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Thermodynamic and base-pairing studies of matched and mismatched DNA dodecamer duplexes containing *cis-syn*, (6-4) and Dewar photoproducts of TT

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ABSTRACT

Cis-syn **dimers, (6-4) products and their Dewar valence isomers are the major photoproducts of DNA and have different mutagenic properties and rates of repair. To begin to understand the physical basis for these differences, the thermal stability and base pairing properties of the corresponding photoproducts of the TT site in d(GAGTATTATGAG) were investigated. The (6-4) and Dewar products destabilize the duplex form by ~6 kcal/mol of free energy at 37°C relative to the parent, whereas a *cis-syn* dimer only destabilizes the duplex form by 1.5 kcal/mol. Duplexes with G opposite the 3′-T of the (6-4) and Dewar products are more stable than those with A by ~0.4 kcal/mol, whereas the *cis-syn* dimer prefers A over G by 0.7 kcal/mol. Proton NMR suggests that wobble base pairing takes place between the 3′-T of the *cis-syn* dimer and an opposed G, whereas there is no evidence of significant H-bonding between these two bases in the (6-4) product. The thermodynamic and H-bonding data for the (6-4) product are consistent with a 4 nt interior loop structure which may facilitate flipping of the photoproduct in and out of the helix.**

INTRODUCTION

The *cis-syn* cyclobutane dimers and the (6-4) pyrimidine-pyrimidone products (Fig. 1) are the major products induced by UVB and C irradiation of DNA and have been correlated with mutations and skin cancer. The (6-4) products are not stable to the UVA and UVB wavelengths in sunlight and are converted to their Dewar valence isomers (1–3). Whereas the *cis-syn* dimers of TT sites are not very mutagenic (1% TT→TC, 5%→TA) (4–7), the (6-4) products of TT sites are highly mutagenic in *Escherichia coli* under SOS conditions, causing 63–91% mutations, 91–95% of which are TT→TC mutations (7,8). The Dewar valence isomer is less mutagenic, only causing 33–42% mutations, 20–30% of which are also TT→TC mutations (7,8). The preferential incorporation of A opposite the 5′-pyrimidine and G opposite the 3′-pyrimidone ring during DNA synthesis bypass, has been attributed to preferential base-pairing interactions (7,8). Support for this proposal comes from a recent study in which primers terminating in G opposite the pyrimidone ring of the (6-4) and Dewar products were found to be more stable than those terminating in A, C or T (9).

The (6-4) and Dewar products are more rapidly repaired than *cis-syn* dimers by excision repair *in vivo*, having a half life of ~4 h in mammalian cells, compared to 24 h for *cis-syn* dimers (10–14). *In vitro*, (6-4) and Dewar products of TT are repaired almost nine times more rapidly than *cis-syn* dimers by *E.coli* uvrABC (15), and are bound much more tightly by the uvrA and XPE-DDB DNA damage recognition proteins than are *cis-syn* dimers (16). In recent experiments with purified human excinuclease, a (6-4) product of TT was repaired only three times more rapidly than the corresponding *cis-syn* dimer (17). Replacing the A opposite the 3′-T of the photoproducts with a G to create a compound lesion increased the rate of *cis-syn* dimer repair four times, whereas it did not affect the rate of (6-4) photoproduct repair. *Cis-syn* dimers and (6-4) products are also directly repaired by highly homologous photolyases, for which base flipping mechanisms have been proposed (18,19). Introducing a double TT mismatch opposite the (6-4) photoproduct of TT was found to increase binding by (6-4) photolyase (19).

To better understand the physical basis for the differences in the mutagenic properties and rates of repair of DNA photoproducts, we have investigated the thermal stability and base pairing properties of dodecamer duplexes containing centrally located *cis-syn*, (6-4) and Dewar products of TT in native and mutated sequence contexts (Fig. 1). The mutated sequence, or compound lesion, in which G is incorporated in place of A opposite the 3′-T of each photoproduct, corresponds to the DNA synthesis bypass product leading to the major mutation induced by (6-4) and Dewar products in *E.coli* under SOS, and a minor mutation induced by *cis-syn* dimers. The duplex with G opposite *cis-syn* dimer of TT also corresponds to a native photoproduct that arises from deamination of a *cis-syn* dimer of a Tm^{e5}C (5-methylcytosine) site, and apart from the methyl group on the 3′-T, is structurally equivalent to the deamination product that would arise at a TC site

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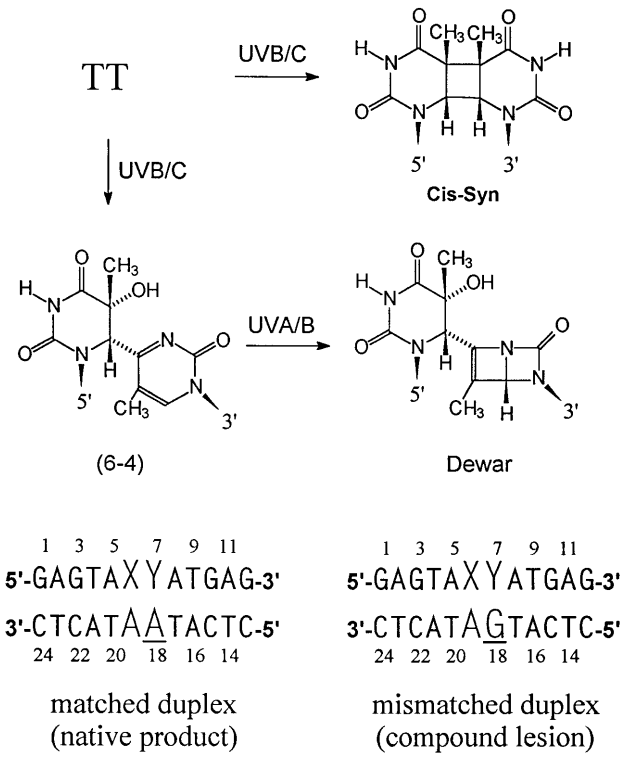

Figure 1. Photochemistry of a TT site, and the matched and mismatched DNA sequences studied, where XY represents the parental TT site, and its *cis-syn*, (6-4) and Dewar photoproducts.

