¹H NMR studies of the 5-(hydroxymethyl)-2'-deoxyuridine containing TF1 binding site

Laura B. Pasternack, Janice Bramham⁺, Luciano Mayol[§], Aldo Galeone[§], Xin Jia[¶] and David R. Kearns*

Department of Chemistry and Biochemistry, University of California, San Diego, La Jolla, CA 92093, USA

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ABSTRACT

The pyrimidine base 5-(hydroxymethyl)-2'-deoxyuridine (HmU) is a common nucleotide in SPO1 phage DNA. Numerous transcriptional proteins bind HmU-containing DNA preferentially implicating a regulatory function of HmU. We have investigated the conformation and dynamics of d-(5'-CHmUCHmUACACGHmUGHmUA-GAG-OH-3')₂ (HmU-DNA). This oligonucleotide mimics the consensus sequence of Transcription Factor 1 (TF1). The HmU-DNA was compared to the thymine-containing oligonucleotide. NOESY and DQF COSY spectroscopy provided resonance assignments of nonexchangeable and exchangeable protons, internucleotide, internucleotide and intrastrand proton-proton distances, and dihedral angle constraints. Methylene protons of the hydroxymethyl group are nonequivalent protons and the hydroxymethyl group is not freely rotating. The hydroxymethyl group adopts a specific orientation with the OH group oriented on the 3' side of the plane of the base. Analysis of imino proton resonances and NOEs indicates additional end base pair fraying and a temperature-induced transition to a conformation in which the internal HmU-A base pairs are disrupted or have reduced lifetimes. Orientation of the hydroxymethyl group indicates the presence of internucleotide intrastrand hydrogen bonding between the HmU12C5 hydroxyl group and A13. All sugars in both DNAs show a C2'endo conformation (typical of B-DNA).

INTRODUCTION

Numerous nucleoside analogs including the pyrimidine base 5-(hydroxymethyl)-2'-deoxyuridine (HmU) demonstrate anti-bacterial, antiviral or mutagenic activity (1–4). Biosynthesis of HmU results in G-C to A-HmU transition (5,6). Excision of HmU is carried out by the activity of HmUra-DNA glycosylase, found to be decreased in Werner's syndrome cells (a disease marked by early symptoms of accelerated aging) indicating a role of HmU accumulation in the aging process (7–9).

While the presence of HmU is cytotoxic, it is present in the DNA of several *Bacillus subtilis* bacteriophages such as SPO1 instead of thymine as a natural nucleotide (10). HmU containing DNA has been shown to be a good template-primer for different DNA and RNA polymerases and may provide a selective advantage over host DNA in transcription or replication (11). In fact, numerous bacteriophage encoded proteins such as the Transcription Factor 1 (TF1) show greater affinity for HmU containing DNA relative to thymine containing DNA (12). These DNA binding proteins are found in only a small subset of bacteriophages and the influence of HmU on the binding of eucaryotic transcription factors is as yet unknown. TF1 is a type II DNA binding protein (DBPII) and shows sequence homology to other DBPII which have been shown to induce DNA bending and compaction. The crystal structure of the protein HU (a *Bacillus stearothermophilus* encoded DBPII) has been solved and a model of the HU-DNA binding has been described (13). DNA binding is proposed to involve two β -ribbon 'arms'. TF1 is a DBPII proposed to have the same binding motif as HU (14). TF1, however, shows greater DNA affinity (specifically for HmU containing DNA) and sequence specificity. This potentially makes TF1 a good candidate for the structural study of the novel β sheet binding motif proposed for DBPII-DNA complexes.

