Preparation and Characterization of a Deoxyoligonucleotide 49-mer Containing a Site-Specific Thymidylyl-(3′,5′)-deoxyadenosine Photoproduct†

Xiaodong Zhao, Jeffrey L.-F. Kao,‡ and John-Stephen Taylor*

The Department of Chemistry, Washington University, St. Louis, Missouri 63130

Received July 20, 1994; Revised Manuscript Received November 1, 1994®

ABSTRACT: Irradiation of d(GTATTATG) with 254 nm light gave rise to four major photoproducts, two of which were readily identified by NMR as the cis−syn cyclobutane dimer and the (6−4) photoproduct of the central TT site. Analysis of the NMR data for the other two photoproducts indicated that they were not any of the other known photoproducts of a TT site and might be TA* photoproducts [Bose, S. N., et al. (1983) *Science 220*, 723−725]. In support of this possibility, the fluorescence spectra of the products of acid hydrolysis of the two photoproducts were very similar to that reported for the hydrolysis product of the TA* photoproduct of TpdA. Only one of the two TA*-containing octamers could be ligated at both ends to form a 49-mer oligonucleotide in the presence of a complementary oligonucleotide scaffold, suggesting that the TA* photoproduct had formed between T5 and A6. The position of the TA* photoproduct was confirmed by mapping the arrest sites for 3′→5′ exonucleolytic degradation of the 49-mer by T4 DNA polymerase and for primer extension opposite the 49-mer by exonuclease deficient Klenow fragment (KF) and Sequenase Version 2.0. The TA* product could also be bypassed by both polymerases, but it was less of a block to KF. Treatment with 1 M aqueous piperidine at 100 °C led to a maximum of about 34% cleavage of the DNA at the site of the TA* product. The ^{1}H NMR signals of d(GTATTA*TG) were assigned by TOCSY, NOESY, and ^{1} H−^{31}P COSY, and the ^{1}H NMR shifts for the TA* photoproduct were comparable to those reported for the dinucleotide photoproduct [Koning, T. M. G., et al. (1990) *Nucleic Acids Res. 18*, 277−284]. The biological implications of this photoproduct are also discussed.

Many of the toxic, mutagenic, and carcinogenic effects of sunlight have been attributed to the DNA photoproducts induced by the UVB portion (280−320 nm) that reaches the earth's surface. Most UV-induced mutations occur at dipyrimidine sites (Hutchinson, 1987; Brash, 1988), which are also the principal sites of cis−syn cyclobutane dimer and (6−4) photoproduct formation (Wang, 1976; Cadet & Vigny, 1990). As a result, most repair and mutagenesis studies have focused on these photoproducts and have largely ignored other classes of photoproducts such as the TA* photoproduct (Figure 1). The TA* photoproduct was first isolated from the irradiation products of the dinucleotide TpdA[1] and was proposed to result from the [2 + 2] cycloaddition between the 5,6-double bonds of the T and the A (Bose et al., 1983). Further structural proof and assignment of the trans−syn stereochemistry of the dinucleotide photoproduct were based on the analysis of 2D NMR data and molecular dynamics simulations (Koning et al., 1990). Because of the unique fluorescence properties of the acid hydrolysis product of TA* (Bose et al., 1983, 1984), it was possible to demonstrate the formation of TA* in duplex poly(dA−dT) and calf thymus DNA irradiated with 254 nm light (Kumar & Davies, 1987). The biological properties of this interesting photoproduct are as yet unknown, primarily because of a lack of substrates containing site-specific TA* photoproducts for precise structure−activity studies. Herein, we report the isolation and spectroscopic characterization of an oligonucleotide octamer containing a site-specific TA* photoproduct and the preparation and enzymatic characterization of a 49-mer containing this product.

MATERIALS AND METHODS

Enzymes, Reagents, and Equipment. Oligonucleotides (Figure 2) were synthesized on an ABI 380B synthesizer by standard β-cyanoethyl phosphoramidite chemistry and purified by either anion-exchange or C-18 reverse phase HPLC. Oligonucleotide concentrations were determined by absorbance at 260 nm using estimated molar extinction coefficients. Sequenase Version 2.0 (a Δ28 3′→5′ exonuclease deficient mutant of T7 DNA polymerase) and the exo$^-$ Klenow fragment of DNA polymerase I were purchased from USB. T4 DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase were purchased from New England Biolabs. [γ-^{32}P]ATP (10 μCi/μL, 2 μM) was purchased from Amersham. Anion-exchange HPLC was carried out on a Nucleogen DEAE 60-7 column. Reverse phase HPLC was carried out on a Dynamax C-18 column (4.6 × 250 mm for analytical work and 21.6 × 250 mm for preparative work). Dideoxy sequencing was carried out with 1 unit of Sequenase Version 2.0 and 100 μM dNTPs (USB), with the eponymous dNTP consisting of a 1:3 mix of ddNTP/dNTP. NMR

† This investigation was supported by PHS Grant Number R01-CA40463, awarded by the National Cancer Institute, DHHS.

* Author to whom correspondence should be addressed.

‡ Washington University High Resolution NMR Facility.

® Abstract published in *Advance ACS Abstracts,* December 15, 1994.

[1] Abbreviations: ATP, adenosine triphosphate; BSA, bovine serum albumin; COSY, correlated spectroscopy; dNTP, 2′-deoxynucleotide; DTT, dithiothreitol; exo$^-$, 3′→5′ exonuclease deficient; NMR, nuclear magnetic resonance; NOESY, nuclear Overhauser effect spectroscopy; TMP, trimethyl phosphate; TOCSY, total correlated spectroscopy; TpdA, thymidylyl-(3′,5′)-deoxyadenosine; TpT, thymidylyl-(3′,5′) thymidine.

