C8-Guanine Adduct-Induced Stabilization of a −1 Frame Shift Intermediate in a Nonrepetitive DNA Sequence†

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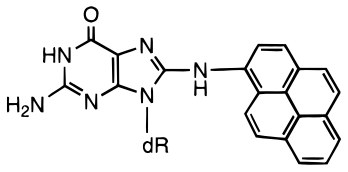
ABSTRACT: The mechanism of frame shift mutagenesis induced by *N*-(deoxyguanosin-8-yl)-1-aminopyrene, the major DNA adduct formed by the carcinogen 1-nitropyrene, was investigated by thermal melting studies of a 13-mer in which the adduct was flanked by a 5′ and a 3′ C. Compared to the unmodified 13-mer, the adduct destabilized the duplex by 4−5 kcal/mol, and the $\Delta\Delta G$ value remained approximately the same regardless of which base was placed opposite the adduct. In contrast, deletion of the base opposite the adduct stabilized the duplex by nearly 4 kcal/mol. The adduct in the same sequence context was inserted into a bacteriophage M13 DNA containing the simian virus 40 origin of replication. The constructed DNA template was replicated in vitro with extracts from normal human fibroblasts. The adduct was not removed from the progeny DNA following bidirectional semiconservative replication, which suggests that it had been bypassed, rather than repaired, by the cell extract. When newly replicated bacteriophage was evaluated for mutations in the region of the modified G, most contained a G at the adduct site, indicating error-free replication. A small number of mutants ($\sim 2 \times 10^{-3}$) were detected, all of which contained a targeted G•C base pair deletion. This suggests a relationship between the thermodynamic stability of the adduct in DNA and the errors that occurred during replicative bypass by the human DNA polymerases.

1-Nitropyrene (1-NP),[1] a ubiquitous environmental carcinogen and the major mutagenic component of diesel exhaust (*1*−*8*), binds covalently to the C8 position of 2′-deoxyguanosine upon reductive activation to form *N*-(deoxyguanosin-8-yl)-1-aminopyrene (dG^{AP}) (*9*−*11*) (Chart 1). Frame-shift mutagenesis induced by dG^{AP} is believed to play a major role in the carcinogenic effects of 1-NP (*12*−*14*). The most commonly accepted mechanism of frame-shift mutagenesis involves a slippage model in repetitive sequences first postulated by Streisinger (*15*−*18*). Experimental evidence for slippage was provided with the major guanine adduct formed by the carcinogen 2-acetylaminofluorene that induces −1 frame shifts in strings of guanines (*19*, *20*). The mechanism of carcinogen-induced frame shifts in nonrepetitive sequences, however, is less certain. DNA sequence context has been demonstrated to play a major role in the types and frequency of mutagenesis (*21*, *22*). Frame-shift mutations induced by dG^{AP}, which include one-base deletion, one-base insertion, and dinucleotide deletion, occur in repetitive guanines (*12*−*14*) and GpC (or CpG) dinucleotide repeats (*23*, *24*) at a high frequency. However, dG^{AP} also induces one-base deletions in nonrepetitive sequences, and a major fraction of the latter contains a 5′ or 3′ cytosine (*14*). To explore the conformations of dG^{AP} in such a DNA sequence, we synthesized an 11-mer, d(CCATCG^{AP}CTACC) (*25*), which was investigated by a combination of NMR and molecular mechanics studies (*26*, *27*). These studies showed that the aminopyrene ring of dG^{AP} is intercalated into the DNA helix between two intact Watson−Crick C•G base pairs flanking the adducted site in the duplex 11-mer, irrespective of whether a cytosine or adenine was placed opposite the adducted base. The intercalation of the aminopyrene ring results in the displacement of the modified dG ring into the major groove. The glycosidic torsion angle of dG^{AP} is in the syn domain. Although there are differences in the orientation of dG in the normal and mismatched duplexes, in each case no hydrogen bond could be detected between

Chart 1: Structure of *N*-(Deoxyguanosin-8-yl)-1-aminopyrene (dG^{AP})



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[1] Abbreviations: 1-NP, 1-nitropyrene; 1-NOP, 1-nitrosopyrene; NHOP, *N*-hydroxy-1-aminopyrene; AP, 1-aminopyrene; dG^{AP}, *N*-(deoxyguanosin-8-yl)-1-aminopyrene; dG, 2′-deoxyguanosine; ss, single-stranded; ds, double-stranded; CD, circular dichroism; M13mp2SV*ori*L, bacteriophage M13mp2 containing the SV40 origin of replication at position 5914−6064; TLS, translesion synthesis.

