Structural features at extra thymidine bulge sites in DNA duplexes have been elucidated from a two-dimensional NMR analysis of through-bond and through-space connectivities in the otherwise self-complementary (d(C-C-G-T-G-A-A-T-T-C-T-C-G-G)) (GTG 13-mer) and (d(C-C-G-G-A-A-T-T-C-T-C-G-G)) (CTC 13-mer) duplexes in aqueous solution. These studies establish that the extra thymidine flanked by guanosines in the GTG 13-mer duplex is in a conformational equilibrium between looped-out and stacked states. The looped-out state is favored at low temperature (0 °C), whereas the equilibrium shifts in favor of the stacked state at elevated temperatures (35 °C) prior to the onset of the duplex-strand transition. By contrast, the extra thymidine flanked by cytidines in the CTC 13-mer duplex is looped out independent of temperature in the duplex state. Our results demonstrate that temperature and flanking sequence modulate the equilibrium between looped-out and stacked conformations of single base thymidine bulges in DNA oligomer duplexes.

Early optical and photochemical research on synthetic RNAs established that extra pyrimidines loop out into solution (1-3). The application of high resolution NMR techniques to nucleic acid oligomer duplexes has provided structural information at the individual base pair level in solution (4, 5) and is readily applicable to pyrimidine bulge containing duplexes. The earliest NMR study established that an extra cytidine in an A-C-A segment loops out of a DNA oligomer duplex (6). These studies were extended to hydrogen-exchange NMR measurements to monitor the migration of a cytidine bulge along a (G)n-(C)n DNA mutational hot spot tract (7). More recent NMR studies on an extra cytidine in a G-C-G segment detected a temperature dependent conformational equilibrium between looped-out (predominant at low temperature) and stacked (predominant at elevated temperature) states at the cytidine bulge site in the duplex state (8). Thus, the conformation at the cytidine bulge site appears to depend on the sequence of the flanking base pairs and temperature.

NMR studies on thymidine/uridine bulges show additional interesting and unexpected conformational features when comparing DNA and RNA oligomers of the same sequence. For identical sequences, the extra thymidine stacks into the DNA helix (9), whereas the extra uridine loops out of the RNA helix (10) at room temperature. It appears that the nature of the helix (B form for DNA and A form for RNA) shifts the equilibrium between stacked and looped out conformations.

Systematic footprinting studies have also been undertaken on bulges in the stem of RNA hairpins (11), demonstrating that structural perturbations are propagated away from the bulge site.

We report below on an NMR study of thymidine bulges as a function of flanking sequence and temperature in self-complementary tridecanucleotide duplexes. This paper compares the structural features of thymidine bulges flanked by guanosines in the G-T-G segment of the otherwise self-complementary (d(C-C-G-T-G-A-A-T-T-C-T-C-G-G)) duplex (designated GTG 13-mer, Scheme 1) and of thymidine bulges flanked by cytidines in the C-T-C segment of the otherwise self-complementary (d(C-C-G-G-A-A-T-T-C-T-C-G-G)) duplex (designated CTC 13-mer, Scheme 2). The extra thymidine at the bulge site is designated TX in Schemes 1 and 2. Our results establish that the flanking residues play an important role in defining the conformation of pyrimidine bulge sites which contrasts with our earlier demonstration that the conformation of purine bulges are independent of flanking sequence in solution (12).

**EXPERIMENTAL PROCEDURES**

**Oligonucleotide Synthesis**—The oligodeoxyribonucleotides (d(C-C-G-G-A-A-T-T-C-T-C-G-G)) and (d(C-C-G-G-A-A-T-T-C-T-C-G-G)) were synthesized by the solution phase phosphotriester approach (13) and deprotected by treatment with 1,1,2,2-tetramethylethylguanidium-syn-2-nitrobenzaldoximate (14), concentrated aqueous ammonia, and finally acetic acid-water. The deprotected DNA was purified by ion exchange high performance liquid chromatography using a Partisil SAX column followed by desalting on a column of Sephadex G25. The oligomer was finally converted to the sodium form by passage through a short column of Dowex 50X8 sodium form cation exchange resin and then lyophilized.

**Sample Preparation**—The NMR spectra of the GTG 13-mer and CTC 13-mer duplexes (250 APmnrn units) were recorded in 0.1 M NaCl, 10 mM phosphate, 1 mM EDTA solutions of either 99.96% D2O or 90% H2O/10% D2O (v/v).

**NMR Experiments**—One- and two-dimensional proton data sets on the thymidine bulge 13-mer duplexes were collected on a Bruker AM 500 spectrometer. Proton chemical shifts are referenced relative to internal sodium 3-trimethylsilyl-(2,2,3,3-d4)-propionate. One- and two-dimensional 121.5 MHz phosphorus data sets on the adenosine...
bulge 13-mer duplexes were collected on a Bruker AM 300 spectrometer. Phosphorus chemical shifts are referenced relative to internal trimethyl phosphate.

Phase-sensitive two-dimensional NOESY\(^*\) spectra of the thymidine bulge 13-mer duplexes in \(\text{H}_2\text{O}\) buffer were collected using quadrature detection and a 120-ms mixing time. A jump-return solvent suppression pulse (15, 16) was used for the detection pulse. The preparation and mixing pulses were reduced to 70° (6.0 μs) diminishing the amount of transverse magnetization due to the solvent at the end of the mixing period. This allowed the jump-return acquisition pulse to achieve greater solvent suppression such that the receiver gain could be increased by at least a factor of two, enhancing the sensitivity of the experiment. The waiting time between the jump and return pulses was optimized for even excitation of the imino, amino, and aromatic protons (65 μs). The carrier frequency was centered on the \(\text{H}_2\text{O}\) resonance, which was irradiated with the decoupler channel. The data sets were collected with 512 \(t_1\) experiments using 1024 complex data points in the \(t_2\) dimension and 32 or 64 scans/\(t_1\) increment. The real and imaginary data sets were collected sequentially and merged to yield 225 complex \(t_1\) increments. The free induction decays were apodized with a 90° shifted sine bell function to reduce truncation and 3-Hz line broadening to improve signal-to-noise in both dimensions. A fifth order polynomial base-line correction was applied in both dimensions.

Phase-sensitive two-dimensional NOESY spectra of the thymidine bulge 13-mer duplexes in \(\text{D}_2\text{O}\) buffer were collected with a repetition delay of 1.5 s, a sweep width of 5000 Hz, and a mixing time of 250 ms. The carrier frequency was positioned on the residual HOD signal. The time domain data sets consist of 1024 complex data points over a sweep width of 10,000 Hz collected over 450 \(t_1\) experiments with a 1.0-s repetition delay and 256 scans/increment. The real and imaginary data sets were collected sequentially and merged to yield 225 complex \(t_1\) increments (17). The free induction decays were apodized with a 90° shifted sine bell function to reduce truncation and 3-Hz line broadening to improve signal-to-noise in both dimensions. A fifth order polynomial base-line correction was applied in both dimensions.

