Stabilities of Nucleotide Loops Bridging the Pyrimidine Strands in DNA Pyrimidine·Purine·Pyrimidine Triplexes: Special Stability of the CTTTG Loop†

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ABSTRACT: Recent studies of DNA hairpin loops have shown considerable dependence of the stability on the sequence of the loop [Senior, M., Jones, R. A., & Breslauer, K. J. (1988a) *Proc. Natl. Acad. Sci. U.S.A. 85,* 6242–6246; Xodo, L. E., Manzini, G., Quadrifoglio, F., van der Marel, G., & van Boom, J. H. (1989) *Biochimie 71,* 793–803; Hirao, I., Nishimura, Y., Tagawa, Y., Watanabe, K., & Miura, K. (1992) *Nucleic Acids Res. 20,* 3891–3896]. Analogous studies have not, until now, been carried out with loops in triple helices. We report the results from experiments in which we examine the relative stabilities of pentanucleotide loops that bridge between the pyrimidine strands in DNA pyr·pur·pyr triple helices. There are two types of loops that are defined by the relative orientation of the purine strand: a 5′-loop and a 3′-loop. The sequences examined in this study are the bimolecular triplexes formed between 5′-dTTCTTTTCL$_1$TTTL$_5$-CTTTTCTT (loop nucleotides are underlined, and L_1 and L_5 represent varied nucleotides) and the two purine strands, 5′-dAAGAAAAG-3′ and 5′-dGAAAAGAA-3′. The first and last nucleotides in the loop are varied, since stacking interactions may be strongest at these positions [Senior et al., 1988a; Senior, M., Jones, R. A., & Breslauer, K. J. (1988b) *Biochemistry 27,* 3879–3885], and we examine 14 sequence combinations for each loop type. Thermal denaturation studies carried out at pH 7.0 indicate considerable variation in the stabilities of these loops. In the 5′-loop series, the strongest complex is formed with the loop having the sequence 5′-CTTTG, with a T_m of 35.1 °C and a free energy (37 °C) of –8.2 kcal mol^{-1}. The least stable complex has the loop sequence 5′-CTTTC, with a T_m of 27.1 °C and a free energy of –4.8 kcal mol^{-1}. Similarly, in the 3′-loop series, the strongest complex is formed with the loops having the sequence 5′-CTTTG, with a T_m of 32.2 °C and a free energy of –6.7 kcal mol^{-1}. The least stable complex has the loop sequence 5′-CTTTC, with a T_m of 25.2 °C and a free energy of –5.0 kcal mol^{-1}. Several conclusions are drawn from the 28 different cases studied: (1) 5′-loops are somewhat more stable than 3′-loops, with an average difference of 0.4 kcal in free energy; (2) purines in both types of loop and at both positions are stabilizing, by an average of 0.5–0.9 kcal each, relative to pyrimidines (presumably, this is due to more efficient base stacking); (3) placement of a purine on the 5′-end of the helix is more favorable than on the 3′-end by an average of 0.6 kcal, thus favoring YTTTR loops over RTTTY loops (this is consistent with the stacking preferences seen for duplex DNA); (4) there is a special stability present in the cases where the first and last bases are complementary in the Watson–Crick sense, suggesting possible pairing interactions across the loop. This interaction adds as much as –0.8 kcal of stability to the 3′-end and –2.1 kcal to the 5′-end, relative to a C–A mismatched case, and thus leads to relatively high stability for complexes with TTTTA and especially those with CTTTG loops. A model for this pairing interaction is proposed.

Recent studies of DNA triple-helical structures have elucidated much about the thermodynamics and kinetics of base pairing in these complexes (Pilch et al., 1990; Plum et al., 1990; Rougée et al., 1992). The most commonly studied form, the termolecular triple helix, is formed when a synthetic oligodeoxynucleotide is targeted to a specific site in duplex DNA (Moser & Dervan, 1987; Le Doan et al., 1987). A few of the more recent studies have focused on the formation of triple helices from two strands of DNA. Such bimolecular triplexes are seen in the structure of H-DNA (Mirkin et al., 1987; Kohwi & Kohwi-Shigematsu, 1988; Shimizu et al., 1989; Hanvey et al., 1989; Htun & Dahlberg, 1989; Belotserkovskii et al., 1990). H-DNA forms from partially unwound duplex DNA, in which one pyrimidine-rich strand (in the pyrimidine motif) folds back to form Hoogsteen-type hydrogen bonds with a purine-rich stretch in the duplex. H-DNA structures can form in vivo and are proposed to be involved in transcriptional regulation and in replication (Shimizu et al., 1989; Ussery & Sinden, 1993). Studies have been carried out on the structural requirements for H-DNA formation and on base pairing strengths and specificities in the triplex (Belotserkovskii et al., 1990; Shimizu et al., 1989).

