Structures of Base Pairs with 5-(Hydroxymethyl)-2′-deoxyuridine in DNA Determined by NMR Spectroscopy†

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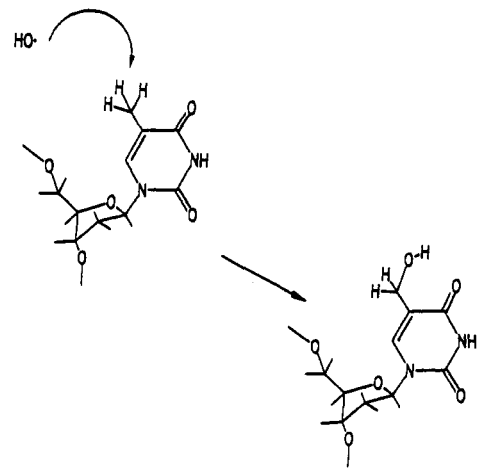
ABSTRACT: Base pairs with 5-(hydroxymethyl)-2′-deoxyuridine (HMdU) opposite either adenine or guanine in a seven-base oligonucleotide duplex have been studied by NMR spectroscopy. When paired with A, the HMdU–A base pair is in Watson–Crick geometry. The hydroxymethyl group maintains a fixed orientation in which the oxygen is on the 5′ side of the base. The energy-minimized structure indicates the presence of a hydrogen bond between the hydroxymethyl group and the N7 of the 5′ guanine residue. When paired with guanine, HMdU–G is in a wobble configuration at low pH. The hydroxymethyl group is on the 3′ side of the base, positioned to form an intramolecular hydrogen bond with its own O4 carbonyl. With increasing pH, HMdU–G is observed to ionize with an apparent p*K* value of 9.7. The high-pH structure is in a Watson–Crick configuration, with the HMdU residue in a position similar to that observed for HMdU–A. It is proposed that interresidue hydrogen bonding of the HMdU residue may stabilize aberrant base-pair configurations.

Oxidation of DNA and its components can cause genetic mutations and chromosomal instability (Cross et al., 1987). Oxidation of the methyl group of thymidine residues in DNA is known to result in the formation of 5-(hydroxymethyl)-2′-deoxyuridine (HMdU),[1] as shown in Scheme I (Teebor et al., 1984; Frenkel et al., 1985, 1991; Cattley et al., 1990). HMdU, when administered to cells in culture, is mutagenic (Bilimoria & Gupta, 1986; Shirnamé-Moré et al., 1987; Boorstein et al., 1988) and cytotoxic (Kahilainen et al., 1966; Boorstein & Teebor, 1989) and has antiviral activity (Shiau et al., 1980). These properties are believed to result, in part, from incorporation into cellular DNA. In vivo, 5-(hydroxymethyl)uracil (HMU) residues are removed from DNA by a specific glycosylase activity (Hollstein et al., 1984; Cannon-Carlson et al., 1989), indicating that HMdU residues are relevant DNA damage adducts and that the presence of HMdU in the DNA of higher organisms has deleterious effects.

In duplex DNA, oxidation of thymidine with formation of HMdU would generate an HMdU–A base pair. The biological consequences of such a modification in higher eucaryotes are as yet unknown. HMdU completely replaces thymidine in some bacteriophages (Kallen et al., 1962), and DNA templates containing HMdU in place of thymidine serve well as substrates for DNA polymerases from several sources (Herrala & Vilpo, 1989). However, it is known that HMdU perturbs DNA protein interactions, as it has been demonstrated that replacement of thymidine residues with HMdU in or around the recognition sequence of several restriction endonucleases inhibits subsequent cleavage (Berkner & Folk, 1979; Bron et al., 1983). Furthermore, administration of HMdU to cells in culture induces chromatid gaps, breaks, and exchanges (Zhang et al., 1993). We have constructed a seven-base-pair oligonucleotide duplex in which the central thymidine residue has been replaced by HMdU, forming an HMdU–A base pair in order to determine the structural consequences of HMdU in DNA. We report here results from one- and two-dimensional proton NMR studies on this system. Structural studies of several other DNA duplexes containing normal and modifying pyrimidines in the same sequence have been previously reported and serve as reference points for this study (Sowers et al., 1987, 1988, 1989; Fazakerley et al., 1987, 1993; Carbonnaux et al., 1990).

Scheme I: Oxidation of a Thymidine Residue with Formation of HMdU



The mechanism for the mutagenicity of HMdU is currently a topic of debate. HMdU has been demonstrated to induce λ prophage and is mutagenic in the Ames assay (Bilimoria & Gupta, 1986; Shirnamé-Moré et al., 1987; Boorstein et al., 1988). Base-substitution errors induced by HMdU are believed to result from the incorporation of HMdU into DNA. In a recent study however, the miscoding properties of an HMdU residue in a model template failed to reveal miscoding

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[1] Abbreviations: DQF-COSY, double-quantum-filtered correlated spectroscopy; HMdU, 5-(hydroxymethyl)-2′-deoxyuridine; HMU, 5-(hydroxymethyl)uracil; NMR, nuclear magnetic resonance spectroscopy; NOE, nuclear Overhauser effect; NOESY, two-dimensional NOE spectroscopy; TOCSY, total correlation spectroscopy.

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above background levels (Levy & Teebor, 1991). The mutagenicity of HMdU has been compared with that of the mutagenic base analogue, 5-bromouracil. Base-substitution errors would then be expected to result from the occasional formation of HMdU–G base pairs during DNA replication. In this study, we have also prepared and examined a DNA duplex containing an HMdU–G base pair in the same sequence as used previously for examination of the mispairing properties of 5-bromouracil (Sowers et al., 1989). We report here our results on this system and propose a hypothesis which may explain some of the mutagenic effects of HMdU in DNA.

EXPERIMENTAL SECTION

The heptanucleotides were synthesized by standard phosphoramidite methods (Gait, 1985). The description of the preparation of the HMdU-containing oligonucleotides has been described previously (Sowers & Beardsley, 1993). Duplexes were formed by combination of appropriate oligonucleotides to form the following duplexes:

5′ C1 A2 G3 HMdU4 G5 G6 C7

3′ G14 T13 C12 X11 C10 C9 G8

in which X11 is either deoxyadenosine or deoxyguanosine. In each case, the strand concentration was 4 mM, 100 mM NaCl (unless otherwise stated), 10 mM phosphate buffer, 0.2 mM EDTA, and 0.4 mM tetramethylammonium chloride as internal reference (3.18 ppm). NMR spectra were recorded in either 99.996% D_2O or 90% H_2O/10% D_2O.