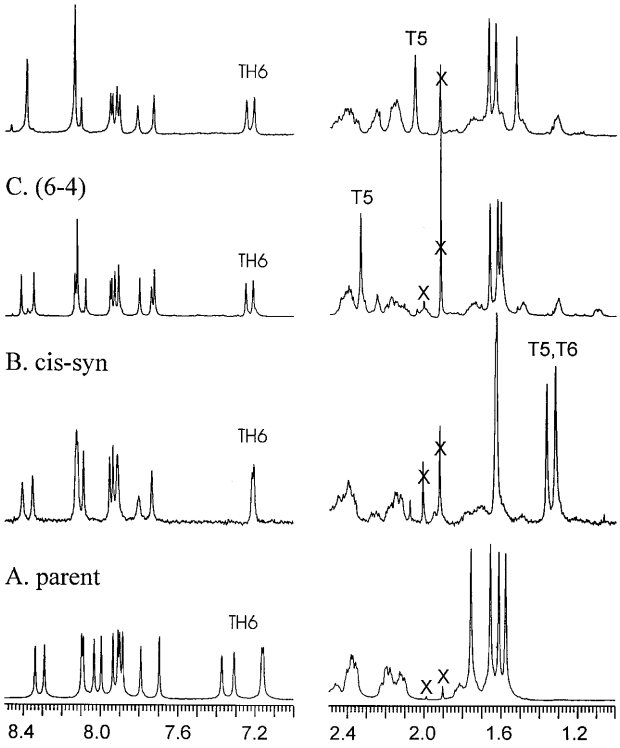

Figure 2. 600 MHz proton NMR spectra of the parental and photodamaged strands in D_2O at 25°C. The crossed out signals correspond to solvent impurities resulting from the HPLC purification. The other minor peaks have not been identified.

(20). These deaminated products are highly mutagenic and cause C→T mutations, the major mutation induced by UV light.

MATERIALS AND METHODS

Preparation and characterization of the photoproduct-containing dodecamers

The undamaged parental and complementary strands were purchased from Integrated DNA Technologies, Inc., and checked for purity by 1D ^{1}H NMR in D_2O before use. The *cis-syn* dimer-containing dodecamer was prepared by automated DNA synthesis with a *cis-syn* thymine dimer building block (21), and purified on a Nucleogen column with a 40 min gradient of 0–1 M KCl in 20% CH_3CN, 20 mM phosphate buffer. The peak at retention time 33 min was collected and desalted by eluting with 50:50 CH_3CN:H_2O from a C18 column that was pre-equilibrated with water. The (6-4) and Dewar products were obtained as previously described for a hexamer (22). Thus d(GAGTATTAT-GAG) was exposed to 254 nm light (1520 µw/cm^2) from a UV-C lamp with a 250–375 nm filter, at 0°C for 2.5 h in 0.373 µmol batches in 30 ml of nitrogen-purged dd H_2O in Petri dishes sealed under nitrogen in a ziplock polyethylene bag. The (6-4) product was isolated by reverse phase HPLC on an analytical C18 column (5 µm particle size, 4.6 mm ID × 25 cm L) with a 60 min 10–40% methanol gradient in 75 mM phosphate buffer (pH 6.6) at flow rate of 0.7 ml/min. The (6-4) product fractions were desalted as described above for the *cis-syn* dimer product. The Dewar product was obtained in >95% yield by exposing a D_2O solution of the (6-4) product in a 5 mm NMR tube to Mylar and Pyrex-filtered 450 W medium pressure mercury arc lamp at distance of 2–3 cm for 30 min, and was not further purified.

Melting temperature studies

The temperature dependence of the absorption of the DNA duplexes at 260 nm in 1 M or 250 mM NaCl, 10 mM sodium cacodylate (pH 7.0) and 0.1 mM EDTA was obtained in 1 cm pathlength cells in a nitrogen purged Cary 1E UV-visible spectrometer fitted with a multicell block with peltier cooling pumps. The samples were thermally annealed in the spectrometer and then denaturation and renaturation curves were collected for each duplex at both 7.5 and 15 µM total strand concentrations with heating and cooling rates of 0.5°C/min. The thermodynamic parameters ΔH° and ΔS° were derived by non-linear least-squares fitting of the UV melting curves to a two state model as previously described (23) with the Kalaidagraph program. The ΔH° parameter calculated in this way is a van't Hoff enthalpy which may or may not be equivalent to the van't Hoff enthalpy calculated by fitting of $\ln(C_T)$ versus $1/T_m$ data, and both of which are often not equivalent to the calorimetric enthalpy (for a discussion see 24). Strand concentrations were calculated according to a standard equation (25) from the absorbance at 260 nm at 25°C that was extrapolated from the upper single strand base lines of the melting curves.

Table 1. Proton NMR data for the photoproduct site in the *cis-syn* and (6-4) mismatch duplexes in comparison to data for matched duplexes

		NH	H6	CH3	H1′	H2′	H2″	H3′
cis-syn	Tp	12.15	4.75	0.39	5.54	na	na	4.89
		(12.02)	(4.44)	(0.57)	(5.57)	(1.97)	(2.63)	(4.75)
	pT	11.37	4.47	1.41	5.45	na	na	4.93
		(13.06)	(4.09)	(1.48)	(5.41)	(2.04)	(2.63)	(4.86)
(6-4)	Tp	13.60	4.71	1.39	5.35	0.89	1.78	3.86
		(13.11)	(4.74)	(1.22)	(5.78)	(0.94)	(1.94)	(3.79)
	pT	–	7.91	2.24	6.41	3.01	2.59	5.02
		–	(7.87)	(2.23)	(5.78)	(2.49)	(2.85)	(4.20)

na, not assigned. Values in parentheses are for d(CGCATTACGC)•d(GCGTAATGCG) (39).