0006-2960/95/0434-1386$09.00/0

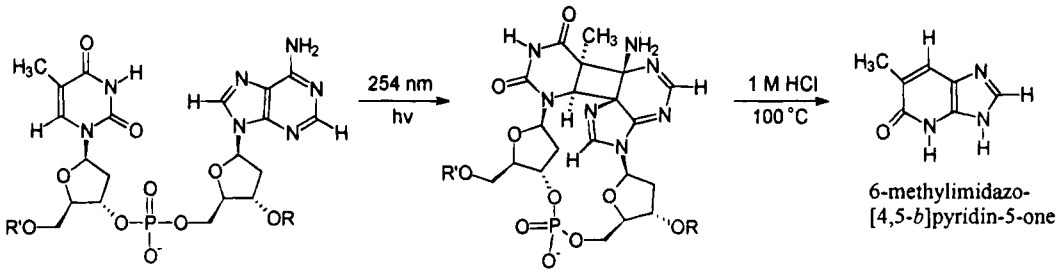

FIGURE 1: Proposed trans−syn I structure for the TA* photoproduct of DNA and its acid degradation product (Bose et al., 1984; Koning et al., 1990).

```
5'-AGCTACCATGCCTGCAC-3'                                    17-mer
              5'-GTATTA*TG-3'                               8-mer
                        5'-CAATTCGTAATCATGGTCATAGCT-3'     24-mer
    3'-GTACGGACGTGCATAATACGTTAAGCATTAGTAC-5'               34-mer
                    20                  40
5'-AGCTACCATGCCTGCACGTATTA*TGCAATTCGTAATCATGGTCATAGCT-3'   49-mer
                        3'-GCATTAGTACCAGTA-5'               15-mer
```

FIGURE 2: Oligonucleotides used in this study.

spectra were obtained on Varian UNITY 600 and VXR-500 spectrometers, and the data were processed on a SPARC 10 computer with VNMR software. The excitation and emission spectra were obtained on a FLUOROLOG II spectrometer (SPEX) with an R928PMT detector. All electrophoresis was carried out on 7 M urea, 0.4 mm thick, 375 mm long, acrylamide gels. DNA fragments were visualized by autoradiography with Kodak XAR-5 film.

*Isolation of d(GTATTA*TG).* d(GTATTATG) (0.8 μmol) was dissolved in 40 mL of water, degassed, and distributed between four Petri dishes. These were separately sealed under argon in Ziplock bags and irradiated with approximately 0.6 kJ of 254 nm light from a Spectronics Corp. (Westbury, NY) UVC BLE-1T155 low-pressure mercury arc lamp. The irradiated solutions were combined, concentrated, and then fractionated by preparative C-18 HPLC with a 40 min 6.25−8.75% gradient of CH_3CN in 75 mM K_2HPO_4/KH_2PO_4 (pH 6.8) at a flow rate of 2.5 mL/min. The crudely separated fractions were concentrated and chromatographed again under the same conditions. The purified fractions were desalted by loading onto the preparative column in water and then washing with water for 20 min at 3 mL/min followed by elution with 50% aqueous acetonitrile. A number of samples were irradiated and processed in a similar fashion to give 0.14 μmol of d(GTATTA*TG) from about 7 μmol of starting material, although no attempt was made to optimize the formation and chromatographic isolation of this product.

*Acidic Hydrolysis of d(GTATTA*TG).* d(GTATTA*TG) (1.16 μM) in 1 M HCl was heated in a boiling water bath for 4 h. The emission spectrum of the hydrolysate was obtained by excitation at 304 nm with a 295 nm cutoff filter, and the excitation spectrum was obtained by monitoring the emission at 404 nm.

*^1H and ^{31}P NMR Assignment of d(GTATTA*TG).* All NMR spectra were acquired on a 0.5 mL sample of 0.23 mM d(GTATTA*TG) in D_2O at 25 °C. The ^1H NMR spectra were referenced to the HOD signal (assigned to 4.80 ppm), and ^{31}P NMR spectra were referenced to external TMP (assigned to 0.00 ppm). All 2D spectra were acquired in the phase sensitive mode by employing the Hypercomplex method (States et al., 1982). The 600 MHz ^1H TOCSY (2D HOHAHA) spectrum (Bax & Davis, 1985) was collected into a 2 × 320 × 2048 data matrix by using a 120 ms MELV-17 mixing sequence that was flanked by two 2 ms trim pulses with 16 scans per t_1 value. Two-dimensional Fourier transformation was carried out following Gaussian line broadening and sine-bell weighting in the t_2 and t_1 dimensions, respectively, to give a 2000 × 2000 data set that was phase and baseline corrected in both dimensions. The 600 MHz ^1H NOESY spectrum (Kumar et al., 1980; Macura et al., 1981) was collected into a 2 × 512 × 2048 data matrix using a 250 ms mixing time and 32 scans per t_1 value and then zero filled to give a 2000 × 2000 data matrix that was processed as described for the TOCSY spectrum. The proton-detected 500 MHz $^1H-^{31}P$ correlation spectrum (Sklenar et al., 1986) was collected into a 2 × 128 × 1024 data matrix with 128 scans per t_1 value and spectral widths of 1600 Hz in f_2 and 750 Hz in f_1. The data was weighted with Gaussian line broadening in both dimensions and zero filled to 512 points in f_1 prior to 2D Fourier transformation.

