the adduct to occur.

The interplay between the structure of a particular mutagenic adduct in DNA and the sequence within which the adduct is situated clearly plays a role in the mechanism of adduct-induced mutagenesis. This relationship has been directly observed for the adducts formed by the potent carcinogen *N*-acetyl-2-aminofluorene (AAF).[1] This carcinogen is known to produce two major adducts in cellular DNA: the *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene adduct (dG-C8-AAF), which is known to be a strong block to DNA synthesis, and the *N*-(deoxyguanosin-8-yl)-2-aminofluorene adduct (dG-C8-AF), which can be much more easily bypassed by all polymerases tested (*1*, *2*). In bacteria, while the aminofluorene (AF) adduct is predominantly a base substitution mutagen (*3*), the AAF adduct produces mostly frameshift mutations that are targeted to a few repetitive sequences (*4*). One such sequence is the *Nar*I restriction enzyme recognition sequence (5′-$G_1G_2CG_3CC$-3′), which has been shown to lead to a GC dinucleotide deletion when the AAF adduct is positioned at the third guanine, G_3, of this sequence (*4*). Similar placement of an AF adduct does not produce this same frameshift mutation (*5*).

It has long been thought that the differing biological properties of the AAF and AF adducts are a result of different structures that these adducts adopt in DNA (*6*, *7*) and that these structures are affected by the DNA sequence context in which the adduct is positioned (*8*). Structural analyses, including solution structure determinations, indicate that the AAF adduct distorts the structure of the duplex DNA more than the corresponding AF adduct (*6*, *7*, *9*, *10*). Moreover, chemical modification (*11*), circular dichroism (*12*), and enzymatic (*13*) studies suggest that the AAF adduct adopts a very different structure when it is positioned in the *Nar*I sequence compared with other nonrepetitive sequences. One inference from these studies is that the structural differences observed for these adducts in DNA lead to different structures within the polymerase active site that, in turn, leads to different mutagenic consequences. In support of this model, it has been found that the dissociation constants for the binding of DNA polymerase I (Klenow fragment) (KF) to a template are very different for an AAF vs an AF adduct (*14*) and also that the AAF adduct strongly inhibits the conformational change to a closed ternary complex while the AF adduct does not (*15*).

Recent structural studies (*16*) on the T7 DNA polymerase, a structural analogue of DNA polymerase I, have shown that when this polymerase is bound to an AAF-modified primer-template, the AAF adduct is positioned in a hydrophobic pocket on the fingers subdomain behind the O helix. This structure locks the polymerase in an open conformation and also pushes the O helix into the active site, partially blocking the nucleotide binding site. Presumably, this structure is the

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[1] Abbreviations: AAF, *N*-acetyl-2-aminofluorene; dG-C8-AAF, *N*-(2′-deoxyguanosin-8-yl)-2-acetylaminofluorene; AF, aminofluorene; KF, Klenow fragment; K_d, dissociation constant; dNTP, deoxyribonucleoside 5′-triphosphate; ddNTP, dideoxyribonucleoside 5′-triphosphate; PAGE, polyacrylamide gel electrophoresis.

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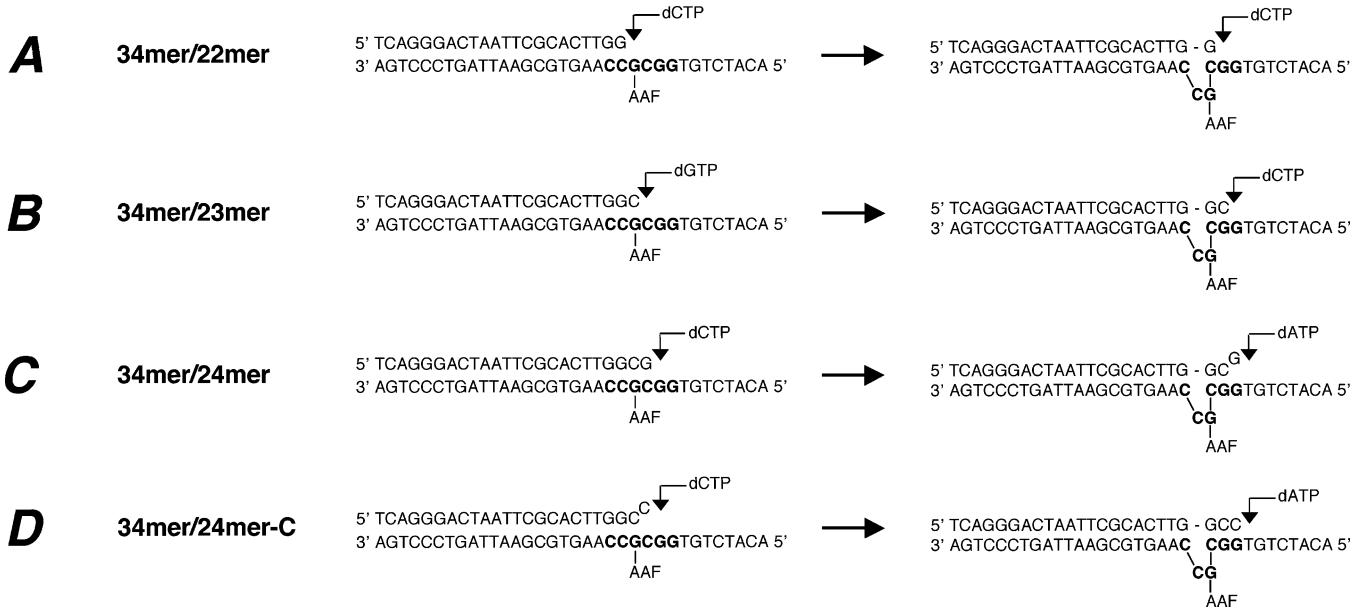