Table 2. Derived thermodynamic data in kcal/mol (± <1%) for formation of the parent, (6-4) and Dewar dodecamer duplexes in 1 M NaCl

Duplex	[NaCl]	ΔH°	ΔS°	ΔG°	$\Delta\Delta G^\circ_{AA}$	$\Delta\Delta G^\circ_{par}$	T_M @ 15 μM
TT•AA	1 M	–76.9	–214.0	–10.6	–	–	49
Calculated[a]		–77.6	–219.2	–9.6	–	–	45
TT•GA	1 M	–78.3	–225.0	–8.6	+2.0	–	40
T[cs]T•AA	1 M	–75.1	–212.9	–9.1	–	+1.5	43
T[cs]T•GA	1 M	–75.8	–217.5	–8.4	+0.7	+0.2	40
T[6-4]T•AA	1 M	–61.3	–183.5	–4.4	–	+6.1	21
T[6-4]T•GA	1 M	–67.1	–200.5	–4.9	–0.5	+3.7	25
T[Dew]T•AA	1 M	–64.0	–191.5	–4.6	–	+5.9	23
T[Dew]T•GA	1 M	–68.2	–204.0	–5.0	–0.3	+3.6	25
TT•AA	250 mM	–79.8	–226.9	–9.5	–	–	44
TT•GA	250 mM	–76.2	–221.4	–7.6	+1.9	–	36
T[cs]T•AA	250 mM	–75.1	–212.9	–8.2	–	+1.3	39
T[cs]T•GA	250 mM	–75.8	–217.5	–7.4	+0.8	+0.2	36

Free energies are calculated for 37°C, and differences are relative to the corresponding AA duplex ($\Delta\Delta G_{AA}$) or the parental duplex ($\Delta\Delta G_{par}$).
[a] Calculated from published parameters (38).

Proton NMR spectra

NMR spectra were obtained on the mismatched parent, *cis-syn* and (6-4) duplexes (0.75, 0.60 and 0.75 mM, respectively) in 0.3 ml of either 100% D_2O or 90% H_2O/D_2O, 10 mM Na/HPO_3 (pD 7.0), 0.01% NaN_3 and either 100 or 250 mM NaCl and referenced to external sodium 3-(trimethylsilyl)-L-propanesulfonate. All the spectra were acquired on Varian Unity 600 and UNITYplus 500 (Varian Assoc., Palo Alto, CA) spectrometers and processed off-line on a SPARC 10 station with VNMR software. 1D NMR experiments of the exchangeable protons were carried out at 1°C using pre-saturation or WATERGATE gradient echo sequences (26) for water suppression. The WATERGATE sequence was carried out with a proton spectral width of 11001 Hz, a 7 μs non-selective 90° pulse, 1 ms 12 G/cm field-gradient pulses, and 256 Hz selective RF fields at the water resonance. Two-dimensional phase sensitive NOESY (27,28) experiments on the exchangeable protons were carried out with 70, 100 and 250 ms mixing times using 1–1 jump return (29,30) or WATERGATE sequences for water suppression at 1°C. A total of two 420 × 4096 data matrices with 128 scans per t1 value were collected and linear prediction was applied to obtain 840 real points in the F1 dimension before Fourier transformation. Digital filtering was used in the 2D processing to minimize the water signal.

RESULTS AND DISCUSSION

The identity and purity of all oligonucleotides used in this study were established by 1D ^1H NMR spectroscopy in D_2O prior to duplex formation (Fig. 2). In particular, all the dithymine photoproduct-containing oligonucleotides could be characterized by the loss of two thymine H6 signals in the 7.1–7.4 p.p.m. range when compared to the parent strand (22). The *cis-syn* dimer was further distinguished by two upfield-shifted methyl signals, and the (6-4) product by a downfield shifted T5 methyl signal and the appearance of a UV absorbance at 320 nm. The Dewar product was distinguished from the (6-4) by a slightly less downfield shifted T5 methyl signal and the loss of the 320 nm absorbance.