An important part of the H-DNA structure is the loop that bridges the outer pyrimidine strands, but there are few reports to date of experiments directed toward loop structural requirements. In one report (Shimizu et al., 1989), the effect of loop size was investigated, and it was shown that a loop size of four nucleotides was preferred over six, eight, or ten nucleotides. In a study of related bimolecular triplexes, we found (Prakash & Kool, 1992) that the intermediate loop size of five nucleotides was preferred over four, six, or ten nucleotides, at least for the sequences studied.

In addition to the H-DNA complex, bimolecular triple helices are also formed in a new motif for nucleic acid recognition, in which single strands are targeted by triplex formation (Kool, 1991; Giovannangeli et al., 1991). In this

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approach, two pyrimidine domains are connected on one or both ends by loops. To our knowledge, no report of investigations on the sequence dependence of the stability of triplex-bridging loops exists in the literature, either for synthetic complexes or for H-DNA structures.

In order to investigate systematically the effects of loop sequence on the stabilities of bimolecular triplexes, we constructed 14 model triplex hairpin-forming oligodeoxynucleotides in which pentanucleotide loops bridge the pyrimidine strands. The first and fifth nucleotides were varied to investigate sequence effects, and we found that relatively large differences in stability occurred with changes in only these two nucleotides. The results have implications in the relative stabilities of H-DNA sequences and in the design of bimolecular and unimolecular triplexes in general.

EXPERIMENTAL PROCEDURES

Oligonucleotide Synthesis. DNA oligonucleotides were synthesized on a Pharmacia LKB automated synthesizer using standard β-cyanoethyl phosphoramidite chemistry (Beaucage & Caruthers, 1981). The oligomers were purified by preparative 20% denaturing polyacrylamide gel electrophoresis and quantitated by absorbance at 260 nm. Molar extinction coefficients for the oligomers were calculated by the nearest-neighbor method (Borer, 1985).

Thermal Denaturation Studies. Solutions for the thermal denaturation studies contained a 1:1 ratio of variable-sequence pyrimidine oligomer and the complementary purine oligomer (1.0 μM each). Solutions were buffered with 10 mM Na·PIPES (1,4-piperazinediethanesulfonate, Sigma) at pH 7.0. This buffer was chosen because of the low dependence of its pK_a on temperature relative to other Good buffers (Good et al., 1966). The buffer pH is that of a 500 mM stock solution at 25 °C; after dilution, the final solution pH was shown to be within 0.1 unit of that of the buffer stock. Also present were 100 mM NaCl and 10 mM $MgCl_2$. After the solutions were prepared, they were heated to 90 °C and allowed to cool slowly to room temperature prior to the melting experiments.

The melting studies were carried out in Teflon-stoppered 1-cm path length quartz cells under a nitrogen atmosphere on a Varian Cary 1 UV–vis spectrophotometer equipped with a thermoprogrammer. Absorbance (260 nm) was monitored while temperature was raised from 5.0 to 50 °C at a rate of 0.5 °C/min. In nearly all cases the complexes displayed sharp, apparently two-state transitions, with monophasic melting from bound complex to free oligomers. Melting temperatures (T_m) are reported as the midpoint of transition, as determined by a computer fit of the first derivative of absorbance with respect to $1/T$. Uncertainty in T_m is estimated at less than ±0.5 °C on the basis of repetition of experiments.

Each reported value for T_m and free energy is an average of 2–4 separate experiments; the repeated T_m values generally agreed within 0.5 °C, and the uncertainty in the averaged value is estimated at ±0.3 °C. Free energy values (37 °C) were derived by computer-fitting the denaturation data using the two-state approximation for melting (Petersheim & Turner, 1983). Fits were excellent, with χ^2 values of 5×10^{-6} or better. The uncertainty in individual free energy measurements is estimated at ±10–15%.

