Effect of Aminofluorene and (Acetylamino)fluorene Adducts on the DNA Replication Mediated by *Escherichia coli* Polymerases I (Klenow Fragment) and III[†]

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ABSTRACT: *N*-(Deoxyguanosin-C8-yl)-2-(acetylamino)fluorene (dG-C8-AAF) and *N*-(deoxyguanosin-C8-yl)-2-aminofluorene (dG-C8-AF) are the two major DNA adducts induced by the chemical carcinogen 2-(acetylamino)fluorene (AAF). Molecular modeling shows that, in the DNA double helix, dG-C8-AF can maintain an *anti*-structure and normal base pairing, while dG-C8-AAF favors a *syn*-structure and causes base displacement. In the ϕX174 RF DNA−*Escherichia coli* transfection system, it has been found that dG-C8-AF is 7−10-fold less lethal than dG-C8-AAF; these results suggest that these two kinds of DNA adducts may have different effects on DNA replication and that they may be repaired by different pathways. We have investigated the effects of these two kinds of adducts on DNA polymerase III holoenzyme (pol III-H) and DNA polymerase I Klenow fragment (pol I-Kf) mediated DNA synthesis by using carcinogen-modified M13 single-stranded DNA hybridized with ^{32}P-labeled primer as templates. We have found that pol III-H and pol I-Kf replicate through dG-C8-AF with 92% and 62% frequency, respectively; in contrast, these two enzymes replicate through dG-C8-AAF with only 38% and 25% frequency, respectively. AF-adducted DNA shows a more profound sequence specificity in blocking DNA synthesis than AAF-adducted DNA, and the sequence specificities in blocking DNA synthesis for both kinds of adducts differ for pol III-H and pol I-Kf.

2-Acetylaminofluorene (AAF)[1] is a strong procarcinogen; metabolically activated AAF reacts with macromolecules such as DNA, RNA, and proteins [for reviews, see Miller (1970) and Miller and Miller (1975)]. It has been shown that *N*-(deoxyguanosin-C8-yl)-2-(acetylamino)fluorene (dG-C8-AAF), *N*-(deoxyguanosin-C8-yl)-2-aminofluorene (dG-C8-AF), and 2-(deoxyguanosin-N^2-yl)-2-(acetylamino)fluorene are the three major DNA adducts formed in AAF-treated animals [for reviews, see Kriek and Westra (1979), Irving (1979), and Miller (1978)]. Although it is believed that AAF-induced damage in DNA initiates carcinogenesis, the role of these three kinds of DNA adducts in carcinogenesis is unclear. In rodent liver dG-C8-AAF is rapidly removed, while dG-C8-AF can remain in the cells for months (Kriek, 1972; Beland et al., 1982). The effects of these two kinds of adducts on DNA replication and how they are repaired may be important in determining their role in carcinogenesis.

Previously, using the ϕX174 RF DNA−*Escherichia coli* cell transfection system, we found that although the repair of both dG-C8-AF and dG-C8-AAF adducts is controlled by the *uvr* nucleotide excision repair system, *uvrA*, *uvrB*, and *uvrC* mutants respond differently to dG-C8-AF but not dG-C8-AAF adducts (Tang et al., 1982). Moreover, we have found that dG-C8-AF is much less lethal for viral DNA survival than dG-C8-AAF in *E. coli* cells (Tang et al., 1982). These results suggest that these two kinds of DNA adducts have different effects on DNA replication and may be repaired by different repair pathways.

In order to investigate the effects of dG-C8-AF and dG-C8-AAF on DNA replication and their relevance to lethality and mutagenicity, we have quantified these effects on the DNA synthesis conducted by DNA polymerase III holoenzyme (pol III-H) and DNA polymerase I (Klenow fragment) since these enzymes are the major DNA replication enzymes in *E. coli* cells (Kornberg & Baker, 1992). We have found that neither dG-C8-AF nor dG-C8-AAF totally blocks DNA synthesis. Calculations based on the Poisson equation show that both pol III-H and pol I-Kf read through dG-C8-AF significantly more efficiently than through dG-C8-AAF. We also found that the DNA sequence plays an important role in adduct-induced DNA synthesis blockage, and this effect is more profound in AF-adducted DNA than in AAF-adducted DNA.

MATERIALS AND METHODS

Materials. [*ring*-^{3}H]NA-AAF (specific activity, 710 Ci/mmol) and [*ring*-^{3}H]N-OH-AF (specific activity, 110 mCi/mmol) were generous gifts from Dr. Fred Beland, National Center for Toxicological Research, Jefferson, AR. *E. coli* DNA polymerase III holoenzymes and single-stranded DNA binding proteins were kindly provided by Dr. Charles McHenry, University of Colorado, Boulder, CO. *E. coli* DNA polymerase I Klenow fragments, restriction enzymes *Hin*dIII, and *Pvu*II, and T4 kinase were purchased from Bethesda Research Laboratories. γ ^{32}P TTP and [α^{32}P]dATP were purchased from DuPont Co.

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[1] Abbreviations: AF, 2-aminofluorene; AAF, 2-(acetylamino)fluorene; N-OH-AF, *N*-hydroxy-2-aminofluorene; NA-AAF, *N*-acetoxy-2-(acetylamino)fluorene; dG-C8-AF, *N*-(deoxyguanosin-C8-yl)-2-aminofluorene; dG-C8-AAF, *N*-(deoxyguanosin-C8-yl)-2-(acetylamino)-fluorene, RF DNA, replicative form DNA; pol III-H, DNA polymerase III holoenzyme; pol I-Kf, DNA polymerase I Klenow fragment; DTT, dithiothreitol; TFA, trifluoroacetic acid.

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N-OH-AF and NA-AAF Modification of M13 DNA. M13mp10 single-stranded DNA (ssDNA) was isolated as described by Messing (1983). To achieve different degrees of carcinogen modification, various amounts of either [*ring*-^{3}H]NA-AAF or [*ring*-^{3}H]N-OH-AF were dissolved in dimethyl sulfoxide or argon-purged ethanol and added to 50 μg of M13mp10 ssDNA in a solution containing 5 mM Tris, 0.5 mM EDTA, and 0.01 M sodium citrate, pH 5.5. Samples were incubated for 3 h at room temperature in the dark, and the unbound N-OH-AF and NA-AAF were removed by diethyl ether extractions followed by ethanol precipitation. The number of adducts per M13 DNA molecule was determined by the ^{3}H specificity in the modified DNA.

Construction of DNA Templates. A chemically synthesized 23-base oligonucleotide, 5′-CCAGTGCCAAGCTTGGGCTGCAG-3′ (RD1), which is complementary to the region containing the unique *Hin*dIII site in M13mp10 DNA, was used as primer. These DNA fragments were purified on a 20% polyacrylamide gel and ^{32}P labeled at the 5′ end with γ-^{32}P TTP using T4 kinase as previously described (Pierce et al., 1989). These 5′-^{32}P-labeled primers were added to M13mp10 ssDNA at a primer to template molar ratio of 6:1. An aliquot of 0.5-μg mixtures of templates and primers of DNA was heated in 10 mM Tris, pH 7.5, and 50 mM NaCl for 1 min at 50 °C and then was cooled to 25 °C over 40 min. The primed DNA templates were frozen at −20 °C until further use.