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the adducted dG and its partner. This is particularly interesting, not only for addressing the mechanism of −1 deletion mutagenesis but also because hydrogen bonding is believed to play a major role in replicative bypass by a DNA polymerase (*28*). Recent studies with difluorotoluene as a nonpolar isostere of thymine suggest that replicative bypass by a DNA polymerase might be possible even in the absence of hydrogen bonds (*29*). Studies with 1,N^6-ethenoadenine in which the etheno ring blocks the Watson−Crick hydrogen-bonding face of adenine also suggest that translesion synthesis is possible without hydrogen bonds (*30*, *31*). In the current work we have examined the thermodynamic stability of the normal as well as various promutagenic intermediates containing dG^{AP}. We present here a link between the thermodynamic data and the biological results when the adducted DNA was replicated in vitro with an extract of human fibroblast cells.

MATERIALS AND METHODS

Materials

1-NP, 1-aminopyrene, and *m*-chloroperoxybenzoic acid were obtained from Aldrich Chemical Co., Milwaukee, WI. [γ-^{32}P]ATP was from Du Pont−New England Nuclear, Boston, MA. T4 polynucleotide kinase was obtained from Bethesda Research Laboratory, Gaithersburg, MD. UvrABC excinuclease was obtained from Dr. Peter Van de Putte, University of Leiden, Leiden, The Netherlands.

Methods

Unlabeled 1-NOP was synthesized according to a published procedure (*10*). Oligodeoxynucleotides were synthesized on an Applied Biosystems, Inc., Model 380B DNA synthesizer by the phosphoramidite method. HPLC separations were performed on reverse-phase columns (Phenomenex C-18, 5 μm particle size, 4.6 × 250 mm).

Synthesis of Adducted Oligonucleotides. The HPLC-purified oligonucleotide (50 nmol) was stirred at ambient temperature with 15 μmol of 1-NOP and 3 μmol of ascorbic acid in 100 μL of DMF−sodium acetate−acetic acid buffer (0.1 M), pH 5.0 (1:9) under nitrogen and protected from light. An additional aliquot of 3 μmol of ascorbic acid was added every 15 min during the first hour, and the reaction was allowed to continue for 16−20 h. Noncovalently bound material was removed by extensive extraction with chloroform. The adducted and unadducted oligonucleotides were separated by reverse-phase HPLC. For the biological studies further purification was carried out by polyacrylamide gel electrophoresis. The oligonucleotides were desalted on a Sephadex G-10 column, dried, and stored at −20 °C until further use.

Circular Dichroism. CD spectra were measured in 10 mM KH_2PO_4, pH 7.0, and 0.2 M NaCl with a Jasco Model J-710 spectropolarimeter equipped with a refrigerated circulating water bath. Spectra were plotted with a Hewlett-Packard 7470A plotter.

Optical Melting Studies. Temperature-dependent absorption changes for oligonucleotide samples were measured in a Hewlett-Packard HP 8452A diode-array spectrophotometer equipped with a Peltier temperature controller. Oligonucleotides were dissolved in 10 mM NaH_2PO_4, pH 7.0, 1 mM EDTA, and 0.2 M NaCl. The mixture was heated to 90 °C and cooled slowly to room temperature before temperature-dependent absorption measurements were carried out. The heating rate of these studies was 0.3 °C/min and measurements were made at 260 nm. Thermodynamic parameters were calculated from van't Hoff plots as described in detail by Marky and Breslauer (*32*). For each data point of the van't Hoff plot at least three experiments were performed and the estimated precision of each point was ±0.5 °C. The standard deviations were calculated essentially as described by Persmerk and Guengerich (*33*).