FIGURE 1: Fully paired and frameshift structures for primer-template combinations showing the next correct nucleotide for incorporation.

reason that the AAF adduct is such a strong block of DNA synthesis.

Interestingly, purified *Escherichia coli* polymerase II has been shown to synthesize past an AAF adduct when it is positioned on G_3 of the *Nar*I sequence to produce a GC dinucleotide deletion (*17*), and genetic evidence has suggested that this polymerase is responsible for this frameshift mutation in vivo (*18*). In this paper we show that KF is also not blocked by an AAF adduct located on G_3 of the *Nar*I sequence and that synthesis past it produces this same GC dinucleotide deletion. Analysis of the synthesis products and dissociation constants (K_d) suggests that following incorporation opposite the AAF adduct the modified template then adopts a looped out structure upstream of the polymerase active site that leads to this deleted product.

MATERIALS AND METHODS

Materials. A plasmid coding for the Klenow fragment (exo-) mutant was provided by Dr. Catherine Joyce of Yale University. The protein that contained the D424A mutation, which eliminates the 3′−5′ exonuclease activity (*19*), was overexpressed and purified as described (*20*). The specific activity of the protein was determined as described (*21*). Protein concentrations were determined colorimetrically by the Bradford assay (*22*) using Bio-Rad laboratory agents. T4 DNA ligase, T4 DNA kinase, and the terminal deoxynucleotidyl transferase were purchased from Amersham Pharmacia Biotech. Oligonucleotides were obtained from Operon Technologies, Inc. All deoxyribonucleoside 5′-triphosphates (dNTP) and dideoxyribonucleoside 5′-triphosphates (ddNTP) were purchased from Amersham Pharmacia Biotech. [γ-^{32}P]ATP was from MP Biomedicals, LLC.

Construction and Purification of AAF-Modified Oligonucleotides. All of the synthetic oligonucleotides used in these studies were first purified by denaturing polyacrylamide gel electrophoresis (PAGE) using the crush and soak method as described (*23*). Phosphorylation using polynucleotide kinase and ligations were performed as described (*23*). The site-specifically modified 12-mer (GTGGCG^{C8-AAF}CCAAGT) was synthesized by solid-phase synthesis (*24*) and purified by HPLC as described (*25*). In constructing the 28-mer containing the *Nar*I sequence modified at G_3, the site-specifically modified *Nar*I 12-mer was ligated to a 5′-phosphorylated 16-mer, using a 21-mer as a scaffold. The 28-mer product containing the AAF adduct was separated from the 21-mer scaffold using a 20% polyacrylamide gel in the presence of 8 M urea. The AAF-modified 28-mer was then recovered from the gel and desalted with a centrifugal filter device, YM-3 (Centricon). To construct the 34-mer containing an AAF modification at the G_3 position in the *Nar*I sequence, the AAF-modified *Nar*I 28-mer was phosphorylated at the 5′-end using polynucleotide kinase and dATP and then ligated with a 6-mer using a 25-mer scaffold. The ligated 34-mer containing the adduct and the complementary 25-mer were denatured and purified using a 20% polyacrylamide gel in the presence of 8 M urea.

Synthesis of the ddNTP-Terminated Primers. The ddNTP-terminated primers were synthesized by extending the corresponding −1 length primer with the desired ddNTP using terminal deoxynucleotidyl transferase. The −1 length primers (4 μM) were incubated with 70 units of terminal deoxynucleotidyl transferase in the presence of the desired ddNTP (350 μM), 100 mM sodium cacodylate, pH 7.2, 2 mM $CoCl_2$, and 0.1 mM 2-mercaptoethanol. The reaction was performed at 37 °C for 5 h, and the resulting dideoxy-terminated primers were purified by 20% denaturing polyacrylamide gel electrophoresis in the presence of 8 M urea.