Pol III-H-Mediated DNA Synthesis and Characterization of the Synthesized Products. A ratio of 70 units of DNA pol III-H to 60 ng of M13mp10 ssDNA primed with 5′-^{32}P-end-labeled RD1 was used with 0.6 μg of *E. coli* single-stranded DNA binding proteins in 50 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.7, 10 mM DTT, 20% glycerol, 0.01% NP40, 100 μg/mL bovine serum albumin, 5 mM $MgCl_2$, 50 mM NaCl, and 150 μM of each of the four deoxyribonucleotide phosphates (dNTPs). Reactions were carried out in a volume of 15 μL for 20 min at 30 °C. Reactions were stopped by adding EDTA (5 mM, pH 7) followed by phenol extractions and then diethyl ether extractions. The synthesized DNA products were then separated by alkaline agarose gel electrophoresis for quantification of full-length DNA and by sequencing gel electrophoresis for characterization of the sequence effect on the blockage of DNA synthesis induced by dG-C8-AF and dG-C8-AAF adducts.

For full-length DNA determination, an aliquot (10 μL) of each sample was denatured by mixing in a denaturing dye solution (30 mM NaOH solution with 10% (v/v) glycerol, 5 mM EDTA, and 1.5% bromophenol blue), heating for 3 min at 95 °C, and then immediately quenching on ice. Similar sample count levels, as determined by Cerenkov counting of the tube before and after loading, were electrophoresed in alkaline agarose gels (0.8% agarose in 30 mM NaOH and 1 mM EDTA) for 18 h at 40 V or 1.14 V/cm in 30 mM NaOH and 1 mM EDTA. Gels were then dried onto DEAE membranes with a Bio-Rad 1125B gel dryer. Gels were counted on a Betagen Betascope and subsequently autoradiographed. Full-length replication products were quantified by the manual quantitation mode. Graphs plotting cpm/mm^2 vs distance migrated were generated with the lane profile mode that takes data points every 0.4 mm down the lane.

For sequence analysis of synthesized products the samples were ethanol precipitated and resuspended in formamide dye solution (98% deionized formamide, 5 mM EDTA, and 0.5% of both bromophenol blue and xylene cyanol) followed by heating for 3 min at 95 °C and quenching on ice. Similar count levels of each sample were electrophoresed in an 8% polyacrylamide/8M urea sequencing gel for 2 h at 50 mA. Sample tubes were counted by Cerenkov counting, both before and after sample loading, to determine the amount loaded. Dideoxy DNA sequencing samples synthesized by pol III-H were run parallel to the samples.

Pol I-Kf-Mediated DNA Synthesis and Characterization of the Synthesized DNA Products. Pol I-Kf reactions were carried out as recommended by the supplier, BRL. The reaction was carried out at 30 °C for 20 min in a 15-μL solution of 7 mM Tris·HCl (pH 7.5), 7 mM $MgCl_2$, 50 mM NaCl, and 6.7 mM DTT containing 1 unit of Pol I-KF and 75 ng of M13mp10 ssDNA template primed with the 5′-^{32}P-labeled 23-base primer, RD1. The reactions were stopped by the addition of NaOH to 30 mM, EDTA to 5 mM, glycerol to 10% (v/v), and bromophenol blue to 1.5%, followed by heating at 95 °C for 3 min. Similar counts were loaded and electrophoresed on alkaline and sequencing gels as described above for quantification of full-length DNA and characterization of the synthesized products.

Isolation of 3′-Labeled 221-Base ssDNA Fragment. In order to determine the extent of adduction of the guanine residues at different positions, template DNAs were 3′ end labeled with ^{32}P as follows: 30 pmol of RD1, complementary to the M13mp10 DNA region containing the unique *Hin*dIII site, was added to 1.3 pmol of carcinogen-modified M13mp10 ssDNA in 150 μL of 50 mM Tris, pH 8.0, 10 mM $MgCl_2$, and 50 mM NaCl. Samples were heated at 50 °C for 1 min and allowed to cool to 25 °C over 30−40 min. The hybrid DNAs were then digested with *Hin*dIII (10 units and 37 °C incubation for 2 h). The template DNAs were then 3′ end labeled by adding 3 pmol of [α-^{32}P]dATP (6000 Ci/mol) and 1 unit of pol I-Kf, and the mixtures were incubated at 22 °C for 30 min. DNAs were precipitated by ethanol with 5 μg of tRNA as a carrier and resuspended in 40 μL of 50 mM Tris, pH 7.4, 6 mM $MgCl_2$, 50 mM KCl, and 50 mM NaCl. To generate a second restriction site, the ^{32}P-labeled DNAs were added to 30 pmol of the 23-base oligonucletide 5′-TGTCGTGCCAGCTGGATTAATGA-3′ (RD2), which is complementary to M13 bases 6045−6067 and contains a *Pvu*II restriction site, and the mixtures were heated at 50 °C and then cooled to 25 °C over 40 min. These DNAs were then digested with *Pvu*II (10 units and 37 °C incubation for 2 h). This treatment generated a 221-base ssDNA fragment and a 10-base ssDNA fragment that was ^{32}P labeled, as depicted in Figure 1B. These products were ethanol precipitated, resuspended in 20 μL of formamide dye solution, and electrophoresed in a 5% polyacrylamide gel, and the 221-bp fragments were isolated.

Determination of Sequence Specificity of Reaction of NA-AAF and N-OH-AF. AF- or AAF-adducted, ^{32}P-labeled, 221-base DNA was treated for various times with 1 M piperidine at 90 °C; this treatment causes strand breakage at the dG-C8-AF and dG-C8-AAF adducts (Bases et al., 1983; Johnson et al., 1987; Pierce et al., 1989). The samples were then transferred to new tubes and frozen on dry ice, and the piperidine was removed *in vacuo* in a Savant Speed Vac concentrator. Samples were resuspended in a formamide dye

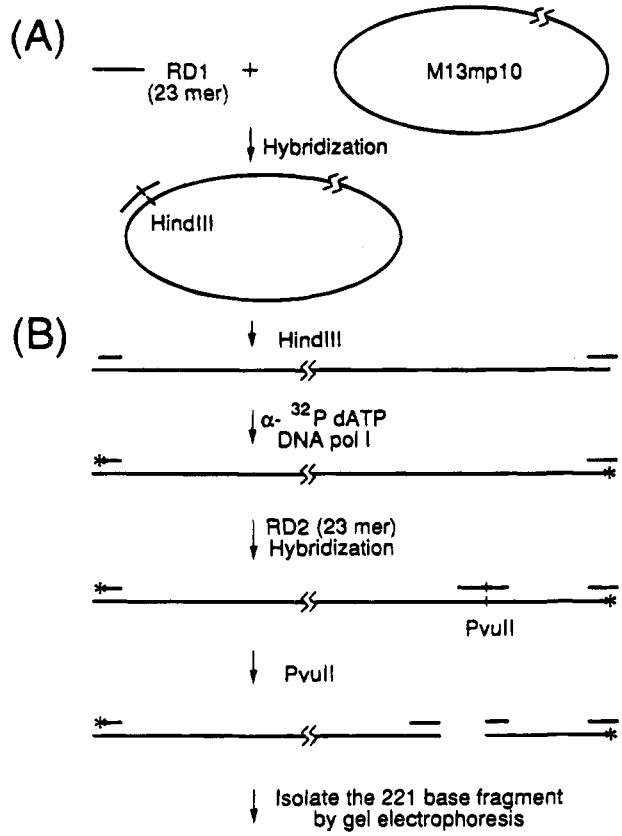

FIGURE 1: Schematic presentation of methods for constructing (A) a template for DNA synthesis & (B) a 3′-end-^{32}P-labeled 221-base M13mp10 ssDNA fragment.

mix and incubated at 37 °C for 30 min, followed by electrophoresis at 50 mA in an 8% polyacrylamide gel containing 8 M urea. Maxam and Gilbert (1980) sequencing reactions were performed on nonadducted 221-base DNA, and these samples were electrophoresed parallel to the carcinogen-modified samples. Gels were dried on 3MM Whatman chromatography paper with a Bio-Rad gel dryer. Following autoradiography with Kodak XAR-2 X-ray film, 20-cm squares were cut and counted on a Betagen Betascope for 4−16 h. Band intensity was quantitated in the manual quantitation mode. Band counts from piperidine-treated, unmodified controls were subtracted from the corresponding piperidine-treated, carcinogen-modified DNA band counts.