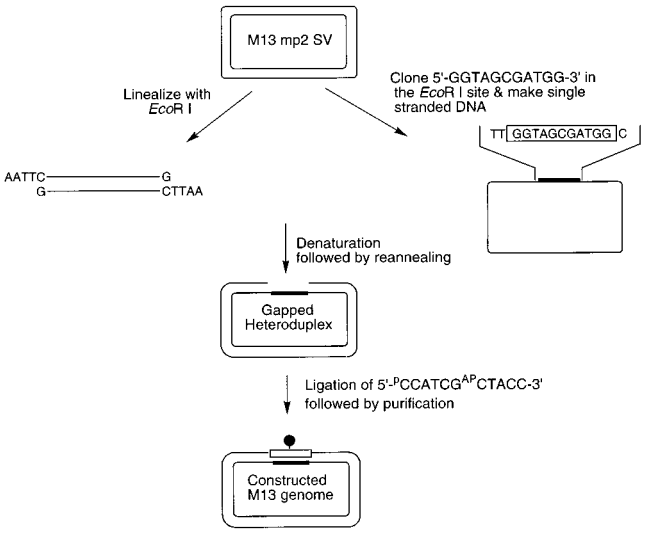

FIGURE 1: Construction scheme of M13mp2SV +11 containing a site-specific dG^{AP}.

Cells and Preparation of Cell Extracts. Cell extracts were derived from MSU1.2 cells. The MSU1.2 cell strain is a karyotypically stable, nontumorigenic, infinite lifespan, excision repair-proficient cell strain derived from the foreskin of a normal neonate (*34*, *35*). The cells were maintained in logarithmic growth in Eagle's MEM supplemented with 10% calf serum (Hyclone), 1 μg/mL hydrocortisone, and antibiotics. Replication-competent cell extracts were prepared according to the method of Li and Kelly (*36*).

Replication Template. The template was derived from M13mp2SV*ori*L (*37*, *38*), which contains the SV40 origin of replication and will undergo semiconservative bidirectional replication in the presence of SV40 large T antigen and replication-competent extracts derived from human cells (*39*). A single dG^{AP} was inserted into the template by the following strategy (Figure 1): A +11 mutant clone of M13mp2SV*ori*L was generated by inserting d(GGTAGCGATGG) into the *Eco*RI site of the multiple cloning region of the bacteriophage. A gapped heteroduplex was formed by denaturation followed by reannealing of the single-stranded +11 clone with *Eco*RI-linearized M13mp2SV*ori*L duplex DNA. Subsequently, 5′-phosphorylated d(CCATCG^{AP}CTACC) or its unmodified analogue was ligated into the gap in the presence of T4 DNA ligase. Covalently closed circular DNA was obtained by electroelution from an 1% agarose gel containing 5.0 μg/mL ethidium bromide. The latter was removed by ion-exchange chromatography, phenol−chloroform extraction, and alcohol precipitation. Although the ligation efficiency was in excess of 35%, the final yield of covalently closed circular DNA was only 8−10%.

Replication Reactions. Reactions, carried out as described previously (*40*), contained 40 ng of the template in a total volume of 25 μL. T antigen was omitted from the control reaction tubes. Following addition of replication-competent cell extract (75−100 μg of protein), the reaction mixtures were incubated for 1 h at 37 °C. An aliquot ($^1/_{10}$ volume) was taken for determination of [α-^{32}P]dCTP incorporation into acid-insoluble material. A ^{32}P-labeled internal standard was added to each sample. The DNA was extracted and treated with *Dpn*I to digest any fully methylated (i.e., unreplicated) templates and with *Mbo*I to digest any fully unmethylated products (i.e., products of more than one round of replication). Aliquots of the samples were electrophoresed on 1% agarose gels containing 0.5 μg/mL ethidium bromide. The density of the bands corresponding to covalently closed circular (form I) DNA was quantified by use of ImageQuant software in a Molecular Dynamics PhosphorImager. The amount of form I synthesis as a percentage of synthesis of DNA from the control (nonadducted) template was corrected for loss of the DNA during the purification procedure by normalizing the density of the form I band to that of the internal control.

UvrABC Reactions and Southern Analysis. To determine if adducts were present in nascent DNA, an aliquot of the purified, newly replicated DNA was treated with UvrABC excinuclease as follows. After treatment with *Dpn*I and *Mbo*I, the replication products were divided into two aliquots, one treated with UvrABC and the other exposed to excinuclease buffer alone. The excinuclease reaction mixture was composed of 17 pmol of each of the three subunits of UvrABC in a final volume of 250 μL of buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 75 mM KCl, 2 mM ATP, 1 mM dithiothreitol, and 1 μg/μL BSA. At the end of 1 h of incubation at 37 °C, the reaction was stopped by adding proteinase K at 0.1 μg/μL and 0.1% SDS to each sample and incubating for 1 h at 42 °C. After alcohol precipitation, the samples were run on a 1% agarose gel containing 0.5 μg/mL ethidium bromide. The gel was dried, and the amount of form I DNA was quantified with a PhosphorImager.