Both dimensions were zero-filled to 1024 complex data points prior to Fourier transformation. The \(t_2\) dimension was apodized by gaussian multiplication of -1.0 Hz and 0.04 broadening. The \(t_1\) dimension was apodized with a 90° shifted sine bell to reduce truncation and 1-Hz line broadening for improved signal-to-noise.

Data Processing—The one- and two-dimensional data sets were processed using PTNRMR software\(^1\) on a VAX 11-780 and a MicroVax II. Streaking in the \(t_2\) dimension was reduced using the \(t_1\) noise reduction routine (20) prior to plotting on either a Zeta 882 plotter or a Hewlett Packard 7475A plotter for analysis, assignment, and publication figures.

RESULTS

GTG 13-mer Duplex

Exchangeable Protons—The imino proton spectra (10.0–14.5 ppm) of the GTG 13-mer duplex in \(\text{H}_2\text{O}\) buffer are plotted as a function of temperature at \(\text{pH} 6.34\) in Fig. 1A and as a function of \(\text{pH}\) at \(-5^\circ\) C in Fig. 1B. The hydrogen-bonded imino protons within base paired regions resonate between 12 and 14 ppm, whereas the imino proton of the unpaired thymidine at the bulge site resonates at ~11 ppm (Figs. 1, A and B).

Expanded regions of the NOESY (120 ms mixing time) spectrum of the GTG 13-mer duplex in \(\text{H}_2\text{O}\) buffer, \(\text{pH} 6.0, 0^\circ\) C are shown as contour plots in Fig. 2. A of Fig. 2 focuses on the distance connectivities among the imino protons and the base and amino protons (4.5–9.0 ppm). The thymidine imino protons exhibit NOE\(\text{s}\) to the adenine \(\text{H}\_2\text{N}\) and amino protons in the \(\text{A}5\text{-T}8\) and \(\text{A}6\text{-T}7\) base pairs, whereas the guanosine imino protons exhibit NOE\(\text{s}\) to the cytidine amino protons in the \(\text{C}1\text{-G}12\), \(\text{C}2\text{-G}11\), \(\text{G}3\text{-C}10\), and \(\text{G}4\text{-C}9\) base pairs. An analysis of the NOE connectivities

\(^1\) The abbreviation used is: NOESY, nuclear Overhauser effect spectroscopy.

\(^2\) D. Hare, unpublished results.
Fig. 2. Expanded phase-sensitive NOESY contour plots (120 ms mixing time) establishing distance connectivities in the symmetrical imino proton region (10.5–14.5 ppm) (A) and between the imino protons (10.5–14.5 ppm) and the base and amino protons (4.5–9.0 ppm) in the GTG 13-mer duplex on 0.1 M NaCl, 10 mM phosphate, H2O, pH 6.0, at 0 °C (B).

The assignments of cross-peaks A to D in A are as follows: NOEs are detected between the imino protons of adjacent G7 and G8 (peak A), adjacent G3 and G4 (peak B), and adjacent G9 and G11 (peak D). The chemical shift of the imino proton of G3 is indicated by a box on the diagonal. The assignments of cross-peaks A to Y in B are as follows: The 14.01-ppm imino proton of T8 exhibits NOE cross-peaks to the H2 proton of A5 and amino protons of A5/A6 (peaks H/F/I). The 13.86-ppm imino proton of T7 exhibits NOE cross-peaks to the hydrogen-bonded amino proton of A5 (peak C) and to the exposed amino protons of A5/A6 (peaks H/I). The 13.26-ppm imino proton of G6 exhibits NOE cross-peaks to the hydrogen-bonded amino proton of G5 (peak P) and to the exposed amino protons of G5/A6 (peaks H/I). The 12.97-ppm imino proton of G5 exhibits NOE cross-peaks to the hydrogen-bonded amino proton of G4 (peak N) and exposed (peak O) amino protons and the H5 proton (peak P) of C10. The 12.61-ppm imino proton of G4 exhibits NOE cross-peaks to the hydrogen-bonded amino proton (peak Q) and exposed (peak S) amino protons and the H5 proton (peak P) of C9 and to the H2 proton of A5 (peak R). Strong exchange cross-peaks with the solvent H2O are detected for the imino protons of G12 (peak V) and TX (peak Y), whereas weak exchange cross-peaks are detected for the imino protons of G3 (peak W) and G4 (peak X).

Table I

| Base pair | Exchangeable proton chemical shifts, °C | ppm |
|----------|----------------------------------------|-----|
|          | C1.G12                                | 13.26 7.91 7.08 |
|          | C2.G11                                | 13.26 8.87 7.10 |
|          | G3.C10                                | 12.97 8.52 7.01 |
| TX.C9    | 10.96                                 | 12.61 8.27 6.80 |
| A5.C8.0  | 14.01                                 | 7.36 5.92 7.15 |
| A6.C7.7  | 13.86                                 | 7.14 6.10 7.64 |

Amino protons.

Base pair Nonsymmetrical imino proton chemical shifts, 25 °C

| Base | H8 | H8 | H6 | H5/CH1 | H1' | H2' | H2'' | H3' |
|------|----|----|----|--------|-----|-----|------|-----|
| C1   | 7.76 5.97 6.01 2.03 2.50 4.66 4.12 |
| C2   | 7.48 5.73 5.59 1.94 2.26 4.81 4.08 |
| G3   | 7.87 | 5.93 2.59 2.73 5.03 4.33 |
| TX   | 7.29 1.54 6.09 2.08 2.57 4.71 4.05 |
| G4   | 6.80 1.57 5.95 2.01 2.59 4.84 4.23 |
| A5   | 8.11 | 6.01 2.57 2.92 5.02 4.43 |
| A6   | 8.10 7.71 | 6.20 2.57 2.93 5.00 4.47 |
| G7   | 7.14 1.28 5.95 2.01 2.59 4.84 4.23 |
| C9   | 7.47 6.66 6.10 1.00 2.37 4.92 4.20 |
| C10  | 7.47 5.73 5.49 1.88 2.21 4.83 4.05 |
| G11  | 7.86 | 5.63 2.65 2.69 4.96 4.31 |
| G12  | 7.84 | 6.17 2.57 2.55 4.66 4.21 |

- 0.1 M NaCl, 10 mM phosphate, H2O, pH 6.0.
- Hydrogen-bonded amino protons.
- Exposed amino protons.
- 0.1 M NaCl, 10 mM phosphate, D2O, pH 6.5.