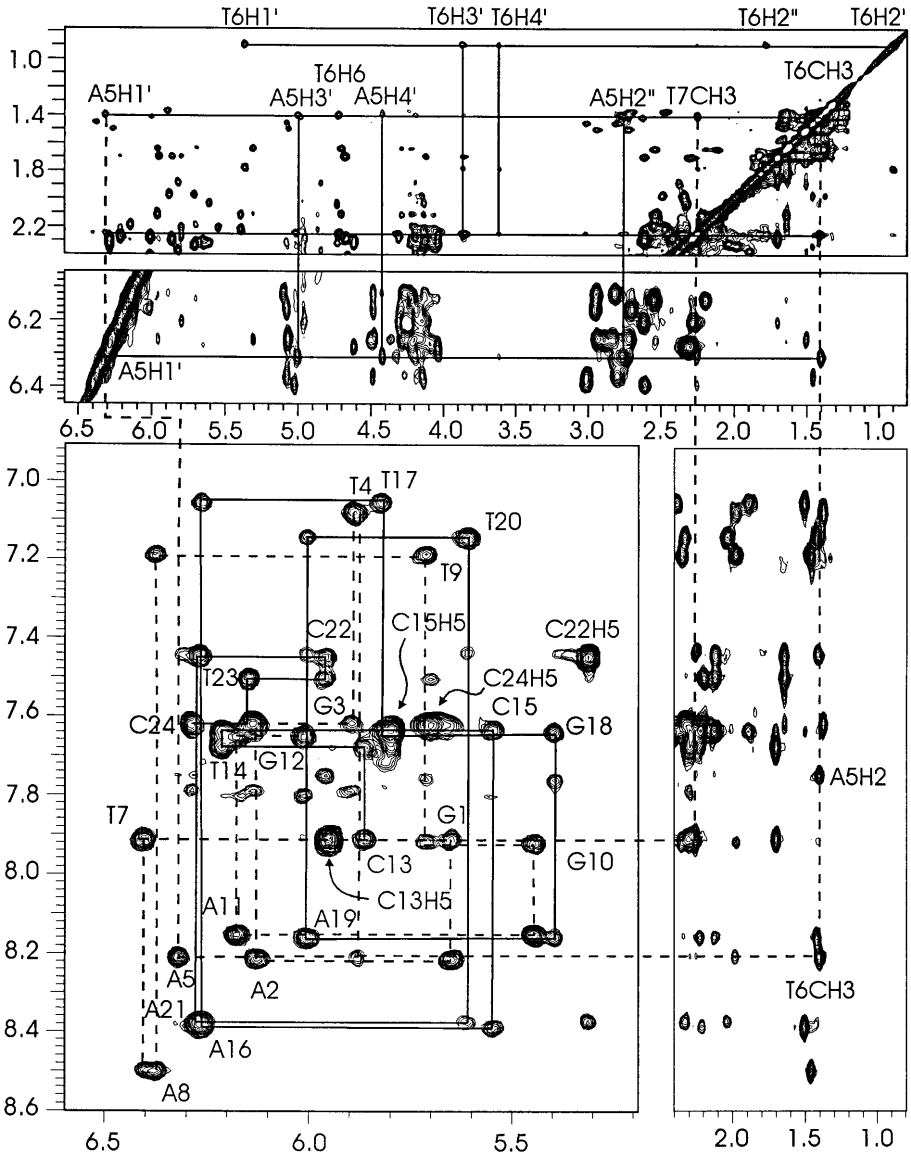

Figure 3. Sections of a 600 MHz 250 ms NOESY spectrum of the mismatched (6-4) duplex at 15°C in D_2O. The 7–8.5 versus 5–6.5 p.p.m. section shows the sequential assignment of the H6/8 and H1′ protons of photoproduct-containing strand (dashed line), and the complementary strand (solid line), in which the intranucleotide crosspeaks are labeled. The other sections show correlations involving the (6-4) product of TT, and an unusual set of crosspeaks between T6CH3 and the protons of A5.

The mismatched *cis-syn* and (6-4) duplexes were also characterized by sequential assignment of the non-exchangeable proton signals by 2D NOESY spectroscopy in D_2O as shown in Figure 3 for the (6-4) product, and the assignments of the photoproduct signals are given in Table 1 in comparison to reported data. Exchangeable proton spectra are shown in Figures 4 and 5 for all mismatched duplexes.

Thermodynamic properties of photodamaged DNA duplexes

The van't Hoff ΔH° and ΔS° for duplex formation in 250 mM and 1 M NaCl (Table 2) were obtained by curve fitting according to a two state, all-or-none model (31,32). Complete melting temperature curves could not be obtained for the (6-4) and Dewar product-containing dodecamers at 250 mM salt, and as a result, the thermodynamic parameters could not be reliably determined under these conditions. For purposes of comparison and discussion, ΔG° for duplex formation at 37°C was calculated from the ΔH° and ΔS° data, though it is understood that free energy differences calculated at temperatures far removed from the melting temperatures may not be very accurate (24). The (6-4) and Dewar products were found to destabilize the duplex form by ~6 kcal/mol of free energy at 37°C relative to the parent duplex in 1 M NaCl. This is much greater than the calculated destabilization of 1.5 kcal/mol caused by a *cis-syn* dimer, which is similar to that of 1.7 and 1.2 kcal/mol previously calculated for dimer-containing octamer (33) and decamer (34) duplexes, respectively. The duplex with G opposite the 3′-T of the (6-4) product is more stable than A by ~0.5 kcal/mol, whereas the Dewar valence isomer shows a lower preference for G over A (0.3 kcal/mol). The observed preference for G over A opposite the 3′-T of the (6-4) and Dewar isomers is