Primer Extension Reactions. Unmodified or AAF-modified templates (2.5 nM) were annealed to the ^{32}P-labeled primer (1 nM) in the presence of 50 mM Tris-HCl, pH 7, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.05 mg/mL bovine serum albumin. The sequences of these primer-templates are shown in Figure 1. The extension reactions were initiated by the addition of 28 nM (full extension) or 0.28 nM (single nucleotide extension) Klenow fragment (exo-) and 0.4 mM desired dNTP to give a final reaction volume of 50 μL. Aliquots (5 μL) were removed at specific time intervals and added to 15 μL of gel loading buffer containing 90% formamide and 5 mg/mL bromophenol blue and xylene cyanol to halt the reaction. The samples were then analyzed

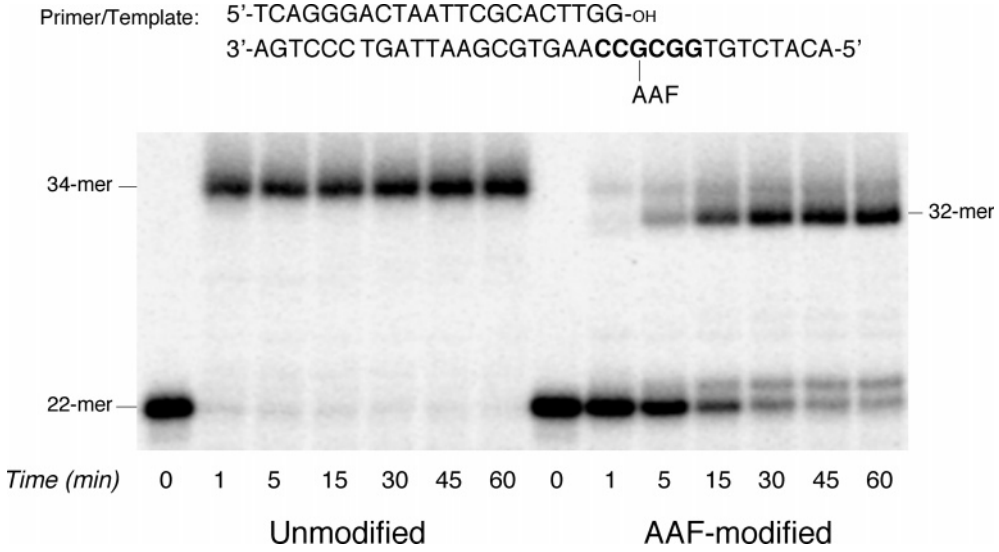

FIGURE 2: DNA synthesis on a template containing an AAF adduct in the *Nar*I sequence gives a dinucleotide deletion. Full extension reactions were carried out using KF on a 34-mer template containing an AAF-dG adduct at the G_3 position of the *Nar*I sequence hybridized to a ^{32}P-labeled 22-mer primer as described in the Materials and Methods section. Aliquots were removed from the reactions at the indicated times and quenched, the products were analyzed on a 20% polyacrylamide gel, and the bands were visualized using a phosphorimager.

using 15% denaturing polyacrylamide gel electrophoresis (0.4 mm), which was fixed with 7% acetic acid and dried. The bands were visualized using a Molecular Dynamics PhosphorImager SF.

Gel Shift Binding Analysis. The equilibrium dissociation constants (K_ds) for the polymerase−primer-template complexes were determined by a method similar to that described by Astatke et al. (*26*) and Dzantiev et al. (*14*). The DNA binding reactions were performed in 50 mM Tris-HCl, pH 7, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.05 mg/mL bovine serum albumin, and 4% glycerol. The binding was carried out at 25 °C for 30 min in a 10 μL reaction containing 10−100 pM labeled duplex DNA, increasing amounts of KF (typically 0−200 nM), and 0.4 mM dNTP (if present). The reaction mixtures were loaded onto a native 7% polyacrylamide gel preequilibrated with 0.4 × TB buffer (0.04 M Tris−borate, pH 8.8), which were run at 4 °C for 30 min at 250 mV. Gels were fixed with 7% acetic acid, dried, and scanned using a Molecular Dynamics PhosphorImager SF. 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 program Ultrafit (Biosoft, Cambridge, U.K.) and fitted to the equation for single-site ligand binding. The values for the $K_d \pm$ SE were obtained from the resulting fit of this equation (*14*).

Nucleotide Incorporation Reactions. Unmodified or AAF-modified templates (2.5 nM) were annealed to the ^{32}P-labeled primer (1 nM) in the presence of 50 mM Tris-HCl, pH 7, containing 10 mM $MgCl_2$, 1 mM dithiothreitol, and 0.05 mg/mL bovine serum albumin. The extension reactions were initiated by the addition of 0.028 nM Klenow fragment (exo-) and 0.4 mM desired dNTP to give a final reaction volume of 10 μL. The unmodified reaction was incubated for 2 min, while the AAF-modified reaction was incubated for 30 min before adding 20 μL of gel loading buffer containing 90% formamide and 5 mg/mL bromophenol blue and xylene cyanol to halt the reaction. The samples were then analyzed using 17% denaturing polyacrylamide gel electrophoresis (0.4 mm), which was fixed with 7% acetic acid and dried. The bands were visualized using a Molecular Dynamics PhosphorImager SF.

RESULTS

*DNA Synthesis on Templates Containing an AAF Adduct in the Nar*I *Sequence.* Prior studies have shown that the AAF adduct is a strong block to DNA synthesis by KF (*14*, *27*) with rates of synthesis across from and past it reduced by 10^6 compared with synthesis on unmodified templates. However, as shown in Figure 2, when the AAF adduct is positioned in the *Nar*I sequence context, efficient bypass synthesis occurs. Moreover, the major product that is produced is two nucleotides shorter than expected if a full-length product was produced, although small amounts of the full-length product are also observed. Sequencing of this shortened product showed that a GC dinucleotide deletion had occurred (Don Johnson, unpublished results), the same deletion that occurs in vivo (*4*).