Observed within each base pair coupled with those NOEs observed to protons of adjacent base pairs provides the specific resonance assignments (Table I) and cross-peak assignments (caption to Fig. 2). These exchangeable proton assignments were obtained following analysis of the NOESY spectrum of the GTG 13-mer duplex acquired in H2O solution.

An NOE cross-peak is detected between the imino protons of G3 and G4 (peak C, Fig. 2A) which flank the extra thymidine, TX, in the d(G3-TX-G4)-d(C9-C10) segment of the GTG 13-mer duplex at 0 °C. The imino proton of TX exchanges quite rapidly with the solvent during the 120-ms mixing time as evidenced by the strong exchange cross-peak with H2O (peak Y, Fig. 2B) and the absence of a diagonal resonance (see box, Fig. 2A). The absence of an NOE between the imino proton of TX and the imino protons of G3 and G4 has been confirmed by one-dimensional NOE experiments, as is the presence of an NOE between the imino protons of G3 and G4 in the GTG 13-mer duplex. Both one- and two-dimensional NOE experiments also establish that the CH2 group of TX, like its imino proton, does not exhibit NOEs to the imino proton of flanking G3-C10 and G4-C9 base pairs in the GTG 13-mer duplex at 5 °C. The exchangeable proton resonance assignments in the GTG 13-mer duplex in H2O buffer, pH 6.0 at 0 °C are listed in Table I.

Once the imino proton assignments have been determined, the effects of temperature and pH at the individual base pair level can be monitored through the imino proton chemical shifts and line widths. Specifically, the imino protons of TX and the terminal C1.G12 base pair broaden out above 20 °C, followed by the onset of broadening of the imino protons of the C2.G11 and G3.C10 base pairs by 40 °C in the GTG 13-mer duplex at pH 6.34 (Fig. 1A). The temperature dependence of the imino proton chemical shifts of TX, G3, and G4 centered about the bulge site in the GTG 13-mer duplex are plotted in Fig. S1A (see Miniprint Section). The imino proton of TX broadens significantly on raising the pH from 5.74 to 7.82, whereas the imino protons of the base pairs are unaffected for the GTG 13-mer duplex at 5 °C (Fig. 1B).

Two-dimensional NOESY spectra of the GTG 13-mer in H2O were also recorded at ambient temperature. The rapid imino proton exchange with H2O successfully competes with the aid of a standard magnifying glass. Full size photocopies are included in the text. Portions of this paper (including Figs. S1-S4) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

Table I

Proton chemical shifts in the GTG 13-mer duplex

| Base pair | Exchangeable proton chemical shifts, °C | ppm |
|----------|----------------------------------------|-----|
|          | C1.G12                                | 13.26 7.91 7.08 |
|          | C2.G11                                | 13.26 8.87 7.10 |
|          | G3.C10                                | 12.97 8.52 7.01 |
| TX.C9    | 10.96                                 | 12.61 8.27 6.80 |
| A5.C8.0  | 14.01                                 | 7.36 5.92 7.15 |
| A6.C7.7  | 13.86                                 | 7.14 6.10 7.64 |

- 0.1 M NaCl, 10 mM phosphate, H2O, pH 6.0.
- Hydrogen-bonded amino protons.
- Exposed amino protons.
- 0.1 M NaCl, 10 mM phosphate, D2O, pH 6.5.
the NOE at this temperature thereby reducing or eliminating
the intensity of many of the cross peaks preventing any
fruitful analysis.

Nonexchangeable Protons—The nonexchangeable protons
in the GTG 13-mer duplex have been monitored in NOESY
(250 ms mixing time) spectra recorded in D$_2$O buffer, pH 6.5
between 5 and 33 °C. An expanded region of the NOESY
spectrum establishing distance connectivities between the
base protons (7.0-8.4 ppm) and the sugar H1' and cytidine
H5 protons (5.2-6.4 ppm) at 5 °C is contour-plotted in Fig.
3A. Cross-peaks are detected between the base (purine H8 or
pyrimidine H6) protons and their own and 5'-sugar H1'
protons, and the observed directionality (21) permits the
chain to be traced from C1 to G12. Several breaks in the
connectivities occur where NOEs are absent between the H6
of TX and the H1’ of G3, between the H8 of G4 and the H1’
of TX, and between the H8 of G11 and the H1’ of C10 in the
5 °C data set (Fig. 3A). These breaks are detected primarily
at the G3-TX-G4 segment centered about the thymidine bulge
site. The same expanded NOESY contour plot for GTG 13-
mer data at 33 °C is shown in Fig. 3B. The breaks detected in
the connectivities at 5 °C are retained at 33 °C with additional
disruptions corresponding to the absence of NOEs between
the H8 of G3 and the H1’ of C2 and also between the H6 of
C2 and the H1’ of G1 at the higher temperature (Fig. 3B).
Severe spectral overlap prevents the monitoring of the poten-
tial NOE between the H6 of C9 and the H5 of C10 within the
C9-C10 step opposite the bulge site in the GTG 13-mer duplex
recorded at 5 °C (Fig. 3A) and 33 °C (Fig. 3B).

An important difference is detected between the NOESY
spectra of the GTG 13-mer duplex recorded at 5 and 33 °C.
An NOE is observed between the H8 of G4 and the H1’
of G3 in the G3-TX-G4 segment at 5 °C (peak G, Fig. 3A) which
is absent in the 33 °C spectrum (Fig. 3B).

The NOESY data sets have also been recorded at short
mixing time (50 ms) on the GTG 13-mer duplex at 5 and
33 °C. The quality of the short mixing time NOESY data sets
are marginal at 33 °C compared with their counterparts at
5 °C. Expanded regions establishing distance connectivities
from the base protons to the sugar H1’ protons and to the
sugar H2’,2” protons are plotted in Figs. S2A and S3A,
respectively (Miniprint Section) for the 5 °C data set.

The sugar H1’, H2’2”, H3’, and H4’ protons within each
nucleotide in the GTG 13-mer duplex can be linked via bond
connectivities in correlated two-dimensional data sets. The
expanded region of the COSY spectrum establishing the cou-
pling connectivities between sugar H1’ (5.2-6.4 ppm) and
sugar H2’,2” (1.6-3.2 ppm) protons in the GTG 13-mer
duplex in D$_2$O at 25 °C is plotted in Fig. 5A. The H1’-H2’
cross-peaks are upfield of the H1’-H2’ cross-peaks except
for an inversion at G3 and terminal G12 residues.