RESULTS

Sequence and Structure Considerations. The present studies were designed to model interactions that occur in a number of different bimolecular and unimolecular triple helices. Figure 1 shows illustrations of several of these triplex forms. In all cases, the outer pyrimidine strands in the triplex are bridged by nucleotide loops. In some of them, the central purine strand continues beyond the loop, which may result in some direct interactions with nucleotides in the loop. In this study, we first sought to measure the intrinsic stabilities of the bridging loops in the absence of an extended central strand. Later studies were then carried out to investigate specific interactions between the loop and an extended central strand (Booher et al., 1994).

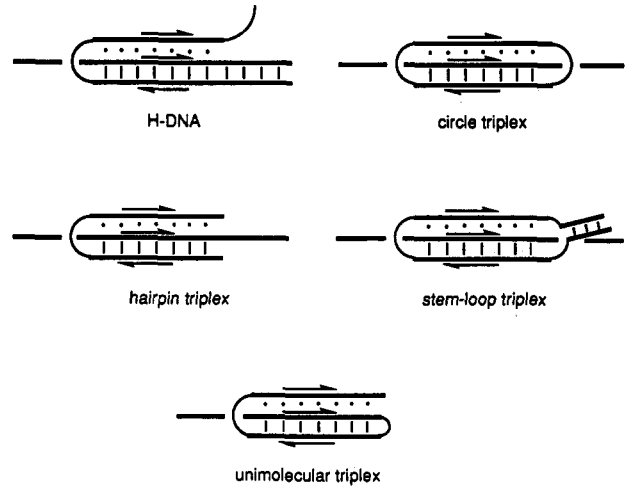
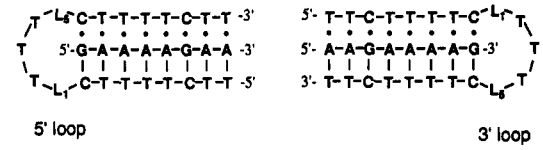


FIGURE 1: Illustrations of four types of bimolecular pyr·pur·pyr triple helices and one unimolecular triplex (arrows denote 5′ to 3′ strand orientation, dots illustrate Hoogsteen bonding, and short lines show Watson–Crick bonding). All of these structures require bridging of the outer pyrimidine domains by a loop, which may be located at either the 5′-end or 3′-end of the complex, as defined by the orientation of the strand containing the purine domain.



Loop sequences:

L_1	L_5	L_1	L_5	L_1	L_5	L_1	L_5
A	A	C	A	G	G	T	A
A	C	C	C	G	C	T	C
A	T	C	G	G	T	T	G
		C	T			T	T

FIGURE 2: DNA sequences used in this study. L_1 and L_5 denote the two varied nucleotides in the loop domains. The 21-nucleotide pyrimidine oligomers bind the sequence 5′-dGAAAAGAA by forming a hairpin-type triplex with a 5′-bridging loop, and they bind the sequence 5′-dAAGAAAAG by forming a triplex with a 3′-bridging loop.

The sequences constructed for these studies are shown in Figure 2. All 14 21-mer oligodeoxynucleotides can bind by triplex formation to two eight-nucleotide purine sequences: 5′-dAAGAAAAG and the sequence-reversed 5′-dGAAAAGAA. When bound to the first target, the five loop nucleotides are situated at the 5′-end of the purine strand, and when bound to the reverse, the loop is situated at the 3′-end of the purine strand. All pyrimidine-rich sequences that form such bimolecular triplexes can bind a given purine sequence or its reverse, simply by exchanging the roles of the Hoogsteen and Watson–Crick domains. In the present case, this bimodal binding allows testing and comparison of the various loop sequences at both ends without construction of twice the number of oligodeoxynucleotides.