Single Nucleotide Extension Reactions. To study the mechanism of incorporation opposite a AAF adduct located in the *Nar*I sequence, primers were constructed that ended before (22-mer), opposite (23-mer), and past (24-mer) the AAF adduct position (Figure 1), and incorporation studies were carried out in the presence of a single nucleotide (Figure 3). When the 22-mer was used as the primer (Figure 3A), a 23-mer was formed in the presence of dCTP when an unmodified template was used but produced a 24-mer on the AAF-modified template. If the AAF adduct, either before or following the incorporation of one C, flips out to form a GC dinucleotide bulge, then the C of the 23-mer product can hydrogen bond to the G two nucleotides past the adduct position, allowing the incorporation of a second C on the subsequent template G (see Figure 1). Extension with nucleotides other than C did not occur using this template (Figure 4).

Single nucleotide extension reactions were carried out using the 23-mer primer in the presence of either dGTP or

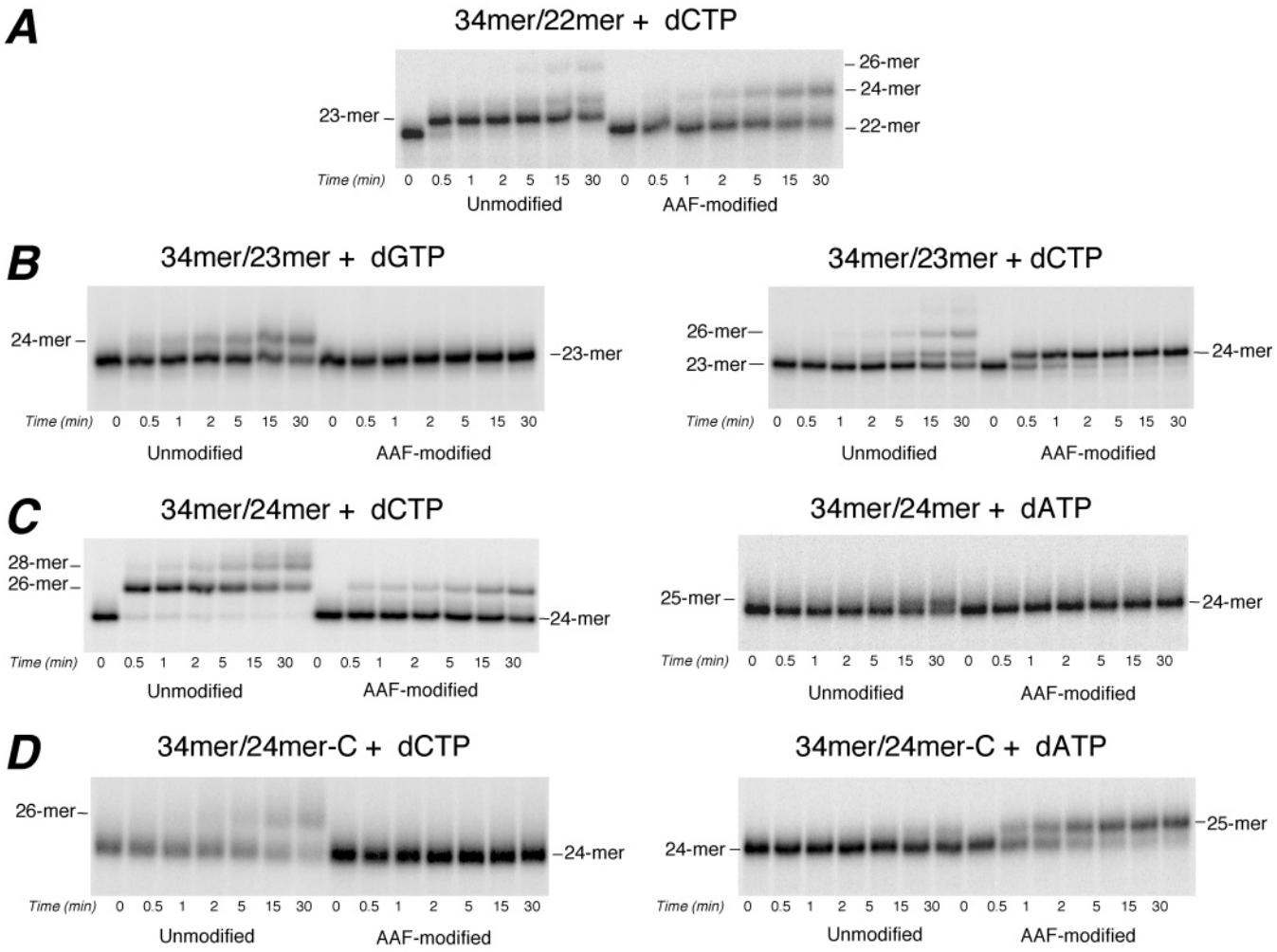

FIGURE 3: Single nucleotide primer extension analysis. ^{32}P-Labeled primers were annealed to unmodified and AAF-modified 34-mer templates and incubated with KF in the presence of the nucleotide shown above each gel panel. Aliquots of the extension mixtures were taken at timed intervals shown and the extension products detected by denaturing PAGE and phosphorimager analysis as described in the Materials and Methods section. (A) Extension products from the ^{32}P-labeled 22-mer primer shown in Figure 