We are studying the structural and dynamic features of the DNA binding site of TF1 (15). High resolution NMR spectroscopy provides a means of studying the structure and dynamics of double-stranded DNA (16–20). In order to simplify structural analysis a single base deletion was made to the consensus sequence of the known TF1 binding site resulting in a self complementary 16 bp DNA containing eight HmU residues. We find that the 16mer establishes a conformation with sugar puckers in the C2'endo configuration (typical of B-DNA). The average orientation of the hydroxymethyl group consistently places the oxygen on the 3' side of the plane of the base in all HmU base pairs. Orientation of the hydroxymethyl group indicates the presence of an intrastrand internucleotide hydrogen bond between the HmU12C5 hydroxyl group and A13. Comparison of several experiments indicates instability of A-HmU basepairing resulting in additional fraying, and a temperature-induced transition to a conformation in which the internal HmU-A base pairs are disrupted or have reduced lifetimes.

* To whom correspondence should be addressed

Present addresses: ⁺Department of Biochemistry, The University Dundee, UK, [§]Dipartimento di Chimica delle Sostanze Naturali, Università di Napoli Federico II, via Domenico Montesano 49, I-80131 Napoli, Italy and [¶]La Jolla Cancer Research Foundation, La Jolla, CA, USA

MATERIALS AND METHODS

Materials

The synthesis of 5-hydroxymethyl-uracil containing oligonucleotides has been previously described (21). Thymine containing 16mer 5'-CTCTACACGTGTAGAG-OH-3' (T-DNA) was purchased from Oligo's, Inc. Wilsonville, OR. Samples were dissolved to 2 mM in 100 mM NaCl in 80% H₂O, 20% D₂O (purchased from Cambridge Isotope Laboratories, Woburn, MA) and titrated to pH 6.2 with NaCl. Samples were dried under a stream of nitrogen and resuspended in D₂O when appropriate.

Melting profiles of the HmU-DNA [d-(5'-CHmUCHmUACA-CGHmUGHmUAGAG-OH-3')] and T-DNA were compared by optical melting curves. The melting temperature of the HmU-DNA is ~10° lower than the T-DNA. The denaturation appears to be a simple two-state process in both the HmU-DNA and T-DNA. No significant melting is apparent prior to 30°C in either oligonucleotide.

Nuclear magnetic resonance spectroscopy

Proton NMR spectra were recorded on a Bruker AMX500 FT-NMR spectrometer (499.896 MHz). NOESY spectra for analysis of exchangeable protons were recorded in 80% H₂O/20% D₂O solvent using a jump-return pulse sequence for water suppression over a spectral width of 10 504 Hz (22). The time domain consisted of 2048 data points along t₂ and 480 data points along t₁ with 96 summed scans for each t₁. Mixing times varied from 80 to 200 ms as noted. A relaxation delay of 30 ms was used (total recycle time of 1.31 s). NOESY spectra for analysis of nonexchangeable protons were recorded in a 100% D₂O solvent over a spectral width of 5000 Hz. The time domain consisted of 2048 data points along t₂ and 480 data points along t₁ with 96 summed scans for each t₁. A relaxation delay of 100 ms was used (total recycle time of 1.38 s). The effect of increasing recycle time on signal intensity was evaluated by 1D spectra of the HmU-DNA. No significant increase (<5%) in signal intensity occurs after 1.3 s. Spectra were analyzed using Felix software. In all cases data workup included a zero fill to 2048 along the t₁ axis and shifted sine bell functions along both axes prior to two dimensional Fourier transformation. Spectra were referenced to H₂O at 4.7 p.p.m.

Double quantum filtered correlated spectroscopy (DQF COSY) data were collected in D₂O over 5000 Hz spectral width with 2048 data points and 80 summed scans per t₁.

One dimensional data was collected on a Bruker AMX 500 FT NMR spectrometer using a jump-return pulse with presaturation (22). 400 transients of 16 K data points were collected over a spectral width of 10 504 Hz. Data processing included shifted sine bell adpodization. All spectra were referenced by designating the center of the spectral width as 4.7 p.p.m.

Restraint determination

Interstrand, internucleotide, intranucleotide and dihedral angle constraints were determined from NOESY spectra taken at 80 ms mixing time in H₂O and D₂O and DQF COSY spectra for both HmU-DNA and T-DNA.