Table 1: Melting Transition Temperatures and Free Energies of Complexation by Hairpin Triplex-Forming 21-mer Oligodeoxynucleotides with 5′-dGAAAAGAA (5′-Loop Series) and 5′-dAAGAAAAG (3′-Loop Series), as a Function of the First (L_1) and Fifth (L_5) Nucleotides in the Loop

	5′-loop		3′-loop	
L_1,L_5	T_m (°C)	$-\Delta G°_{37}$ (kcal mol^{-1})	T_m (°C)	$-\Delta G°_{37}$ (kcal mol^{-1})
A,A	31.3	7.1	28.6	5.9
A,C	27.9	5.0	26.2	5.3
A,T	27.7	5.8	26.4	5.5
C,A	29.7	5.3	28.2	5.5
C,C	27.1	4.8	25.2	5.0
C,G	35.1	8.2	32.2	6.7
C,T	26.0	5.7	25.1	5.2
G,C	30.7	6.3	29.4	5.6
G,G	32.8	7.1	31.6	6.7
G,T	30.4	6.3	27.1	5.7
T,A	32.2	7.0	28.8	6.3
T,C	27.0	5.5	25.4	5.5
T,G	29.7	5.8	25.3	5.7
T,T	26.9	5.6	24.9	5.5

With five-nucleotide loops, there are 1024 possible sequences for a loop at a given end of the helix. To limit the possibilities for this study, we varied only the first and fifth nucleotides (shown as L_1 and L_5 in Figure 2), keeping the second, third, and fourth positions constant with the sequence TTT. We chose to vary these two positions initially because base stacking effects might be expected to be strongest at positions directly adjacent to the helix. In addition, it was anticipated that the interactions of an extended purine strand with these two positions might be greatest (Booher et al., 1994). We initially focused on sequence combinations in which at least one pyrimidine was present; when experiments began to indicate that purines added extra stability, we extended the study to include the L_1,L_5 combinations G,G and A,A as well.

Results of Thermal Denaturations. Thermal denaturation studies were carried out at pH 7.0 with the 14 21-mer sequences and the two 8-mer target purine strands. Each experiment was carried out 2–4 times, with the T_m and free energy values being averaged for each sequence (Table 1). The complexes were also measured at pH 5.5 (data not shown), and as expected for DNA triplexes involving C-G-C triads, the complexes are stronger at this lower pH (Xodo et al., 1990). Similar sequence-dependent results were seen, and so we chose to pursue the bulk of the experiments at the biologically more relevant higher pH value. Results show (Table 1) that there is a considerable dependence of stability on changes in only these two nucleotides, even though they are not directly involved in triplex pairing. The T_m's range from 24.9 to 35.1 °C, and the free energies vary by as much as 3.4 kcal overall.

Figure 3 gives a plot of T_m versus free energy data for the complexes and allows a ranking of the complexes by T_m or by $-\Delta G°_{37}$. Results show that similar conclusions would be reached using either value, with a roughly linear correlation between them. Of particular note is the isolated point at the upper right, which is the complex where L_1 and L_5 are C and G, respectively, in the 5′-loop. This is the strongest complex by a wide margin. Among the next most stable group are complexes having (L_1,L_5) of (5′G,G), (5′A,A), (5′T,A), (3′G,G), and (3′C,G). The weakest complexes in free energy terms are found when both L_1 and L_5 are C. Considering the 28 stability measurements in total, several correlations can be drawn from the varied nucleotides involved (Table 2).

Differences between 5′-Loops and 3′-Loops. Comparing the results from the experiments involving a 5′-loop versus a

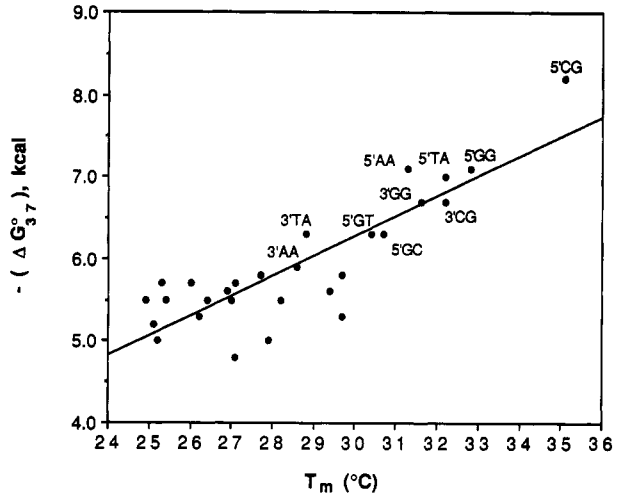


FIGURE 3: Plot of melting temperature (T_m, °C) versus free energy ($-\Delta G°_{37}$, kcal) at pH 7.0, in 100 mM NaCl and 10 mM $MgCl_2$ for the 28 complexes in this study. Selected points are labeled with the corresponding loop sequence (e.g., 5′CG = 5′-CTTTG).