Preparation of the 5'-End-Labeled TpA-Containing 49-mer Substrate.* d(GTATTA*TG) and 24-mer (1.5 nmol each) were phosphorylated separately with 10 units of T4 polynucleotide kinase and 25 nmol of ATP in 20 μL of kinase buffer (70 mM Tris·HCl, 10 mM $MgCl_2$, and 5 mM DTT, pH 7.6). After incubation at 37 °C for 1 h, the reactions were stopped by heating in a boiling water bath for 7 min to inactivate the kinase. The phosphorylated 8-mer and 24-mer were added to a tube containing 1.5 nmol each of 17-mer and 34-mer. The mixtures were annealed, lyophilized, and then dissolved in 30 μL of ligase buffer (50 mM Tris·HCl, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, and 50 μg/mL BSA, pH 7.8) containing 800 units of T4 DNA ligase. The reaction was incubated on ice for 20 h, during which time the temperature of the bath rose to 15 °C. The reaction mixture was then lyophilized, dissolved in 95% aqueous formamide, electrophoresed, excised, eluted, and dialyzed. The ligation yield was about 23%. The 49-mer (3 pmol) was 5'-end-labeled by incubation with 10 μCi (2 pmol) of [γ-^{32}P]ATP and 10 units of T4 polynucleotide kinase in total volume of 10 μL of kinase buffer at 37 °C for 1 h, followed by heating in a boiling water bath for 7 min to inactivate the kinase.

3'→5' Exonucleolytic Cleavage by T4 DNA Polymerase. 5'-End-labeled 49-mer (0.3 pmol) in 15 μL of 50 mM NaCl, 10 mM Tris·HCl, 10 mM $MgCl_2$, and 1 mM DTT (pH 7.8) containing 1 unit of T4 polymerase was incubated at 37 °C. After 5, 30, and 60 min, 5 μL samples were removed and quenched by addition to an equal amount of 95% aqueous

formamide, electrophoresed, and autoradiographed.

Hot Piperidine Treatment. 5′-End-labeled 49-mer (0.3 pmol) in 15 μL of 1 M aqueous piperidine was incubated in a boiling water bath. Samples (5 μL) were then removed after 5, 30, and 60 min, concentrated, redissolved in 10 μL of water, concentrated again, dissolved in loading buffer, electrophoresed, and autoradiographed.

Primer Extension Study opposite the TpA-Containing 49-mer.* A 5′-end-labeled primer was prepared by incubating 20 pmol of 15-mer in 20 μL of kinase buffer containing 10 units of T4 polynucleotide kinase and 20 μCi (4 pmol) of [γ-^{32}P]ATP at 37 °C for 45 min. Primer extension reactions were carried out with 1 pmol of annealed template/primer in 20 μL of polymerase buffer containing 1, 10, or 100 μM in each dNTP and 1 unit of the corresponding polymerase and were incubated for 40 min at 37 °C. The buffer used for the Sequenase Version 2.0 reactions was 40 mM Tris·HCl, 20 mM $MgCl_2$, 50 mM NaCl, and 20 mM DTT (pH 7.6), and for the exo$^-$ KF reactions the buffer was 50 mM Tris·HCl, 10 mM $MgCl_2$, and 10 mM DTT (pH 7.6). Following incubation, the reactions were quenched by adding an equal volume of 95% aqueous formamide, electrophoresed, and autoradiographed.

RESULTS AND DISCUSSION

Our original intention in irradiating d(GTATTATG) with 254 nm light was to produce the (6−4) photoproduct-containing octamer for high-field 2D NMR studies, and in the process, we discovered that TA*-containing products were also produced. We had previously prepared the (6−4) photoproduct of d(AATTAA) by 254 nm irradiation (Smith & Taylor, 1993) and had not isolated any TA*-containing products, suggesting that TA* photoproduct formation may be highly sequence dependent. When we irradiated the same sequence flanked by G's, i.e., d(GAATTAAG), a very complicated mixture of products was produced, presumably due to the formation of photoproducts of the two AA sites (Kumar et al., 1991). It is for this reason that we turned our attention to irradiating d(GTATTATG), which has a unique dipyrimidine site and is devoid of complicating AA sites.

Photochemistry of d(GTATTATG). Irradiation of d(GTATTATG) with 254 nm light gave four major photoproducts and unreacted starting material in a ratio of approximately 2:1:2:4:8 in their order of elution from a C-18 column (Figure 3). The third of these products to elute could be readily identified by ^{1}H NMR as the cis−syn dimer-containing octamer, d(GTAT[c,s]TATG), by comparison with the spectra of other dimer-containing oligonucleotides (Kemmink et al., 1987; Taylor et al., 1990). The fourth product to elute could be identified as the (6−4) product-containing octamer, d(GTAT[6,4]TATG), on the basis of the characteristic absorption maximum at about 325 nm and by comparison with the ^{1}H NMR data of d(AAT[6,4]TAA) (Smith & Taylor, 1993). The ^{1}H NMR data of the other two major products did not correspond to what would be expected for any of the other known photoproducts of the TpT site, such as the trans−syn I, Dewar, or spore photoproducts (Cadet & Vigny, 1990) or the recently isolated trans−syn II photoproduct (Kao et al., 1993), leading us to suspect that they were the photoproducts of some other site. A prime candidate was the TA* photoproduct (Bose et al., 1983), for which there are two sites in d(GTATTATG). Acidic hydrolysis of these two octamer photoproducts gave products whose fluorescence and emission spectra in 1 N HCl were very similar to those reported for the TA* photoproduct of the dinucleotide TpdA at pH 7 (Bose et al., 1983) (Figure 4). Subsequent work (vide infra) established that the first major peak to elute from the column was d(GTATTA*TG) and the second was d(GTA*TTATG).