Distance constraints were estimated using very strong (1.8–2.2 Å), strong (2.2–2.5 Å), medium-strong (2.5–2.9 Å), medium (2.9–3.3 Å), weak-medium (3.3–3.7 Å), weak (3.7–4.1 Å) and very weak

Figure 1. Expanded NOESY (80 ms mixing time) contour plots of the HmU-DNA duplex acquired in D₂O at 20°C displaying intraresidue and interresidue distance connectivities between the nonequivalent methylene protons and base protons.

(4.1–6.0 Å) (23). Cross peak intensities were categorized by measurement of crosspeak volumes in NOESY spectra acquired at 80 ms mixing time and application of the relationship $r_{ij} = r_{\text{ref}}(R_{\text{ref}}/R_{ij})^{1/6}$ where (r_{ij}) is the proton–proton distance, (r_{ref}) is the fixed reference distance (cytosine H5-H6 = 2.46 Å) and R_{ref}/R_{ij} is the relative initial cross-relaxation rate. NOE buildup curves were used in order to determine the mixing time appropriate for the ‘two spin’ approximation used in molecular dynamics.

The pseudorotational angle was determined by a combination of H1'-H4', H1'-H2' and H4'-H2'' measured distances (24). Individual torsional angles for all dihedral restraints within the sugar ring were determined from the pseudorotational angle (25). The angle (5'-O-5'-C-4'-C-3'-C) was determined from the $\Sigma I_{H4'}$ measured by DQF COSY (18). The angle [C4'-C3'-O3'-(n+1)P] was determined from the $\Sigma I_{H3'}$ (18). Glycosidic angle (4'-O-1'-C-1N-2C) for pyrimidines and (4'-O-1'-C-9N-4C) for purines was determined by H8/H6-H1' distances (24). Hydrogen bond restraints were added in order to maintain base pairing.

Molecular modeling

Starting structures for molecular modeling ranging from B-DNA to A-DNA were generated using the Insight software. Five starting structures having an average RMSD of 3.42 Å (all atom) were generated by building the 16mer duplex of T-DNA (used directly for T-DNA modeling) and modifying the thymine bases to reflect the 5-hydroxymethyl group (for HmU-DNA modeling). Molecular modeling was performed using Biosym software on a Silicon Graphics Iris workstation. All calculations were carried out on a SGI Challenge L compute server. Counterions were not included, however, their effects were mimicked using distance-dependent dielectric constant with $\epsilon = r_{ij}$ (26). Molecular dynamics was carried out with a simulated annealing protocol utilizing the Amber forcefield (26). Molecular dynamics included (i) energy minimization until a root mean square of 0.1 kcal/mol Å was achieved, (ii) temperature increase to 800 K, (iii) ramping of force constants at 800 K followed by 14 phases of molecular dynamics

Table 1. Proton chemical shifts of HmU-DNA and T-DNA (in parentheses) at 30°C. Chemical shifts differing by >0.1 p.p.m. are in bold face