3′-loop (Table 1), we find that in 10 of the 14 cases where the same sequence was studied, the 5′-loop complex is more stable. The remaining four cases give stabilities that are within 0.3 kcal of each other. Taking the total averaged 5′-loop results, we find a T_m of 29.6 °C and a free energy value of –6.1 kcal mol^{-1}. The corresponding 3′-loop values are 27.5 °C and –5.7 kcal mol^{-1}. Thus, for the sequences shown, a complex bridged by a 5′-loop is more stable, on average, by 2.1 °C in T_m and 0.4 kcal in free energy. Since both 5′-loop and 3′-loop complexes have cytosine as the last helix base adjacent to the loops, the nearest neighbors are the same in both complexes, and so we conclude that this difference in stability may arise from the orientation of the purine strand alone.

Effects of Purine Substitution. The strongest complexes found in this study involve loops that contain one or two purines at the first (L_1) and fifth (L_5) positions (see Figure 3). To investigate whether there might be a systematic correlation of stability with purine content, we plotted average free energy versus the number of purines at the L_1 and L_5 positions for both types of loops (Figure 4). The results show that there is a direct, linear correlation of stability with the number of purines. On the 5′-loop end, the linear fit (r^2 = 0.999) gives a slope of 0.87, indicating that, on average, each purine adds 0.9 kcal of stability to the loop. On the 3′-loop end, the fit is equally good (r^2 = 1.000), but the slope is smaller (0.51); on that end, each purine adds an average of 0.5 kcal of stability to the overall complex, relative to pyrimidines.

Effects of Purine Placement. The finding of an advantage for purines in the first and fifth positions of the loops suggests that an advantage in base stacking might be at least partly responsible for this stabilization. Breslauer reported (Senior et al., 1988b) that base stacking in DNA duplexes, as measured by dangling end effects, is greater at the 5′-end of a helix than at the 3′-end. Although the effects may be different in triple helices, we thought it possible that there might be a selective preference for purine placement at either the first (L_1) or fifth (L_5) position, which might arise from differences in base stacking at the 5′- or 3′-end of a strand in the helix. In the complexes studied here, the first (L_1) position is located at the 3′-end of a pyrimidine strand, following a C in the triplex. The fifth (L_5) position is located at the 5′-end of the other pyrimidine strand, preceding a C in the triplex.

When the 16 cases that involve one purine and one pyrimidine at the L_1 and L_5 positions are examined, a

Table 2: Averaged Values for Melting Transitions and Free Energies of Complexation Associated with Structural Motifs in the Loops of the 21-mer Hairpin Triplex-Forming Oligomers

	5′-loop		3′-loop		all loops	
structural feature	T_m (°C)	$-\Delta G°_{37}$ (kcal mol^{-1})	T_m (°C)	$-\Delta G°_{37}$ (kcal mol^{-1})	T_m (°C)	$-\Delta G°_{37}$ (kcal mol^{-1})
number of purines						
0	26.3	5.4	25.2	5.3	25.8	5.4
1	30.4	6.2	28.0	5.8	29.2	6.0
2	32.1	7.1	30.1	6.3	31.1	6.7
5′ or 3′ placement of purine against helix						
5′ stack	31.7	6.6	28.6	6.1	30.1	6.3
3′ stack	29.2	5.9	27.3	5.5	28.3	5.7
complementarity of L_1,L_5						
C,A or A,C	28.8	5.2	27.2	5.4	28.0	5.3
T,G or G,T	30.1	6.1	26.2	5.7	28.2	5.9
T,A or A,T	30.0	6.4	27.6	5.9	28.8	6.2
C,G or G,C	32.9	7.3	30.8	6.2	31.9	6.8