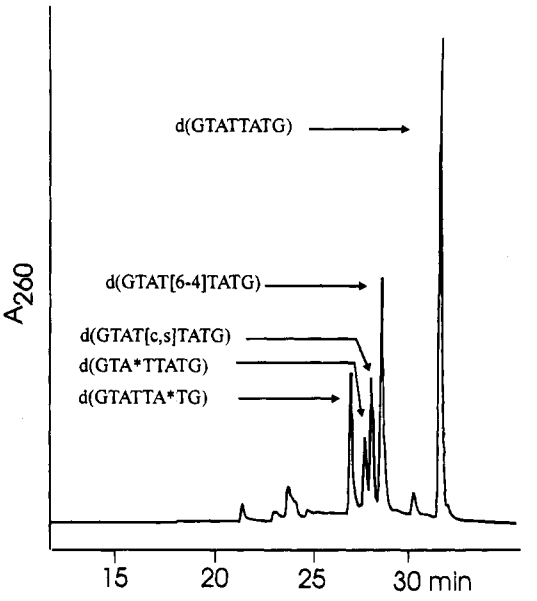

FIGURE 3: C-18 HPLC chromatogram of d(GTATTATG) following 254 nm irradiation.

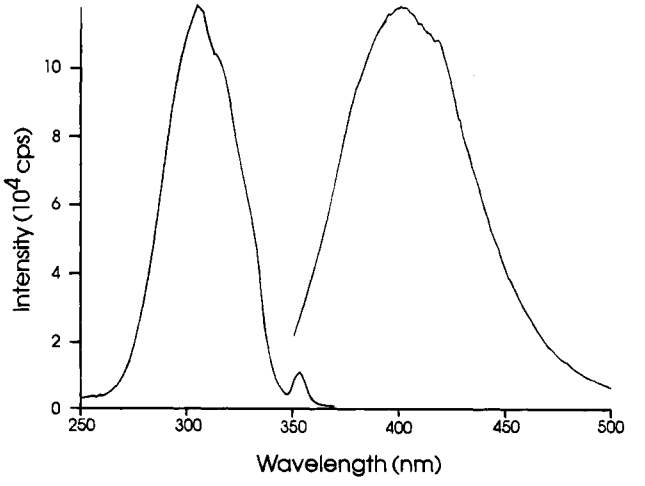

FIGURE 4: Excitation spectrum with detection at 404 nm (left) and emission spectrum with excitation at 304 nm (right) of the products of acidic hydrolysis of d(GTATTA*TG).

Analysis of the 1D ^{1}H NMR spectra of the two octamer photoproducts (only one of which is shown, Figure 5) also supported the possibility that they both contained TA* photoproducts. In the aromatic region, the undamaged octamer has 10 signals, which correspond to the four TH6, two GH8, two AH8, and two AH2 protons. The two octamer photoproducts have nine signals, just like the (6−4) product-containing octamer, due to saturation of one of the TH6 protons (Figure 5). Because cyclobutane formation saturates two TH6 protons, cis−syn and trans−syn photoproducts have only eight aromatic signals. Compared to the starting material, only one methyl signal of the two octamer photoproducts shifts upfield to 1.6 ppm. This is similar to

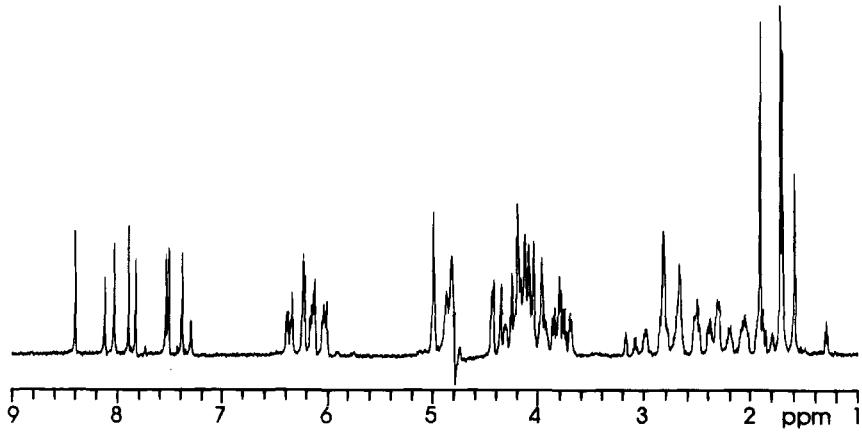

FIGURE 5: 600 MHz ^{1}H NMR spectrum of d(GTATTA*TG) in D_2O.

what is observed for (6−4) photoproduct formation, which causes the T4 methyl signal to shift upfield to 1.6 ppm, because the carbon to which the methyl group is attached changes the hybridization from sp^2 to sp^3.

Structure and Stereochemistry of the TA Photoproduct in d(GTATTA*TG).* Detailed 2D NMR studies were only carried out on d(GTATTA*TG) because, of the two TA* octamers, it was the only one that could be incorporated into a 49-mer oligonucleotide (see the following section). Proton NMR signals could be assigned to individual deoxyribose rings by the TOCSY experiment (Figure 6A), as well as to the four pairs of H6 and methyl signals of the undamaged thymidines. With the exception of the TA* site, the intranucleotide NOESY crosspeaks between H6/8 and H1′ were of sufficient intensity to allow assignment of the signals due to the bases and deoxyribose rings of individual nucleosides. The eight sets of nucleoside ^{1}H assignments were then assigned to specific nucleotides in the sequence by way of crosspeaks in the ^{1}H−^{31}P COSY spectrum corresponding to through-bond (*n*)H3′−P−(*n* + 1)H5′ correlations. A strong, but atypical crosspeak between a proton signal at 2.73 ppm and a phosphorus signal at −3.19 (Figure 6B) could be unambiguously assigned to TH3′ and the internucleotide phosphate of the TA* photoproduct, consistent with the previous ^{1}H NMR assignment of the dinucleotide photoproduct (Table 1) (Koning et al., 1990). Some of the crosspeaks originally used to assign trans−syn I stereochemistry of the TA* photoproduct of TpdA were also present in the octamer, such as crosspeaks between A6H8 and both A6H2′ and H2″. A crosspeak between T5H6 and T5H1′, indicative of a *syn*-glycosyl conformation, was not observed nor, however, were any crosspeaks between T5H6 and T5H2′ or H2″ that would be indicative of an anti conformation.

