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NMR evidence of the stabilisation by the carcinogen *N*-2-acetylaminofluorene of a frameshift mutagenesis intermediate

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ABSTRACT

Two heteroduplexes d($C_1A_2C_3T_4C_5G_6C_7A_8C_9A_{10}C_{11}$)–d($G_{12}T_{13}G_{14}T_{15}G_{16}G_{17}A_{18}G_{19}T_{20}G_{21}$) containing a bulged guanine either unmodified or modified with the carcinogen *N*-2-acetylaminofluorene (AAF) have been studied by nuclear magnetic resonance (NMR) as models of slipped mutagenic intermediates (SMI). Conformational equilibria are observed in both the unmodified and the AAF-modified heteroduplexes. The major conformation of the unmodified duplex is one where the extra guanine is stacked in the helix and the major conformation of the AAF-modified heteroduplex is one where the AAF is external to the helix. Unusual sugar proton chemical shifts of C5– and G6–AAF indicate that the AAF ring is pointing out in the 5′ direction. A strong increase in the modified heteroduplex melting temperature (+15°C) is observed. Moreover, in contrast to the unmodified heteroduplex, which shows extensive melting in the vicinity of the bulged guanine, the base pairs around the bulge in the AAF-modified heteroduplex remain paired at temperatures up to 30°C. This exceptional stability of the site around the bulged modified guanine is suggested to be responsible for the high rate of –1 mutation induced by AAF at repetitive sequences.

INTRODUCTION

Spontaneous frameshift mutation hotspots are found to occur within sequences containing short repetition of mono-, di- and trinucleotides. The frameshift mutations occurring at these sites usually involve the loss or the gain of a single repeated unit. Streisinger and co-workers have suggested that these events result from strand slippage within such sequences during DNA replication producing intermediate structures containing bulges (1). Chemical carcinogens such as AAF were found to increase the frequency of –1 frameshift events within short runs of guanines (2) by three to four orders of magnitude over the spontaneous frequency (3). Direct experimental evidence suggests that these mutations occur in a two-step process; incorporation of a cytosine residue across from the guanine-C(8)–AAF adduct followed by a misalignment step during which this cytosine residue slips onto the guanine residue located immediately after the adduct on the template strand (3). It has recently been shown (4) that the covalent binding of an AAF residue to a bulged guanine increases the melting temperature of the heteroduplex by 6–10°C depending on the position of the modified guanine in the run (4). Moreover, in the unmodified bulged oligonucleotide, the bases within the run are found to be highly reactive toward chemical probes, suggesting the occurrence of a transient single-stranded state delocalized throughout the length of the run (4). In contrast, when the bulged guanine residue carries an AAF adduct, only minor reactivity towards chemical probes was observed, indicating that the AAF adduct induces a structural organisation of the bulge (4). In order to investigate the structural and dynamic properties of such slipped mutagenic intermediates (SMIs), we studied two 11mer::10mer heteroduplexes by NMR spectroscopy. Each contained a bulged guanine; one was unmodified, the other was modified with AAF (Figure 1).

MATERIALS AND METHODS

Sample preparation

Oligonucleotides were synthesised on an automated Applied Biosystems 380B instrument using standard phosphoramidite chemistry and were purified twice by reverse-phase HPLC on a C18 column. The synthesis, purification and characterisation of the modified oligonucleotides have been previously described (5). Equimolarity of the two strands was determined by changes in the hypochromicity at 260 nm when adding aliquots of the complementary strand to a single-stranded solution. NMR samples were prepared by dissolving about 100 units of 260 nm absorbance of the heteroduplex in 500 µl phosphate buffer, pH 7, 90% H_2O/10% D_2O or 99.996% D_2O. DSS was added as an internal standard.

Melting temperature determination

A sample of heteroduplex, prepared in 100 mM phosphate buffer, pH 7, has a measured absorbance of 0.9 at 260 nm and 20°C.

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The melting of the duplex was followed by monitoring the optical density of the solution at 260 nm as a function of temperature from 5 to 90°C.

NMR experiments

NMR experiments were performed on a Bruker AMX 500, 500 MHz spectrometer.