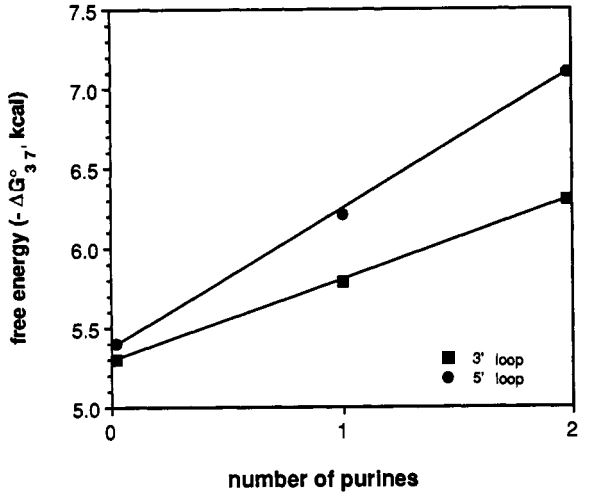


FIGURE 4: Plots of stability ($-\Delta G°_{37}$) versus the number of purines at the first and fifth loop nucleotide positions, averaged from the 14 complexes involving 5′-loops (●) and the 14 complexes involving 3′-loops (■).

significant correlation is found between stability and the placement of the purine (Table 2). When the purine is positioned at the 5′-end of a strand in the helix (the L_5 position), the average T_m is 30.1 °C and the average free energy (37 °C) is –6.3 kcal mol^{-1}. When the orientation is reversed, placing the purine at the 3′-end of the helix, the average values are found to be 28.3 °C and –5.7 kcal mol^{-1}; thus, the total average advantage for 5′ purine placement is 1.8 °C in T_m and 0.6 kcal in free energy. This advantage of the YTTTR loop sequence over the RTTTY sequence is seen in six of the eight sequence combinations for both the 5′- and 3′-loop types. Thus, it would appear that, in this type of DNA triplex, purine stacking may be more favorable on the 5′-end than on the 3′-end of the pyrimidine strands of the triple helix.

Effects of Complementarity between Positions 1 and 5. It is interesting to note that, for the cases involving one purine, the average free energy of complexation is the result of widely varying values (with a range of 3.2 kcal) from individual cases. This is in contrast to the cases involving zero or two purines, which are much closer in value, having ranges of 0.9 or 1.2 kcal, respectively. Closer examination of the single-purine cases shows that there appear to be two distinct levels of stability, depending on the nature of the purine and pyrimidine in the L_1 and L_5 positions (Table 2). For the 5′-loop orientation, the cases in which the two bases are complementary in the Watson–Crick sense have an average T_m of 31.5 °C and a free energy of –6.9 kcal mol^{-1}. When the two bases are not complementary, the stability is significantly lower, with an average T_m of 29.5 °C and a free energy of –5.7 kcal mol^{-1}. For the 3′-loop orientation, a similar effect is seen: complementarity between L_1 and L_5 nucleotides gives a T_m of 29.2 °C and a free energy of –6.5 kcal mol^{-1}, while noncomplementary pairs have values of 28.1 °C and –5.6 kcal mol^{-1}. Thus, on average, Watson–Crick complementarity gives an advantage in T_m of 1.1–2.0 °C and in free energy of 0.9–1.2 kcal mol^{-1}.

If the results are broken down further into specific pairs of nucleotides for the L_1 and L_5 positions (Table 2), the order of decreasing stability is (C,G) > (T,A) ≥ (T,G) > (C,A). This is the same order found for the stabilities of these pairs in Watson–Crick duplex DNA (Aboul-ela et al., 1985; Kool, 1991). This finding leads us to propose that there are specific base pairing interactions between the first (L_1) and fifth (L_5) positions that stabilize the complex when L_1 and L_5 are complementary (see below).

DISCUSSION

Differences between 5′-Loops and 3′-Loops. The stability of the 5′-loop orientation relative to the 3′-loop orientation, a difference of 2.1 °C in T_m and 0.4 kcal mol^{-1} in free energy, is small, but it appears to be significant, since the advantage appears in 10 of the 14 sequence combinations studied. It is unclear at present what the origin of the advantage is; however, it seems possible that the orientation of the central strand affects the base stacking of the L_1 and L_5 bases in the loop. Models indicate (Figure 5) that in the 5′-loop these two nucleotides may be stacked partly over the 5′ base in the central purine strand, whereas in the 3′-loop they are stacked at the 3′-end of the central strand. The result would thus be consistent with the preference for 5′ stacking over 3′ stacking in this triplex and in DNA duplexes in general (Senior et al., 1988b).