1A that has the 3′-terminus positioned one nucleotide before the adduct site. (B) Extension products from the ^{32}P-labeled 23-mer primer shown in Figure 1B that has the 3′-terminus positioned across from the adduct site. (C) Extension products from a ^{32}P-labeled 24-mer primer shown in Figure 1C that has the 3′-terminal G positioned one nucleotide after the adduct site. (D) Extension products from a ^{32}P-labeled 24-mer primer shown in Figure 1D that has the 3′-terminal C positioned one nucleotide after the adduct site. This primer forms the frameshift structure without mispairing at the terminal nucleotide.

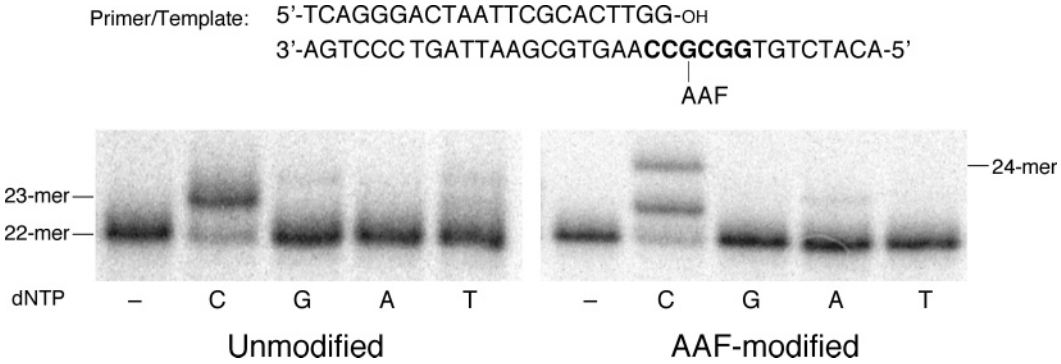

FIGURE 4: Nucleotide incorporation opposite an AAF-dG adduct in the *Nar*I site. Nucleotide incorporation was measured in the presence of the indicated dNTP on either an unmodified or AAF-modified template shown above using the procedure described in the Materials and Methods section. Reaction mixtures using unmodified templates were incubated for 2 min while those that contained AAF-modified templates were incubated for 30 min before terminating the reaction.

dCTP. On an unmodified template, dGTP was found to be incorporated (Figure 3B), suggesting that the primer-template was fully paired (see Figure 1B). However, on the AAF-modified template, no dGTP incorporation was observed, suggesting that the fully paired primer-template structure was not present in the polymerase active site. When this same experiment was carried out in the presence of dCTP, low levels of both 24- and 26-mer products were observed on