NOESY, HOHAHA and ROESY spectra in D_2O were recorded using the TPPI method (6) at temperatures and mixing times specified in the legends. A total of 2048 data points were collected in the *t2* dimension, 512 *t1* values were collected for each data set and 32 transients were collected for each *t1* value. The spectral width was set to 5 kHz. The relaxation delay was set to 3 s. Residual signal of HDO was removed by low power continuous selective irradiation during the preparation period.

Spectra in H_2O were recorded by using a 'Jump−Return' excitation pulse sequence (7) over a 10 kHz spectral width. The excitation maximum of the 'Jump−Return' sequence was set on the imino protons region (60 ms delay between the two 'Jump−Return' pulses).

2D processing was achieved on a X32 computer using UXNMR software (Bruker) or on an IBM RS6000 workstation using the programme FELIX 2.01 (Hare Research Inc.). The final size of the matrices was 2K*2K real points. Prior to Fourier transform, the signal was multiplied in both dimensions by a 80° shifted square sine−bell function. In dimension 1, the signal was zero-filled in order to get 2K real points in the final 2D map.

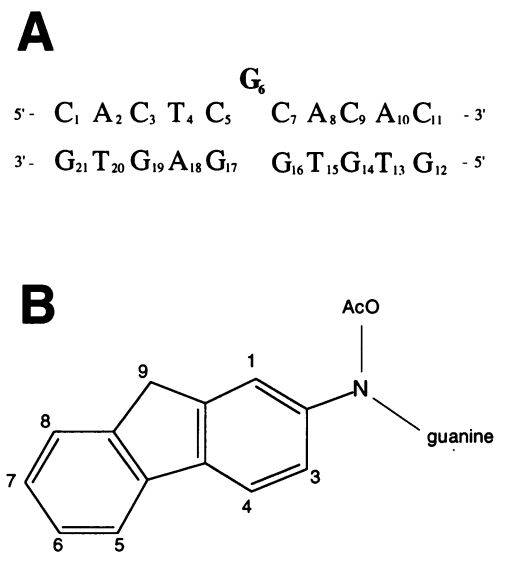

Figure 1. (**A**) Sequence of the heteroduplex DNA proposed to mimic a slipped mutagenic intermediate. The bold guanine 6 is the modified guanine in the modified heteroduplex. (**B**) Chemical structure of *N*-2-acetylaminofluorene (AAF).

RESULTS AND DISCUSSION

Melting temperatures: stabilization of the modified heteroduplex

The melting temperatures of the unmodified and modified heteroduplexes were found to be 31 ± 1 and 46 ± 1°C respectively. The strong increase in melting temperature (+15°C) of the AAF-modified heteroduplex is in agreement with the stabilization previously observed in a different sequence context (4). In the previous work, the G−AAF adduct was located in a run of guanines (GGG) while in the present work, the adduct is located at the G residue within the local sequence CGC. This latter sequence was chosen for sake of simplicity of preparation and purification of the AAF-modified oligonucleotide.

Unmodified heteroduplex

Structural features: an equilibrium between stacked in and looped out guanine conformations. The assignment of the unmodified oligonucleotide proton resonances, based on HOHAHA and NOESY spectra, is straightforward for temperatures ranging from

Table 1. Chemical shift assignments in p.p.m. of the non-exchangeable and exchangeable protons of the unmodified heteroduplex in the G-in and G-out (in brackets) conformations at 10°C