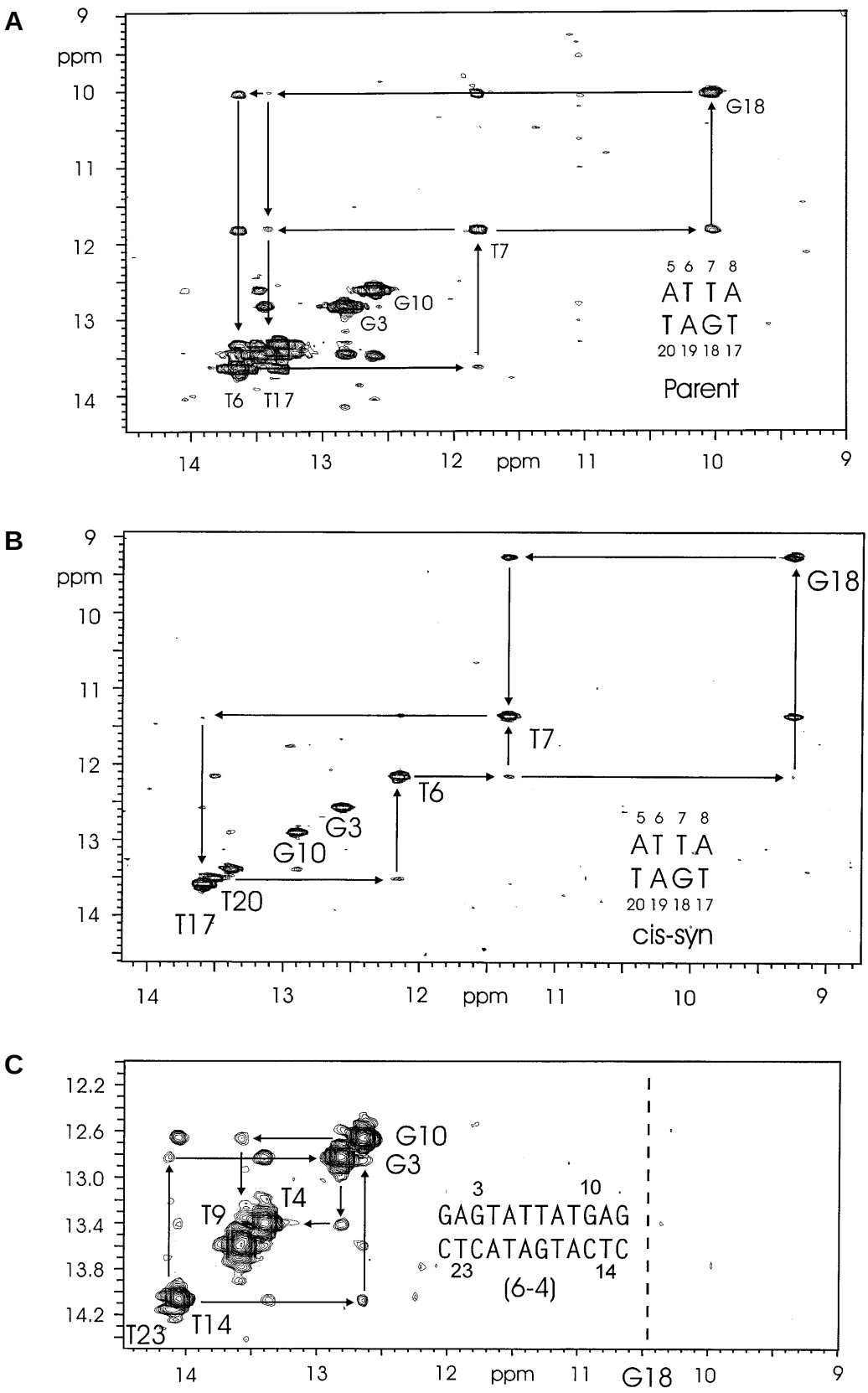

Figure 4. Section of the 600 MHz NOESY spectra in 90% H_2O/D_2O at 1°C, showing the correlations between the imino protons in the mismatched (**A**) parent and (**B**) *cis-syn* duplexes in 100 mM NaCl and (**C**) the mismatched (6-4) duplex in 250 mM NaCl. A 1–1 jump return water suppression method and a 250 ms buildup time was used for all the spectra. No correlations with the imino proton signal of G18 (dashed line) were observed in the other half of the NOESY spectrum of (C) or when WATERGATE suppression was used.

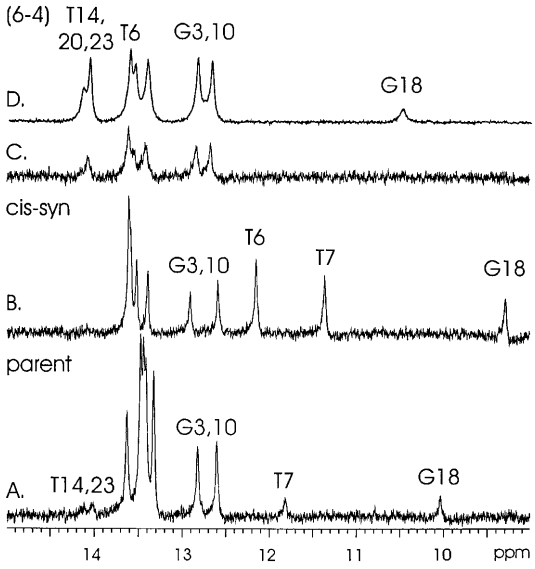

Figure 5. 600 MHz spectra of the imino protons in 90% H_2O/D_2O at 1°C for the mismatched (**A**) parent and (**B**) *cis-syn* duplexes in 100 mM NaCl, and (**C**) the mismatched (6-4) duplex in 250 mM NaCl utilizing pre-irradiation to suppress the water peak. In (**D**) the WATERGATE suppression method was used at 500 MHz.

consistent with values of 0.6 and 0.3 kcal/mol, respectively, for primers terminating in these nucleotides opposite the 3′-T of the (6-4) and Dewar product-containing templates (9). In contrast, changing the A opposite the 3′-T of the photoproduct site to a G in the parent duplex, destabilizes the duplex form by 2.0 kcal/mol at 37°C, which is comparable to 1.8 kcal/mol previously reported for the same change in the same local sequence context (35). Surprisingly, changing the A to a G opposite the 3′-T of the *cis-syn* dimer destabilizes the duplex by only 0.7 kcal/mol.

The 6 kcal/mol destabilization of duplex formation caused by the (6-4) product is greater than that of 4.4 kcal/mol caused by replacing the T in an A•T base pair with an abasic site analog (as calculated in the way described herein from data in 36). Calculations based on thermodynamic parameters for predicting nucleic acid duplex stability would further suggest that the central 4 nt of the duplex behave similarly to an interior loop structure. Though parameters for the free energy of formation of a 4 nt interior loop structure in DNA are not available, it is estimated to be about +1.7 kcal/mol for RNA at 298°K (37). Combining this value with recently optimized nearest neighbor parameters for DNA duplex stability (38) leads to a predicted free energy of formation of –5.9 kcal/mol for the dodecamer containing a 4 nt interior loop at the site of the (6-4) and Dewar products (i.e. the two Ts of the photoproduct and the AA or GA in the opposite strand). This calculated value is only slightly more negative than the experimental values of –4.4 to –5.0 kcal/mol (Table 2). The additional loss of stability may be due to the fact that unlike undamaged nucleotides in an interior loop structure, the (6-4) and Dewar products may not able to stack as well with the flanking bases.