An expanded region of the magnitude RELAY COSY spec-
trum of the GTG 13-mer duplex in D$_2$O buffer at 25 °C is
plotted in Fig. 5B. The cross-peaks grouped on the left side
 correspond to the four-bond relay connectivities between the
sugar H1’ and H3’ protons, whereas the cross-peaks grouped on
the right side corresponding to the three-bond connectiv-
ties between the sugar H3’ and H4’ protons (Fig. 5B). We do
not detect a four bond relay cross-peak between the H1’
and H4’ of C9 nor do we detect a three-bond cross-peak between
the H3’ and H4’ of the same residue in the GTG 13-mer
duplex at 25 °C.

The regions of the NOESY spectrum (250 ms mixing time)
establishing the distance connectivities between the base pro-
tons and the sugar H2’,2” protons similarly lack NOEs
between the H6 proton of TX and the H2’,2” protons of G3
and between the H8 protons of G4 and the H2’,2” protons
of TX in the G3-TX-G4 segment at 5 °C (Fig. 4A) and 33 °C
(Fig. 4B). NOEs are also absent between the H6 proton of
TX and the H8 protons of either G3 or G4 at both 5 and
33 °C.

Fig. 3. Expanded phase-sensitive NOESY (250 ms mixing
time) contour plots establishing distance connectivities be-
tween the base protons (7.0-8.2 ppm) and the sugar H1’
and cytidine H5 protons (5.4-6.3 ppm) in the GTG 13-mer
duplex in 0.1 M NaCl, 10 mM phosphate, D$_2$O, pH 6.5, at 5 °C (A) and
33 °C (B). The cross peaks between the H8 and H6 protons of
cytidine are designated by asterisks. The labeled NOE cross-peaks
are listed below. A, C1(H6) to C2(H5); B, T8(H6) to C9(H5); C,
A6(H2) to T7(H1’); D, A6(H2) to T6(H1’); E, A5(H2) to C9(H1’);
F, A5(H2) to A6(H1’); G, G4(H8) to G3(H1’).

Fig. 4. Expanded phase-sensitive NOESY (250 ms mixing
time) contour plots establishing distance connectivities be-
tween the base protons (7.0-8.2 ppm) and the sugar H2’,2”
and thymidine CH3 protons (1.2-3.0 ppm) in the GTG 13-mer
duplex in 0.1 M NaCl, 10 mM phosphate, D$_2$O, pH 6.5, at 5 °C
(A) and 33 °C (B). The cross-peaks between the H6 and CH3
protons of thymidine are designated by asterisks. The labeled cross-
peaks correspond to NOEs between base and their own sugar H2’,2”
protons in the (G3-TX-G4)-(C9-C10) segment.
Thymidine Bulge-containing Deoxytridecanucleotide Duplexes

L---R-J;

6.4 6.0 5.6

H1' H3' H4'

FIG. 5. Expanded magnitude contour plots establishing coupling connectivities in the GTG 13-mer duplex in 0.1 M NaCl, 10 mM phosphate, D2O, pH 6.5, at 25 °C. A, COSY plot between sugar H1' protons (5.2-6.4 ppm) and the sugar H2',2'' protons (1.8-3.0 ppm). B, RELAY-COSY plot between sugar H1', H3', and H4' protons (4.0-6.4 ppm) and sugar H3' and H4' protons (4.0-5.4 ppm).

The above analysis of the NOESY, COSY, and RELAY COSY data sets of the GTG 13-mer duplex in D2O at 25 °C yield the nonexchangeable base and sugar proton chemical shifts listed in Table I.

Phosphorus Spectra—The phosphorus spectrum of the GTG 13-mer in D2O buffer at 25 °C is plotted in Fig. 6A. The majority of the phosphorus resonances are dispersed between −3.9 and −4.4 ppm with one phosphorus resonating downfield at −3.77 ppm. The phosphorus spectrum has been assigned by correlating the phosphorus resonances to the known sugar H3' and H2',2'' proton resonances in a phosphorus (observed)-proton COSY experiment. The contour plot (positive levels only) is shown in Fig. 6B and permits a complete assignment of the phosphorus spectrum of the GTG 13-mer duplex (Table II). A slice taken through the −3.77 ppm phosphorus resonance exhibits through bond correlations to the H2', H3', and H4' protons of G3 and the H4' proton of TX (Fig. 6C) permitting assignment to the G3-TX step.

CTC 13-mer Duplex

Exchangeable Protons—The imino proton spectra of the CTC 13-mer duplex in H2O buffer as a function of temperature at pH 6.42 is plotted in Fig. 7A and as a function of pH at −5 °C is plotted in Fig. 7B. The base-paired imino protons have been assigned following an analysis of the NOESY (120 ms mixing time) spectrum of the CTC 13-mer duplex in H2O buffer, pH 6.0, at 0 °C (Fig. 8, for assignments see the figure legend). The imino protons of the C1-G12, C2-G11, and G3-C10 base pairs are superpositioned at 13.15 ppm, whereas the imino proton of G4-C9 resonates upfield at 12.53 ppm. An NOE is detected between the superpositioned imino protons at 13.15 ppm and the 12.53-ppm imino proton of G4 (peak C, Fig. 8B), whereas no NOEs are detected between these imino protons and the imino proton of the extra thymidine which

FIG. 6. The phosphorus spectrum (A) is recorded above the contour plot. B, Two-dimensional heteronuclear phosphorus (observed)-proton COSY contour plot of the GTG 13-mer duplex, 0.1 M NaCl, 10 mM phosphate, D2O, pH 6.5, at 25 °C. Only positive contour levels are recorded for clarity. C, a slice through the −3.77 ppm phosphorus resonance establishing direct and relay connectivities to the sugar protons in the 05' and 03' directions.

TABLE II

| Phosphate | P | H3' | H4'' | H4' | H1''' | H4'''' |
|-----------|---|-----|------|-----|-------|-------|
| ppm       |   | 3'- | 3''- |     | 5'-   | 5''-  |
| C1-C2     | −4.05 | 4.67 | 4.11 | 2.02 | 2.48  |
| C2-G3     | −4.88 | 4.80 | 4.09 | 1.93 | 2.25  | 5.58  |
| G1-TX     | 3.77  | 5.02 | 2.67 | 2.73 | 4.07  | 4.21  |
| TX-G4     | −4.16 | 4.75 | 4.05 | 2.07 | 2.30  |
| G4-A5     | −4.31 | 4.85 | 2.40 | 2.62 | 4.43  |
| A5-A6     | −4.35 | 5.02 | 4.43 | 4.49 |       |
| A6-T7     | −4.35 | 5.02 | 4.45 | 4.40 |       |
| T7-T8     | −4.40 | 4.85 | 4.25 | 4.25 |       |
| T8-C9     | −4.30 | 4.90 | 4.19 | 2.20 | 2.58  | 4.19  |
| C9-C10    | −4.09 | 4.82 | 1.98 | 2.34 | 4.05  |
| C10-G11   | −3.86 | 4.80 | 4.05 | 1.86 | 2.22  | 5.58  | 4.31  |
| G11-G12   | −3.92 | 4.97 | 4.31 | 2.62 |       | 4.20  |

* 03'-linked sugar proton correlations.
* 05'-linked sugar proton correlations.
resonate at 11.13 ppm (Fig. 8A). These observations were independently confirmed by one-dimensional NOE experiments where the extra thymidine imino and C3 protons do not exhibit distance connectivities to the imino protons of flanking G3-C10 and G4-C9 base pairs.