The similarity between the chemical shifts and NOESY spectra of the TA* product in the octamer and dinucleotide (Koning et al., 1990) suggests that the TA* photoproduct is rather rigid and that its conformation is not strongly influenced by incorporation into a single-stranded oligonucleotide. The trans−syn I cyclobutane stereochemistry (Liu & Yang, 1978) formally results from a [2 + 2] photocycloaddition reaction in which the T is in a *syn*-glycosyl conformation and the A is in an *anti*-glycosyl conformation. That only the trans−syn I TA* product would be formed is rather puzzling in light of the fact that pyrimidine-containing nucleosides prefer the *anti*-glycosyl conformation, which would lead to a cis−syn stereochemistry as observed for thymine dimer formation. When the octamer oligonucleotide was irradiated under the same conditions, the cis−syn, (6−4), and TA* photoproducts were produced in comparable yields, but no trans−syn I dimer of the TT site was detected. Clearly more information will be needed to rigorously establish the structure and stereochemistry of the TA* product and its mechanistic origin.

Mapping of TA Photoproducts in DNA.* The accurate mapping and quantification of individual photoproducts in both single- and double-stranded DNA are critical to assessing their biological significance and properties. To evaluate methods for mapping TA* products, a TA*-containing 49-mer substrate was prepared in 23% yield by ligating a 17-mer to the 5′-end and a 24-mer to the 3′-end of d(GTATTA*TG) in the presence of a complementary 34-mer as a ligation scaffold. A 49-mer containing the undamaged octamer was prepared in a comparable yield of 28%. d(GTA*TTATG) failed to ligate, probably because the photodamage site was too close to the end of the octamer and may have interfered with binding, catalysis, or both by T4 ligase. The gel-purified TA*-containing 49-mer appeared to be contaminated with about 14% of a series of bands migrating between 23 and 27 nucleotides in length. In a second preparation, in which care was taken not to unnecessarily heat the sample, only 3% of the degradation products was observed. Just recently, it was reported that poly(dT−dA) irradiated with 254 nm light was susceptible to cleavage by hot piperidine, leading to the suggestion that cleavage was occurring at the sites of TA* products (Hejmadi et al., 1994). Heating the TA* 49-mer with 1 M piperidine at 100 °C for 5 min resulted in about a 35% yield of a number of cleavage products (Figure 7). Increasing the incubation time to 60 min did not lead to an increase in cleavage, suggesting that about 65% of the TA* product had been converted to another product that was stable to cleavage. While our results confirm that hot piperidine can cleave DNA at a TA* product, it would appear to be difficult to map and quantify TA* products by this method.

In spite of the presence of a cyclobutane ring, the TA* product did not appear to be a substrate for T4 denV endonuclease V under conditions that cleave a cis−syn dimer readily (Smith & Taylor, 1993) (data not shown). In cases where damage-specific chemical or endonuclease assays are lacking, DNA damage can often be conveniently mapped by the positions of bands that result from the arrest of the