Determination of Mutation Frequencies. An aliquot of the purified, newly replicated DNA was electroporated into *E. coli* strain 9162, which is deficient in mismatch repair (*mutS*) to avoid correction of heteroduplex DNA. Immediately after electroporation, these bacteria were coplated with an indicator strain, *E. coli* CSH50. The transfected bacteria replicate the bacteriophage and infect the surrounding indicator bacteria, resulting in colorless plaques in the lawn of indicator bacteria. The assay scores only newly replicated, covalently closed circular DNA molecules. After overnight incubation at 37 °C, the plates were overlaid with nylon membranes (Boerhinger Mannheim) and the plaques were screened for mutations in the region of the dG^{AP} by hybridization. The nylon membranes were hybridized with the 5′-^{32}P-end-labeled 19-mer d(AGTGCCATCGCTACCAATT) overnight at 30 °C in 0.5 M sodium phosphate (pH 7.5), 1 mM EDTA, and 7% SDS. The membranes were washed twice for 30 min at room temperature in 40 mM sodium phosphate (pH 7.5), 1 mM EDTA, and 5% SDS, followed by two washes at 53 °C for 30 min each in 40 mM sodium phosphate (pH 7.5), 1 mM EDTA, and 1% SDS. The washing temperature of 53 °C was determined, by use of MacVector software, to be the temperature at which the 19-mer would anneal to its complementary sequence but single-base mismatches would fail to anneal. Hybridization was detected by PhosphorImager analysis. Bacteriophage DNA was isolated from plaques that failed to hybridize, or did so weakly, and the DNA was sequenced with a Visible Genetics automated sequencer and a fluorescently labeled M13 universal forward sequencing primer. The mutation frequency is defined as the number of sequence-confirmed mutants divided by the total number of plaques.

RESULTS

Thermodynamic Stability of Oligonucleotides Containing dG^{AP}. For the thermodynamic studies we employed a 13-mer, d(CCCATCG^{AP}CTACCC), which contained the 11-mer sequence used for our earlier NMR experiments (*26*, *27*), except that we added a C at each end of the oligonucleotide to increase the stability of the duplex DNA. Duplex stability with a series of different oligonucleotides opposite the control and adducted 13-mer was examined by UV melting measurements. Representative melting curves and smooth first derivatives are shown in Figure 2. Thermodynamic parameters were calculated from van't Hoff plot in which melting transition (T_m) is measured as a function of concentration (C_t) for the oligonucleotide duplexes by plotting $1/T_m$ vs ln C_t (Figure 3). We used T_{max}, the highest point on the derivative curve, rather than T_m, since the former is a more accurate measure of duplex stability than the latter (*32*). For the unmodified duplex, C opposite G was the most stable pair with a ΔG −17.7 kcal/mol at 37 °C, followed by T, G, and A, as expected (Table 1). For dG^{AP}, however, thermodynamic stability opposite each of the four bases appeared to be nearly the same, and the difference in free energy between the least stable (G^{AP}·C) and the most stable (G^{AP}·G) pairs was only about 0.6 kcal/mol (Table 1). What appears to be most interesting, however, is that deletion of the base opposite G weakened the unmodified duplex significantly, with a free energy difference of approximately 6 kcal/mol relative to the parent G·C pair, whereas for the modified duplex, deletion of the base opposite dG^{AP} made it more stable by 3.7 kcal/mol compared to the G^{AP}·C pair (Table 1, Figure 3). Indeed, the one-base deletion pair containing dG^{AP} was nearly as stable as the unmodified G·C duplex. It appears that in the unmodified 13-mer·12-mer duplex the base in the middle that lacked its partner in the complementary strand induced a highly destabilizing effect. Likewise, any unpaired base opposite dG^{AP} had a similar destabilizing effect, which can be avoided by deleting the partner nucleoside. The stacking interactions of the aminopyrene moiety effectively compensated for the loss of hydrogen-bonding interactions of a G·C pair. However, deletion of a neighboring nucleoside in the complementary strand, rather than the partner, failed to stabilize the duplex. It is also noteworthy that an added base opposite dG^{AP} in the complementary strand, which represents the promutagenic intermediate of the one-base insertion, had a destabilizing effect on the DNA helix. This suggests that one-base insertions would not be thermodynamically favored in the current sequence.