The exchangeable proton chemical shifts in the CTC 13-mer duplex in D2O buffer, pH 6.2, at 5 and 25 °C have been identified following an analysis of the magnitude COSY (Fig. 11A) and the mag.

**Fig. 7.** Imino proton spectra (10.0–14.5 ppm) of the CTC 13-mer duplex in 0.1 M NaCl, 10 mM phosphate, H2O, A, temperature dependence between -5 and 40 °C at pH 6.42. B, pH dependence between pH 5.83 and pH 7.88 at -5 °C.

**Fig. 8.** Expanded phase-sensitive NOESY contour plots (120 ms mixing time) establishing distance connectivities in the symmetrical imino proton region (10.5 to 14.5 ppm) (A) and between the imino protons (10.5–14.5 ppm) and the base and amino protons (4.5–9.0 ppm) in the CTC 13-mer duplex in 0.1 M NaCl, 10 mM phosphate, H2O, pH 6.0 at 0 °C (B). The assignments of cross-peaks A–C in A are as follows: NOEs are detected between the imino protons of adjacent T7 and T8 (peak A), adjacent G4 and T8 (peak B), and adjacent G3 and G4 (peak C). The chemical shift of the imino proton of TX is indicated by a box on the diagonal. The assignments of cross-peaks A–Y in B are as follows: The 13.58-ppm imino proton of T8 exhibits NOE cross-peaks to the H2 protons of A6 (peak C) and A6 (peak A) and to the hydrogen-bonded amino protons of A5 (peak F) and A6 (peak E) and exposed amino protons of A5 (peak D) and A6 (peak D). The 13.67-ppm imino proton of T7 exhibits NOE cross-peaks to the hydrogen-bonded amino protons of A5 (peak F) and A5 (peak G) and to the hydrogen-bonded amino protons of A6 (peak K) and A6 (peak H) and exposed amino protons of A6 (peak J) and A6 (peak I). The 13.11-ppm imino proton of G11 exhibits NOE cross-peaks to the hydrogen-bonded amino protons and to the H5 proton (peak M) of C2. The 13.11-ppm imino proton of G3 exhibits NOE cross-peaks to the hydrogen-bonded amino protons and to the hydrogen-bonded amino protons of A5 (peak N) and exposed amino protons of A5 (peak O) and exposed amino protons of A5 (peak P) and exposed amino protons of C9 and to the H2 proton of A5 (peak K). The cross-peak I may correspond to an NOE between amino and imino protons of G4. Strong exchange cross-peaks with the solvent H2O are detected for the imino protons of G12 (peak V) and TX (peak Y), whereas a weak exchange cross-peak is detected for the imino protons of G4 (peak X).

**Fig. 8.** Expanded phase-sensitive NOESY contour plots (120 ms mixing time) establishing distance connectivities in the symmetrical imino proton region (10.5 to 14.5 ppm) (A) and between the imino protons (10.5–14.5 ppm) and the base and amino protons (4.5–9.0 ppm) in the CTC 13-mer duplex in 0.1 M NaCl, 10 mM phosphate, H2O, pH 6.0 at 0 °C (B). The assignments of cross-peaks A–C in A are as follows: NOEs are detected between the imino protons of adjacent T7 and T8 (peak A), adjacent G4 and T8 (peak B), and adjacent G3 and G4 (peak C). The chemical shift of the imino proton of TX is indicated by a box on the diagonal. The assignments of cross-peaks A–Y in B are as follows: The 13.58-ppm imino proton of T8 exhibits NOE cross-peaks to the H2 protons of A6 (peak C) and A6 (peak A) and to the hydrogen-bonded amino protons of A5 (peak F) and A6 (peak E) and exposed amino protons of A5 (peak D) and A6 (peak D). The 13.67-ppm imino proton of T7 exhibits NOE cross-peaks to the hydrogen-bonded amino protons of A5 (peak F) and A5 (peak G) and to the hydrogen-bonded amino protons of A6 (peak K) and A6 (peak H) and exposed amino protons of A6 (peak J) and A6 (peak I). The 13.11-ppm imino proton of G11 exhibits NOE cross-peaks to the hydrogen-bonded amino protons and to the H5 proton (peak M) of C2. The 13.11-ppm imino proton of G3 exhibits NOE cross-peaks to the hydrogen-bonded amino protons and to the hydrogen-bonded amino protons of A5 (peak N) and exposed amino protons of A5 (peak O) and exposed amino protons of A5 (peak P) and exposed amino protons of C9 and to the H2 proton of A5 (peak K). The cross-peak I may correspond to an NOE between amino and imino protons of G4. Strong exchange cross-peaks with the solvent H2O are detected for the imino protons of G12 (peak V) and TX (peak Y), whereas a weak exchange cross-peak is detected for the imino protons of G4 (peak X).
TABLE III
Proton chemical shifts in the CTC 13-mer duplex

| Base pair | Exchangeable proton chemical shifts, 0 °C |
|-----------|------------------------------------------|
|           | ppm                                      |
| G3-C10   | 13.15 7.89 7.03                          |
| C6-T5    | 13.13 8.40 7.00                          |
| TX-G4    | 11.13 8.43 6.88                          |
| A6-T10   | 13.93 5.84 7.26                          |
| A5-T8    | 13.87 5.84 7.26                          |

Nonexchangeable proton chemical shifts, 25 °C

| Base | H4 | H2 | H6 | H5/CH3 | H1' | H2' | H2'' |
|------|----|----|----|--------|-----|-----|------|
| C1   | 7.71| 5.93| 5.06| 2.00   | 2.45| 4.62| 4.10 |
| C2   | 7.50| 5.72| 5.54| 2.02   | 2.34| 4.78| 4.09 |
| G3   | 7.77|     |     |        |     |     |      |
| G4   | 7.73|     |     |        |     |     |      |
| A5   | 8.16| 7.36|     |        |     |     |      |
| A6   | 8.12| 7.62|     |        |     |     |      |
| T7   | 7.10| 1.90| 5.68| 1.66   | 2.53| 4.81| 4.31 |
| T8   | 7.27| 1.56| 6.11| 2.11   | 2.46| 4.89| 4.17 |
| C9   | 7.53| 5.69| 6.11| 2.55   | 3.59| 4.93| 4.16 |
| C10  | 7.51| 5.76| 6.03| 1.43   | 2.20| 4.64| 4.24 |
| G11  | 7.87|     |     |        |     |     |      |
| G12  | 7.94|     |     |        |     |     |      |

* 0.1 M NaCl, 10 mM phosphate, H2O, pH 6.0.
* Exposed amino protons.
* Hydrogen-bonded amino protons.
* 0.1 M NaCl, 10 mM phosphate, D2O, pH 6.2.