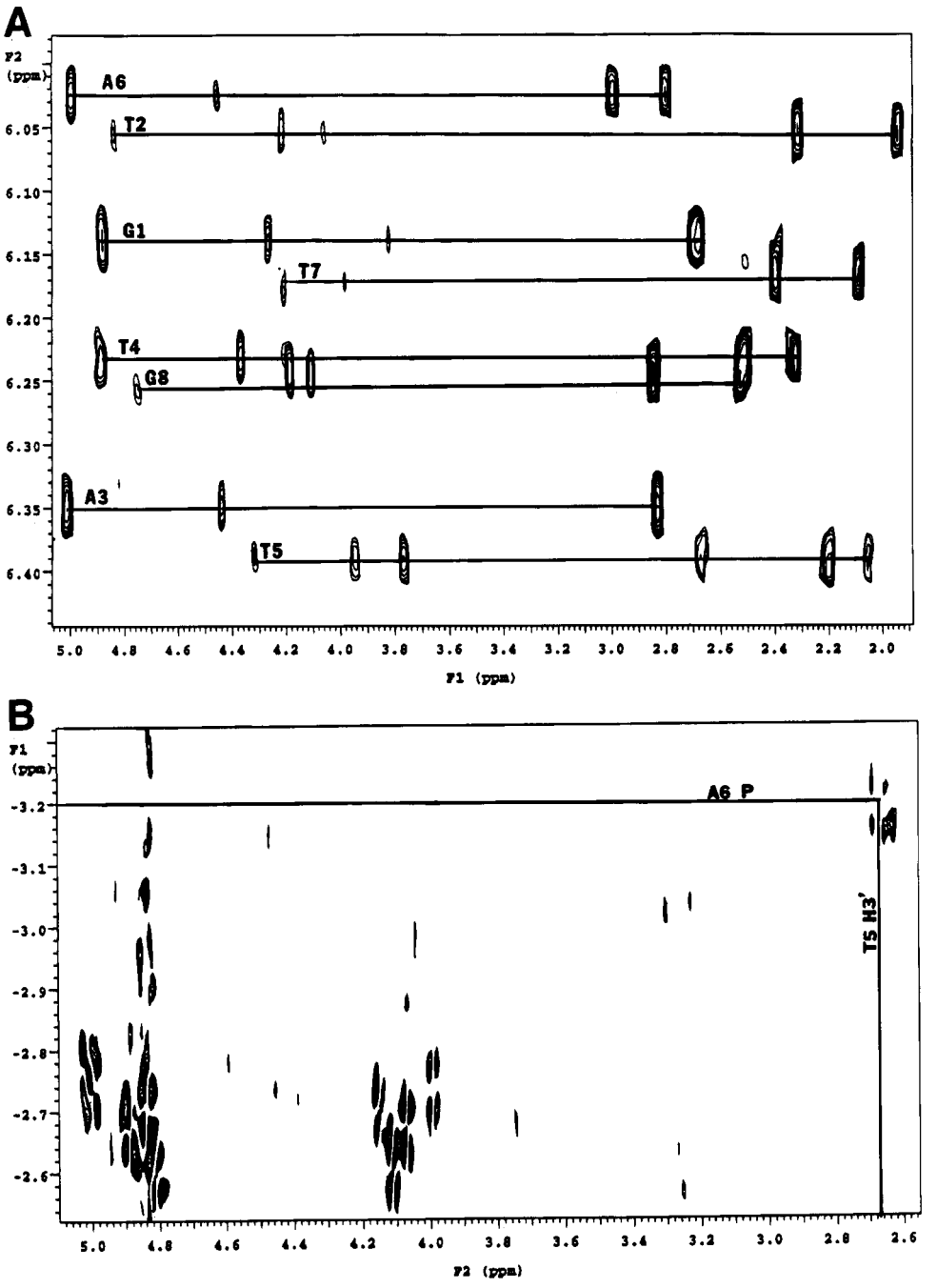

FIGURE 6: (A) 600 MHz ^{1}H TOCSY spectrum of d(GTATTA*TG), showing assignments of the sugar protons, and (B) 500 MHz ^{1}H−^{31}P heterocorrelated NMR spectrum showing the atypical crosspeak at 2.73,−3.19 ppm that could be assigned to T5H3′,A6P of the TA* photoproduct.

3′→5′ exonuclease activity of T4 polymerase (Doetsch et al., 1985). In a recent study, we found that exonucleolytic degradation of cis−syn and trans−syn dimer-containing 49-mers by T4 polymerase led to two products that could be attributed to termination prior to the dimer and to subsequent cleavage of the intradimer phosphodiester bond (Smith & Taylor, 1993). The TA* product appears to behave similarly, leading to one product that terminates before the A of the TA* product and another that migrates more quickly (Figure 7).

DNA Synthesis opposite the TA Product.* Whether a DNA polymerase is able to synthesize past damaged DNA is of interest for two principal reasons. The first relates to assessing the mutagenic potential of the damaged DNA, which should be greater, the more easily the damage is bypassed. The second relates to mapping and quantifying the damage sites by way of the ability of the damaged DNA to block polymerase-catalyzed primer extension reactions. When primer extension opposite the TA* 49-mer was carried out with the exo$^-$ T7 DNA polymerase Sequenase Version 2.0 (SQV2) at 1 M dNTPs, synthesis terminated almost exclusively opposite the T of the 3′-side of the TA* product (Figure 8, Table 2). At higher dNTP concentrations, termination also occurred opposite the A of the TA* product, and a small amount of bypass product was observed. The inability of SQV2 to bypass the TA* product efficiently at high dNTP concentrations, and instead arrest primarily opposite the A of the TA* product, is consistent with the trans−syn I stereochemistry proposed for this photoproduct (Koning et al., 1990). In the trans−syn I stereochemistry,

Table 1: ^{1}H and ^{31}P NMR Assignments of d(GTATTA*TG) and Comparison to Data in Parentheses for the TA* Photoproduct of the Dinucleotide TpdA (Koning et al., 1990)

	G1	T2	A3	T4	T5	A6	T7	G8
H1′	6.13	6.06	6.34	6.22	6.39 (6.40)	6.02 (6.01)	6.16	6.24
H2′	2.69	1.94	2.83	2.33	2.06 (2.12)	3.00 (3.01)	2.08	2.85
H2″	2.69	2.32	2.83	2.52	2.21 (2.28)	2.80 (2.67)	2.40	2.53
H3′	4.88	4.82	5.02	4.89	2.68 (2.73)	5.00 (4.78)	4.79	4.19
H4′	4.26	4.22	4.44	4.37	3.77 (3.67)	4.46 (4.26)	3.99	4.10
H5′	3.82	4.37	4.15	4.22	4.32 (3.87)	3.88 (3.86)	4.20	4.75
H5″	3.82	4.06	4.15	4.16	3.95 (3.70)	3.72 (3.86)	4.20	4.75
H6/H8	8.13	7.38	8.41	7.52	5.01 (5.03)	7.84 (7.83)	7.55	8.03
H2			7.90[a]			7.31[a] (7.28)		
$C5CH_3$		1.74		1.72	1.60 (1.58)		1.92	
5′-P		−2.68	−2.71	−2.79	−2.68	−3.19	−2.74	−2.61

[a] Assigned by comparison to the NMR shifts for the TA* photoproduct of TpdA.