Circular dichroism indicated that each of the modified duplexes maintained a B-DNA structure (see Supporting Information). Bulged duplexes containing the DNA adducts formed by the carcinogens benzo[*a*]pyrene and 2-aminofluorene have been investigated by NMR studies (for a review,

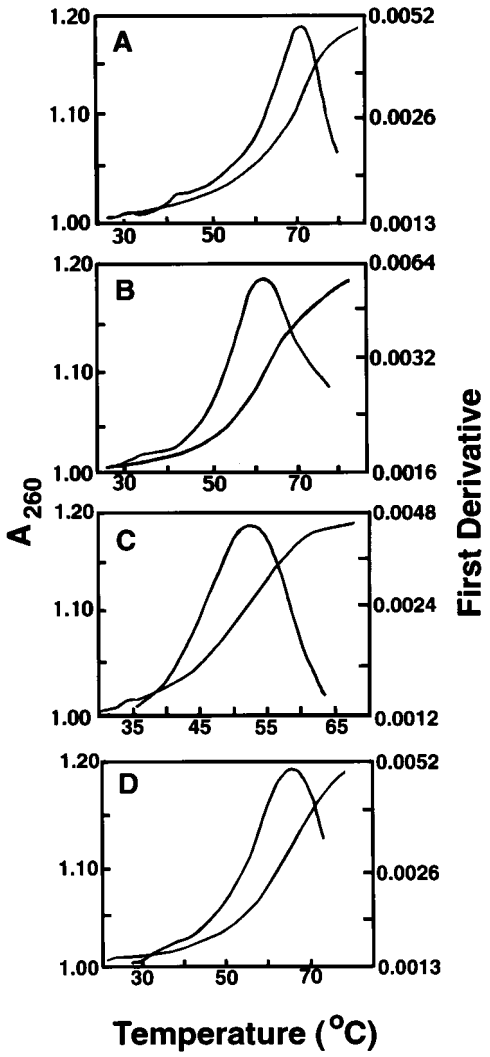

FIGURE 2: Typical UV thermal denaturation profiles and first derivatives for d(CCCATCG^{AP}CTACCC) and its unmodified analogue annealed to a 13-mer or a 12-mer. Panels A and B represent unmodified and modified 13-mer, respectively, annealed to the complementary 13-mer d(GGGTAGCGATGGG). Panels C and D represent unmodified and modified 13-mer, respectively, annealed to the 12-mer d(GGGTAGGATGGG). Oligonucleotide concentration in each case was 30 μM.

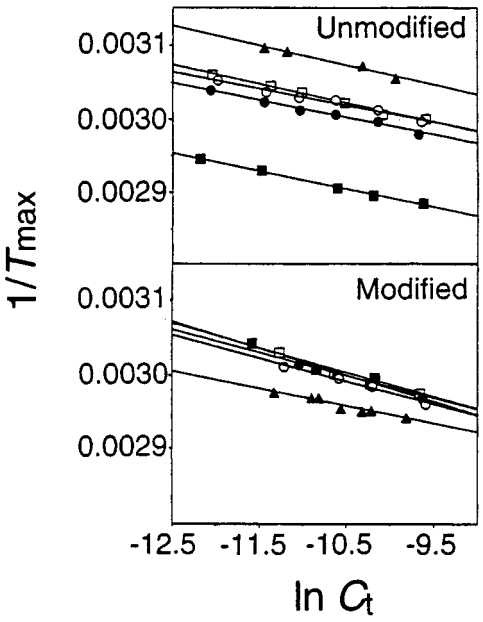

FIGURE 3: van't Hoff plots of $1/T_{max}$ vs ln C_t for helix-to-coil transitions of d(CCCATCG^{AP}CTACCC) and its unmodified analogue annealed either with the 13-mer, d(GGGTAGNGATGGG) [where N denotes C (■), T (●), A (□), or G (○)], or with the 12-mer, d(GGGTAGGATGGG) (▲). Each data point represents an average of at least three determinations, and the estimated precision of each point is ±0.5 °C. Lines were fit by least-squares linear regression analysis.

see refs *41* and *42*). These studies show that large planar aromatic rings intercalate to avoid solvent and achieve favorable stacking interaction at the expense of a lost Watson−Crick base pair. It is noteworthy that Ya et al. (*43*) observed that the guanine−N^2 adduct formed by (+)-*cis*-benzo[*a*]pyrene diol epoxide, in which the pyrene moiety assumes an intercalative conformation, provides significant stabilization of an oligonucleotide duplex in the same sequence context as in the current study when the partner base is missing relative to the duplex with a C opposite the adduct. Other studies with nucleoside analogues have shown that stability in DNA can be achieved without hydrogen bonds (*44*).