Fig. 9. Expanded phase-sensitive NOESY (250 ms mixing time) contour plots establishing distance connectivities between the base protons (7.0–8.2 ppm) and the sugar H1' and cytidine H5 protons (5.2–6.4 ppm) in the CTC 13-mer duplex in 0.1 M NaCl, 10 mM phosphate, D2O, pH 6.2, at 5 °C (A) and 25 °C (B). The cross-peaks between the H6 and H5 protons of cytidine are designated by asterisks. The labeled NOE cross-peaks are listed below. A, C1(H6) to C2(H5); B, T5(H6) to C9(H5); C, A6(H2) to T7(H1'); D, A6(H2) to T8(H1'); E, A5(H2) to C9(H1'); F, A5(H2) to A6(H1').

Fig. 10. Expanded phase-sensitive NOESY (250 ms mixing time) contour plots establishing distance connectivities between the base protons (7.0–8.2 ppm) and the sugar H2',2'' and thymidine CH3 protons (1.2–3.0 ppm) in the CTC 13-mer duplex in 0.1 M NaCl, 10 mM phosphate, D2O, pH 6.2, at 5 °C (A) and 25 °C (B). The cross-peaks between the H6 and CH3 protons of thymidine are designated by asterisks. The labeled cross-peaks correspond to NOEs between base and their own sugar H2',2'' protons in the C3-G4.(C9-TX-C10) segment.

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FIG. 11. Expanded magnitude contour plots establishing coupling connectivities in the CTC 13-mer duplex in 0.1 M NaCl, 10 mM phosphate, D_2O, pH 6.2, at 25 °C. A, COSY plot between sugar H_1' protons (5.2-6.4 ppm) and the sugar H_2',2'' protons (1.4-3.0 ppm). B, RELAY-COSY plot between sugar H_1', H_3', and H_4' protons (4.0-6.4 ppm) and sugar H_3' and H_4' protons (4.0-5.4 ppm).

ciated with the strand to duplex transition and then exhibit temperature-independent chemical shifts between 40 and 5 °C in the duplex state (Fig. 13).

The CH_3 protons of TX in the GTG 13-mer shift upfield on lowering the temperature from 70 to 40 °C but then shift dramatically downfield on lowering the temperature from 40 to 5 °C (Fig. 13A). By contrast, the CH_3 protons of TX in the CTC 13-mer exhibit a temperature-independent chemical shift between 70 and 5 °C (Fig. 13B).

DISCUSSION

Our previous NMR study focused on single adenosine bulges in the GAG 13-mer and the CAC 13-mer duplexes which concluded that the extra adenosine stacks into the duplex independent of temperature and flanking sequence (12). The current study focuses on the role of flanking sequence and temperature on the conformation of thymidine bulge sites in the GTG 13-mer and CTC 13-mer duplexes. The bases flanking the thymidine bulge were chosen to be either both purines or both pyrimidines to cover the two extremes.

Sequence-dependent Stability of the Duplex—The CH_3 proton of T8 located towards the center of the GTG 13-mer and CTC 13-mer duplexes provides a convenient monitor of the relative stabilities of the two self-complementary duplexes through the helix-coil transition. The GTG 13-mer duplex (T_m = 47.5 °C) is more stable than the CTC 13-mer duplex (T_m = 42.5 °C) in the 0.1 M NaCl solution (Fig. S4) emphasizing the importance of the residues flanking the thymidine bulge site to the stability of the entire helix. The same trend is observed in the temperature-dependent line width behavior of the imino protons, where the imino protons of the CTC 13-mer duplex, pH 6.42 (Fig. 7A), are broadened to a greater extent at a given elevated temperature than the same imino

FIG. 12. The phosphorus spectrum (A) is recorded above the contour plot. B, two-dimensional heteronuclear phosphorus (observed)-proton COSY contour plot of the CTC 13-mer duplex, 0.1 M NaCl, 10 mM phosphate, D_2O, pH 6.2, at 25 °C. Only positive contour levels are plotted for clarity. C, a slice through the -3.73-ppm phosphorus resonance establishing direct and relay connectivities to the sugar protons in the 05' and 03' directions.

### TABLE IV

| Phosphate | P 3' H3' H4' H2' 2'' H1' H4'' | Chemical shift (ppm) |
|-----------|-------------------------------|---------------------|
| C1-C2     | -4.03 4.64 4.11 2.01, 2.46    | 5.98                |
| C2-G3     | -3.89 4.79 4.06 2.02, 2.34    |                    |
| G3-G4     | -4.12 4.92                     | 2.55                |
| G4-A5     | -4.09 4.98 2.55, 2.66          | 4.44 4.20          |
| A5-A6     | -4.93 5.07 4.47                | 4.96                |
| A6-T7     | -4.58 5.01                     | 4.18                |
| T7-T8     | -4.47 4.80 4.18 1.96, 2.54     | 4.07                |
| T8-C9     | -4.26 4.89 4.18 2.11, 2.46     | 4.18                |
| C9-TX     | -3.73 4.93 4.17 2.34           | 4.29 4.11           |
| TX-C10    | -3.89 4.82 4.29 2.34, 2.51     | 6.28 4.24           |
| C10 G11   | -3.88 4.66 4.24 1.43, 2.30     | 6.64 4.29 4.06      |
| G11-G12   | -3.78 4.93 2.62                | 4.20 4.05           |

* 03'-linked sugar proton correlations.
* 05'-linked sugar proton correlations.
protons of the GTG 13-mer duplex, pH 6.34 (Fig. 1A).

The greater stability of the GTG 13-mer relative to the CTC 13-mer duplex parallels the earlier result of greater stability of the GAG 13-mer relative to the CAC 13-mer duplex (12). Thus, purines flanking an extra base are more stabilizing than pyrimidines flanking an extra base in the interior of DNA oligomer duplexes.