	H1′	H2′	H2″	H3′	H2 or H5 or Me	H6 or H8	NH or NH_2 b/f
C1	5.67 (5.59)	1.92	2.41	4.72	5.96	7.71 (7.80)	8.21/7.08
A2	6.28	2.83	2.93	5.06	7.93	8.43 (8.40)	
C3	5.93	2.05	2.56	4.70	5.32 (5.32)	7.44	8.06/6.87
T4	6.04	2.06	2.45	4.84	1.59	7.42	14.08 (14.01)
C5	5.86 (6.02)	2.01	2.19	4.83	5.67 (5.50)	7.48 (7.62)	8.54/7.11
G6	5.90 (6.14)	2.53	2.63	4.06		7.85 (8.05)	N.D.
C7	5.19 (5.47)	2.15	2.33	4.76	5.56	7.59 (7.25)	8.16/6.68
A8	6.21	2.74	2.87	5.03	7.80	8.30 (8.28)	
C9	5.50 (5.69)	2.03	2.35	4.81	5.37 (5.30)	7.36 (7.23)	8.30/6.70
A10	6.25 (6.14)	2.67	2.88	4.25	7.78	8.24 (8.04)	
C11	6.05	2.10	2.25	4.47	5.30 (5.61)	7.34 (7.57)	8.19/6.84
G12	6.01 (6.14)	2.68	2.83	4.83		7.97 (8.07)	N.D.
T13	5.92	2.27	2.55	4.93	1.35	7.42	13.76 (13.62)
G14	6.01 (6.14)	2.61	2.78	4.38		7.93	12.62 (12.86)
T15	5.74 (5.90)	1.83	2.23	4.83	1.40	7.15	13.83 (13.76)
G16	5.76 (5.90)	2.41	2.54	4.03		7.78 (7.36)	12.81
G17	5.44 (5.60)	2.67	2.76	4.33		7.91	12.78
A18	6.08 (5.98)	2.76	2.88	5.06	7.65	8.20 (8.18)	
G19	5.84	2.45	2.60	5.07		7.63 (7.72)	12.84 (12.77)
T20	5.85 (6.01)	1.87	2.35	4.80	1.36	7.12 (7.19)	13.90 (13.95)
G21	6.18	2.36	2.63	4.69		7.92	N.D.

NH are imino protons of thymines or guanines. NH_2 b/f are the bound and free amino protons of cytosines. N.D.: not determined.

5 to 25°C (Figure 2, Table 1). All sequential connectivities characteristic of right-handed B-form DNA are observed (8). The sequential NOE's are weaker at the C5−G6, G6−C7 and G16−G17 steps, due to the presence of the extra guanine. Observation of C5−G6 and G6−C7 connectivities indicate that the extra guanine is stacked in the helix and induces only slight deformations, in agreement with previous observations made on a bulged guanine in another sequence context (9). In the present paper, this conformation will be referred to as the 'stacked in guanine' conformation (G-in). In the proton NMR spectra, a second set of smaller peaks in slow exchange with the G-in set of resonances is observed for nearly all bases. These resonances were assigned through the off-diagonal exchange peaks observed in NOESY and ROESY spectra (Figure 2). Both the chemical shifts of H8 and H1′ of G6 are down-field shifted by 0.2 p.p.m. as compared to those observed for G-in conformation. This suggests that G6 is looped out from the helix, experiencing a smaller ring current shift effect from its neighbours. The second conformation, hereafter referred to as G-out, is present at the level of 30% at 5°C and decreases to 15% at 20°C. Given the small concentration of the G-out conformation, no proper NOE can be observed which would allow further characterisation of its structure.

The displacement of the equilibrium towards the G-in conformation when the temperature increases is in agreement with previously observed conformational transitions between stacked and looped out extra bases. In sequences containing an extra cytosine (10) or an extra thymine (11), flanked by guanines, the looped out state is favoured at low temperature (0°C), whereas the equilibrium shifts in favour of the stacked state at elevated temperatures (35°C). In these sequences, conformational changes induced by the equilibrium are localised within a few bases around the extra base. In contrast, we have observed that the exchange between the G-in conformation and G-out conformation affects the entire oligonucleotide. Nearly all H1′ protons from C1 to G21 exhibit two resonances. This difference may be related to differences in sequence; in the sequences studied by Kalnik *et al.* (10) (11), the extra residues are pyrimidines, whereas, in the present work, the extra residue is the more bulky purine base.

The local conformational change produced by the in and out states of the extra guanine is coupled to remote conformational changes in the oligonucleotide, as revealed by the multiples resonances of protons all along the sequence. This behaviour can be related to the so-called teleaction observed in other studies. It has been shown that an AT-rich sequence can cause structural distortion of Z-DNA in plasmids up to 10 bp from the AT tract (12). An oligopurine track situated in the neighbourhood of a pseudo *Eco*RI site alters the DNA conformation and enables nicking of the restriction site by *Eco*RI (13).