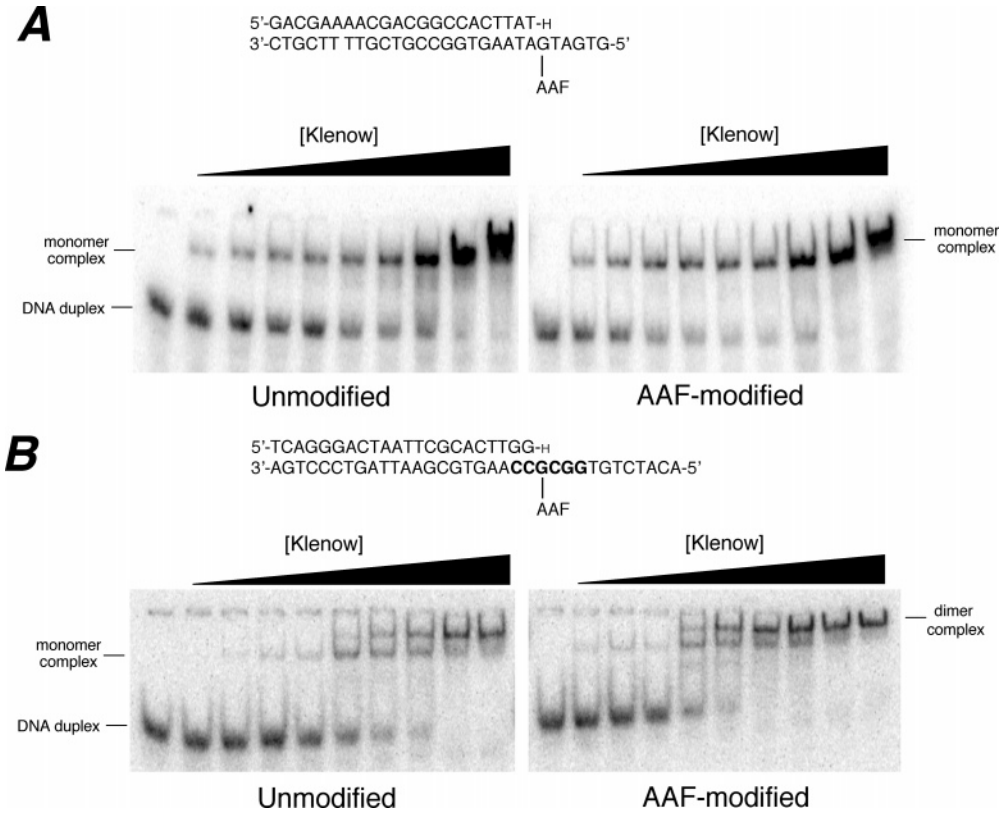

FIGURE 5: Gel shift binding analysis. The primer-templates shown above each gel were incubated with increasing concentrations of KF, and the binding was measured as described in the Materials and Methods section. (A) Unmodified or AAF-modified non-*Nar*I templates hybridized to primers that terminated one nucleotide before the adduct position. (B) Unmodified or AAF-modified *Nar*I templates hybridized to primers that terminated one nucleotide before the adduct position.

the unmodified template, produced possibly by misincorporation of C opposite the template C followed by extension on the two template Gs. However, on the AAF-modified template rapid incorporation of C was observed to form a 24-mer, suggesting again that the AAF adduct was inducing the looping out of the GC dinucleotide on this template.

Two different 24-mer primers were used to explore incorporation at the next position in the template. In the first set of experiments, a 24-mer primer that terminated with a 3′ G was used. In this case the primer is fully paired with the template. Synthesis in the presence of dCTP on the unmodified template produced primarily a 26-mer (Figure 3C), indicating a fully paired primer-template in the polymerase active site. The smaller amount of 28-mer product at later reaction times is presumably produced by a misincorporation of dCTP across from the template T. When this primer is paired with the AAF-modified template, low levels of a 26-mer product are observed (Figure 3C). This result suggests that at least a portion of the primer is fully paired with the template, which would result in a minor bypass product that does not involve the formation of a dinucleotide looped structure (see Figure 1C). dATP was not incorporated on the AAF-modified template, and the weak incorporation at later times on the unmodified template is probably due to misincorporation at this site.

Finally, when the primer was terminated with a 3′ C, this C would be able to pair with the template G if a GC dinucleotide loop formed upstream of the polymerase active site (Figure 1D). In this case low levels of a 26-mer were observed for incorporation on the unmodified when dCTP was present, presumably resulting from weak pairing of the terminal C with the template C (Figure 3D). No incorporation was observed in this case for the AAF-modified template. However, when dATP was added, the 25-mer was efficiently produced on the AAF-modified template, indicating that the GC dinucleotide loop was forming (Figure 3D). This addition did not occur if the template was unmodified.

Binding of KF to Primer-Templates Containing an AAF Adduct in the NarI Sequence. Prior studies have shown that the presence of the AAF adduct in the active site of KF resulted in an enhancement of binding strength compared with binding to an unmodified template and an inhibition of the polymerase to undergo a conformational change to the catalytically active closed complex (*14*, *15*). Crystal structure analysis of the close homologue, T7 DNA polymerase, bound to an AAF-modified template suggested that these properties resulted from the positioning of the AAF moiety behind and in close association with the O helix of the polymerase (*16*). To determine if stronger binding also occurred when the AAF adduct was positioned in the *Nar*I sequence, the dissociation constants were determined for binding of KF to a primer-template shown in Figure 1A. For these analyses, primer-templates were prepared such that the 3′-end of the primer was terminated with a dideoxynucleotide, and gel shift analysis was carried out as previously described (*14*) (Figure 5). For the analyses using the *Nar*I sequence, it is interesting to note that both monomeric and dimeric complexes of the polymerase were observed. These results have been observed using other sequences (*28*) and may be caused by the longer single-stranded region that extends from the template in this sequence or could be dependent on the sequence of the template at and around the binding site of the polymerase.

Table 1: Binding of the KF to a Primer-Template Containing a *Nar*I Sequence[a]

		K_d (nM)			
template		dCTP	dGTP	dATP	dTTP
unmodified	1.8 ± 0.6	0.3 ± 0.1	15.1 ± 5.5	17.5 ± 7.9	2.1 ± 1.2
AAF modified	3.2 ± 1.4	3.3 ± 2.0	5.7 ± 2.9	2.2 ± 0.8	3.5 ± 1.3

[a] Dissociation constants were determined with the 34-mer/22-mer template-primer using the experimental procedure described under Materials and Methods.