High-Performance Liquid Chromatography (HPLC) Analysis of N-OH-AF- and NA-AAF-Modified M13mp10 ssDNA. HPLC analysis was performed according to the method of Tang and Lieberman (1983). Modified DNA was solvolyzed in trifluoroacetic acid (TFA) and analyzed on a C18 column with a gradient of 21−70% methanol in 8 mM $(NH)_4H_2PO_4$ (pH 3.8, 20 °C) over 48 min at a flow rate of 1 mL/min. Fractions were counted in a scintillation counter to identify fractions with ^{3}H-labeled adducts.

Quantitations. In order to quantify the effect of AF−DNA and AAF−DNA adducts on DNA synthesis, a theoretical curve based on the Poisson equation and representing the percentage of full-length DNA that would be synthesized if every AF−DNA and AAF−DNA adduct were to block DNA synthesis completely was constructed. In brief, the percentage of unmodified template DNA which will allow full-length DNA synthesis is equal to $e^{-n} \times 100\%$, where n is the average number of AF−DNA or AAF−DNA adducts per template DNA molecule. The percentage of full-length DNA synthesized using AF- and AAF-modified templates was calculated, and these values were compared to the theoretical curve to obtain the equivalent number of modifications per DNA template in the theoretical curve. The percentage of AF−DNA and AAF−DNA adducts being bypassed by the pol III-H- or pol I-Kf-mediated DNA synthesis was then calculated by using the equation $[(A - T)/A] \times 100\%$, where A is the actual number of AF−DNA or AAF−DNA adducts per template DNA (obtained by the specific activity of ^{3}H-AF or -AAF in DNA), and T is the equivalent theoretical number of AF or AAF modifications per template, which would render the same percentage of full-length DNA synthesized, assuming that every AF−DNA and AAF−DNA adduct blocked DNA synthesis completely.

RESULTS

Since molecular modeling shows that dG-C8-AF favors the *anti*-structure with the AF moiety protruding into the major groove, and consequently maintaining the normal base pairing in the DNA helix, while dG-C8-AAF favors the *syn*-structure with the AAF moiety stacking within the DNA helix, causing helix distortion and base displacement (Fuchs & Duane, 1974; Grunberger & Weinstein, 1978; Hingerty & Broyde, 1982; Lipkowitz et al., 1982; Leng et al., 1980), it is possible that NA-AAF-modified ϕX174 DNAs have lower transfectivity than N-OH-AF-modified DNAs because the DNA replication machinery in *E. coli* is able to replicate through dG-C8-AF more efficiently than through dG-C8-AAF. In order to test this possibility, we have determined the efficiency of two major DNA replication enzymes in *E. coli* cells—pol III-H and polymerase I—in replicating DNA templates containing either dG-C8-AF or dG-C8-AAF. For experimental convenience, we used pol I-Kf instead of polymerase I. M13mp10 ssDNA containing different numbers of dG-C8-AF or dG-C8-AAF was hybridized with ^{32}P-labeled primers as depicted in Figure 1A; these partial duplex DNAs were then used as templates for pol I-Kf- or pol III-H-mediated DNA replication under the conditions described in Materials and Methods. The synthesized DNA products were subsequently either separated by alkaline agarose gel electrophoresis for the purpose of quantifying the full-length DNAs to determine the effect of dG-C8-AF and dG-C8-AAF on DNA replication (Figures 2 and 3), or separated by sequencing gel electrophoresis for analyzing the effects of sequence context on the replication of adducted bases (Figures 4, 5, and 6).

Effects of AF and AAF Modification on DNA Replication. The size distributions of the pol III-H- and Pol I-Kf-synthesized DNAs separated by alkaline agarose gel electrophoresis are shown in Figure 2A,B, and these results demonstrate that these two enzymes have greatly different processivities in DNA synthesis; using unmodified M13 ssDNAs as templates, >90% of DNA synthesized by pol III-H was full length. In contrast, less than 10% of DNA synthesized by pol I-Kf was full length (compare lanes 1 and 5 to 12 and 20 in Figure 2A,B, C). Figure 2A,B also shows that the relative amount of the synthesized DNA in full-length DNA decreases when M13 ssDNAs containing dG-C8-AF or dG-C8-AAF were used as template, and this decrease apparently is dependent on the number of adducts in the template (compare lane 2 to 4, 6 to 11, 13 to 15, and 16 to 19 in Figure 2). From visual inspection it appears that the dG-C8-AAF adducts have a greater effect than the

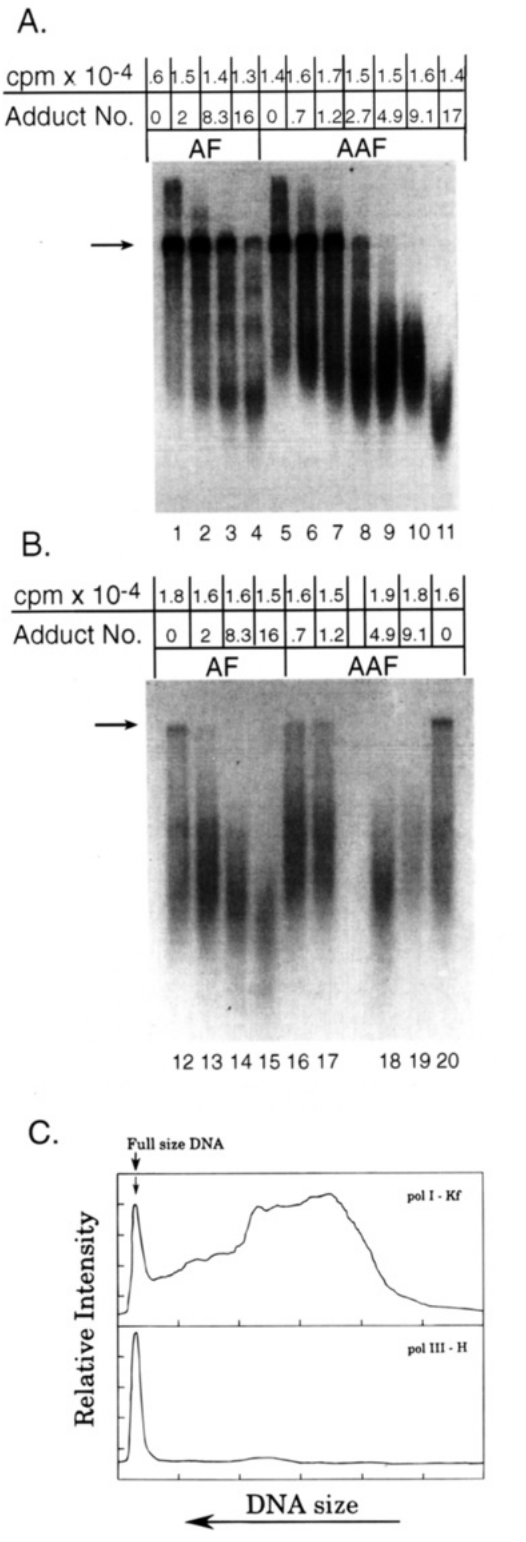

FIGURE 2: Typical autoradiograph of the electrophoretically separated DNA synthesized by DNA pol III-H (A) and DNA pol I-Kf (B) using DNA templates (as shown in Figure 1) modified with different concentrations of N-OH-AF or NA-AAF. The position of the full-length DNA is indicated by an arrow in both panels. The numbers of DNA adduct per DNA molecule and the amount of DNA (cpm) applied to each lane are indicated at the top of each panel. The methods for carcinogen DNA modifications and the conditions for DNA synthesis are described in Materials and Methods. (C) Size distribution of synthesized DNA mediated by pol I-Kf and pol III-H using unmodified DNA templates. The results were obtained by densitometer scanning of lane 1 in (A) and lane 20 in (B). The position of full-size M13 ssDNA is indicated by an arrow.