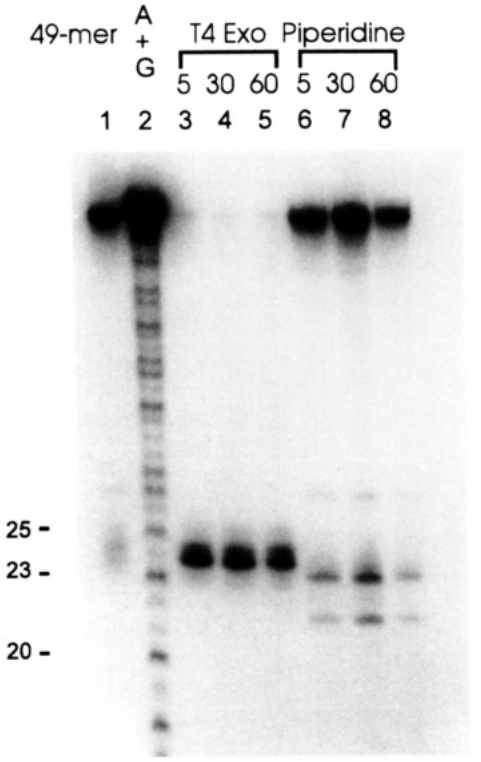

FIGURE 7: Autoradiogram of a denaturing acrylamide gel of the degradation products of the exonuclease activity of T4 DNA polymerase (T4 exo) and hot piperidine of 5′-end-labeled TA* 49-mer for the incubation times shown in minutes. For sequence alignment a Maxam−Gilbert A+G reaction was also run, which leads to loss of the reacted nucleoside and a 5′-^{32}P-labeled product with a 3′-terminal phosphate.

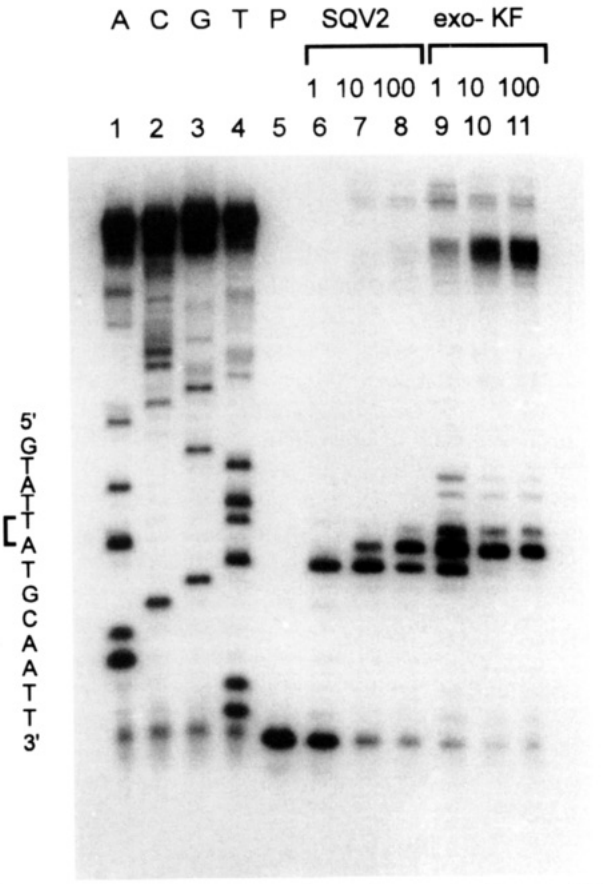

FIGURE 8: Autoradiogram of a denaturing acrylamide gel of the products of Sequenase Version 2.0 (SQV2) and 3′→5′ exonuclease deficient Klenow fragment (exo$^-$ KF)-catalyzed extension products of the 5′-end-labeled 15-mer primer (P) opposite the TA* 49-mer for the dNTP concentrations shown in molar for 40 min. Dideoxy sequencing reactions are shown to the left, with the lane headings referring to the base complementary to the dideoxynucleotide used. The uppermost bands appear to be due to incompletely denatured duplex form.

the T is in a syn conformation, which places the bulky, hydrophobic methyl group in the region of B DNA that is involved in Watson−Crick hydrogen bond base pairing, thereby greatly interfering with nucleotide incorporation.

In contrast, the less processive, exonuclease deficient Klenow fragment (exo$^-$ KF) was unexpectedly able to bypass the TA* product rather efficiently, even at 1 M dNTPs. As observed for SQV2 at high dNTPs, DNA synthesis was primarily arrested opposite the A of the TA* product, indicating that the rate-determining step in the bypass is the subsequent incorporation of a nucleotide opposite the T of the TA* product. Two minor products were also observed that terminated two and three nucleotides past the TA* site, suggesting that the TA* product may interfere with elongation steps even at some distance from the actual damage site. The ease with which exo$^-$ KF was able to bypass the TA* product relative to SQV2 was unexpected, however, because it is opposite what we have found to be the case for bypass of the cis−syn, trans−syn, (6−4), and Dewar photoproducts (O'Day, 1989; Smith, 1993). It is interesting to note that a *cis*-platinum(II) adduct of a GG site, another type of dinucleotide adduct, was found in one study to be more easily bypassed by exo$^-$ KF than by SQV2 (Belguise-Valladier et al., 1994), whereas in another study little difference was observed between the two polymerases in bypassing the same adduct in a different sequence context (Comess et al., 1992). The origin of the differences in bypass ability is not

Table 2: Product Distributions Resulting from the Primer Extension opposite the TA*-Containing Template by Sequenase Version 2.0 (SQV2) and the exo$^-$ Klenow Fragment (KF)

	SQV2			KF		
polymerase	1 μM dNTPs	10 μM dNTPs	100 μM dNTPs	1 μM dNTPs	10 μM dNTPs	100 μM dNTPs
1 prior	92	54	28	19		
1 opposite		26	40	30	26	21
2 opposite			13	14	12	12
1 past				3	3	2
2 past				5	2	2
bypass	8	15	19	29	57	63

understood at this moment, it but must be due to sequence dependent differences in the interactions of different polymerases with different types of damage.