Construction of an M13mp2SVoriL Vector Containing a Single dG^{AP}. To determine the biological consequence of an adduct like dG^{AP} in which the guanine is rotated to syn alignment and lacks hydrogen bonding with its partner base, we inserted the adducted 11-mer into a bacteriophage M13mp2 DNA containing the simian virus 40 origin of replication (M13mp2SV*ori*L). An 11-mer containing a single dG^{AP} was ligated to a gapped heteroduplex by a strategy that has been used extensively by us and others (see refs *45* and *46* and references therein). The adduct dG^{AP} was situated in the (−) strand of the viral DNA at position 6287 (Figure 4). Highly purified covalently closed circular DNA was obtained by electroelution from an agarose gel containing 5.0 μg/mL ethidium bromide, which was subjected to ion-exchange chromatography, phenol−chloroform extraction, and alcohol precipitation. A control construct was similarly prepared by ligating an unmodified 11-mer into the M13 genome followed by purification as described above.

Effect of the dG^{AP} on the Ability of Extracts from Human Fibroblasts To Replicate DNA Templates. Upon replication, the yield of products was consistently decreased by the presence of the adduct in the replication template. As shown in Table 2, there was an average of 48% fewer nascent form I molecules produced when the adducted template was used in the reactions. This suggests that dG^{AP} stalls DNA replication by human cell extracts. Analysis of newly replicated DNA showed that approximately half of the nascent molecules were incised by UvrABC, suggesting the presence of dG^{AP} (data not shown). The simplest explanation for this is that the adduct was bypassed during DNA replication, and dG^{AP} appeared to have stalled but did not completely block translesion synthesis.

Sequence Analysis of dG^{AP}-Induced Mutations. The adduct was located 298 base pairs from the center of the SV40 *ori*, so one replication fork has to travel this far to reach the adducted guanine, but the other fork must travel over 10 times as far to reach the damage. Since the replication forks proceed at the same rate from the center of the origin (*36*), the mode of replication of the strand containing the adduct can be inferred. In the template used in these studies, with the SV40 origin of replication to the immediate left of the

Table 1: Melting Temperature and Free Energies for DNA Duplexes Containing a G or G^{AP}

DNA duplex	T_{max} (°C) [a]	$-\Delta G^{\circ}_{37}$ (kcal) [b]
5'-CCCAT CGC TACCC 3'-GGGTA GCG ATGGG	73.8	17.7±0.3
5'-CCCAT CG*C TACCC 3'-GGGTA GCG ATGGG	61.6	13.0±0.9
5'-CCCAT CGC TACCC 3'-GGGTA GTG ATGGG	62.9	15.7±0.5
5'-CCCAT CG*C TACCC 3'-GGGTA GTG ATGGG	60.5	13.5±1.4
5'-CCCAT CGC TACCC 3'-GGGTA GGG ATGGG	61.1	15.6±0.6
5'-CCCAT CG*C TACCC 3'-GGGTA GGG ATGGG	61.9	13.6±0.5
5'-CCCAT CGC TACCC 3'-GGGTA GAG ATGGG	59.9	14.4±0.4
5'-CCCAT CG*C TACCC 3'-GGGTA GAG ATGGG	60.5	13.1±0.9
5'-CCCAT C G C TACCC 3'-GGGTAG G ATGGG	52.4	11.4±0.6
5'-CCCAT C G* C TACCC 3'-GGGTAG G ATGGG	65.8	16.7±1.1
5'-CCCA T G C TACCC 3'-GGGT A C G ATGGG	53.6[c]	-
5'-CCCA T G C TACCC 3'-GGGT A C G ATGGG	55.0[c]	-
5'-CCCATC G T ACCC 3'-GGGTAG CA TGGG	54.0[c]	-
5'-CCCATC G* T ACCC 3'-GGGTAG C A TGGG	54.8[c]	-
5'-CCCAT C GC TACCC 3'-GGGTAG G CG ATGGG A	60.8[c]	-
5'-CCCAT C G*C TACCC 3'-GGGTA G CG ATGGG A	50.2[c]	-
5'-CCCAT CG C TACCC 3'-GGGTA GC G ATGGG A	59.3[c]	-
5'-CCCAT CG* C TACCC 3'-GGGTA GC G ATGGG A	49.7[c]	-