dG-C8-AF adducts in reducing DNA synthesis to completion, and this effect was observed in both pol III-H- and pol I-Kf-mediated DNA syntheses. To quantify this effect, the amount of radioactivity in the full length of the M13mp10 DNA region was counted and its amount relative to total synthesized DNA was determined. Figure 3 shows that the percent reduction of full-length DNA synthesized is exponentially proportional to the number of adducts in template DNA, and the slope is much steeper in those using dG-C8-AAF-containing templates than in those using dG-C8-AF-containing templates. A theoretical line was calculated on the basis of the Poisson distribution equation, assuming that every AF and AAF modification site would completely block DNA replication, as shown in Figure 3. By comparing the slopes of lines obtained experimentally with the slope of the theoretical line, we have found that (1) pol III-H replicates through dG-C8-AF with 92% frequency, but it replicates through dG-C8-AAF with only 38% frequency; and (2) pol I-Kf replicates through dG-C8-AF with 62% frequency, but it replicates through dG-C8-AAF with 25% frequency. These results demonstrate that pol III-H is more efficient in reading through the AF- and AAF-modified guanines.

Effects of Sequence Context on AF- and AAF-Modification-Induced Blockage of DNA Synthesis. The different efficiencies of dG-C8-AF and dG-C8-AAF in blocking DNA synthesis are likely due to the intrinsic properties of these two adducts that affect base pairing and DNA synthesis. These efficiencies may vary in different sequence contexts since the structures of these two kinds of adducts at different sequences may not necessarily be uniform. To test this possibility, we quantified both the relative efficiency of the replication blockage at guanine residues at different sequences by dG-C8-AF and dG-C8-AAF and the relative frequency of dG-C8-AF and dG-C8-AAF formed at these sequences.

The DNA fragments shown in Figure 2A,B, which were synthesized by pol III-H and pol I-Kf using AF- or AAF-adducted templates, were separated by denaturing polyacrylamide gel electrophoresis for sequence analysis (Figure 4). Bands that were associated with the blocking of DNA synthesis at virtually every guanine were observed. For both AF- and AAF-adducted templates pol III-H produced bands which are 1 base lower than the guanine bands in the sequencing lane (lanes 2, 3, 8, and 9 in Figure 4B); since N-OH-AF and NA-AAF only modify guanine residues, and since no such band is produced when unmodified DNAs are used as templates, these results suggest that pol III-H stalls one base before the AF- or AAF-adducted guanine. In contrast, the stalling positions relative to AF- and AAF-adducted guanines produced by pol I-Kf are different. Using AF-adducted DNA as templates, pol I-Kf produced bands at the guanine positions as well as the positions one base prior to each of the guanines (lanes 4, 5, and 6 in Figure 4A); using AAF-adducted templates, pol I-Kf produced bands corresponding to the position one base prior to each of the guanine positions (lanes 2 and 3 in Figure 4A). These results suggest that pol I-Kf stalls at or one base prior to the AF-adducted guanine and one base prior to the AAF-adducted guanine. Similar results, which show that DNA polymerases pause at or one base before AF−DNA and AAF−DNA adducts, have been obtained by several laboratories (Moore et al., 1982; Michaels et al., 1987; Belguise-Valladier et al., 1994).

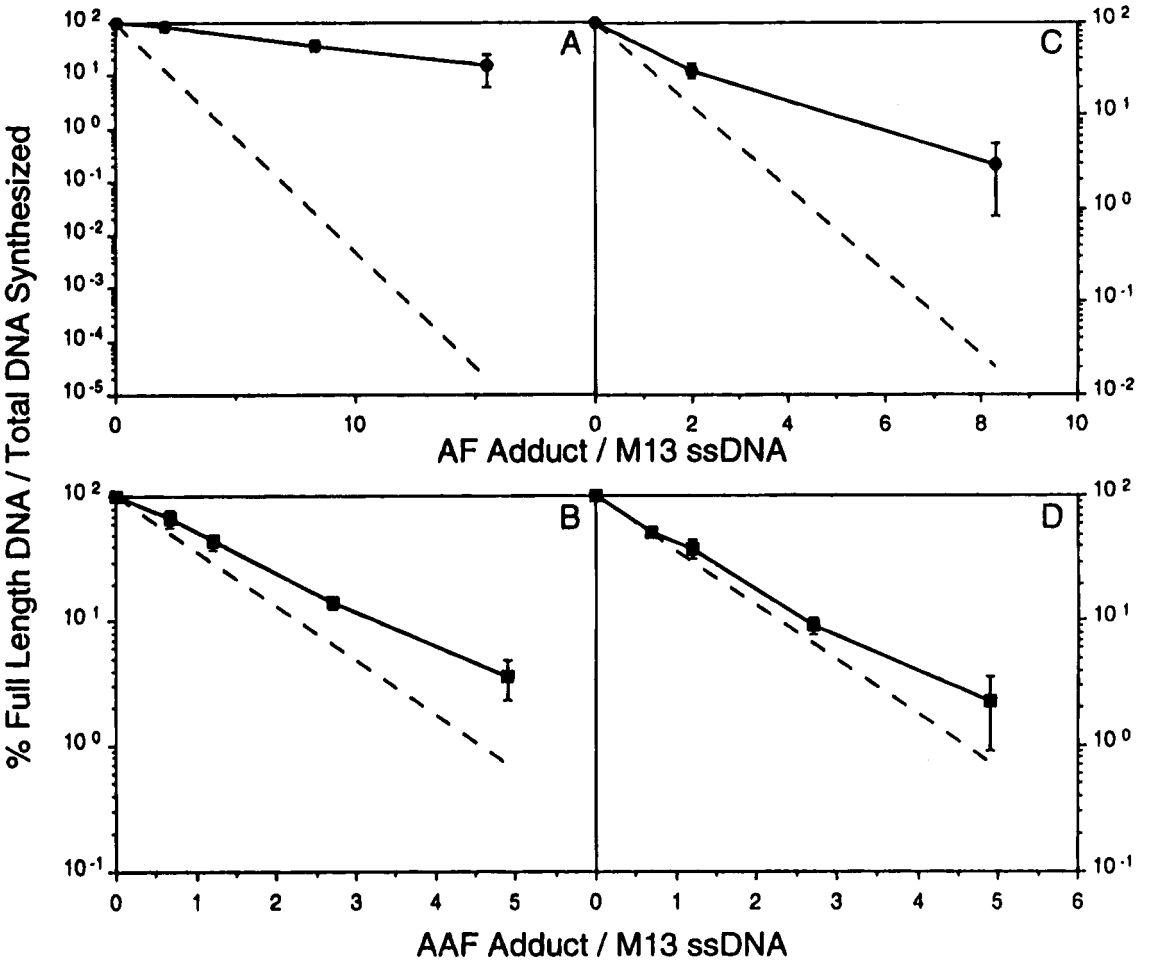

FIGURE 3: Quantification of the effects of N-OH-AF (A and C) and NA-AAF (B & D) modifications on DNA synthesis mediated by DNA pol III-H (A and B) and DNA pol I (C and D). The full-length DNA synthesized by pol III-H or pol I-Kf using N-OH-AF- and NA-AAF-modified templates as shown in Figure 2 was scanned, and its fraction in the total synthesized DNA was calculated. The effects of the AF−DNA and AAF−DNA adducts on DNA synthesis were calculated on the basis of the assignment of the fraction of full-length DNA synthesized using unmodified DNA templates as 100%. The dashed lines represent the theoretical values calculated on the basis of the Poisson distribution equation, assuming that each AF−DNA and AAF−DNA adduct would block the DNA synthesis completely. The results are the averages of two experiments.