The exchangeable imino and amino protons have been assigned using their dipolar connectivities in a 2D NOESY spectrum (Table 1). At 5°C, as observed for non-exchangeable protons, the 1D spectrum of imino protons of the unmodified duplex exhibits two distinct sets of peaks for most of the imino protons (Figure 3A), confirming the presence of two conformations for the whole oligonucleotide. The peaks characterising the G-in conformation are sharp, except for the two terminal guanines G12 and G21 which are more accessible to solvent than the others. All the chemical shifts are in the range of a right-handed DNA, indicating that only slight distortions are induced by the extra guanine. The imino proton resonance of G6 could not be identified because of extensive overlap. The peak at 13 p.p.m. might be one of the G12, G6 or G21 imino protons resonance.

In the G-out conformation, very broad and up-field shifted imino proton resonances, indicating fast exchange with solvent

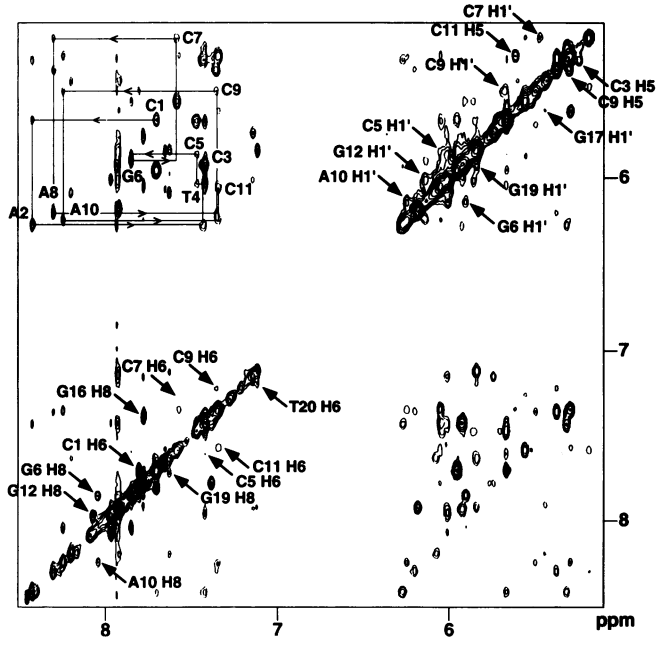

Figure 2. NOESY spectrum of the unmodified heteroduplex recorded at 30°C. The sequential assignment of the upper strand is depicted. The labelled cross peaks indicate the intraresidual H1′−H6/8 connectivity. Arrows labelled with the name of the proton indicate exchange peaks observed in a ROESY spectrum.

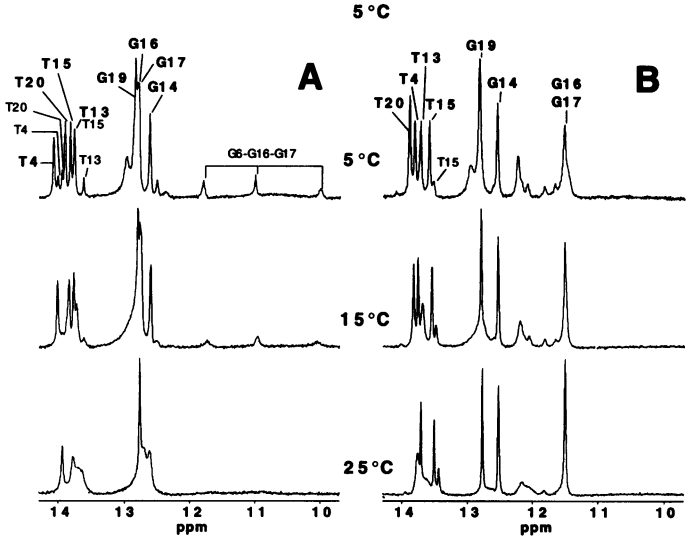

Figure 3. Imino protons 1D spectra as a function of the temperature of the unmodified duplex (A) and of the AAF-modified duplex (B). (A) The large type labels represent the G-in conformation, whereas the small type labels correspond to the G-out conformation. (B) The large type labels represent the AAF-out conformation, whereas the small type label corresponds to the AAF-in conformation.

and short pairing lifetimes, were assigned by deduction to guanines G6, G16 and G17. This observation indicates that the extra guanine, when looped out of the helix, induces a deformation such that the C5−G17 and C7−G16 base pairs are either broken or have a very short lifetime. Other signals of the G-out conformation are either up-field or down-field shifted compared to the G-in conformation, indicating structural rearrangement of the double helix along the sequence.