Base pairing of *cis-syn* thymine dimers

Previous studies of the base pairing of the *cis-syn* thymine dimer opposite AA in octamer and decamer duplexes have concluded that base pairs are formed between both Ts of the photodimer, though base pairing may be weaker with the 5′-T based on the greater upfield shift of the imino proton signal (33,39). An NOE observed between the imino proton of T6 and the H2 of A19 in the mismatched dimer duplex suggests that the 5′-T is also involved in Watson–Crick base pairing with the A (Fig. 6A). Base pairing between the 3′-T of the *cis-syn* thymine dimer in the mismatched dodecamer duplex appears to be quite similar to that observed for the parent mismatched duplex based on similar sets of NOEs (Fig. 4A and B). The NOEs observed in the mismatched parent are in turn quite similar to those previously reported for the self complementary dodecamer duplex of d(CGCGAGCTTG-CG), for which it was concluded that the two G•T mismatches were engaged in wobble base pairing (Fig. 6C) (40). As in the previous study of a G•T mismatch, NOEs were observed between imino proton of G18 and the imino protons of both T6, T7 and T17 in the mismatched parent dodecamer (Fig. 4A). In addition, the imino proton signals of both G18 and T7 were shifted upfield. Likewise, in the mismatched *cis-syn* duplex, NOEs between the imino proton of G18 and the imino protons of both T6 and T7 were observed (Fig. 4B), suggesting that G18 was also engaged in wobble base pairing with T7 (Fig. 6D). The imino signals of T7 and G18 were also upfield shifted, though to a greater extent than observed in the mismatched parent duplex. In the mismatched parent duplex, the imino signals of T7 and G18 are not as strong as those of G3 and G10 at 1°C when the water peak was pre-saturated (Fig. 5A). With WATERGATE water suppression, the T7 and G18 signals persist up to 20 and 25°C, respectively, whereas the G3 and G10 signals and the other internal T signals persist up to 35°C. These results might suggest that wobble base pairing is not as strong as normal Watson–Crick base pairs. In contrast, the imino proton signals of both T7 and G18 were as strong as those of G3 and G10 at 1°C when the water peak was pre-saturated (Fig. 5B), and all were observable along with the other internal T signals up to 30°C when the WATERGATE suppression method was used. These results would suggest that wobble base pairing with the 3′-T of the dimer is possibly as strong as a Watson–Crick base pair and is consistent with the smaller free energy difference (0.7 kcal/mol) for exchanging G for A than observed for the parent duplex (2.0 kcal/mol) (Table 2). The persistence of the imino signals of T7 and G18 in the mismatched dimer duplex at higher temperatures than observed for this signal in the mismatched parent duplex might also be explained in part by the higher pK_a of the imino proton of a thymine dimer (41,42).

Base pairing with (6-4) products

In previous studies of the (6-4) and Dewar products opposite AA, in which only base pairing with the 5′-T could be monitored, a weak NOE was detected between the imino proton of the 5′-T and the opposed A in the (6-4) duplex, but not in the Dewar duplex (39,43). The observed NOE is indicative of Watson–Crick base pairing (Fig. 6B) and was also observed in the mismatched (6-4) dodecamer duplex. Because of the structural rearrangement of the two thymines leading to the (6-4) and Dewar products, the imino proton of T7 in the parent is no longer present in the (6-4) and Dewar products, and is therefore unavailable for monitoring H-bonding interactions with the A by NOE. In the mismatched dodecamer duplex, however, the NMR properties of the imino proton of G18 could be used to monitor H-bonding to the 3′-T of