base	H1'	H2'	H2''	H3'	H4'	H5'/5''	G-H1 A-H2 HmU/T-H3	C/HmU/T- H6 A/G-H8	H5	H4(1)	H4(2)
C1	5.84(5.86)	2.13(2.21)	2.50(2.55)	ND(ND)	ND(4.28)	3.73/4.05(ND)	--	7.72(7.86)	(5.93)	ND(ND)	ND(ND)
HmU/T2	6.09(6.14)	2.28(2.27)	2.56(2.58)	4.86(4.84)	4.23(ND)	ND(4.05)	ND(ND)	7.72(7.62)	4.03/4.15(1.66)	--	--
C3	6.01(5.97)	2.05(2.08)	2.47(2.47)	ND(ND)	4.18(4.16)	ND(ND)	--	7.51(7.60)	5.69(5.66)	8.49(8.40)	6.83(7.00)
HmU/T4	5.57(5.56)	2.03(2.06)	2.38(2.39)	4.84(4.56)	4.11(ND)	ND(ND)	13.71(13.55)	7.51(7.34)	4.01/4.16(ND)	--	--
A5	6.13(6.11)	2.65(2.66)	2.79(2.80)	5.00(4.97)	4.38(4.34)	ND(ND)	7.33(7.35)	8.27(8.26)	--	--	--
C6	5.43(5.38)	1.82(1.88)	2.24(2.26)	ND(5.28)	ND(ND)	ND(ND)	--	7.16(7.22)	5.24(5.28)	8.00(8.05)	6.42(6.42)
A7	6.07(6.09)	2.57(2.60)	2.75(2.80)	4.94(4.93)	4.31(ND)	ND(4.09)	7.45(7.51)	8.11(8.12)	--	--	--
C8	5.50(5.50)	1.89(1.93)	2.24(2.28)	ND(5.17)	4.08(ND)	ND(ND)	--	7.12(7.16)	5.13(5.16)	7.92(7.97)	6.32(6.34)
G9	5.90(5.87)	2.49(2.50)	2.73(2.70)	4.88(4.89)	4.29(4.07)	ND(ND)	12.58(12.56)	7.70(7.74)	--	--	--
HmU/T10	5.68(5.74)	2.01(2.05)	2.41(2.42)	ND(ND)	ND(4.14)	ND(ND)	13.61(13.42)	7.20(7.10)	3.63/3.90(1.35)	--	--
G11	5.90(5.85)	2.48(2.52)	2.71(2.69)	4.90(4.88)	4.31(4.29)	ND(4.51)	12.40(12.37)	7.76(7.79)	--	--	--
HmU/T12	5.53(5.53)	1.85(1.91)	2.24(2.28)	ND(ND)	4.07(ND)	ND(ND)	13.54(13.38)	7.25(7.15)	3.69/3.86(1.39)	--	--
A13	5.86(5.85)	2.59(2.61)	2.73(2.78)	4.97(4.99)	4.31(4.30)	ND(ND)	7.27(7.28)	8.15(8.10)	--	--	--
G14	5.34(5.33)	2.45(2.47)	2.58(2.60)	4.91(4.90)	4.24(4.11)	ND(4.23)	12.54(12.56)	7.63(7.65)	--	--	--
A15	6.01(6.05)	2.50(2.55)	2.73(2.81)	4.94(4.95)	4.34(ND)	ND(4.10)	ND(7.75)	7.94(7.99)	--	--	--
G16	5.95(5.96)	2.23(2.22)	2.35(2.35)	ND(4.57)	4.11(4.10)	4.18/4.06(ND)	ND(ND)	7.57(7.58)	--	--	--

--, not applicable.

ND, not detected.

with a temperature reduction from 800 to 100 K, and (iv) minimization until a root mean square gradient of 0.03 kcal/mol Å was achieved. Twenty-nine final structures were obtained which converged to an RMSD of 0.50 Å (all atom) for the internal 12 bp.

RESULTS

Resonance assignment of nonexchangeable protons

Two dimensional ¹H NMR NOESY spectra of nonexchangeable protons of HmU-DNA and T-DNA in D₂O were recorded at 30°C. Sequence specific assignment has been carried out and the chemical shifts are listed in Table 1. The upfield region of the NOESY of HmU-DNA shows the connectivities of the H2'/H2'' nonexchangeable protons. Examination of the H8/H6-H2'/H2'' NOEs and absence of H2''-H4' NOEs confirms the sugar pucker conformation to be C2'endo, typical of B-DNA (27). DQF COSY spectra of T-DNA and HmU-DNA identify through bond connectivities of H1' with H2'/H2''. Characteristic couplings of these resonances assign the H2', H2'' and H1' protons.