The intensity of these bands varies significantly at different sequences. The most striking differences occurred at two regions: 6178/6179 and 6116/6120 (Figure 4A). At the former positions no bands were observed for AAF-adducted templates, and at the latter no bands were observed for AF-adducted templates. The intensities of these bands should represent the frequencies of the replication stallings. In principle these different replication stallings could be due to variations of adduct formation and/or different efficiencies of blockage of DNA synthesis at different sequences.

Effects of Sequence Context on the Efficiency of AF−DNA and AAF−DNA Adduct Formation. It has been shown that hot alkaline treatments induce phosphodiester bond breakage at the 5′ side of AF- and AAF-modified guanines (Bases et al., 1983; Johnson et at., 1987; Pierce et al., 1989). The chemistry leading to piperidine-induced strand cleavage at the site of dG-C8-AF and dG-C8-AAF adducts has not been fully elucidated. Furthermore, the kinetics of this reaction may not be simple pseudo-first-order (Johnson et al., 1987). If we ignore this potential complication and assume that the fraction of adducts hydrolyzed for any given time is independent of sequence context, then the intensity of strand breaks reflects the level of adduction. It is currently impossible to assess the validity of this assumption, but it seems intuitively reasonable.

In order to distinguish between the aforementioned two possibilities, we determined the effects of DNA sequence on the formation of dG-C8-AF and dG-C8-AAF adducts by hot alkaline methods. In brief, the N-OH-AF- and NA-AAF-modified templates were 3′ end labeled with ^{32}P as depicted schematically in Figure 1B. These DNA fragments were then treated with 1 M piperidine for different periods of time at 90 °C and separated by sequencing gel electrophoresis. Results in Figure 5 show that piperidine treatment up to 1 h produces no bands in unmodified DNA. However, this treatment, for both N-OH-AF- and NA-AAF-modified DNA, produces breakage at positions identical to the guanine positions in the Maxam and Gilbert (1980) sequencing lane. Figure 5 also shows that the DNA modified with higher concentrations of N-OH-AF and NA-AAF produces stronger piperidine bands than the DNA modified with lower N-OH-AF and NA-AAF concentrations (compare lane 2 to lane 6 and lane 19 to lane 23), and this increment of piperidine band intensity is independent of sequence. Given these results, and making the assumption cited above, the results in Figure 5 suggest that piperidine-induced breakage in both AF- and AAF-modified DNA represents the extent of the

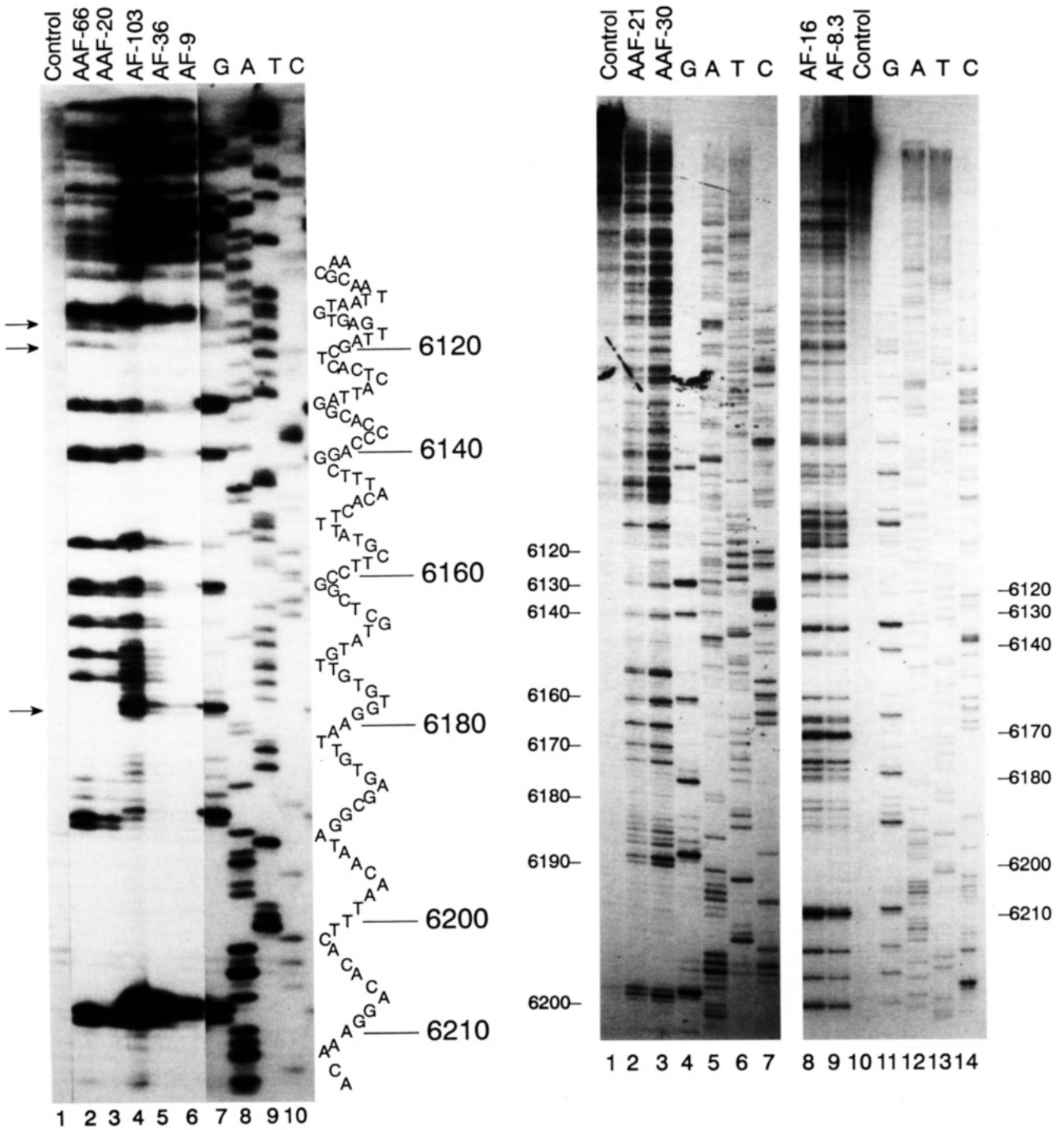

FIGURE 4: Sequencing gel electrophoresis of the DNA synthesized by pol I-Kf (A) and pol III-H (B) using N-OH-AF- or NA-AAF-modified M13mp10 templates. The details of DNA modifications and DNA synthesis are described in Materials and Methods. The standard dideoxyribose sequencing reactions (G, A, T, and C) and the numbers of chemical carcinogen modifications per M13mp10 DNA are presented at the top of the panel (AAF-66 represents 66 AAF-DNA adducts per M13mp10 DNA template, and so on). The DNA sequences and the numbers of the sequences are presented at the side of the panel. The arrows indicate sequences of interest which are discussed in the text.