Observation of base pairing as a function of temperature. Various degrees of base pairing strength may be observed through the resonances of exchangeable amino and imino protons. When the base pair forms, the exchange rate of imino and amino protons decreases and their resonances can be observed. As the lifetime of the base pair increases, these resonances get sharper and NOE between protons belonging to the two bases of the pair develop, for example, between the imino proton of thymine and the H2 proton of adenine, and between the imino proton of guanine and amino protons of cytosine. For very stable base pairs, an NOE between the imino proton of guanine and the H5 proton of cytosine, relayed by the amino protons of cytosine, may be observed.

When increasing the temperature, many peaks of the unmodified 11mer::10mer heteroduplex in the G-in conformation become very broad (Figure 3A). At 25°C, only C3−G19 and T4−A18 remain as fully intact pairs; the base pairings around the bulge (C5−G17 and C7−G16) are progressively disrupted. At 25°C only the cytosine amino proton resonances are observed, however, without any NOE to the guanine imino protons. High temperatures increase the fraying at the ends of the duplex but also the 'breathing' of the molecule around the bulge. The base pairing pattern and its evolution with temperature is sketched in Figure 4A, using the scale of base pair strength depicted above.

Table 2. Chemical shifts assignments in p.p.m. of the AAF protons in the AAF-out and AAF-in conformation at 30°C

	AAF-out	AAF-in
H1	7.39	6.96
H3	7.33	6.62
H4	7.17	5.60
H5	7.54	6.20
H6	7.86	5.14
H7	7.48	6.63
H8	7.60	5.61

AAF-modified heteroduplex

AAF position: another equilibrium between AAF-out and AAF-in conformations. The presence of two sets of AAF proton resonances about 1 p.p.m. apart in the aromatic region of the NMR spectrum indicates conformational exchange between two species of the AAF-modified heteroduplex (Table 2). The low field AAF spin system (between 7 and 8 p.p.m.) was easily assigned to the AAF moiety lying outside the helix (AAF-out conformation) because of similarity of its chemical shifts range with an AAF-modified guanine monomer (14) and absence of NOE with DNA protons. The high field AAF spin system (between 5 and 7 p.p.m.) is characteristic of AAF inserted in the helix (AAF-in), as previously observed with other AAF-modified oligonucleotides (15). The relative population of AAF-out is about 70% and does not change with temperature, indicating that the enthalpy change of the in−out exchange of AAF is small. The AAF proton resonances of the AAF-in conformation are strongly dependent on temperature (data not shown). At low temperature (5°C), a set of very broad peaks is observed for each AAF proton. When the temperature is increased to 30°C, each set of peaks merges into a single narrower peak, indicating that AAF does not adopt a unique AAF-in conformation. At low temperature, the rate of exchange between the various AAF-in conformations is in the intermediate range on the NMR time scale, whereas at elevated temperature, the chemical shifts are averaged and the equilibrium is in the fast exchange range (16).

Table 3. Chemical shift assignments in p.p.m. of the non-exchangeable and exchangeable protons of the AAF-modified heteroduplex in the AAF-out and AAF-in (in brackets) conformation at 30°C