The region of the spectrum associated with the methylene protons of two of the hydroxymethyl groups of HmU-DNA is shown in Figure 1. The two methylene protons (HM1 and HM2) are nonequivalent indicating that the hydroxymethyl group is not freely rotating. These resonances are doublets due to the spin coupling of the two methylene protons which show a strong NOE to each other (data not shown). Each proton has two significant NOEs, one intranucleotide to the H6 and one internucleotide to the neighboring base H8. Thus for each HmU residue four distances (six when there is cytosine 5' of the HmU) can be determined. The orientation of the hydroxymethyl group can be seen by inspection of the 80 ms NOESY (Fig. 1). The two protons show similar NOEs to the neighboring (n-1)H8 proton and very dissimilar NOEs to the intranucleotide H6; one strong and one weak NOE. This defines the orientation of the hydroxymethyl group in space. Assignment of these methylene protons was accomplished by trial molecular dynamics, once with M1 assigned as the upfield resonance and once with M1 assigned as the downfield resonance. Intra- and interresidue distances in the final models were compared with the experimentally determined distances. In the model calculated with M1 assigned as the upfield resonance the

two interresidue distances involving the methylene protons are quite different from each other (3.95 and 2.83 Å) while the experimentally measured interresidue distances are similar at 3.39 and 3.28 Å. However, in the model calculated where M1 is assigned as the downfield resonance the two interresidue distances involving the methylene protons are quite similar at 3.09 and 3.29 Å and close to the experimentally determined distances. Thus, in this case, assignment of M1 as the downfield resonance results in an orientation of the hydroxymethyl group that more closely adheres to the experimental data. Each HmU residue was evaluated in this manner and in each case calculations performed with M1 assigned as the downfield resonance resulted in an orientation of the hydroxymethyl group that best satisfies the experimentally determined distances. Molecular dynamics calculations with M1 assigned as the upfield resonance result in deviations of up to 0.71 Å (average overall |deviation| = 0.45 Å) from the measured values of the M1 and M2 associated interproton distances described above. Calculations with M1 as the downfield resonance, however, result in maximum deviation of only 0.45 Å (average overall |deviation| = 0.25 Å) from the measured distances.

Resonance assignment of exchangeable protons

Two dimensional ¹H NOESY NMR spectra of HmU-DNA and T-DNA in 80% H₂O/20% D₂O were recorded at 200 ms mixing time at 10°C. Sequence specific assignment of the exchangeable protons has been carried out and their chemical shifts listed in Table 1.

Temperature dependence of imino proton resonances

One dimensional ¹H NMR and two dimensional ¹H NOESY spectra were acquired of HmU-DNA and T-DNA in 80% H₂O/20% D₂O at increasing temperatures. At low field, imino proton resonances associated with HmU/T and guanine can be observed. In HmU-DNA loss of resonances associated with the internal A-HmU base pair imino protons can be seen as early as 15°C. The melting profile obtained from 1D NMR measurements of HmU-DNA shows the downfield shift typical of imino protons at higher temperatures (data not shown). At 30°C a distinct difference can be seen between the 1D resonances of the imino

Figure 2. Comparison of imino proton resonances at 30°C. 1D and NOESY spectra were acquired at 30°C with a Bruker AMX500 at 200 ms mixing time. Samples are 2 mM DNA, 100 mM NaCl and in 80% H₂O/20% D₂O. Spectra depict both (A) 1D and (B) NOESY data for T-DNA (top) and HmU-DNA (bottom).

protons of T-DNA and HmU-DNA (Fig. 2A). In addition, distinct differences are seen in the NOEs associated with A-T/HmU and G-C base pairing between the two DNAs (Fig. 2B). NOEs associated with the A-HmU base pairing in HmU-DNA are smaller than those in T-DNA indicating a temperature-dependent transition to a conformation in which these base pairs are disrupted or have reduced lifetimes. In addition, spectra acquired at 10°C show that while a large NOE is observed for T2H3-A15H2 of T-DNA the corresponding NOE observed for HmU2H3-A15H2 is quite small (Fig. 3). Thus, while T-DNA demonstrates a typical fraying profile for DNA duplexes at 10°C, HmU-DNA shows excess fraying at 10°C.