NMR spectra were recorded on either Bruker AMX500 or AMX600 spectrometers. NOESY spectra were recorded in the phase-sensitive mode (Bodenhausen et al., 1984). The residual H_2O signal was presaturated during the relaxation delay. The NOESY spectra were recorded with mixing times of 40, 60, 80, and 400 ms in D_2O and 250 ms in H_2O. The data were multiplied by a slightly shifted sine bell in both dimensions prior to Fourier transformation. Additional 1D difference spectra were recorded with presaturation times in the range 30–60 ms. For the 1D and 2D spectra in H_2O, a jump and return sequence was used to suppress the water signal (Plateau & Guéron, 1982). The pulse maximum was at 15 ppm.

TOCSY spectra were recorded in the phase-sensitive mode with a mixing time of 25 ms (Davis & Bax, 1985). DQF-COSY spectra were recorded with 2048 data points in the t2 dimension with a sweep width of 3500 Hz.

Molecular modeling was performed with the Hyperchem package (Autodesk, Inc., Sausalito, CA). The starting point for the structure of the HMdU residue was the geometry of the HMdU monomer in the crystal structure (Birnbaum et al., 1980). Approximate partial charges were obtained using the semiempirical method PM3 (Stewart, 1989). The energy-minimized structures of the seven-base duplexes were obtained using the AMBER potential (Weiner et al., 1984).

RESULTS

Duplex HMdU–A Nonexchangeable Protons. The spectrum of the oligonucleotide duplex containing HMdU–A does not vary over the pH range 5.5–10.8. Above pH 10.8, the aromatic resonances shift downfield, indicative of alkaline denaturation. The pH and the temperature were chosen for the best resolution in the spectra.

Figure 1 shows the H8/H6-H1′/H5 region of the 400-ms-mixing-time NOESY spectrum. We can follow the connectivities for each strand through HMdU4 and A11. All the interactions involving the central trinucleotide appear

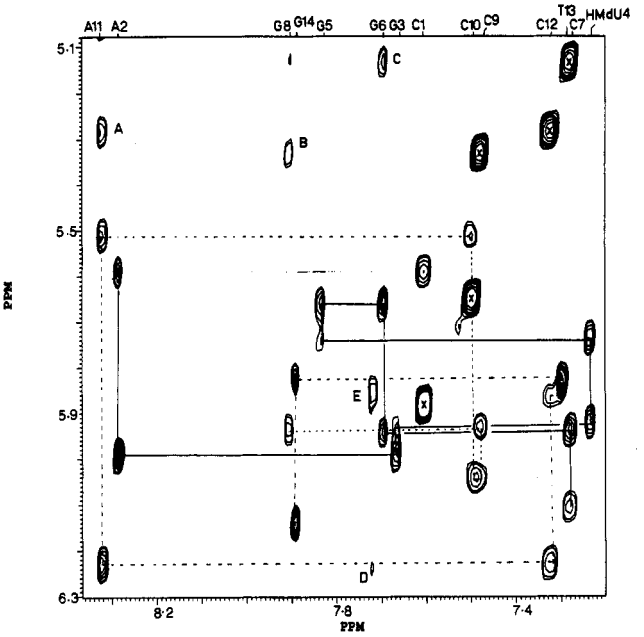

FIGURE 1: Expanded contour plot of the H6/H8-H1′/CH5 region of the NOESY spectrum (400-ms mixing time, in D_2O) of the HMdU–A duplex 5′d(C A G HMdU G G C)·3′d(G T C A C C G) at pH 7.5, 23 °C. This region shows the connectivities between the base protons for the first strand (solid line) and the second strand (broken line). The H6–H5 cross-peak interactions are marked X.

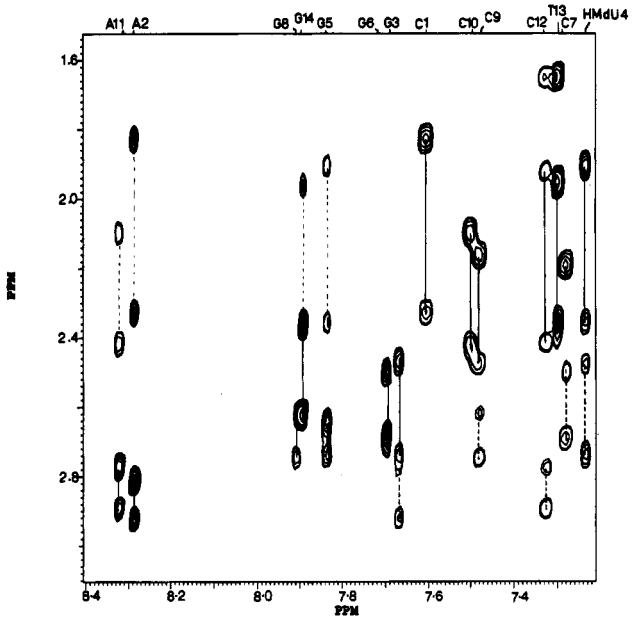

FIGURE 2: Expanded contour plot of the H6/H8-H2′/H2″ region of the 400-ms NOESY spectrum. This region shows the intraresidue (solid lines) and the interresidue (broken lines) interactions between the aromatic and the H2′/H2″ protons.

normal. Three interbase crosspeaks are observed, peaks A–C, and two arising from A11 H2, peaks D and E. All bases are intrahelical, and the magnitudes of the NOEs are indicative of a normal B-form DNA.

The global B-conformation is confirmed by the H8/H6-H2′/H2″ interactions shown in Figure 2. Quantitative analysis of NOESY spectra recorded with short mixing times (Cuniasse et al., 1987) shows that there are no significant deviations from classical B DNA. The H3′ and H4′ and most of the H5′ and H5″ resonances have been assigned from the NOESY and TOCSY spectra. The observed chemical shifts are listed in Table I. All sugar puckers were found to be predominantly C2′ endo.