Table 2: Binding of KF to *Nar*I Templates Hybridized to Primers Terminating before, Across from, and Past the AAF Adduct[a]

Primer-Template		Nucleotide				
Fully paired	-2 Deletion	–	dCTP	dGTP	dATP	dTTP
		Kd (nM)				
...TTGGC ...AACCGCGGTGTCTACA		3.4 ± 1.6	–	1.0 ± 0.4	–	–
...TTGGC ...AACCGCGGTGTCTACA AAF	...TTG GC ...AAC CGGTGTCTACA CG AAF	6.8 ± 1.6	9.8 ± 2.5	49 ± 24	20 ± 11	47 ± 21
...TTGGCG ...AACCGCGGTGTCTACA		2.7 ± 0.9	0.2 ± 0.01	–	–	–
...TTGGCG ...AACCGCGGTGTCTACA AAF	...TTG GCG ...AAC CGGTGTCTACA CG AAF	10.2 ± 2.7	13.8 ± 3.1	42 ± 21	81 ± 21	20.2 ± 6.9
...TTGGCC ...AACCGCGGTGTCTACA AAF	... TTG GCC ...AAC CGGTGTCTACA CG AAF	7.6 ± 1.7	46 ± 14	247 ± 98	12.6 ± 4.9	20.6 ± 6.5

[a] Dissociation constants were determined using the experimental procedure described under Materials and Methods.

The dissociation constants that were calculated were for the first binding event, and it is assumed that the binding of the second polymerase resulted from protein−protein interactions rather than two-site ligand binding.

Table 1 shows the dissociation constants for the unmodified and AAF-modified *Nar*I primer-templates. In general, the binding strengths were weaker to templates containing the *Nar*I sequence in the active site. Moreover, unlike what was observed for binding to an AAF-modified non-*Nar*I sequence (*14*), the binding strength to the modified *Nar*I DNA was not enhanced compared with binding to the unmodified template. However, similar to what was observed for AAF adducts in non-*Nar*I sequence contexts, no enhancement or reduction of binding strength was observed when any of the four dNTPs were added (Table 1), indicating that the conformational change to a closed ternary complex was inhibited on this *Nar*I template. This result is consistent with the primer-template being fully paired at this point in synthesis because had a GC bulge formed, then the addition of dNTP would be expected to result in an enhancement of binding by the conversion to a closed ternary complex (*14*, *29*).

Walking the Primer Past the AAF Adduct in the NarI Sequence. To explore the mechanism for the GC dinucleotide deletion, gel shift analyses were performed using templates in which the primer ended across from or one nucleotide past the adduct position. When the primer is extended opposite the adduct (23-mer), it was found that the addition of dGTP, dATP, and dTTP all resulted in a weaker binding while the addition of dCTP caused no change in the K_d (Table 2). The weaker binding for a noncomplementary dNTP suggests that a conformational change in the polymerase structure was initiated but that a closed complex could not form because of a steric clash in the active site (*29*). Using this primer-template construction, dCTP is the correctly paired nucleotide if a GC dinucleotide loop is present upstream of the active site while dGTP is the correct nucleotide if the primer and template are fully paired (see structures shown in Table 2). Thus, it appears that the GC dinucleotide loop is the most likely structure for this primer-template construction since dCTP results in the strongest polymerase−template complex.

Two primer-template constructions were used for positioning the end of the primer at the +1 position (Figure 1C,D). In the first case (Figure 1C), the primer sequence was complementary to the template if no GC dinucleotide loop formed, while in the second case (Figure 1D), the sequence was complementary if the GC loop formed. As expected, in the first case, where dCTP is the correct nucleotide, dCTP addition resulted in the most stable polymerase−primer-template complex while dATP provided the most stable complex in the second case (Table 2). The former result is consistent with the formation of a fully paired primer-template that allows for the production of bypass products observed in Figures 2 and 3C. Comparison of the two cases indicated that the stability of the two complexes (i.e., dCTP

addition in the first case and dATP addition in the second case) was approximately the same.

Taken together, the dissociation constants observed in these studies suggest that the interactions between the KF and a primer-template containing an AAF adduct in the *Nar*I sequence are very different from those observed when the adduct is positioned in a non-*Nar*I sequence. Not only is it likely that the AAF adduct is positioned very differently in the active site of the KF when located in a *Nar*I sequence, but an AAF adduct in this sequence appears to lead to a GC dinucleotide loop structure that presumably leads to the −2 deletion frameshift mutation.

DISCUSSION

The AAF modification at the G_3 position of the *Nar*I sequence ($G_1G_2CG_3CC$) results specifically in a GC dinucleotide deletion in bacteria (*4*, *5*, *30*). This mutational event has been reproduced in vitro using purified *E. coli* polymerase II and templates containing an AAF adduct positioned at G_3 of the *Nar*I sequence (*17*). In the present study we found that KF was also able to generate the GC deletion as the major extension product when the AAF adduct is on G_3 (Figure 2). This result suggests that the structure that forms to produce this mutation is not specific for Pol II and may be a more general phenomenon relating to the structure of the AAF adduct in this sequence. In either of these cases, the molecular mechanism that leads to this unusual mutational consequence is unknown. The fact that the positioning of an AAF adduct within this sequence leads to unusual structures in duplex DNA (*31*) has led to the speculation that this structural distortion may somehow be maintained in the polymerase active site. Similar arguments have been made when comparing the mutational and structural differences between an AAF and AF adduct. Thus, the AAF adduct linked at the C8 position of guanine is a frameshift mutagen in bacteria (*4*) and is known to distort the structure of the DNA duplex (*9*, *11*, *32*, *33*) while the related AF adduct causes base substitution mutations (*3*) and is known to cause much less distortion (*6*, *10*, *34*). Recent crystallographic studies (*16*, *35*) have shown that the AAF and AF adducts do in fact have very different orientations within the active site of the T7 DNA polymerase, structures that may be related to the orientations of the adducts in DNA.