carcinogen modification at guanine residues. If this is the case, then the results in Figure 5 also suggest that both N-OH-AF and NA-AAF modify guanines at different positions with different efficiencies, and this sequence specificity of modification apparently is different for these two carcinogens. In general, there are less sequence-dependent variations of modification by NA-AAF than by N-OH-AF; there are certain positions which have high affinity for both chemicals, and there are sequences that these two chemicals modify with dramatically different efficiencies. For example, guanines at positions 6176, 6178, and 6179 are modified by N-OH-AF but are not significantly modified by NA-AAF. Therefore, the lack of DNA synthesis stalling at these positions for AAF-adducted templates shown in Figure 4A is likely due to lack of modifications at these positions. On the other hand, both N-OH-AF and NA-AAF modify guanines at positions 6116/6120, and thus the stallings of pol I-Kf-mediated DNA synthesis at these positions using AAF-adducted templates but not AF-adducted templates, as shown in Figure 4A, indicate that the structures of the AF

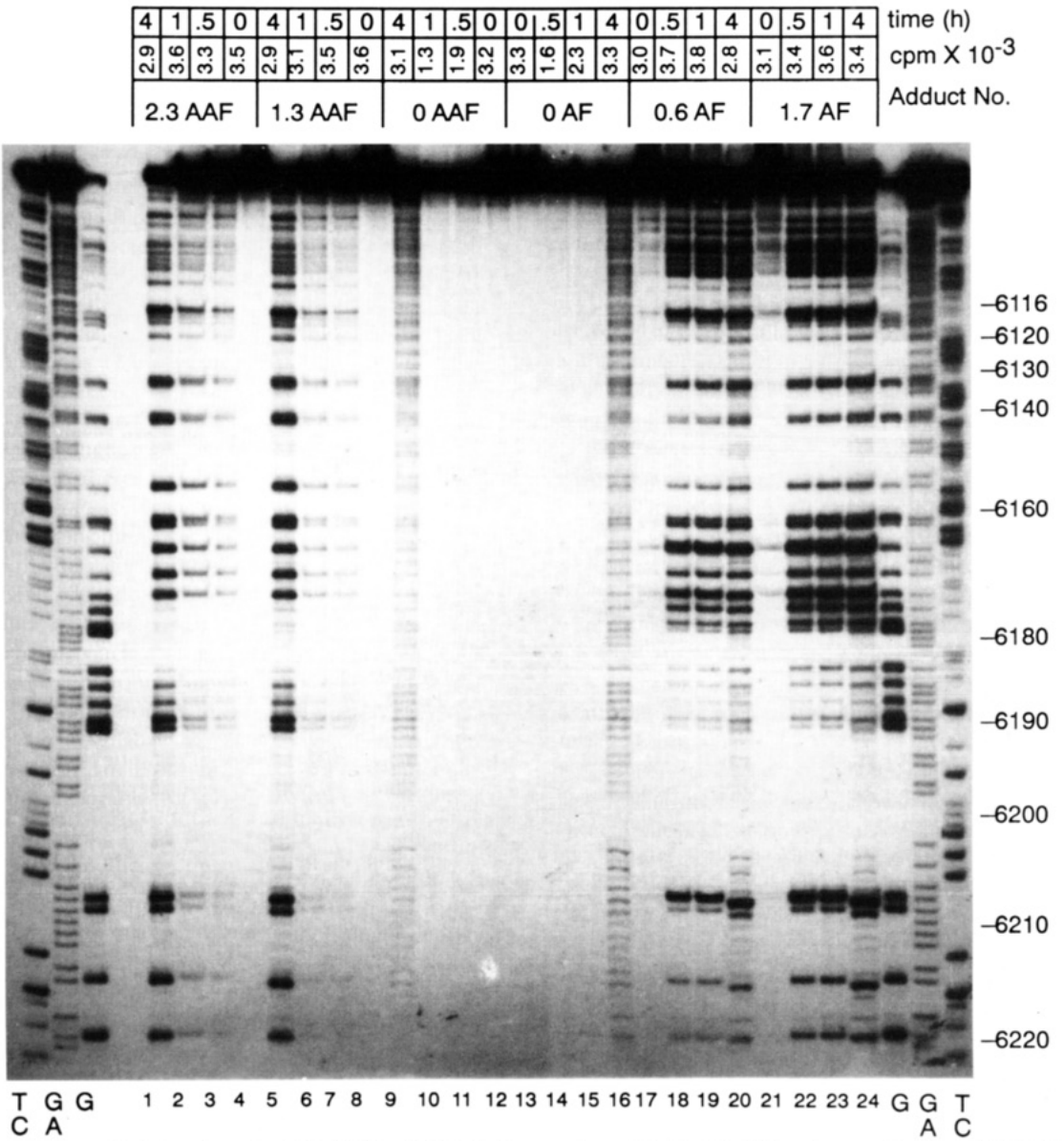

FIGURE 5: Sequencing gel electrophoresis of the M13mp10 DNA fragments modified with different concentrations of N-OH-AF or NA-AAF followed by treatment of piperidine at 90 °C for different periods of time. The numbers of chemical carcinogen modifications per M13mp10 DNA, the time periods for piperidine treatment, and the amount of DNA (cpm) applied to each lane are described at the top of the panel. The standard Maxam and Gilbert sequencing reactions (G, GA, and TC) are indicated at the bottom. The numbers of the sequences are presented at the side of the panel.

adducts at these positions may be significantly different from those at other sequences.

Relative Efficiency of AF- and AAF-Modified Guanines in Different Contexts in Blocking pol III-H- and pol I-Kf-Mediated DNA Replication. Since the band intensity in Figure 5 represents the relative frequency of AF and AAF adduction at different sequences, and since the band intensity in Figure 4 represents the relative frequency of DNA replication stalling at different sequences, the ratio of these two values should represent the efficiency of dG-C8-AF and dG-C8-AAF at different positions in blocking DNA replication. The ratios of the frequency of DNA synthesis blockage to the frequency of DNA adduct formation (B/P) for sequences from 6150 to 6210 were calculated because sequences in this region were better separated in the sequencing gel. Figure 6 shows that both enzymes have more sites with a high B/P ratio for NA-AAF-modified templates than for N-OH-AF-modified templates; these results suggest that there are more strong blockage sites for DNA synthesis in the former than in the latter since the results in Figure 2 demonstrate that AAF adducts block DNA synthesis more substantially than AF adducts. Although the B/P patterns of pol I-Kf and pol III-H are similar at most sequences for both AF- and AAF-adducted templates, there are a few exceptions: If we assume that the highest value of the B/P ratio along the sequences represents the total blockage of DNA synthesis, then the results in Figure 6 indicate that most of the dG-C8-AF adducts in the sequences were read through by both DNA polymerases with frequencies of 0.9 to 0.6 with few exceptions. At positions 6209 and 6208 the pol I-Kf was blocked 70−100%, but pol III-H was blocked only 30%; at position 6162 pol III-H was blocked 100%, but pol I-Kf was hardly blocked at all. In contrast, most of dG-C8-AAF adducts were read through by both polymerases with lower frequency. At positions 6156, 6176, and 6208 these two polymerases showed greatly different efficiencies in replicating through the dG-C8-AAF adduct. It is likely that the efficiency of stalling DNA