Conformational analysis

NOESY spectra of HmU-DNA were acquired with increasing mixing times and NOE buildup curves were constructed in order to determine the effect of mixing time on estimated interproton distances. A mixing time of 80 ms was chosen for the qualitative estimation of interproton distances for both HmU-DNA and T-DNA. Data were acquired at 20°C in order to reduce the effects of fraying and premelting phenomena noted in the previous sections. All conformational analysis described below was restricted to the internal 14 bp as, even at 20°C, considerable fraying is apparent at the last base pair.

The pseudorotational angle of $P = 160^\circ$ was determined by combination of H1'-H4' (3.21 Å), H1'-H2' (2.91 Å) and H4'-H2'

Figure 3. Comparison of interstrand NOEs at 10°C. NOESY spectra were acquired at 10°C with a Bruker 500AMX at 200 ms mixing time. Samples are 2 mM DNA, 100 mM NaCl and in 80% H₂O/20% D₂O. The spectra display the T/HmUH3-AH2 crosspeaks of (A) T-DNA and (B) HmU-DNA. Both figures are at the same contour level relative to the H5-H6 crosspeak.

(>4.0 Å), distances measured from ^1H - ^1H NOESY data acquired at 80 ms mixing time (24). Individual torsional angles for all dihedral restraints within the sugar ring were determined from the pseudorotational angle based on an average of 12 structures of $P = 163^\circ \pm 10^\circ$ (25). The glycosidic torsion angle ($4'\text{O}-1'\text{C}-\text{IN}-2\text{C}$) for pyrimidines and ($4'\text{O}-1'\text{C}-9\text{N}-4\text{C}$) for purines was constrained to $-125^\circ \pm 35^\circ$ and $125^\circ \pm 75^\circ$ respectively based on the experimentally determined H6/H8-H1' distances (24). The angle ($5'\text{O}-5'\text{C}-4'\text{C}-3'\text{C}$) was established by the $\Sigma J_{\text{H}4'}$ determined from DQF COSY. The average total coupling of the six discernible H4' resonances was 8.1 Hz. This limits the possible range of angle ($5'\text{O}-5'\text{C}-4'\text{C}-3'\text{C}$) to $60^\circ \pm 30^\circ$ or $240^\circ \pm 30^\circ$ (18). Comparison of the relative intensities of H6/H8-H5'/H5'', H6/H8-H1', H2'-H5'/H5'' and H2'-H1' in the NOESY spectrum eliminates the $240^\circ \pm 30^\circ$ possibility. Thus angle ($5'\text{O}-5'\text{C}-4'\text{C}-3'\text{C}$) is determined to be $60^\circ \pm 30^\circ$. Distance bounds of 2.28–2.70 Å were set for H4'-H5'/H5'' based on this angle (18). Based on the pseudorotational angle and ($5'\text{O}-5'\text{C}-4'\text{C}-3'\text{C}$), additional distance constraints involving the H5'/H5'' backbone protons were determined (24). This was verified by close inspection of NOESY spectra acquired at 80 and 200 ms that show all H1'-H5'/H5'' distances to be >3 Å. Imposing a lower bound of 3 Å on all H1'-H5'/H5'' (interresidue and intraresidue) effectively restricts the torsional angles ($3'\text{O}-\text{P}-5'\text{O}-5'\text{C}$) and ($3'\text{C}-3'\text{O}-\text{P}-5'\text{O}$) (18). The dihedral angle $[n4'\text{C}-n3'\text{C}-n3'\text{O}-(n+1)\text{P}]$ was restricted to 140 – 360° based on the sum of the H3' coupling constants ($\Sigma J_{\text{H}3'}$). With this angle restriction a distance constraint of 2.8–3.25 Å is imposed for $n\text{H}3'-(n+1)\text{P}$ (18). Similar measurements were made from the T-DNA.