Table I: Chemical Shifts of Nonexchangeable Protons at 23 °C and of Exchangeable Protons at 1 °C for the Duplexes HMdU–A and HMdU–G, pH 7.5, and for the Central Base Pairs of HMdU–G (Marked with an Asterisk)[a]

	H6/H8	H5/CH_3/CH_2OH	H1′	H2′	H2″	H3′	H4′	H5′/H5″	imino/H2	amino	
C1	7.61	5.86	5.59	1.81	2.32	4.67	4.02	3.70		7.00	8.28
A2	8.28		5.99	2.80	2.92	5.02	4.36	4.11/3.98	7.72	7.85	6.52
G3	7.78		5.93	2.47	2.74	4.93	4.40	4.22	12.76		
G3*	7.64		5.92	2.40	2.67	4.96	4.41	3.70	12.96		
H4	7.24	3.60/3.88	5.74	1.91	2.35	4.81	4.12	n.o.	14.01		
H4*	7.30	4.01/4.20	5.45	1.91	2.16	4.81	4.07	3.90	11.79		
G5	7.83		5.66	2.64	2.71	4.96	4.34	4.00	12.97		
G5*	7.88		5.73	2.72	2.77	4.98	4.36	4.08/3.90	13.37		
G6	7.69		5.94	2.50	2.68	4.95	4.35	4.17	13.10		
C7	7.27	5.13	6.02	2.18	2.18	4.48	4.24	3.98		8.18	6.45
G8	7.91		5.92	2.62	2.74	4.81	4.25	3.72	12.93	6.06	6.06
C9	7.48	5.32	6.04	2.16	2.45	4.85	4.22	4.12		8.26	6.41
C10	7.50	5.65	5.51	2.10	2.42	4.86	4.09	n.o.		8.55	6.85
C10*	7.57	5.68	5.74	2.22	2.50	4.88	4.15	4.08		8.56	6.88
A11	8.32		6.23	2.76	2.90	5.03	4.43	4.15	7.72	7.75	6.30
G11	7.91		5.99	2.60	2.73	4.97	4.41	4.14	10.51	6.06	6.06
C12	7.33	5.28	5.87	1.92	2.40	4.71	4.18	4.30		8.04	6.82
C12*	7.38	5.37	5.88	1.92	2.42	4.76	4.30	4.15		8.16	6.36
T13	7.30	1.65	5.81	1.96	2.34	4.84	4.09	n.o.	14.13		
G14	7.89		6.14	2.60	2.34	4.67	4.17	n.o.	12.84		

[a] n.o. designates resonances which could not be unambiguously assigned.

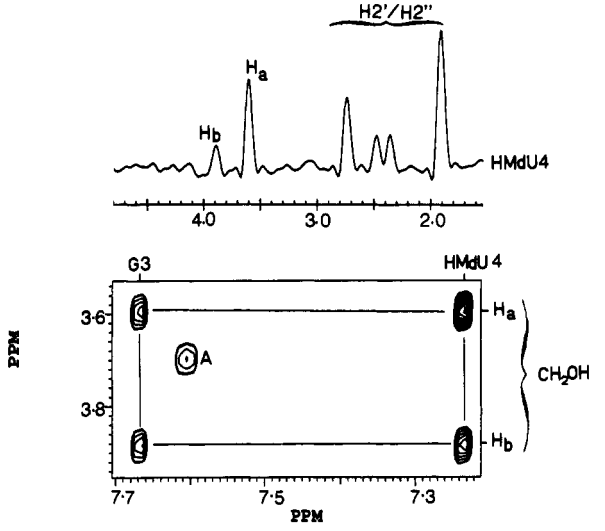

FIGURE 3: Expanded contour plot showing the interactions between the G3 and HMdU aromatic protons and the two nonexchangeable protons of the hydroxymethyl group of the 400-ms NOESY spectrum. Peak A corresponds to the interaction between C1 H6 and its H5′/H5″ protons. Above is shown the matrix column through HMdU H6 of the 60-ms NOESY spectrum.

A group of four crosspeaks to high field of the H5′ and H5″ resonances are observed (Figure 3). These arise from interactions with HMdU4 H6 and G3 H8 and must be assigned to the nonequivalent methylene protons of the 5-CH_2OH group of HMdU. The orientation of this group relative to the HMdU base was determined by analysis of short-mixing-time spectra and 1D difference spectra recorded with presaturation times in the range 30–60 ms. Figure 3 shows the matrix column through HMdU H6 of a 60-ms NOESY spectrum, showing that the NOEs to Ha and Hb are very different in intensity. From the distances observed between HMdU H6 and the hydroxymethyl protons, two orientations of this group are possible. However, the high-field resonance, Ha, is closer to both HMdU H6 and G3 H8 than is Hb. This puts both Ha and the hydroxymethyl oxygen atom on the 5′ side of HMdU. The calculated interproton distances are shown in Table II. These distances are consistent with a single conformation for the hydroxymethyl group in which the oxygen atom of the hydroxymethyl group points away from the HMdU H6 proton and toward the G3 residue on the 5′ side of the HMdU base

Table II: Distances (Å) between Aromatic Protons of G3 and HMdU4 and the Nonexchangeable Protons Ha and Hb of the Hydroxymethyl Group Obtained by NOE Intensities of a Small-Mixing-Time Matrix[a]

	HMdU–A	HMdU–G(neutral)	HMdU–G(ionized)
HMdU4 Ha	2.2–2.8 (2.3)	2.2–2.8 (2.3)	2.5–3.1 (3.2)
HMdU4 Hb	2.8–3.4 (3.2)	2.8–3.4 (3.3)	2.1–2.7 (2.3)
G3 Ha	2.9–3.5 (3.3)	n.d.	n.d.
G3 Hb	n.d.	2.4–3.0 (2.9)	2.8–3.4 (2.9)

[a] Numbers in parentheses are derived from the energy-minimized models presented in Figure 10. n.d. indicates not determined.

plane. Analysis of the DQF-COSY spectrum gave, for all nonterminal base residues, H1′–H2′ coupling constants in the range 8.5–10 Hz and for H1′–H2″, 5.5–6.5 Hz. This data confirm that these residues adopt a predominantly C2′ endo conformation.

Exchangeable Protons at pH 5.5, 1 °C. As we observed that the spectrum does not change as a function of pH, we recorded a NOESY spectrum in H_2O at lower pH to reduce the rate of proton exchange with bulk solvent. Regions of the 250-ms-mixing-time spectrum are shown in Figure 4. In the lower part of the figure we can follow the imino–imino connectivities from T13 to G5. These assignments and those of the remaining imino resonances are confirmed by examination of the upper part of Figure 4 from the interactions, via spin diffusion, with the CH5 resonances previously assigned. Only one of the G amino resonances can be assigned, that of the terminal G8, which rotates rapidly due to fraying of the terminal base pair. All other amino resonances have been located. The pairs of resonances give strong cross peaks in the amino–amino region (not shown).