**Conformational Equilibria in Duplex State**—The temperature dependence of the chemical shift of the CH₃ protons of TX in the temperature range (5–40 °C) prior to the onset of the melting transition provides an excellent marker for monitoring the potential changes in the conformational equilibria at the bulge site in the duplex state. The CH₃ proton of TX in the GTG 13-mer duplex exhibits a chemical shift at 5 °C which is very close to its strand value (Fig. 13A) indicating that the extra thymidine is looped out of the helix. By contrast, this CH₃ resonance shifts upfield by 0.2 ppm on raising the temperature to 35 °C (Fig. 13A), indicating that the extra thymidine is stacked into the GTG 13-mer duplex and experiencing the ring current contributions to the chemical shift from the flanking GC pairs. The H6 proton of TX similarly shifts upfield on raising the temperature from 5 °C (Fig. 13A) to 35 °C (Fig. 13B) in the NOESY spectra of the GTG 13-mer duplex. These results indicate that the extra thymidine equilibrates between a looped-out conformation predominant at low temperature (5 °C) and a stacked conformation predominant at elevated temperatures (40 °C) in the GTG 13-mer duplex prior to the onset of the melting transition.

In striking contrast, the CH₃ protons of TX in the CTC 13-mer duplex exhibit an essentially temperature-independent chemical shift between 5 and 65 °C (Fig. 13B). Thus, the similarity in the chemical shift of the CH₃ protons of TX in the strand state (5 °C) and the duplex state (5–35 °C) indicates that TX is looped-out both at low (5 °C) and elevated (35 °C) temperatures prior to the onset of the melting transition. This conclusion is further supported by the identical chemical shift of the H6 proton of TX at 5 °C (Fig. 13A) and at 25 °C (Fig. 13B) in the expanded NOESY spectra of the CTC 13-mer duplex.

The results of these studies on the GTG 13-mer and CTC 13-mer duplexes establish the importance of flanking sequence and temperature in defining the conformation at thymidine bulge sites. A previous NMR study on an extra thymidine located in a G-T-G segment established that this thymidine stacked into the DNA oligomer duplex at ambient temperature (9). Our research predicts that this thymidine will loop out of the duplex if the studies (9) are extended to low temperature.

**Right-handed Helices with Watson-Crick Pairing**—The local and global features of the GTG 13-mer and CTC 13-mer duplexes are reflected in the observed NOE and chemical shift parameters. The NOEs detected between thymidine imino and adenosine H2 protons establish Watson-Crick A-T pairing, whereas the observed NOEs between guanosine imino and cytidine amino protons establish Watson-Crick G-C pairing in the extra thymidine 13-mer duplexes (Figs. 2B and 8B). Furthermore, intact G3-C10 and G4-C9 base pairs are detectable flanking the extra thymidine sites for the GTG 13-mer and CTC 13-mer duplexes (Figs. 1 and 6).

The observed directionality of the intrastrand NOEs between base protons and their own and 5′-flanking sugar H2, H2′, and H3′ protons establish that the GTG 13-mer (Fig. 3) and the CTC 13-mer (Fig. 9) form right-handed duplexes in solution. In addition, all glycosidic torsion angles in base-paired regions are anti (interproton separation ~3.7 Å), since the base-to-sugar H1′ NOE cross-peaks are much weaker than the H5 to H6 cytidine NOE cross-peaks (fixed 2.45 Å separation) in the GTG 13-mer (Fig. S2A) and CTC 13-mer (Fig. S2B) NOESY spectra at short (50 ms) mixing times recorded at 5 °C.

These results establish anti-glycosidic torsion angles with Watson-Crick pairing for all base pairs along the entire length of right-handed GTG 13-mer and CTC 13-mer duplexes.

**Thymidine Imino Proton Exchange**—We observe faster exchange rates at the imino proton of the extra thymidine compared with the thymidines paired with adenosines in A-T base pairs in the GTG 13-mer and the CTC 13-mer duplexes at low temperature. This is reflected in the observed increase in line width of the extra thymidine imino proton with increasing pH in both the GTG 13-mer duplex (Fig. 1A) and the CTC 13-mer duplex (Fig. 7B) at ~5 °C. Further support for this conclusion follows from the absence of a diagonal peak (boxed region) and the presence of an exchange cross-peak with solvent H2O (peak Y) for the extra thymidine imino proton in NOESY plots of the GTG 13-mer duplex (Fig. 2) and the CTC 13-mer duplex (Fig. 8) at 0 °C. The absence of the diagonal peak reflects complete chemical exchange of the thymidine imino proton with solvent H2O during the 120-ms mixing time period of the NOESY experiment. Hydrogen exchange studies on thymidine imino protons have established previously that exchange lifetimes for internal A·T pairs are on the order of 250 ms at pH 6.5 and 0 °C (22, 23). Thus, the thymidine imino protons in A·T base pairs are observable along the diagonal in the 120-ms mixing time NOESY spectra of the two duplexes, whereas the extra thymidine imino proton whose exchange lifetime is much shorter than 120 ms is not detected.

**Thymidine Bulge Site in the GTG 13-mer Duplex**—The temperature-dependent chemical shift data of the CH₃ protons of TX in the GTG 13-mer indicate a predominant looped-out conformation for TX at 5 °C and a predominant stacked conformation for TX at 35 °C with rapid equilibrium between these forms at intermediate temperature values. The thymidine CH₃ of TX shifts as a narrow average resonance over the 0.2-ppm chemical shift separation so that the exchange rate of the extra thymidine between the looped-out and stacked states is much greater than 600 s⁻¹ [k >>2π(ΔΔ)].

Both conformations exhibit interruptions in the NOE connectivities between base protons and their 5′-flanking sugar H1′ protons in the G3-TX and TX-G4 steps in the GTG 13-mer duplex (Fig. 3, A and B). Furthermore, both conformations show no NOE between the H8 proton of G3 and the H5...
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Thymidine Bulge Sites in CTC 13-mer Duplex—Several lines of evidence establish that the extra thymidine loops out of the CTC 13-mer duplex at low temperature. The chemical shift of the CH$_{3}$ protons of TX at 5 °C are similar to their value in the strand state at 65 °C (Fig. 13B). An NOE is detected between the imino protons of the G3-C10 and G4-C9 base pairs (peak C, Fig. 8) but not between these protons and the imino protons of TX in the d(G3-G4)-d(C9-TX-C10) segment.