	H1′	H2′	H2″	H3′	H5 or Me	H8 or H6	NH or NH_2 b/f
C1	5.70	1.85	2.38	4.71	5.96	7.68	8.20/7.08
A2	6.24	2.80	2.90	5.04		8.38	
C3	5.82 (5.75)	1.95	2.45	4.60	5.30	7.35	8.04/6.83
T4	5.99	1.81	2.20	5.02	1.46	7.26	13.82
C5	5.58 (5.74)	0.59	1.96	4.94	5.30	7.27	8.04/7.04
G6	6.21 (5.88)	2.54	3.93	N.D.			N.D.
C7	5.78 (5,74)	1.99	2.43	4.78	5.58	7.79	7.68/5.89
A8	6.12	2.63	2.82	4.96		8.19 (8.26)	
C9	5.50	1.99	2.32	4.79	5.30	7.28	8.29/6.72
A10	6.23	2.64	2.82	5.01		8.21 (8.26)	
C11	6.08	2.11	2.11	4.47	5.39	7.37	8.16/6.82
G12	6.01	2.65	2.79	4.82		7.95	N.D.
T13	5.94	2.26	2.57	4.93	1.38	7.39	13.69
G14	6.00	2.62	2.78	4.97		7.88	12.54
T15	5.87 (5.69)	2.09	2.52	4.91	1.47	7.19 (7.18)	13.55
G16	5.91	2.31	2.47	5.07		7.79 (7.84)	11.54
G17	5.51	2.75	2.75	5.01		8.03	11.53
A18	6.06 (6.14)	2.71	2.88	5.05		8.11 (8.19)	
G19	5.82 (5.33)	2.40	2.65	4.89		7.55 (7.57)	12.80
T20	5.86	1.85	2.32	4.82	1.35	7.10	13.76
G21	6.16 (5.90)	2.36	2.59	4.67		7.90	N.D.

NH are imino protons of thymines or guanines. NH_2 b/f are the bound and free amino protons of cytosines. N.D.: not determined.

High conformational heterogeneity around the modified base has recently been observed in 2-aminofluorene (AF)-modified homoduplexes (17,18). In both cases, the AF moiety was found in equilibrium between a looped out and a stacked in conformation. It has been suggested that this high heterogeneity may play a significant role in mutagenesis (18).