We observe that the HMdU4 imino proton gives NOEs not only with adjacent imino protons but also with the adjacent C amino and H5 protons. Those observed with the non-hydrogen-bonded amino protons are of very similar intensity, indicating a similar helical twist on both sides of the central base pair, as these interactions are little influenced by spin diffusion. Base pairing is clearly Watson–Crick.

Duplex HMdU–G Spectra as a Function of pH. Unlike the system HMdU–A, that of HMdU–G is pH sensitive. Chemical shift changes are observed for the base protons of the central trinucleotide when the pH is varied. These

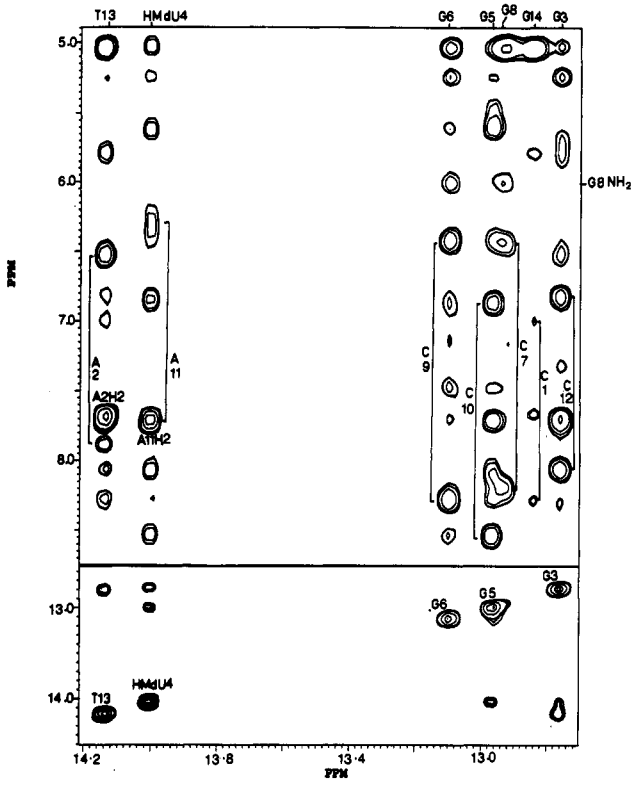

FIGURE 4: Expanded contour plots of the NOESY spectrum (250-ms mixing time) of the duplex HMdU–A at pH 5.5 and 1 °C in H_2O. Interactions between the imino protons are shown in the lower part, and between the imino and the amino/H2/CH5 protons, in the upper part. The two protons of the amino groups of cytosine or adenosine are connected by a solid line.

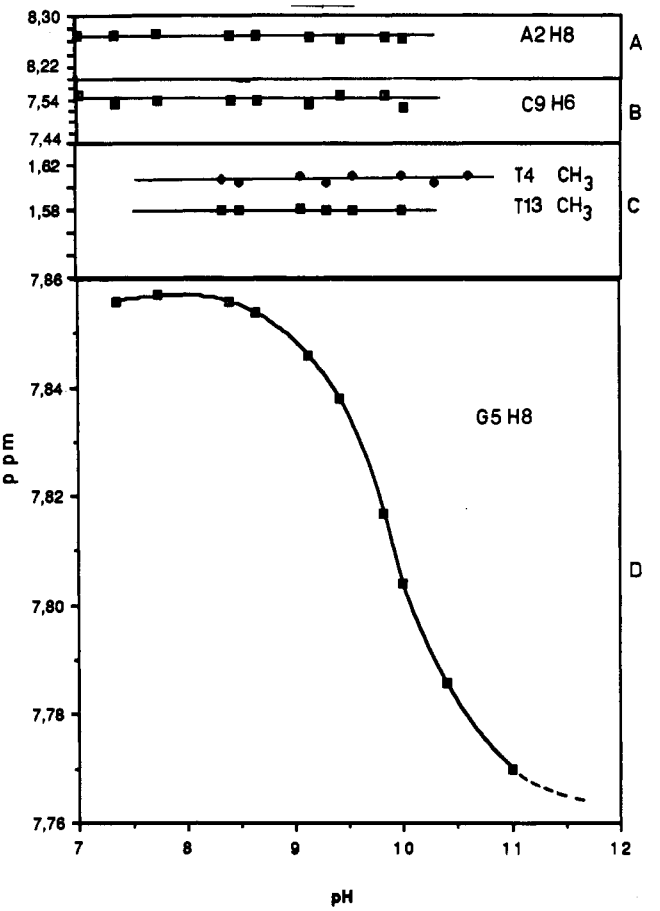

FIGURE 5: Lower part (D), chemical shift dependence of the G5 H8 proton of the HMdU–G duplex 5′d(C A G HMdU G G C)·3′d(G T C G C C G), 150 mM NaCl as a function of pH. Panels A and B, chemical shifts of A2 H8 and C9 H6, respectively. Panel C, comparison of the effect of pH on the CH_3 resonances of the duplex in which HMdU4 has been replaced by thymine.

variations must reflect changes in the structure of the central base pair.

Figure 5 shows the variation of the chemical shift of the G5 H8 proton with pH, which has the form of a normal titration curve. We could not follow accurately shifts for protons of the mismatched base pair either because the shifts are too small or because of resonance overlap. The observed p*K*, ca. 9.7, shows that a transition exists between two different species. Significant chemical shift changes are only observed for the three central base pairs. Figure 5 shows that the chemical shifts of A2 H8 and C9 H6 are unchanged with pH. We have verified that this observed effect is not due to alkaline denaturation of the duplex by carrying out the titration on a closely related duplex, the same six Watson–Crick base pairs with a central G–T wobble pair. For resonances of the central part of the duplex containing normal bases, no chemical shift changes are observed up to pH 10.8 (Figure 5), above which the entire duplex starts to denature starting with the ends of the helix. We can thus conclude that the HMdU–G base pair undergoes deprotonation at high pH, prior to helix denaturation. We have, therefore, carried out two series of experiments corresponding to the two ionization states of the HMdU–G base pairs in the duplex, at pH 7.5 and at pH 10.55.