Two possibilities are envisioned for the mutational mechanism by which an AAF adduct generates frameshift mutations in general and specifically how its presence in the *Nar*I sequence results in a GC dinucleotide deletion. Either the polymerase incorporates a nucleotide opposite the adduct position followed by a rearrangement to a structure that contains a two-nucleotide bulge or the adduct induces the formation of this bulge structure first, followed by incorporation of a nucleotide directly at the position one nucleotide past the adduct. To distinguish between these possibilities, a template was constructed containing an AAF adduct situated at the G_3 position of the *Nar*I sequence, and polymerase extension and binding interactions were measured when the template was hybridized to primers that end before, across from, and past the adduct site. As indicated in the following analysis, our results are best explained by the mechanism in which nucleotide incorporation precedes bulge formation.

Single nucleotide extension from a primer that ends one nucleotide prior to the adduct position indicated that C was preferentially incorporated (Figure 4), but these types of studies cannot be used to distinguish between the two possible mechanisms for deletion formation. This is due to the fact that a C is the correct nucleotide for incorporation for either possible structure. Thus, in addition to extension studies we have also measured the ability of the polymerase to undergo a conformational change to a closed complex as an indication of the structure of this template in the polymerase active site.

Prior studies have shown that measurements of the polymerase dissociation constants can be useful in predicting the structure of the adducts in the polymerase active site. Thus, we found that the polymerase was bound more tightly to a template containing an AAF adduct in the active site (*14*), a fact that was explained by the crystal structure studies to result from an intercalation of the adduct behind the O helix (*16*). Moreover, our interpretation that an AAF adduct inhibited closed complex formation, based on the fact that the addition of any dNTP did not cause an increased polymerase binding strength (*14*), was confirmed by both protease digestion studies (*15*) and by the position of the O helix in the crystal structure (*16*).

The dissociation constants that were determined for the complexes in which the primer ends prior to the adduct in the *Nar*I sequence (Table 1) indicate that the adduct is inhibiting the conformational change, corresponding to what is found when the adduct is positioned in a non-*Nar*I sequence (*14*). Although this result does not completely rule out a dNTP-stabilized misalignment model, it is most consistent with a structure in which the primer is fully paired with the template in the active site and the adduct is positioned in a way that inhibits the conformational change, just as is found for non-*Nar*I sequences (*15*). Had the adduct caused the template to loop out to form the GC dinucleotide bulge, then the addition of dCTP would have been expected to result in tighter polymerase binding following the formation of the closed ternary complex (*14*). The facts that the binding is significantly weaker than observed with templates containing an AAF adduct in a non-*Nar*I sequence and is not stronger compared with an unmodified primer template may indicate that a different structure is formed in the *Nar*I case, one where the adduct is not intercalated behind the O helix.

When the primer was extended to a position across from the adduct, extension studies (Figure 3) and dissociation constants (Table 2) are both consistent with a structure where the GC dinucleotide bulge has formed upstream of the active site. In this situation, if the primer is fully paired, then dGTP is the correct nucleotide for incorporation across from the C that follows the adduct position. However, the results shown in Figure 3 and Table 2 indicate that dCTP, not dGTP, is efficiently incorporated in this situation, and the presence of this nucleotide results in the strongest polymerase binding. Both results are consistent with the pairing of the primer terminus with the G two nucleotides past the adduct position (see Figure 1B).

To extend the primer to the next position, two different primers needed to be used because the sequence at this position was different depending on the structure of the complex (see Figure 1C,D). Using the fully paired primer-

template both extension (Figure 3C) and the strongest binding (Table 2) occurred when dCTP, the correct nucleotide for extension at the next position, was added. Alternatively, if a primer sequence was used that allowed correct pairing with a GC bulge (Figure 1D), then dATP, the correct nucleotide at the next position in this structure, allowed extension to occur (Figure 3D) and provided the strongest binding (Table 2). Taken together, these results indicate that having one nucleotide correctly paired past the adduct position could result in a structure that the polymerase could utilize without a frameshift structure forming but that if the frameshift structure was present, it also could be efficiently extended.

These studies clearly show that an AAF adduct in the *Nar*I sequence results in a very different structure for the primer-template in the polymerase active site compared with a non-*Nar*I sequence. Presumably, it is this difference that leads to the propensity of this sequence to generate a GC dinucleotide deletion. Both extension and binding experiments indicated that with the *Nar*I-modified template a dinucleotide bulge can form upstream of the polymerase active site but, interestingly, once the adduct is bypassed by one nucleotide in the fully paired structure, then the deletion mutation does not occur. In addition, these results suggest that the frameshift structure forms only after the incorporation of a C across from the AAF-modified G and thus support a two-step mechanism for trans-lesion synthesis (*36*, *37*) in which incorporation and extension might be performed by two different polymerases.

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