The looped-out conformation at the thymidine bulge site is retained on raising the temperature prior to the onset of the melting transition. Thus, the CH$_{3}$ (Fig. 13B) and H6 chemical shifts of TX are independent of temperature in the range 5–35 °C and identical to their values at 65 °C. Further evidence for the looped-out conformation of TX in the CTC 13-mer at elevated temperatures is based on NOESY data collected at 25 °C. An NOE is detected between the H5 proton of C10 and the H9′,2′′ protons of C9 at the C9-TX-C10 bulge site, whereas no NOEs are detected between adjacent protons in the pyrimidine H6 (3′–5′) pyrimidine H5 C9-TX and TX-C10 step at 25 °C.

The structural perturbation of the helix at the looped out bulge site is reflected in the absence of NOEs between the base H6 proton and its 5′-flanking sugar H1′ proton for the C9-TX and TX-C10 steps in both 5 °C (Fig. 9A) and 25 °C (Fig. 9B). This perturbation is also reflected in the large chemical shift differences at the H2′,2′′ protons of C10 but not C9 in the CTC 13-mer duplex at 25 °C (Fig. 11A) and at 5 °C.

Measurement of volume integrals in the 50-ms NOESY spectrum of the CTC 13-mer duplex at 5 °C establishes that the H6–H1′ NOE cross-peak of TX exhibits an intensity that is approximately 0.12 that of the H6–H5 of cytidines (Fig. S2B). This translates qualitatively into TX adopting a glycosidic torsion angle weighted towards the anti-orientation in the CTC 13-mer at 5 °C. A similar measurement could not be undertaken at higher temperature since the signal-to-noise was weak in the 50 ms NOE plot of the CTC 13-mer duplex at 25 °C.

Comparative Conformational Features—The phosphorus spectra of the GTG 13-mer and the CTC 13-mer duplexes exhibit resonances downfield of the −4.0 to −4.5 ppm spectral dispersion characteristic of unperturbed duplexes. The most downfield phosphorus resonance in the GTG 13-mer duplex is assigned to the G3-TX phosphodiester linkage (Fig. 6), whereas the most downfield phosphorus resonance in the CTC 13-mer duplex is assigned to the C9-TX phosphodiester linkage (Fig. 12). The observed perturbation in the phosphodiester linkage 5′ to the TX residue in both duplexes coupled with the break in the base-sugar proton distance connectivities centered about the TX site in both duplexes demonstrates that the major perturbation in the helix occurs on the strand containing the thymidine bulge site in both the GTG 13-mer and CTC 13-mer duplexes.

The temperature dependence of the TX imino proton chemical shifts can be monitored in the GTG 13-mer (Fig. 6) and the CTC 13-mer (Fig. 12) duplexes. The TX imino proton in the GTG 13-mer undergoes a larger upfield shift than its counterpart in the CTC 13-mer with increasing temperature. This is consistent with stacking contributions to the chemical shift for TX in the GTG 13-mer as the equilibrium shifts from a looped-out to a stacked state at the bulge site with increasing temperature.

By contrast, the imino proton of G3 shifts upfield with increasing temperature between −5 and 35 °C in the CTC 13-mer duplex (Fig. S1B) but is perturbed to a lesser extent in the GTG 13-mer duplex (Fig. S1A). This observation suggests a greater disruption of the G3-C10 base pair in the CTC 13-mer duplex compared to the GTG 13-mer duplex with increasing temperature. This instability of the G3-C10 base pair is further reflected in the broad line width of the G3 imino proton resonance (peaks N and O, Fig. 7) in the CTC 13-mer duplex relative to the line width of the G3 imino proton resonance (peaks N and O, Fig. 2) in the GTG 13-mer duplex.

We have no definitive explanation for the absence of a few cross-peaks in the Relay COSY experiment for the GTG 13-mer duplex (Fig. 5B) and the CTC 13-mer duplex (Fig. 11B). This could reflect changes in sugar pucker but the absence of individual cross-peaks do not provide definitive insights into sugar conformation.

We have not attempted to measure qualitative estimates of glycosidic torsion angles and/or sugar puckers based on NOEs between base and sugar H1′, H2′,2′′ protons on the same and 5′-flanking residues due to the overlap of the NOE cross-peaks for residues centered about the lesion site.
Summary—The present NMR studies on the role of flanking sequence and temperature on the conformation of thymidine bulge sites contrasts dramatically with related studies on the conformation of adenosine bulge sites at the DNA oligomer level in aqueous solution. The earlier studies established that an extra adenosine stacks into the duplex independent of flanking sequence and temperature in aqueous solution (12). By contrast, a greater conformational variability is detected for pyrimidine bulge sites. The present NMR study on DNA oligomers demonstrates that for extra thymidines flanked by purines, the pyrimidine base is looped out at low temperature but stacks into the duplex at elevated temperature prior to the onset of the melting transition. By contrast, extra thymidines flanked by pyrimidines are looped out independent of temperature in the duplex state. These observations and the demonstration of a subtle balance between a stacked extra thymidine in B-DNA (9) and a looped out extra uridine in A-RNA (10) at room temperature establish that a range of parameters modulate the equilibrium between stacked and looped states at pyrimidine bulge sites in solution.

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Supplementary Material to
Conformational Transitions in Thymidine Bulge-containing Deoxytridecanucleotide Duplexes:
Role of flanking sequences and temperature in modulating the equilibrium between looped and stacked thymidine bulge states.

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Figure 31: Temperature dependence of the thymine proton chemical shifts of TX and flanking G/C pairs in (A) the GTG 13-mer duplex, pH 6.3 and (B) the CTC 13-mer duplex, pH 6.42 in 0.1M NaCl, 10mM phosphate, pH 7. The shift value is expressed as the difference between 25°C and 40°C.

Figure 32: Expanded thymine proton NMR (50 ms mixing time) contour plots showing distance correlations between the base protons (4.8 to 6.2 ppm) and the sugar H1' and cytidine H3 protons (5.4 to 6.3 ppm) in (A) the CTC 13-mer duplex, pH 6.5, 5°C and (B) the CTC 13-mer duplex, pH 6.2, 5°C in 0.1M NaCl, 10mM phosphate, pH 7.
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Figure S8. Temperature dependence of the CH chemical shifts of T8 in the parent 12-mer (C1), GTG 13-mer (A) and CTC 13-mer (C) in 0.5M NaCl, 10mM phosphate, pH 7.

Figure S9. Temperature dependence of the (CH) chemical shifts of T8 in the parent 12-mer (C1), GTG 13-mer (A) and CTC 13-mer (C) in 0.5M NaCl, 10mM phosphate, pH 7, between 5°C and 60°C.
Conformational transitions in thymidine bulge-containing deoxytridecanucleotide duplexes. Role of flanking sequence and temperature in modulating the equilibrium between looped out and stacked thymidine bulge states.

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