Duplex HMdU–G at Neutral pH: Nonexchangeable Proton Spectra in D_2O at 23 °C. Resonance assignment was carried out as described above for the HMdU–A system. In the NOESY spectrum recorded with a 400-ms mixing time, all the aromatic-H1′ connectivities can be followed (not shown). Figure 6 shows the H8/H6-H2′/H2″/CH_3 region of this spectrum. The interresidue interactions show that the HMdU and G11 residues are intrahelical and that there are no major changes relative to a B DNA. All sugars are observed in a predominantly C2′ endo conformation from analysis of the

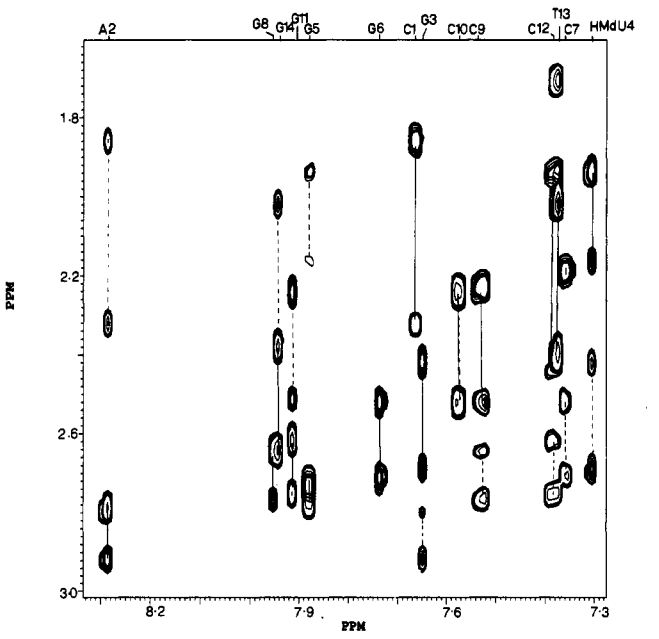

FIGURE 6: Part of the 400-ms-mixing-time NOESY spectrum of the HMdU–G duplex recorded at pH 7.4, 23 °C corresponding to the H8/H6-H2′/H2″ interactions. Intraresidue interactions are connected with solid lines.

DQF-COSY spectrum. Examination of the short-mixing-time experiments reveals two sets of interactions which, nevertheless, differ from those of HMdU–A. The interresidue NOEs G5 H8–HMdU H2′/H2″ are weak, and these two

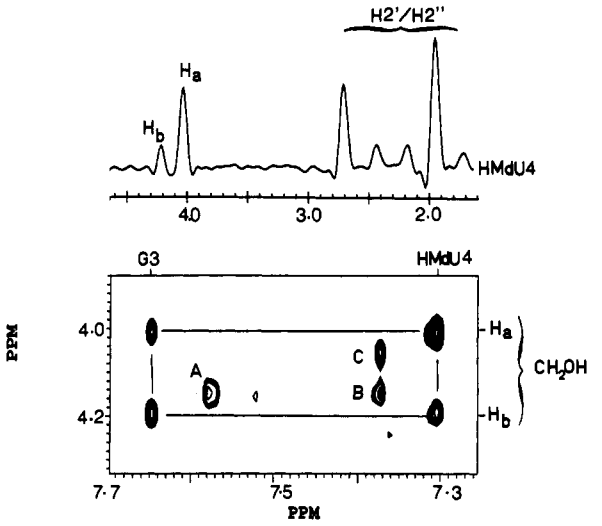

FIGURE 7: Part of the 400-ms-mixing-time NOESY spectrum of the HMdU–G duplex recorded at pH 7.4, 23 °C showing the interactions between G3 H8 and HMdU4 H6 with the hydroxymethyl protons. Peaks A–C arise from interactions involving C10, C12, and T13, respectively. Above is shown the matrix column through HMdU H6 of the 60-ms NOESY spectrum.

interactions are of similar intensity. The same is observed for the interactions of G11 H8–C10 H2′/H2″. For B DNA the interresidue NOE to the H2′ proton is very much weaker than that with the H2″ proton. We have previously observed this changed pattern of interactions in wobble structures (Carbonnaux et al., 1990).

Figure 7 shows the region of the 400-ms NOESY spectrum involving interactions with the CH_2OH group and the matrix column through HMdU H6 of a 60-ms NOESY spectrum. The proton designated Ha is closer to the HMdU H6 than is Hb. However, unlike the system HMdU–A, Hb is closer to G3 H8. Calculated distances determined from short-mixing-time experiments are shown in Table II. These distance measurements show that the oxygen atom of the hydroxymethyl group still points away from the HMdU H6; however, it is now on the 3′ side of the HMdU base plane.

Exchangeable Protons in H_2O, pH 5.5, 1 °C. Figure 8 shows two regions of the NOESY spectrum recorded with a 250-ms mixing time. In the lower part the connectivities can be followed from the T13 imino resonance to that of G6 via either of the two high-field resonances at 11.81 and 10.5 ppm. The imino protons of the Watson–Crick base pairs show normal interactions in the upper part of Figure 8. The chemical shift of these two high-field resonances is typical for N—H···O=C hydrogen bonding as found in wobble structures (Carbonnaux et al., 1990). This base pairing is confirmed by the observation of a very strong cross peak at 6.00 ppm for which no interaction is observed in the amino–amino region (not shown). This resonance corresponds to the G11 amino group which is not hydrogen bonded in a wobble structure and is in rapid rotation on a proton NMR time scale. This is further confirmed in the lower part of Figure 8, where we observe that the NOE between the two high-field resonances is much stronger than those between imino protons of adjacent base pairs. The relative assignment follows from two observations. Of the two high-field resonances, that at 11.81 ppm shows the strongest NOEs with the hydrogen-bonded amino protons of C10 and C12, showing that it is on the major-groove side and corresponds to the imino proton of HMdU. Further, the imino proton at 10.50 ppm shows the strongest interaction with the amino group at 6.00 ppm, and this confirms the assignment to G11. Observed chemical shifts are given in Table I.