Initial models for the T-DNA were generated as described in Materials and Methods. The methyl group of T-DNA was replaced with a hydroxymethyl group. Five starting structures ranging from B-DNA to A-DNA and having an average RMSD of 3.42 Å were subjected to molecular dynamics refinement using simulated annealing protocol and the Discover program as described in the Materials and Methods section. A total of 24 interstrand, 147

Figure 4. Model of T-DNA and HmU-DNA. (A) T-DNA model was determined using 24 interstrand, 142 internucleotide, 497 intranucleotide, 34 hydrogen bond and 221 dihedral angle constraints with a final RMSD = 0.40 Å (all atom) for the internal 12 bp. (B) HmU-DNA model was determined using 24 interstrand, 147 internucleotide, 502 intranucleotide, 34 hydrogen bond and 221 dihedral angle constraints with a final RMSD = 0.50 Å (all atom) for the internal 12 bp.

internucleotide, 502 intranucleotide, and 34 hydrogen bond distances and 221 dihedral angle constraints were used to generate the ultimate refined model of the HmU-DNA for which a root mean square gradient of 0.03 kcal/mol Å was achieved. Twenty-nine final structures were obtained from the five starting templates with an average RMSD of 0.50 Å (all atom) for the internal 12 bp. As typical, the external base pairs have a greater RMSD due to the lack of neighboring constraints and are not included in RMSD evaluation.

The T-DNA model was calculated as described for the HmU-DNA with a total of 24 interstrand, 142 internucleotide, 497 intranucleotide, and 34 hydrogen bond distances and 221 dihedral angle constraints. An RMSD of 0.40 Å was achieved for the final model. The overall structure of the T-DNA did not show a significant variation from that of the HmU-DNA.

The models of the T-DNA and HmU-DNA are depicted in Figure 4. The conformation of all bases is found to be C2'endo, typical of the B-DNA family. Specific orientation of the hydroxymethyl groups is consistent for all internal base pairs and places the hydroxyl group 3' and both methylene protons 5' to the plane of the base (Fig. 5). The conformation of this group can be described by the torsional angle (6C-5C-7C-7O) which is $70^\circ \pm 4^\circ$ for HmU4, $79^\circ \pm 10^\circ$ for HmU10 and $105^\circ \pm 3^\circ$ for HmU12. While the hydroxyl proton is not apparent in NOESY spectra, it is included in the modeling as unrestrained. In the final model the distance from the hydroxyl proton to the intranucleotide O4 carbonyl ranges from 3.2 to 4.6 Å and to any neighboring residue ranges from 2.2 to 4.8 Å. The 5'-GHmUAG-OH-3' sequence shows potential intrastrand internucleotide hydrogen bonding between the 5'HmUC5 hydroxyl proton and the neighboring A13 (Fig. 5).

DISCUSSION

We have studied the HmU-DNA containing TF1 binding site and its corresponding T-DNA. Sequence specific assignment of the nonexchangeable and exchangeable protons of HmU-DNA and

Figure 5. Bases of the 5'-GHmUAG-OH-3' sequence depicting the orientation of the hydroxymethyl group and the possible intrastrand interresidue hydrogen bonding between the 5C hydroxyl of HmU12 and the neighboring adenine.

T-DNA has been carried out, models developed, and exchangeable protons analyzed for base pair stability.