While H-DNA may, in principle, occur in two orientations, generating either a 5′-type bridging loop (the H-y3 conformer) or a 3′-bridging loop (the H-y5 conformer), in practice only one of these orientations is found (Shimizu et al., 1989). Interestingly, the orientation observed is that which generates a 5′-bridging loop, indicating that there is an energetic preference for this orientation. This fact is consistent with the findings of the present study, suggesting that the differential loop stability may contribute to the H-DNA preference. With an extended central strand, however, it is unlikely that stacking effects would contribute as much (Booher et al., 1994). Additionally, an energetic difference of 0.4 kcal may not be large enough alone to account for the observed preference in H-DNA, and it seems likely that differential relaxation of

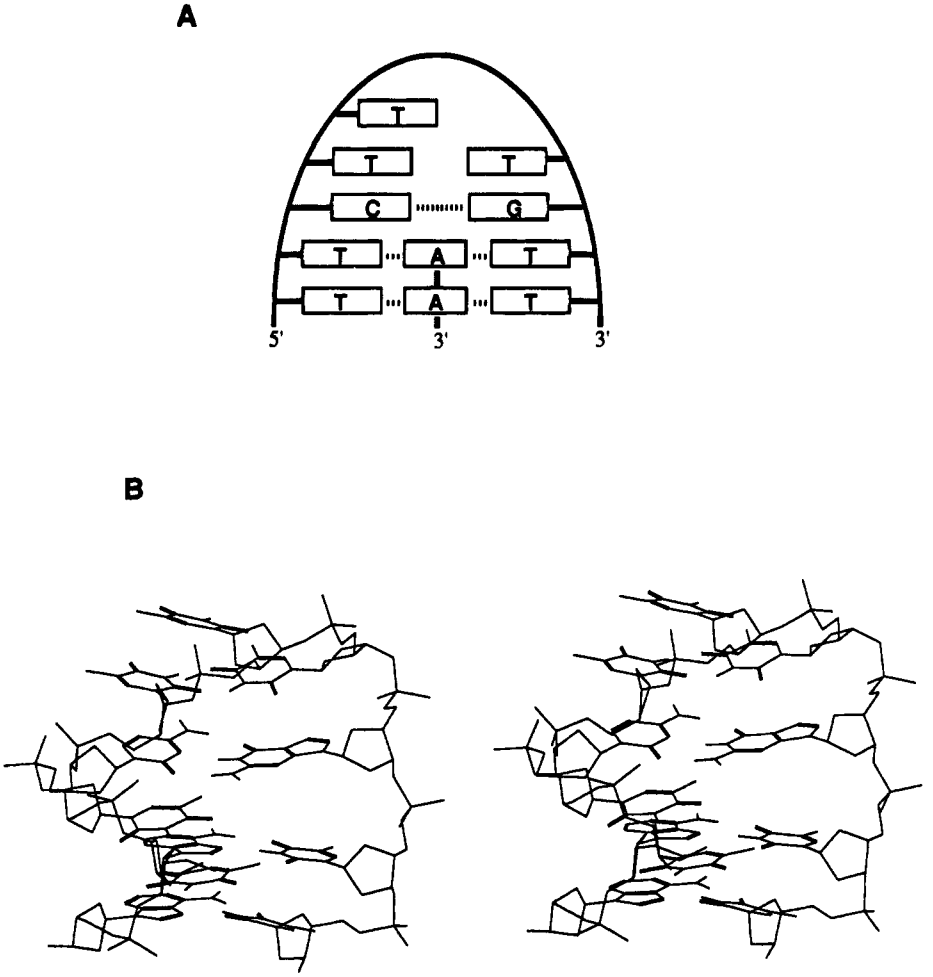


FIGURE 5: Schematic diagram (A) and stereodiagram of a computer-generated model (B) for a short triple helix bridged by a 3,2-stacked CTTTG loop in the 5′ orientation, illustrating the proposed base pairing between the C and G bases. The structure was designed using a published loop model (Harvey et al., 1988) as a guideline and was built and energy-minimized using MacroModel v3.5a (C. Still, Columbia University) with the AMBER force field.

negative supercoiling (Htun & Dahlberg, 1989) may have a larger influence on this selectivity.