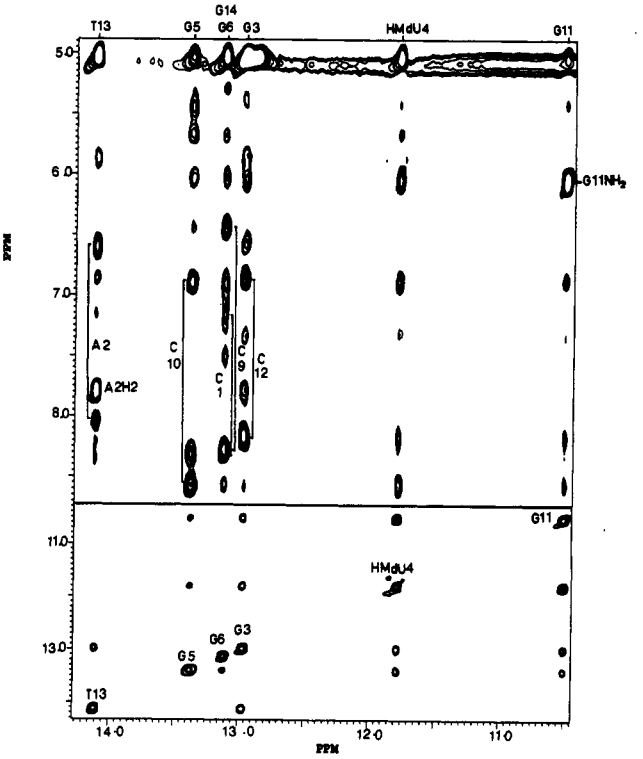

FIGURE 8: Two regions of the NOESY spectrum of HMdU–G recorded in H_2O at pH 5.5, 1 °C with a mixing time of 250 ms.

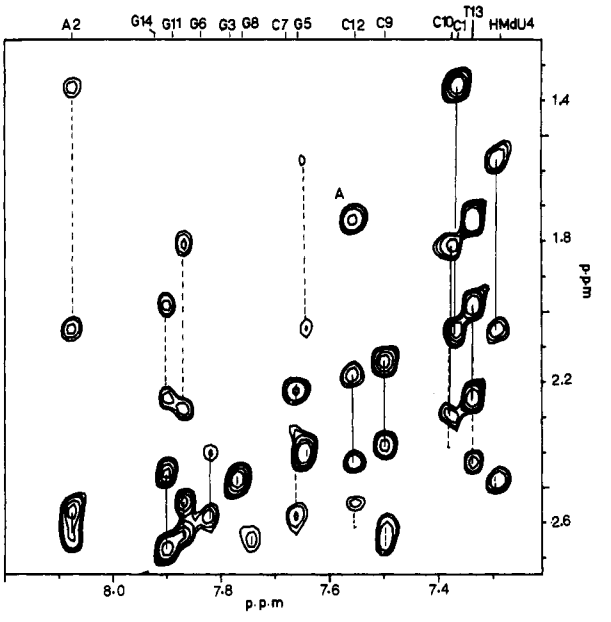

FIGURE 9: Part of the NOESY spectrum of HMdU–G corresponding to the interactions between aromatic protons and H2′/H2″/CH_3 protons. The spectrum was recorded at pH 10.55, 7 °C in 1 M NaCl, with a mixing time of 400 ms.

Duplex HMdU–G at pH 10.55, 7 °C. To obtain 80–90% of the HMdU–G basic form, we had to record spectra at high pH. The optimum pH was found to be 10.55. This is slightly less than 1 pH unit from the observed pK but is already close to that of denaturation. To stabilize the duplex, we added 1 M NaCl to the system and recorded spectra at relatively low temperature. Under these conditions, the proton resonances are much broader than those for the other systems studied.

Figure 9 shows a region of the 400-ms-mixing-time NOESY spectrum. The connectivities can be unambiguously followed from G3 to G5 and C10 to C12, showing that the mismatched bases lie within the helix. Some of the interresidue cross peaks appear to be absent in Figure 9, but they are found by

Table III: Chemical Shifts of Nonexchangeable Protons at 7 °C and pH 10.55[a]

	H6/H8	H5/CH_3CH_2OH	H1′	H2′	H2″	H3′	H4′	H5′/H5″
C1	7.38	5.84	5.86	1.36	2.06	4.50	3.96	3.58/3.60
A2	8.09		6.09	2.57	2.64	4.92	4.30	n.o.
G3	7.78		5.87	2.46	2.48	4.90	4.29	4.08/4.15
H4	7.30	4.10/4.15	6.01	1.57	2.05	4.57	4.09	n.o.
G5	7.65		6.04	2.38	2.39	4.22	4.08	3.93
G6	7.83		5.86	2.39	2.59	4.94	4.34	4.09
C7	7.66	5.80	6.16	2.21	2.30	4.51	4.11	4.25
G8	7.75		6.13	2.64	2.64	3.75	3.75	n.o.
C9	7.51	5.70	6.07	2.14	2.39	5.31	4.21	4.12
C10	7.39	5.77	6.05	1.81	2.31	4.70	4.12	n.o.
G11	7.87		6.00	2.54	2.63	4.90	4.31	4.04
C12	7.56	5.73	6.11	2.18	2.41	4.78	4.22	4.03
T13	7.35	1.74	6.19	1.99	2.22	4.69	4.11	4.01/3.93
G14	7.90		6.16	2.46	2.68	4.68	4.16	4.02

[a] n.o. indicates resonances which could not be unambiguously assigned.

examination of the matrix slices. All sugar puckers are found to be predominantly C2′ endo from analysis of the DQF-COSY spectrum. The connectivities are observed for both strands in the H8/H6-H1′ region (not shown).

Analysis of short-mixing-time NOESY spectra shows that the anomalous base proton–H2′/H2″ interactions observed for this system at neutral pH are absent. Within experimental error, the interresidue cross peak volumes have the same pattern as was observed for the HMdU–A system. This strongly suggests that the ionized HMU–G base pair adopts Watson–Crick geometry. The proton Hb of the hydroxymethyl group is found to be closest both to HMdU H6 and to G3 H8. This, as we observed with HMdU–A, puts the oxygen on the 5′ side of the base. The calculated distances are shown in Table II, and chemical shifts, in Table III. Due to rapid exchange with bulk solvent, we are unable to observe the exchangeable protons at this high pH.

DISCUSSION

HMdU–A Base Pair. The data presented here show that both the HMdU and A residues of the HMdU–A base pair are intrahelical and paired in Watson–Crick geometry. The global conformation of the duplex is normal B-form, and no unusual distortions of helix geometry are observed. The observation that replacement of T by HMdU does not significantly alter helix or base pair conformations is not an unexpected result, as thymidine residues are completely replaced by HMdU in some bacteriophages (Kallen et al., 1962) and HMdU-containing oligonucleotides serve well as templates for DNA polymerases from several sources (Herrala & Vilpo, 1989).