Biological Implications. It was originally estimated that the quantum yield of TA* induction per TA site in duplex DNA by 254 nm light was about 0.0001 (Bose & Davies, 1984), which is about 1 and 2 orders of magnitude less than the values of 0.019 and 0.0013 estimated for TT cis–syn and TC (6–4) photoproduct induction, respectively (Patrick, 1977). We find that the rates of TT (6–4) and TA* photoproduct formation in d(GTATTATG) are similar and consistent with a quantum yield of 0.0001–0.0002 for (6–4) product formation in TpT (Lemaire & Ruzsicska, 1993) and the observation that the quantum yield for TA* photoproduct formation goes up by a factor of 5 upon denaturing DNA (Bose et al., 1983). Although the TA* photoproduct is not as abundant as other photoproducts, Davies and coworkers noted that the highly conserved TATA sequence of promoters has four potential sites for TA* photoproduct formation that could lead to mutations that permanently interfere with expression of the downstream gene (Bose et al., 1983; Bose & Davies, 1984). Now that oligonucleotides containing TA* photoproducts can be isolated in high purity and ligated into longer substrates, the biological properties of this largely ignored photoproduct can be studied.

ACKNOWLEDGMENT

We thank Steve Gentemann for help with the fluorescence measurements.

REFERENCES

Bax, A., & Davis, D. G. (1985) *J. Magn. Reson. 65*, 355–360.
Belguise-Valladier, P., Maki, J., Sekiguchi, M., & Fuchs, R. P. P. (1994) *J. Mol. Biol. 236*, 151–164.
Bose, S. N., & Davies, R. J. H. (1984) *Nucleic Acids Res. 12*, 7903–7914.
Bose, S. N., Davies, R. J. H., Sethi, S. K., & McCloskey, J. A. (1983) *Science 220*, 723–725.
Bose, S. N., Kumar, S., Davies, R. J. H., Sethi, S. K., & McCloskey, J. A. (1984) *Nucleic Acids Res. 12*, 7929–7947.
Brash, D. E. (1988) *Photochem. Photobiol. 48*, 59–66.
Cadet, J., & Vigny, P. (1990) in *Bioorganic Photochemistry* (Morrison, H., Ed.) Vol. 1, Chapter 1, pp 1–272, John Wiley & Sons, New York.
Comess, K. M., Burstyn, J. N., Essigmann, J. M., & Lippard, S. J. (1992) *Biochemistry 31*, 3975–3990.
Doetsch, P. W., Chan, G. L., & Haseltine, W. A. (1985) *Nucleic Acids Res. 13*, 3285–3304.
Hejmadi, V., Stevenson, C., Kumar, S., & Davies, J. H. (1994) *Photochem. Photobiol. 59*, 197–203.
Hutchinson, F. (1987) *Photochem. Photobiol. 45*, 897–903.
Kao, J. L.-F., Nadji, S., & Taylor, J.-S. (1993) *Chem. Res. Toxicol. 6*, 561–567.
Kemmink, J., Boelens, R., Koning, T. M. G., Kaptein, R., van der Marel, G. A., & van Boom, J. H. (1987) *Eur. J. Biochem. 162*, 37–43.
Koning, T. M. G., Davies, R. J. H., & Kaptein, R. (1990) *Nucleic Acids Res. 18*, 277–284.
Kumar, S., & Davies, R. J. H. (1987) *Photochem. Photobiol. 45*, 571–574.
Kumar, A., Ernst, R. R., & Wuthrich, K. (1980) *Biochem. Biophys. Res. Commun. 95*, 1.
Kumar, S., Joshi, P. C., Sharma, N. D., Bose, S. N., Davies, R. J. H., Takeda, N., & McCloskey, J. A. (1991) *Nucleic Acids Res. 19*, 2841–2847.
Lemaire, D. G. E., & Ruzsicska, B. P. (1993) *Photochem. Photobiol. 57*, 755–769.
Liu, F.-T., & Yang, N. C. (1978) *Biochemistry 17*, 4865–4876.
Macura, S., Huang, Y., Suter, D., & Ernst, R. R. (1981) *J. Magn. Reson. 43*, 259–281.
O'Day, C. L. (1989) Synthesis and structure-activity relationships of site-specific cis-syn and trans-syn thymine dimers, Ph.D. Dissertation, Washington University, St. Louis, MO.
Patrick, M. H. (1977) *Photochem. Photobiol. 25*, 357–372.
Sklenar, V., Miyashiro, H., Zon, G., Miles, H. T., & Bax, A. (1986) *FEBS Lett. 208*, 94–98.
Smith, C. A. (1993) In vitro and in vivo mutation spectra of the cis-syn, trans-syn-I, (6,4), and Dewar photoproducts of TpT at a unique site, Ph.D. Dissertation, Washington University, St. Louis, MO.
Smith, C. A., & Taylor, J.-S. (1993) *J. Biol. Chem. 268*, 11143–11151.
States, D. J., Haberkorn, R. A., & Ruben, D. J. (1982) *J. Magn. Reson. 48*, 286–292.
Taylor, J.-S., Garrett, D. S., Brockie, I. R., Svoboda, D. L., & Telser, J. (1990) *Biochemistry 29*, 8858–8866.
Wang, S. Y., Ed. (1976) *Photochemistry and Photobiology of Nucleic Acids Volume I Chemistry*, Academic Press, Inc., New York.

BI941650G