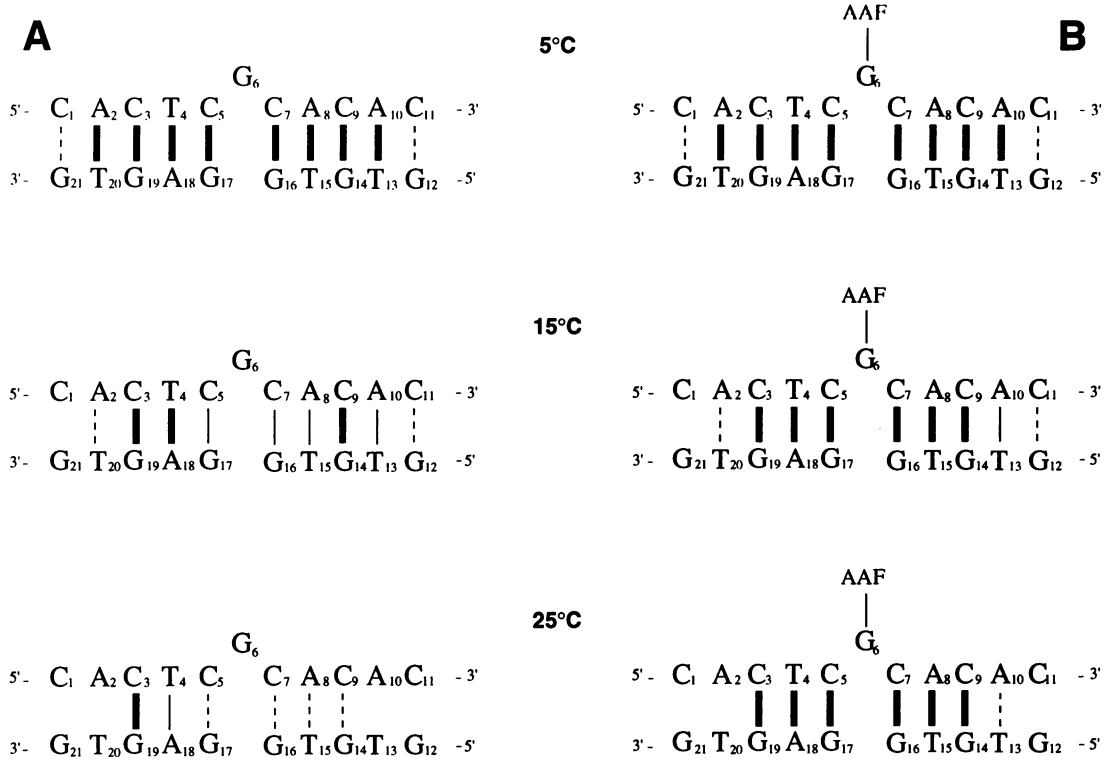

Figure 4. Base pairing as a function of temperature of the unmodified duplex (**A**) and of the AAF-modified duplex (**B**). The sign materialising the base pair is a function of the lifetime of the base pairing state: ■ Indicates that significant NOE are observed between imino protons and amino and H5 of cytosines for G−C pairs or H2 of adenines and H1′ of thymines for A−T pairs (full base pairing). | Means that NOE are observed between imino proton of guanine and amino protons of cytosine in GC pairs or imino proton of thymine and H2 proton of adenine in AT pairs (weaker base pairing). ¦ Means that imino and amino protons are observed without exchanging any NOE (poor base pairing). No symbol at all means that exchange is too fast to observe any exchangeable protons even on a 1D spectrum (no base pairing).

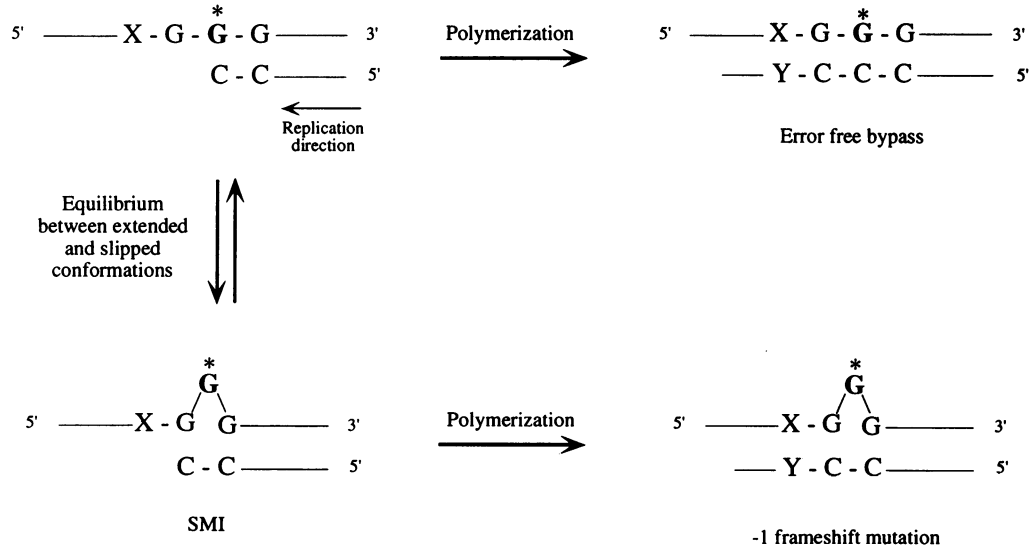

Figure 5. Scheme for the extended and slipped conformations equilibrium during DNA replication: error-free bypass of the AAF adduct or formation of a SMI leading to a −1 frameshift mutation.

Structural features of the AAF-modified heteroduplex. Sequential assignment of the modified heteroduplex in the AAF-out conformation is straightforward for residues 1–4, 7–11, 12–16 and 17–21 and resembles that of the unmodified G-in heteroduplex (Table 3). Due to the lack of H8 proton of G6–AAF and because of conformational equilibrium, the assignment around the adduct is difficult. No sequential H1′–H6/8 connectivity is observed at T4–C5, G6–AAF–C7 and G16–G17 steps. This indicates that the distance or the base stacking have changed compared to the unmodified heteroduplex. On the other hand, strong sequential H1′–H1′ NOE are observed at T4–C5, C5–G6–AAF and G6–AAF–C7 steps. This kind of sequential connectivity is unusual in a B-type DNA as the distance between two adjacent H1′ protons is about 5Å. This suggests that the sugar–phosphate backbone adopts a compact bent structure around the bulged guanine, which brings the sugar moieties close together.