The hydroxymethyl group of HMdU lies in the major groove of a B-form DNA duplex. Unlike the methyl group of T, however, the hydroxymethyl group of HMdU is not freely rotating. Inspection of the matrix column through HMdU H6 in the 60-ms NOESY spectrum (Figure 3) indicates that the methylene protons of HMdU are asymmetrically positioned with respect to the base plane. The methylene proton which is closer to HMdU H6 is also closer to the H8 proton of G3, which establishes the orientation of the HMdU hydroxyl group. Approximate intermolecular distances obtained from NOESY data (Table II) confirm that the hydroxymethyl group is on the 5′ side of the HMdU residue and that the hydroxymethyl oxygen atom points away from the HMdU H6 proton.

The NMR date presented here indicate a preferred orientation for the hydroxymethyl group of HMdU. However, because the hydroxyl proton exchanges rapidly with solvent protons, the hydroxyl proton is not observed in the NMR spectrum. Thus potential hydrogen-bond formation by the hydroxymethyl group cannot be determined on the basis of NMR data. Because potential hydrogen-bond formation by the hydroxymethyl group is one of the features which distinguish T and HMdU, we have attempted to probe potential hydrogen-bond formation of HMdU with molecular modeling studies as described below.

When the HMdU hydroxymethyl group is on the 5′ side of the HMdU residue, it could form either an intramolecular hydrogen bond with its own O4 carbonyl or an intermolecular hydrogen bond with N7 of G3. Comparison of the two possible structures using the AMBER potential suggests that the configuration with the intermolecular hydrogen bond is more likely. This structure is shown in Figure 10 (left), and in this orientation, the hydroxyl proton of HMdU is within 1.8 Å of N7 whereas it is 2.9 Å from its own carbonyl. The distances obtained from the model-building study fit well with the experimentally determined values (Table II).

The close proximity of the hydroxymethyl group of HMdU and the N7 of the adjacent G residue can be easily accommodated without helix distortion. In B-form DNA, the purine H8 proton could approach within 2.6 Å of the T methyl group for a GpT step (Feigon et al., 1983; Weiss et al., 1984). Although the bond angles for the interresidue-hydrogen-bonded configuration are less than ideal for a hydrogen bond involving the N7 lone pair electrons, the hydrogen bonding between HMdU and the adjacent G residue may involve the aromatic ring of the G residue, as has been described for interactions between water and aromatic molecules (Levitt & Perutz, 1988; Suzuki et al., 1992). A model-building study of another thymidine oxidation product, thymidine glycol, similarly indicates the formation of an intrastrand interresidue hydrogen bond between the C6 hydroxyl of thymidine glycol and the N7 of the adjacent purine residue (Clark et al., 1987).

Oxidation of T to HMdU in a DNA duplex does not alter the overall conformation of the base pair or the surrounding helix. However, the replacement of the T methyl group with the hydroxymethyl group in the major groove of the helix may significantly modify interactions with sequence-specific DNA binding proteins and in part explain the cytotoxicity of HMdU in higher eucaryotes. The importance of the thymidine methyl group in sequence-specific DNA–protein interaction has been previously discussed (Ivarie, 1987; Pu & Struhl, 1992). The hydroxymethyl group, which is significantly larger than a methyl group, would be expected to interfere with proteins containing methyl-binding clefts. Interbase hydrogen-bond formation by HMdU could also obscure other contact points. As noted in the introduction, replacement of T by HMdU inhibits cleavage of DNA by restriction endonucleases

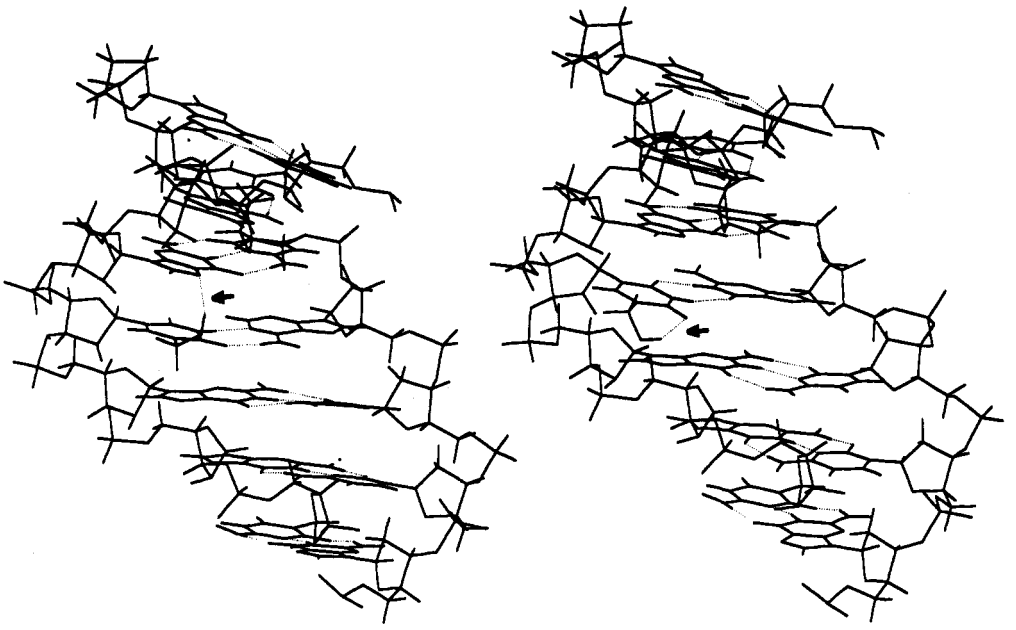

FIGURE 10: Molecular models of HMdU-containing seven-base-pair oligonucleotides studied here: (left) HMdU–A Watson–Crick base pair in which the HMdU residue forms an intermolecular hydrogen bond with G3; (right) HMdU–G wobble pair in which the hydroxymethyl group forms an intermolecular hydrogen bond with the O4 carbonyl. Arrows indicate the presumptive inter- and intramolecular hydrogen bonds, respectively.

which contain T residues at or near the recognition site (Berkner & Folk, 1979; Bron et al., 1983). Recently, it has been demonstrated that substitution of T by a similar oxidized base, 5-hydroxy-2′-deoxyuridine, at specific sites can inhibit binding of the 434 repressor to the O_R1 binding sequence (Mascareñas et al., 1993).