[a] Conditions: 10 mM NaH_2PO, pH 7.0, 1 mM EDTA, 0.2 M NaCl, and 30 μm DNA. Each data point represents an average of at least three determinations, and the estimated precision was ±0.5 °C. [b] Obtained by plotting $1/T_{max}$ vs ln C_t; estimated precision ±10%. [c] Melting transitions determined for one or two concentrations only.

target sequence (Figure 4), the strand containing the dG^{AP} is the template for leading-strand synthesis. It is formally possible that there is complete arrest of this fork at the site of the adduct and that the opposite fork traverses 7111 base pairs to complete the synthesis. In any case, however, the adduct must be bypassed because the assay only scores nascent covalently closed circular (form I) molecules, and preliminary experiments showed that there was no detectable removal of the adduct by nucleotide excision repair over the 1 h time course of the replication reactions (data not shown). As shown in Table 2, 4320 progeny phage derived from replication of the control (nonadducted) template were evaluated for mutations in the region of the target sequence. Fourteen plaques were identified that hybridized weakly to the oligonucleotide probe, but each sequence was determined

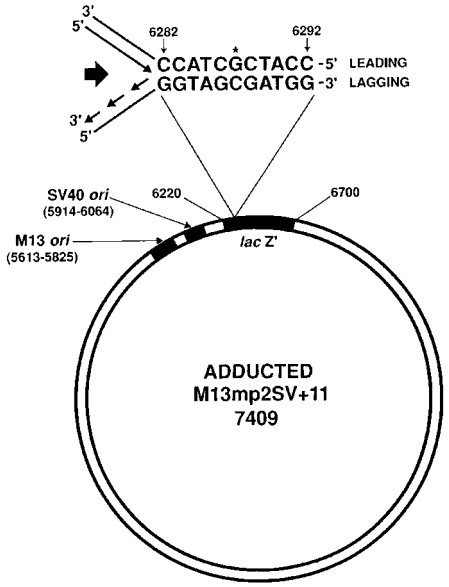

FIGURE 4: Replication of the DNA by human cell extracts initiates at the center of the simian virus 40 origin of replication (SV40 *ori*), such that two replication forks proceed to the left and to the right of position 5989. The adduct dG^{AP} is situated in the (−) strand of the viral DNA at position 6287, such that the right fork traverses only 298 bases before encountering the adduct. In this orientation, dG^{AP} is in the template for leading-strand synthesis. It is formally possible that there is complete arrest of the right fork at this point and synthesis is completed by the left fork; in this scenario the adduct would be in the lagging-strand template 7111 bases from the origin of left fork. We consider this unlikely because a cyclobutane pyrimidine dimer on the leading-strand template, which blocks replication at least as efficiently as dG^{AP}, is directly bypassed (*61*). The replication fidelity assay only scores newly replicated covalently closed circular DNA.

by DNA sequence analysis to be wild type. Among progeny phage derived from replication of the adducted template, 5525 were evaluated for mutations in the target sequence. Nineteen were found to hybridize weakly or not at all. Of these, nine were found to have a deletion of the base pair that originally contained the dG^{AP}•C pair in the parent genome. The mutation frequency at the site of the dG^{AP} was therefore only 9 out of 5525 bypass events, or $\sim 2/10^{-3}$.

Could the G•C base pair deletion observed in this study be due to a contaminating 10-mer in the adducted 11-mer? We believe this is extremely unlikely because both the control and modified oligonucleotides originated from the same batch. For the biological studies, the unmodified 11-mer was first purified by HPLC, which was taken through the steps of adduction and subsequent purification. Prior to ligation into the M13 genome, the control and adducted 11-mers were again rigorously purified by gel electrophoresis. More important than the multiple purification steps, however, is the absence of −1 deletions in the control, which strongly suggests that both the control and adducted oligonucleotides did not contain any contaminating decamer.