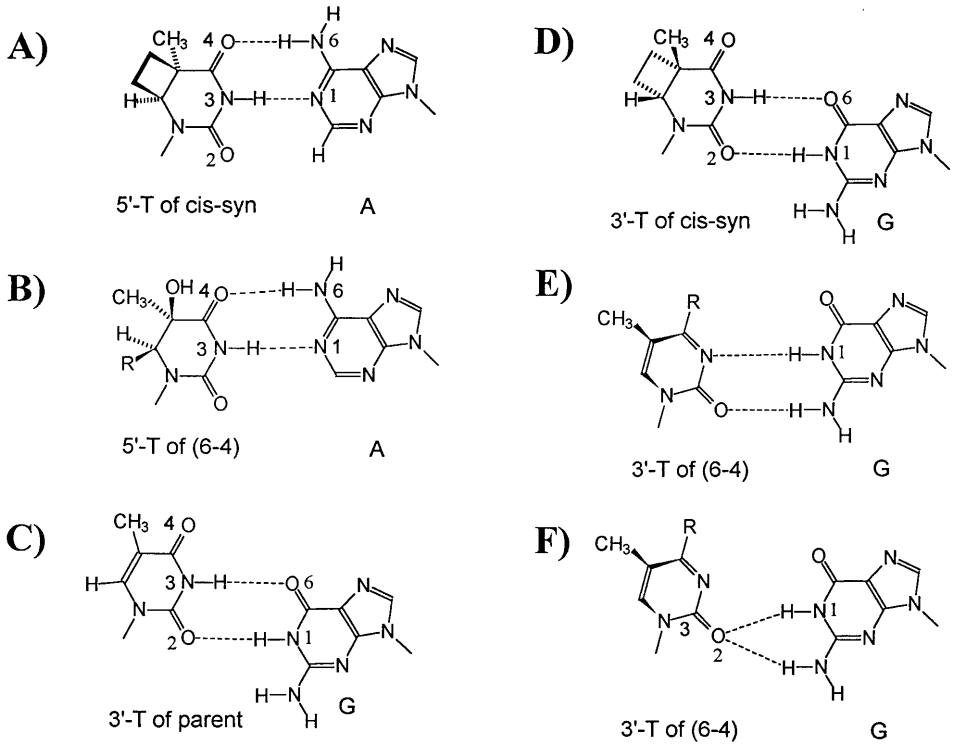

Figure 6. Possible H-bonding interactions in the mismatched *cis-syn* and (6-4) product-containing duplexes. (**A** and **B**) Watson–Crick base pairing of the 5′-T and (**C** and **D**) wobble base pairing of the 3′-T. The base pairing shown in (**E**) (8,44) and (**F**) (7) were previously proposed to account for the origin of C→T mutation induced by (6-4) products. The base pairing in (E) was also proposed to account for the greater stability of template-primers terminating in G opposite (6-4) products (9).

the (6-4) product. Whereas correlations between imino protons could be detected and assigned to G3 and G10, no correlations between imino protons could be assigned to G18 under conditions that worked for the parent and *cis-syn* mismatched duplexes. To increase the stability of the (6-4) duplex, the salt concentration was raised from 100 to 250 mM NaCl, and NOESY spectra were acquired with both 100 and 250 ms mixing times. Under these conditions, the imino protons of G3 and G10 could be readily assigned (Fig. 4C) and confirmed by correlations to the base-paired CH5 protons that were assigned from the non-exchangeable spectra (Fig. 3). No correlations could be observed for the G18 imino proton, however, even when the WATERGATE water suppression method was used.

In sharp contrast to the behavior of imino proton signal of G18 in the mismatched parent and dimer duplexes, the imino proton signal in the (6-4) duplex could only be detected when the WATERGATE suppression was used and could not be detected at 1°C when the water signal was pre-saturated (Fig. 5C and D). With WATERGATE suppression, the G18 imino signal could be observed up to 10°C, whereas the G3 and G10 signals persisted up to 25°C. The rapid exchange of this proton with solvent is highly indicative of little or no H-bonding interactions between G18 and the pyrimidone ring of the (6-4) product of the types that have previously been proposed to account for the origin of T→C mutations (Fig. 6E) (8,44) and (Fig. 6F) (7), or for the thermal stability of primers terminating in G opposite the 3′-T of the (6-4) product (9). Likewise, the upfield shifted value of 10.5 p.p.m. observed for the imino proton of G18 is almost identical to that in duplexes containing G•A mismatches, for which it has been concluded that the imino proton of G is not involved in base-pairing and is instead exposed to water (45,46). Shift alone does not appear to be good indicator of H-bonding, however, as shifts of 10.0 and 9.3 were observed for the imino proton of G18 in the mismatched parent and *cis-syn* duplexes. Values of 10.3–10.4 p.p.m. have also been reported for the imino proton of G involved in base pairing with G or an N6-benzopyrene adduct of A (47,48).

Given that the NMR experiments indicate that there is little or no H-bonding between the imino proton of G and the 3′-T of the (6-4) product, the greater stability of G over A at this site is more likely to be due to some other type of interaction. Stacking of dangling bases which have no base pairing partners have been shown to increase the thermodynamic stability of DNA and RNA duplexes (36,37,49). Though the internucleotide crosspeak between the T17 and G18 could not be resolved due to signal overlap, sequential NOEs were detected between the H1′ and H6/8 protons in the G18-A19-T20-A21-C22-T23 section of DNA (Fig. 3), suggesting that the A and G opposite the (6-4) product are stacked upon each other and within the helix to a large degree. Thus, the increased stability of the G opposite the 3′-T of the (6-4) product may be due to better pi-stacking of a G than an A with the flanking bases.

Structural implications

It now appears from a number of previous physical studies that *cis-syn* dimer formation has only a modest effect on DNA duplex structure. It has been found that *cis-syn* dimers only unwind DNA

by ~15° (50) and bend DNA by 7° (51,52). An NOE-constrained molecular dynamics study of a *cis-syn* thymine dimer opposite AA in a duplex decamer also concluded that the DNA structure is not greatly distorted and that base-pairing is generally maintained (39). It was found, however, that the structure in the immediate vicinity of the dimer is somewhat disrupted and base pairing with the 5′-T is distorted. A similar conclusion was also reached by unrestrained molecular dynamics calculations (53). The relatively small structural changes induced by dimer formation would explain the relatively small drop in duplex stability of 1–2 kcal/mol that has been observed for three different dimer-containing duplexes (33,34). When the A opposite the 3′-T of the dimer is replaced by G, the stability of the duplex decreases, but not as much as seen for the parent duplex. This, together with the exchangeable proton data, suggests that the wobble base pair with the 3′-T of the dimer is stronger than in the parent, but that both occur at the expense of unfavorable changes in pi-stacking and conformation.

A structure for the (6-4) product of TT opposite AA in d(CGCATTACGC)•d(GCGTAATGCG) was proposed on the basis of NOE-constrained molecular dynamics calculations in which pi-stacking between the 3′-T and the flanking A is lost, and the 5′-T of the (6-4) product base pairs with the opposed A, but the 3′-T does not (39). Similar conclusions were reached by unrestrained molecular dynamics calculations on the same duplex though a weak H-bond was detected between the carbonyl of the 3′-T and the amino group of the A (53). The precipitous drop in thermal stability in going from the matched or mismatched parental duplexes to the (6-4) or Dewar product-containing duplexes is consistent with an interior loop structure in which base pairing and pi-stacking at the site of the photoproducts are greatly diminished.