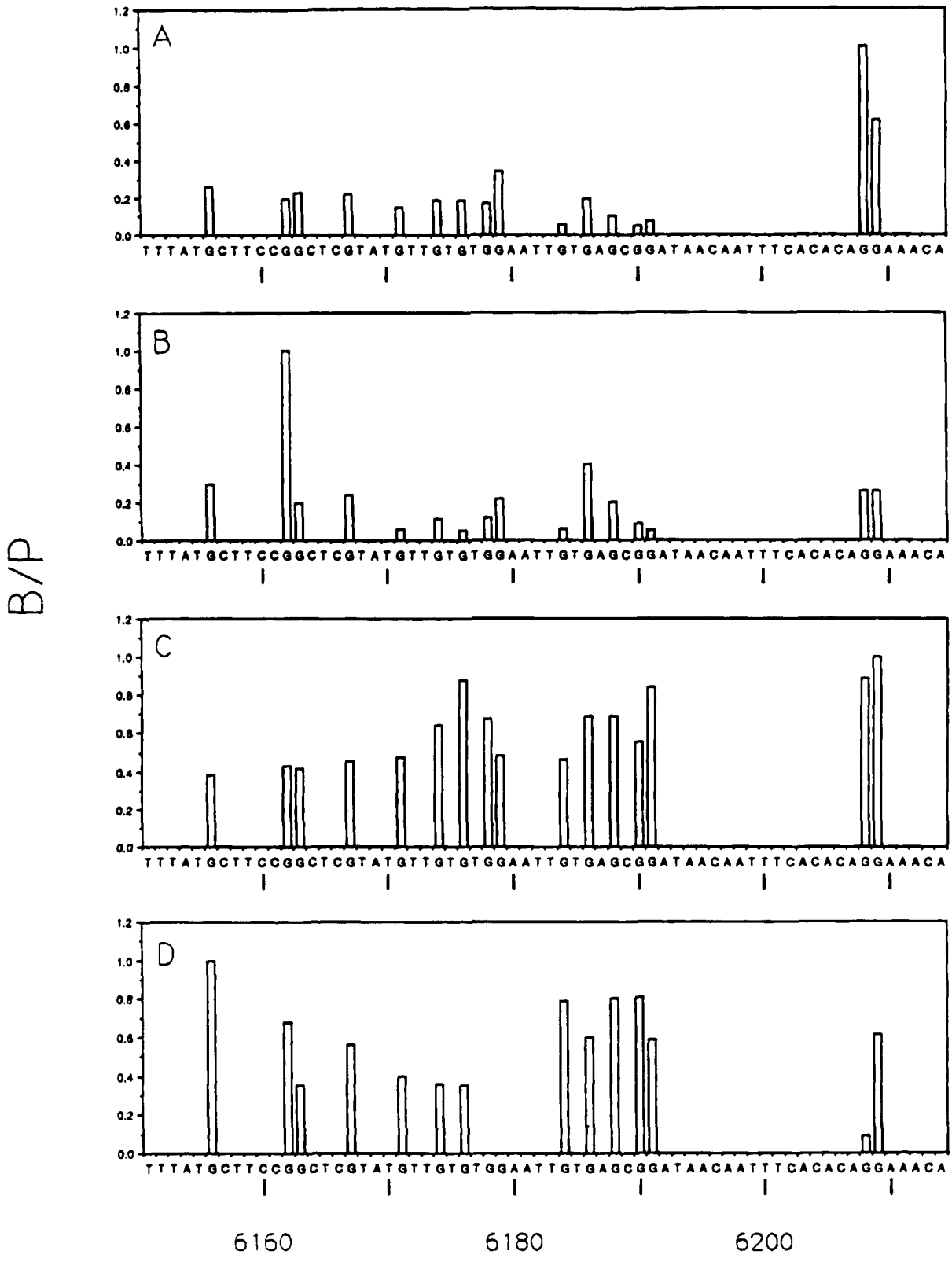

FIGURE 6: Effects of DNA sequence on the N-OH-AF (A and B) or NAAAF (C and D) modification induced DNA synthesis stalling. The autoradiographs of Figures 4 and 5 were scanned by a densitometer, and the relative degree of DNA synthesis stalling (B), and chemical carcinogen modifications (P) at different guanine positions were calculated. For pol III-H (B and D), which stalls one base prior to the AF- and AAF-modified guanine, B represents the degree of stoppage one base prior to the guanine position. For pol I-Kf (A and C), a single AF-modified base induces two stoppage sites; B represents the sum of these two stoppages. For a run of two G's, it is theoretically impossible to quantify B for each G. However, since the stoppage at the AF-G is significantly more prominent than that prior to the AF-G, for simplicity, B represents the stoppage at the AF-modified G's. For an AAF-modified guanine, B represents the stoppage one base prior to the guanine position for either a single G or a run of two G's. The ratio of these two values (B/P) represents the relative efficiency of the chemical modification in inhibiting the DNA synthesis mediated by pol I-Kf (A and C) and pol III-H (B and D).