DISCUSSION

The present study indicates that the dG^{AP} adduct is a block to DNA replication and that bypass of the adduct is almost always error-free. Mutations resulted in only about 2 out of every 1000 bypass events. These results are consistent with the observations of other investigators who have found that eukaryotic cells, including human cells, have devised strate-

Table 2: Yield of Replication Products and Types and Frequency of Mutagenesis after in Vitro Replication of Templates Containing a G or G^{AP} by Extracts from Human Fibroblasts

template	amount of dNTP incorporated[a]	form I (% of control)[b]	no. of plaques scored		type of mutation	sequence
			mutants	total		
M13mp2SV	86 ± 14	100	0	4320		
M13mp2SV-dG^{AP}	45 ± 12	52 ± 27	9	5525	Δ*G*	GCG^{AP}CT

[a] Mean ± SD of four experiments determined from the amount of [α-^{32}P]dCTP incorporated into acid-precipitable material. Incorporation in the absence of T antigen has been subtracted. [b] Determined from gel analysis of replication products.

gies for tolerating persistent DNA damage that rarely result in mutations. Carty et al. (*47*) showed that in vitro replication past a *cis*−*syn* thymine−thymine dimer or a 6-4 photoproduct resulted in fewer than 2 mutations per 1000 bypass events. Thomas et al. (*48*, *49*) showed that *N*-acetyl-2-aminofluorene adducts resulted in targeted mutations in approximately 10 of every 1000 bypass events, and their results suggested that the undamaged strand was copied preferentially when the plasmid was replicated.

It is generally accepted that the majority of mutations induced by a genotoxic agent such as reductively activated 1-nitropyrene arise as the result of translesion synthesis (TLS), i.e., replication past template damage that interferes with fork progression. Eukaryotic cells, including human cells, have evolved highly conserved strategies to overcome this blockade. Recent data indicate that specialized DNA polymerases may be required to accomplish direct TLS. DNA polymerase *η* can efficiently bypass a *cis*−*syn* thymine−thymine dimer in vitro and does so in an error-free manner (*50*). A defect in this enzyme has been shown to underlie the xeroderma pigmentosum variant syndrome (*51*−*53*), which is characterized by UV hypermutability and a highly aberrant UV-induced mutation spectrum (*54*, *55*). A second pathway involves mutagenic TLS by DNA polymerase *ζ*. In *Saccharomyces cerevisiae* this enzyme is required for the induction of mutations by a wide variety of DNA damaging agents, but it is not required for any other known repair, replication, or recombination function. Abrogation of the function of the human homologue greatly reduces the UV-induced mutant frequency (*56*), suggesting that translesion synthesis strategies have been conserved.

In addition to TLS, an error-free mechanism of bypass exists that involves selective copying of the undamaged strand. The mechanism has not been fully elucidated, but such "damage avoidance" accounts for the great majority of bypass events (*57*), and human homologues to yeast genes in this pathway have been identified (*58*, *59*). The regulatory pathways that govern that mode of DNA polymerization past blocking lesions are not known, but the low frequency of mutagenic events found in the present study and in studies with other replication blocking lesions suggest that the error-prone pathway is minor.

Many studies imply that the structure and conformation of a lesion in DNA influence the error rate during TLS (discussed in ref *60*). Yet the relative effects of hydrogen bonding, thermodynamic stability, and base rotation on the frequency of errors by the DNA polymerase remain unclear. It is particularly a daunting task to investigate the structural features of a lesion in a replication fork. As a result, most studies of a lesion embedded in a duplex region of DNA are carried out with the assumption that similar structures would predominate in a fork during replication, although in a limited number of studies the single-strand−double-strand junction containing a DNA adduct has been examined by NMR (reviewed in ref *41*). In the current work we have determined the thermodynamic stability of a series of promutagenic intermediates containing dG^{AP}. In parallel, we determined the biological effect of the lesion in the same sequence context following replication in an extract of human fibroblast cells. We demonstrated that in comparison to the normal partner C opposite dG^{AP}, the one-base deletion intermediate is significantly more stable. While the quantitative difference in thermodynamic stability of an actual deletion intermediate in a replication fork with and without the adduct may not be as pronounced, there is little doubt that dG^{AP} would allow formation of a −1 deletion intermediate at a much higher frequency than an unmodified dG during replication. It is intriguing that the rate of incorporation of the correct base is nonetheless highly efficient, which probably reflects the consequence of an error-free pathway of DNA replication in human cells rather than efficient repair. Even so, the only errors detected in this study involved the −1 frame-shift mutagenesis, which can be qualitatively rationalized by the thermodynamic properties of the base pairs.

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SUPPORTING INFORMATION AVAILABLE

Two figures showing circular dichroism of the modified 13-mer duplexes with dG^{AP} opposite G, C, A, or T or the −1 deletion intermediate. This material is available free of charge via the Internet at http://pubs.acs.org.

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