Effects of Purine Substitution and Placement. The preference for purines at the first and last positions is quite substantial, and the correlation is surprisingly linear (see Figure 4). The simplest explanation for this observation is that the purines stabilize the complexes by more favorable base stacking interactions. This explanation is consistent with the finding that purines undergo more energetically favorable stacking at the ends of helices in RNA (Turner et al., 1988). The finding of a lower slope for purine substitution in the 3′-loop relative to the 5′-loop may be explained by stronger stacking interactions with the 5′-end of the central purine strand (see below).

The present studies also show a clear preference for the placement of purines at the 5′-end of a pyrimidine strand in the helix rather than at the 3′-end. This effect is consistent with more effective base stacking at this position in the complex (Senior et al., 1988b). A possible alternative explanation to be considered in the present case is that, rather than stabilization by purines, it is the pyrimidines that prefer placement at the 3′-end of helices. However, this is not consistent with the clear overall stabilization seen by purines, regardless of placement, in these complexes. Thus, the simplest explanation is superior 5′-end stacking by purines relative to pyrimidines.

Base Pairing across the Loop. The most stable complexes in the study are those with the loop sequence CTTTG, and the relative order of stability of the loops in general (Figure 3) appears to depend significantly on the complementarity between the first and fifth nucleotides. In fact, complementarity is the single largest factor found in stability, as exemplified by comparison of the CTTTG and CTTTA loops, which differ by only one nucleotide, but differ in stability by 1.2–2.9 kcal mol^{-1}. The apparent preference for purine 5′ placement relative to 3′ placement gives the CTTTG loop a 1.1–1.9 kcal mol^{-1} advantage over the GTTTC loop, and a similar effect is seen for the TTTTA loop relative to the ATTTT loop.

As a result of these experiments, we propose that there are specific pairing interactions between the first and fifth positions in the loop. Models built using the CTTTG loop with a 3,2 stacking arrangement (Harvey et al., 1988) (Figure 5) indicate that close placement between these positions is possible when the bases are placed in an *anti* conformation, which would allow for standard Watson–Crick pairing bridged by a three-base hairpin loop of TTT. Although the relative stabilities of the loops appear to follow standard Watson–Crick pairing stabilities, the present results cannot rule out pairing that involves one or both bases in a *syn* conformation, forming a Hoogsteen, reverse Watson–Crick, or reverse Hoogsteen pair. This pairing interaction does indicate, however, that triplexes and duplexes may exist coaxially without intervening nucleotides to alleviate differences in geometry. The result, while unprecedented, is reasonable considering that the relative

distances between phosphates in duplexes and triplexes are not great, at ~17.4 and 19.8 Å, respectively (Drew et al., 1981; Harvey et al., 1988).

In nucleic acid duplex-bridging loops, special stability has been reported for GNRA and UUCG tetraloops in RNA hairpins (Groebe & Uhlenbeck, 1988; Heus & Pardi, 1991; SantaLucia et al., 1992) and for GAAA tetraloops in DNA hairpins (Hirao et al., 1992). In at least some of these cases, specific noncovalent bonding interactions occur across the loop, and this appears to hold true for the triplex loops in this study as well. Although it remains to be seen in the present case how the three central nucleotides affect the stability of pentanucleotide triplex-bridging loops, the CTTTG triplex loop does have special stability among the loops studied here, thus adding a new stabilizing motif for triplex hairpin loops similar to that seen in duplex hairpins.

CONCLUSIONS

The results show that triplex-bridging loop stabilities in DNA depend considerably on the first and last nucleotides in the loop. In general, 5′-loops are more stable than 3′-loops, possibly providing a partial explanation for the analogous preference seen for H-DNA. In addition, purines in the loop are favored to stack on the 5′-end of a helix more than on the 3′-end. In general, purines are preferred over pyrimidines at all four positions. Finally, there is special stability gained when the first and last nucleotides are complementary in the Watson–Crick sense, with the CTTTG loop showing the greatest stability of all of the loops studied. This special pairing interaction is worth up to 3 kcal of extra stability in these complexes. The result also suggests that, in general, it may be possible for triplexes to form directly adjacent to duplexes without intervening nucleotides.

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