An interior loop structure in which there is diminished H-bonding and pi-stacking would also account for the rapid exchange of the imino proton of G18 opposite the (6-4) product in the dodecamer duplex. It is also consistent with experimental data that suggests that a (6-4) product unwinds DNA six and a half times more than does the *cis-syn* dimer in supercoiled DNA (54), or ~2.5 bp based on an experimentally determined unwinding angle of 15° for a *cis-syn* dimer (50,52). A structure with disrupted pi-stacking would also explain the observation that the hypochromicity of the (6-4) and Dewar product-containing duplexes in 1 M salt appears to be less that of the corresponding *cis-syn* dimer-containing duplexes (data not shown). Additionally, when the salt concentration was lowered from 1 M to 250 mM, the hypochromicity of the (6-4) and Dewar product-containing samples is less than half that observed at 1 M. Because these curves appear to begin to bottom out at the low temperature end, they are more suggestive of the presence of only half a duplex, i.e. the duplex to either side of the photoproduct, rather than the presence of equal amounts of full duplex and single stranded forms. The failure to propagate a duplex at low salt would be consistent with substantial disruption of the helix at the site of the (6-4) and Dewar products.

An unusual set of NOEs between T6CH3 and the A5H1′, H2″, H3′, H4′, H2 and H8 in the (6-4) mismatch duplex at 150 and 250 ms (Fig. 3) buildup times is similar to that reported for a native (6-4) decamer duplex, in which NOEs were reported between the 5′-TCH3 and all sugar protons of the 5′-flanking A (39). It is hard to envision one structure that could account for all these NOEs simultaneously, though simple model building suggests that they could be accounted for by equilibrating (flipping) the (6-4) photoproduct between inside and outside conformations. Photoproduct flipping would also provide a mechanism for the rapid exchangeability of the G18 imino proton.

Implications for photoproduct recognition and repair

(6-4) and Dewar products are repaired about nine times faster than *cis-syn* dimers by the uvr(A)BC excinuclease system (15). The differences in uvrABC excision rates correlate with differences in binding affinity of the uvrA DNA damage recognition subunit which binds the (6-4), Dewar and *cis-syn* dimer-containing duplex 49mers with K_as of 2.4×10^9, 1×10^9 and 2.6×10^8 M^{-1} respectively (16). A human DNA damage recognition protein, XPE-DDB, behaves similarly with K_as of 1.6×10^{10}, 4.7×10^9 and 1.7×10^9 M^{-1}, respectively (16). The rate at which RecA protein binds to DNA has also been found to be faster for (6-4) products than for *cis-syn* dimers in supercoiled DNA, which was correlated with their greater degree of unwinding (54). In this regard, it has been found that binding of uvrA and uvrB to damaged DNA is coupled with unwinding of the DNA duplex (55). Thus, the greater degree of unwinding coupled with the lower thermal stability of the (6-4) and Dewar duplexes relative to *cis-syn* dimers may explain in part why the former products are more rapidly repaired by uvr(A)BC. The data also suggest that *cis-syn* dimers are not as readily recognized and repaired by excision repair systems as the (6-4) and Dewar photoproducts because they do not substantially disrupt the structure and base pairing properties of the DNA duplex.

In an early study with human cell free extracts, (6-4) products were found to be repaired at least 10 times faster by the human excision repair system (14), though in a more recent study with both cell free extracts and purified human excinuclease, the rate difference appears to be only three times greater (17). When compound lesions resulting from the replacement of the A opposite the 3′-T of the photoproducts with G were examined, *cis-syn* dimer repair was stimulated 4-fold, whereas (6-4) product repair was not. Thus, it would appear that the absolute rates of excision repair are not directly correlated with the thermodynamic stabilities of the duplexes, as a *cis-syn* dimer with a G opposite the 3′-T is ~3 kcal/mol more stable than a (6-4) product and yet it is repaired at about the same rate. It may be, however, that the rate limiting step in excision repair initially involves a step that is related to the thermodynamic stability of the duplex up to a certain point, but then switches to an unrelated step. This would explain why further increasing the number of mismatches opposite or adjacent to a *cis-syn* dimer did not further increase the rate of repair, and that the maximal rate was similar to that for a (6-4) product or a mismatched *cis*-platin adduct.

The relatively non-perturbing nature of *cis-syn* dimers in a native sequence context would explain why some organisms have evolved *cis-syn* dimer-specific repair enzymes typified by T4 *denV* endonuclease V (56) and *E.coli* photolyase (57). A crystal structure shows that T4 endonuclease binds to a thymine dimer-containing duplex by destacking the base pair flanking the 5′-side of the dimer and flipping out the base opposite the 5′-T of the dimer (58). This binding mode is consistent with the lower stability of dimer-containing duplexes, and the distorted nature of the 5′-T of the *cis-syn* dimer which disrupts the pi-stacking to the 5′-side of the dimer and base pairing with the opposed A (39,53).

Such a disruption should also facilitate flipping of the dimer itself, as has been proposed for repair by *E.coli cis-syn* photolyase (18). The high degree of homology between the *cis-syn* and (6-4) photolyases (59,60) and recent experiments suggest that the *cis-syn* and (6-4) photolyases may be binding in a similar manner (19). In support of a photoproduct flipping mechanism it was found that the (6-4) photolyase binds single strand and double T mismatch duplex substrates better than a matched double stranded substrate (19). Another line of evidence that a (6-4) product can adopt an extrahelical conformation is the finding that polyclonal antibodies elicited against a dinucleotide (6-4) product antigen bind (6-4) products in both single and double stranded DNA equally well (X.Zhao and J.S.Taylor, unpublished results). The low thermodynamic stability of (6-4) duplexes, together with the rapid exchange of the imino proton of an opposed G, and the unusual set of NOEs between the methyl of the 5′-T and flanking A, also support the notion that (6-4) products are conformationally flexible and able to flip out of the helix.

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