Inspection of the imino (exchangeable protons) region of the ^1H NOESY NMR spectra indicate a temperature-dependent transition to a conformation in which these base pairs are disrupted or have reduced lifetimes. Exchangeable protons associated with the end base pairs of both the HmU-DNA and T-DNA are not detectable. This is typical of short oligonucleotides and is commonly referred to as 'fraying'. A NOE is observed for T2H3-A15H2 of T-DNA (Fig. 3A) indicating that the second base pair is substantially annealed. In contrast, only a small NOE is observed for HmU2H3-A15H2 (Fig. 3B) suggesting significant fraying through the second base pair in HmU-DNA. It is also interesting to note that additional NOEs between HmU12H3-A13H2 and HmU4H3-A5H2 are apparent in the NOESY spectrum of the HmU-DNA that do not appear in the spectrum of T-DNA (Fig. 3). These connections are between adjacent base pairs of the same strand indicating that at 10°C there is a slight tilt of neighboring 5'-HmUA-OH-3' base pairs relative to 5'-TA-OH-3' base pairs. At 10°C imino proton resonances and NOEs of the internal base pairs are quite similar between the two oligomers while at 30°C a distinct difference can be seen between the imino proton resonances associated with A-T and A-HmU base pairs (Fig. 2). This may account for the lower T_m determined for HmU-DNA relative to T-DNA. Lower T_m values for DNA containing HmU residues was first reported by Kallen in 1962 (10). Previous studies, however, suggest that the presence of HmU in DNA does not change the torsional flexibility of the whole molecule (28). In these studies steady state fluorescence polarization anisotropy (FPA) of intercalated ethidium was used to compare the torsional flexibility of HmU-containing SPO1 DNA and thymine-containing DNA. The data indicate that the rigidity is the same in both DNAs. Reduction in DNA flexibility is, however, apparent upon binding to type II DNA binding proteins (28). Thus, while potential DNA flexibility may be important in TF1 binding, sequence-dependent charge distribution and local structural fluctuations may also play an important role in the increased affinity of HmU-DNA to TF1.

It is obvious from 2D NOESY data that the hydroxymethyl group in HmU containing DNA is not freely rotating. Thus, a specific orientation of the hydroxymethyl group can be determined. Inspection of the models obtained from restrained molecular dynamics shows that the hydroxymethyl group consistently adopts an orientation with the hydroxyl on the 3' side and the methylene protons on the 5' side of the plane of the HmU base. In a previous report the hydroxyl group of a 5'-HmUG-OH-3'

sequence was determined to be on the 5' side of the plane of the ring and a hydrogen bond proposed between the HmU and G residues (29). Indeed both intra- and interresidue hydrogen bonding was possible depending on the nature of the HmU base pair (29). It should be noted that in the HmU-DNA of the study presented here orientation the hydroxyl group is too far from its own O4 carbonyl for any intrasidue hydrogen bonding in all HmU residues. The potential for interresidue hydrogen bonding, however, appears to be sequence-dependent. Structural analysis of the 5'-CHmUAC-OH-3' and 5'-GHmUG-OH-3' sequences do not indicate interresidue hydrogen bonding between neighboring base pairs. The orientation of the hydroxyl group in 5'-GHmUAG-OH-3' sequence, however, enables intrastrand interresidue hydrogen bonding to the neighboring adenine N7 (Fig. 5). Presence of the hydroxyl group and intrastrand hydrogen bond formation would most likely render the interstrand base pair hydrogen bonding associated with the HmU residue less energetically favorable. This may cause the instability of the HmU-A base pairs apparent in the data presented here and result in a local potential flexibility of the DNA proposed to influence the affinity of TF1 for HmU-containing DNA, as discussed above.

In conclusion, we find that both the HmU-DNA and T-DNA establishes a conformation with sugar puckers in the C2'endo configuration (typical of B-DNA). In HmU-DNA the average orientation of the hydroxymethyl group consistently places the oxygen on the 3' side of the plane of the base in all HmU base pairs. The orientation of the hydroxyl group in the 5'-GHmUAG-OH-3' sequences is positioned such that intrastrand internucleotide hydrogen bonding is possible between the hydroxyl proton and the neighboring adenine N7. In addition, there is evidence of instability of A-HmU basepairing resulting in additional fraying, and a temperature-induced transition to a conformation in which the A-HmU base pairs are disrupted or have reduced lifetimes.

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