The proton chemical shifts of the modified duplex are significantly different from those observed for the unmodified duplex within the region which includes two base pairs on each side of the modified G; the C5 and G6–AAF sugar protons are the most affected (Tables 1 and 2). These very strong shifts (more than 1 p.p.m.) are probably induced by the ring current effects of AAF; they are consistent with the AAF moiety pointing out in the 5′ direction of G6–AAF, where the chemical shift variations are larger. In the rest of the molecule, the proton chemical shifts are very similar between the AAF-out conformation of the modified duplex and the G-in conformation of the unmodified duplex, suggesting a high similarity of conformation. This is also reflected in the imino proton resonances of G14, G19 and all thymines, which are identical between the two duplexes. The G16 and G17 imino protons are up-field shifted by at least 1 p.p.m. As these peaks are still sharp at 25°C, one can conclude that the up-field shifts are not due to an increase in the exchange with solvent but to a structural feature.

A second set of peaks is observed for protons of residues near the modified guanine: A8H8, G16H8, A18H8, T15H6 and T4NH. Peak intensity measurements confirm that 70% of the molecules are in the AAF-out conformation. The striking feature here is that, in contrast to the unmodified duplex, the conformational equilibrium is restricted to a region of two base pairs on each side of the extra guanine.

Strong base pairing as a stabilising factor. The resonances of the exchangeable amino and imino protons of the modified duplex were assigned following the procedure used for the unmodified heteroduplex (Table 3). When increasing the temperature from 5 to 30°C, many resonances of the exchangeable protons to the AAF-out duplex remain sharp (Figure 3B). Only the terminal resonances broaden (Figure 3B). Even at 30°C, melting around the bulged guanine is not observed. The resonances of the G16 and G17 imino protons are still intense and give significant NOE with the amino protons of the opposite cytosines (data not shown), indicating that, up to 30°C, the bulge flanking base pairs still have a long lifetime (Figure 4B).

The stabilisation of base pairing around the modified bulged guanine is responsible for the higher stability of the modified heteroduplex as compared to the unmodified one. The fact that AAF is outside the helix and that the chemical shifts of the modified guanine are strongly shifted suggests that the whole AAF–G moiety is extruded from the double helix. This may allow a better adjustment of the helical structure through the lesion, perhaps by inducing a bend around the modified bulged guanine. This is not the case with the unmodified heteroduplex, where the guanine is accommodated inside the helix, destabilising its neighbouring base pairs.

CONCLUSION: BIOLOGICAL RELEVANCE

The biological relevance of these results is related to the observation that, in runs of guanines, AAF induces a 10^3- to 10^4-fold increase in the spontaneous frequency of −1 frameshift mutations. The behaviour of a bulged guanine in a CGC context, deduced from the results of the present study, and in a repetitive sequence GGG (4) appear to be very similar. The AAF modification of the bulged guanine in CGC increases the stability of the heteroduplex, as observed in the GGG context. Moreover, the stabilisation of the base pairs around the modified bulged guanine observed by NMR in the CGC sequence explains the weak reactivity to chemical probes of the bases surrounding the bulge in the repetitive sequence GGG (4).

The fact that −1 frameshift mutation can occur spontaneously at repetitive sequences suggests that slipped structures can form during replication (1), following the thermodynamic equilibrium between slipped and extended conformations depicted in Figure 5. The binding of AAF to the guanine displaces the equilibrium towards the slipped state. Moreover, the formation of slipped structures during replication might be kinetically favoured; the rate of incorporation by DNA polymerase of cytosine opposite the adduct and also of the following complementary base is considerably reduced (19), opening a time window for slippage to occur. The newly incorporated cytosine, now paired with an unmodified guanine in a canonical Watson–Crick base pair, will produce a −1 frameshift intermediate upon elongation of DNA synthesis.

Understanding the origin of the stabilising effect of the AAF modification on the bulged guanine requires an analysis of the various interactions between the bases and the AAF in an energetic model. A molecular dynamics analysis in which NMR constraints have been incorporated is in progress.

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