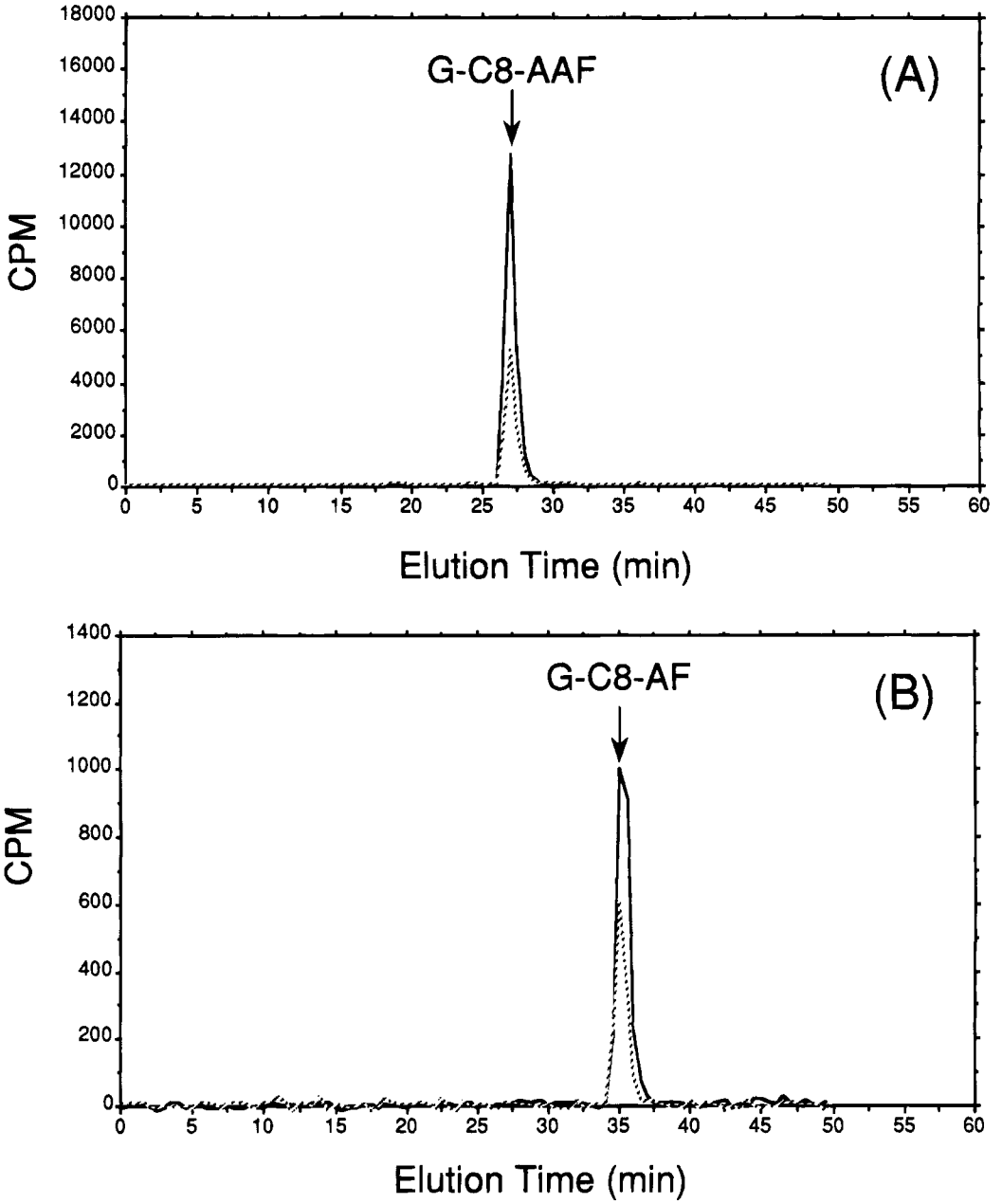

FIGURE 7: HPLC profiles of NA-AAF (A) and N-OH-AF (B) modified M13mp10 ssDNA digested in anhydrous TFA. The phage DNA was modified with [^{3}H]N-OH-AF or -NA-AAF, solvolyzed by TFA, and chromatographed as described in the text. The arrows indicate the positions of elution of the internal standard G-C8-AF and G-C8-AAF. Solid and dotted lines represent results from modified DNA templates before and after using DNA for synthesis. The variations in peak height are due to differences in the amount of sample applied for HPLC analysis.

synthesis is dependent on the structure of the adduct; if this is the case, then the result that the B/P of pol I-Kf does not coincide with the B/P of pol III-H for both N-OH-AF- and NA-AAF-modified templates at certain sequences raises the possibility that these two DNA polymerases may affect the structures of the adducts differently at these sequences.

DISCUSSION

In this report we have presented evidence to demonstrate that both pol III-H and pol I-Kf can replicate through aminofluorene-adducted guanines with relatively high frequency, 92% for the former and 62% for the latter. In contrast, these two enzymes replicate through (acetylamino)-fluorene-adducted guanines with a much lower frequency, 38% for pol III-H and 25% for pol I-Kf. Sequence analyses of the synthesized DNA products demonstrate that AAF-adducted templates indeed cause more strong blocking sites for DNA synthesis than AF-adducted templates do. Previously, we found that the transfectivity of AAF-modified ϕX174 RF DNAs is 7−10-fold less than the transfectivity of AF-modified ϕX174 RF DNAs in *E. coli* cells (Tang et al., 1982). Our current results suggest that AF-modified DNA's higher transfectivity as compared to that of AAF-modified DNA may be a reflection of the different efficiency of interference with DNA replication by these two kinds of adducts; that is, dG-C8-AAF adducts block DNA replication more extensively than dG-C8-AF adducts do. These results are also consistent with the prediction from molecular modeling that shows that dG-C8-AF adducts can maintain *anti*-structures and normal base pairing; in contrast, dG-C8-AAF adducts favor *syn*-structures and cause base displace-

ment and disruption of base pairing (Fuchs & Daune, 1974; Grunberger & Weinstein, 1978). These models suggest that the dG-C8-AF adducts may allow the DNA replication machinery to read through, and dG-C8-AAF adducts may stall DNA replication.

The reasons why dG-C8-AAF adducts are unable to block DNA replication totally and why dG-C8-AF adducts are unable to allow replication to be read through totally are unclear. Although it has been shown that dG-C8-AAF adducts under certain conditions will convert to dG-C8-AF adducts (Kriek & Westra, 1980; Tang & Lieberman, 1983), we have ruled out the possibility that the residual replication read through in AAF-adducted templates is due to this adduct conversion since under our replication conditions both kinds of adducts remain unchanged (Figure 7). It has been proposed, that in addition to the *anti*-structure, dG-C8-AF can exist in the *syn*-structure with low probability (Van Houte et al., 1987). It is possible that when dG-C8-AF is in the *syn*-conformation, it may block DNA synthesis with the same frequency as dG-C8-AAF in the *syn*-conformation does. On the other hand, it also has been proposed that besides in the *syn*-structure, dG-C8-AAF can exist in the *anti*-structure with low probability. It is also possible that when dG-C8-AAF is in the *anti*-conformation, it may not block DNA replication as it does in the *syn*-conformation. These possibilities provide a plausible explanation for the residual DNA replication on dG-C8-AAF and the residual DNA replication blockage on dG-C8-AF. Interestingly, Norman et al. (1989) have demonstrated that (AF)-G(*syn*)-A(*anti*) pair formation occurs in an 11-mer oligonucleotide at neutral pH and speculated that similar pairing can form even when AF is substituted by AAF; they further suggested that this (AF)-G(*syn*)-A(*anti*) pair formation may be one mechanism that enables DNA synthesis to bypass the AF-G and induces G•C → T•A transversion. However, G•C → T•A transversion is a rare event (10^{-5}), and it is unlikely that this (AF)-G(*syn*)-A(*anti*) pair formation can account for the 92% and 62% frequency of bypass DNA synthesis of AF-G mediated by pol III-H and pol I-Kf.

Apparently not every dG-C8-AAF in the sequence blocks DNA replication with the same frequency; Figure 6 shows that adducts formed at different sequences show significant variation in blocking DNA replication. If the DNA replication machinery reads through dG-C8-AAF only when this adduct is in the *anti*-conformation, then these results indicate that sequences dictate whether dG-C8-AAF is in the *syn*- or *anti*-conformation and that the majority of the adducts are in the *syn*-conformation. The results in Figure 6 show that dG-C8-AF formed at different sequences block DNA synthesis with significant variation. If dG-C8-AF blocks DNA replication only when it is in the *syn*-conformation, then these results suggest that sequences also dictate that the frequencies of dG-C8-AF adducts are in the *syn*- or *anti*-conformation and that the majority of the adducts are in the *syn*-conformation. Interestingly, using one- and two-dimensional NMR, Eckel and Krugh (1994a,b) and Cho et al. (1994) recently have found that the AF moiety of dG-C8-AF in a oligomer duplex is either inserted in the duplex, disrupting base pairing, or positioned externally to the major groove, without disrupting base pairing. In these two structures the AF-G residue with its glycosidic torsion angle is likely in an *anti* domain, and these two structures are interchangeable and exist with equal probability. These results suggest that besides the glycosidic torsion angle, there are other factors which contribute to determining whether AF- and AAF-adducted guanine residues can maintain base pairing and, consequently, whether DNA replication is allowed to bypass these residues.

We observed that pol III-H reads through AF−DNA adducts more often than pol I-Kf does—92% vs 62%. It is possible that the higher processivity of pol III-H than that of pol I-Kf may be one of the reasons that, in general, the former has higher efficiency in conducting translesion synthesis. Using site-specific AF-modified M13mp9 template, Michaels et al. (1987) have demonstrated translesion DNA synthesis mediated by pol I-Kf and T7 DNA polymerase with frequencies of 28% for the former and 47% for the latter. The relatively low frequency of bypassing DNA synthesis of AF-G lesions observed by these workers could be attributed to sequence effects, as we presented in Figure 6, and/or the low processivity of these two enzymes.

Strauss and Wang (1990) have shown that partial deacetylation of AAF-G DNA adducts by alkaline treatment in M13mp2 DNA templates modified with (acetylamino)-fluorene enhances translesion DNA synthesis mediated by T7 polymerase, T4 polymerase, pol I-Kf, and AMV and HIV transcriptases. Our results and those of Michaels et al. (1987) and Strauss and Wang (1990) together suggest that the more efficient translesion synthesis on AF-modified templates than on AAF-modified templates is likely universal for DNA polymerases.

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