HMdU–G Base Mispair. Biological studies suggest that base substitution mutations induced by HMdU would arise via the formation of intermediate HMdU–G base pairs during DNA replication. In analogy with the mutagenic base analogue BrdU, one would expect HMdU to have an increased tendency to assume an unpreferred tautomeric form or to have an increased tendency to ionize when paired with G, compared with T in the same base-pairing environment (Levy & Teebor, 1991). Studies of the HMdU–G pair reported here were conducted primarily to investigate potential mechanisms for the observed mutagenicity of HMdU.

We observe that at low pH, both HMdU and G residues are intrahelical in wobble geometry, with helical distortions localized to the vicinity of the mismatched base pair. We have examined several other pyrimidine–G wobble base pairs including T–G within the same base sequence (Sowers et al., 1988, 1989; Carbonnaux et al., 1990), and we observe no significant differences between HMdU–G and other wobble pairs. The hallmarks of the wobble configuration include two high-field imino resonances, a freely rotating G amino group, and perturbation of interactions between purine H8 and adjacent H2′/H2″ protons. At physiological pH, we observe no substantial difference between HMdU–G and T–G wobble pairs when examined within the same surrounding base sequence. This suggests that HMdU does not have an enhanced tendency to mispair via formation of an enol tautomeric form.

As with HMdU–A above, the hydroxymethyl group of HMdU when paired with G assumes a fixed orientation. Inspection of the matrix column through HMdU H6 in the 60-ms NOESY spectrum (Figure 5) shows that the methylene protons of HMdU, when paired with G, are asymmetric with respect to the base plane. In contrast to HMdU–A, however, the methylene proton which is closer to HMdU H6 in the HMdU–G wobble is now the proton further from G3 H8. Interproton distances obtained from NOESY data (Table III) indicate that the hydroxyl group of HMdU, when paired with G, is on the 3′ side of the HMdU residue.

Model-building studies of the helix containing the HMdU–G wobble, as shown in Figure 10 (right), suggest the formation of an intramolecular hydrogen bond between the hydroxymethyl group and the C4 carbonyl of HMdU. Relative to the position of HMdU in the Watson–Crick HMdU–A base pair, the HMdU residue in the HMdU–G wobble pair has rotated toward the major groove, increasing the distance between the hydroxyl of HMdU and the N7 of G3. The hydroxyl of HMdU in the wobble configuration is closer to its own C4 carbonyl, which likely explains formation of the intramolecular hydrogen bond indicated in Figure 10 (right). While the intramolecular hydrogen bond could presumably form on either the 3′ or 5′ side of the HMdU residue, the experimental data indicates a preferential formation on the 3′ side of HMdU.

Previously we demonstrated that the significant difference between BrU–G and T–G mispairs (Sowers et al., 1988), in the same sequence examined in the present study, was ionization of the BrU residue and formation of an ionized BrU–G base pair in Watson–Crick geometry. It is likely that ionization of BrdU accounts for its mispairing potential, as it has been shown that the mispairing frequency of BrU residues during enzymatic DNA replication increases with increasing solvent pH (Driggers & Beattie, 1988). We therefore investigated the behavior of the HMdU–G mispair as a function of pH.

With increasing solvent pH, changes in proton chemical shifts in the vicinity of the HMdU–G mismatch demonstrate that the HMdU–G base pair ionizes with an apparent pK of 9.7 (Figure 5). In contrast, neither T–G, U–G (Carbonnaux et al., 1990), nor HMdU–A (discussed above) undergoes ionization with increasing pH prior to alkaline denaturation of the helix. In order to examine the high-pH structure of the HMdU–G mispair, spectra were obtained at a pH sufficiently high (pH 10.55) that the predominant structure was ionized. While structure determination by proton NMR at high pH is inherently much more difficult because of hydroxide-catalyzed proton exchange, certain details of the high-pH HMdU–G structure may be obtained. NOESY data confirm

that, in the ionized structure, both the HMdU and G bases are intrahelical and that the helix is still predominantly B-form.

In contrast to HMdU–G at low pH, the anomalous base proton–H2′/H2″ NOEs are absent in the high-pH structure, indicating that both bases have relaxed to a conformation consistent with Watson–Crick geometry. Interproton distances obtained from NOESY data indicate that the hydroxyl group of HMdU has returned to the 5′ side of HMdU at high pH, as observed for HMdU–A. These data indicate that, in the ionized HMdU–G structure, HMdU and G are paired in Watson–Crick geometry. Because the hydroxymethyl proton exchanges rapidly with solvent water, it is not observed in the spectrum. Therefore, the orientation of the hydroxyl group, and potential hydrogen-bonding sites, cannot be determined on the basis of experimental data.

Model-building studies of the ionized HMdU–G mispair indicate that the HMdU residue is in essentially the same position as when paired with A, again forming a base pair in Watson–Crick configuration and an interbase hydrogen bond with the adjacent G residue (not shown). It is proposed that the ionized HMdU–G base pair may be stabilized by interbase-hydrogen-bond formation. On the basis of the observed pK of 9.7, the ionized Watson–Crick configuration of the HMdU–G mispair would be present at a level of roughly 1 in 300 at physiological pH. Spontaneous base-substitution mutations involving the T–G mispair are presumed to result from formation of unpreferred tautomeric forms which are estimated to occur at a level of 5 in 100 000 (Topal & Fresco, 1986).

HMdU may therefore mispair with G, in Watson–Crick geometry as a consequence of ionization, between 1 and 2 orders of magnitude more frequently than T. If the ionized configuration of the HMdU–G mispair is stabilized by interbase-hydrogen-bond formation, mispairing of HMdU may be highly sequence-specific. We noted previously that a recent biochemical study utilizing HMdU in a template for DNA polymerase did not demonstrate enhanced mispairing by HMdU (Levy & Teebor, 1991). In the sequence used in that study, however, the HMdU residue was flanked on both sides by cytosine residues. The proposed intermolecular hydrogen bond formed between HMdU and the guanine residue in the 5′ direction would not form when HMdU was adjacent to C. A more complete explanation of the mispairing properties of HMdU will require further biochemical studies; however, the data presented here clearly indicate that selection of the base sequence and pH will be critical factors.

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