Stability of 3' double nucleotide overhangs that model the 3' ends of siRNA

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ABSTRACT

Thermodynamic parameters are reported for duplex formation in 1 M NaCl for 16 RNA sequences, each containing a core tetramer duplex, GGCC, and a 3' overhang consisting of two bases. The results indicate additional double-helical stability is conferred by the double 3' terminal overhang relative to the single 3' terminal overhang. A nearest-neighbor analysis of the data indicates that the free energy contribution at 37°C of the second base in the double 3' terminal overhang varies from 0 to 0.7 kcal/mol. The second base in the 3' double overhang can contribute nearly the same stability to a duplex as a base pair or a 3' dangling overhang. Stability contribution of a dangling base, two nucleotides removed from the 3' end of a duplex, is dependent upon both the identity of the base as well as that of the dangling base that it neighbors. A second dangling base only increases the stability of the duplex when it is neighboring a 3' purine dangling nucleotide. Furthermore, a second dangling pyrimidine provides a greater contribution to duplex stability than a purine. A nearest-neighbor model was developed to predict the influence of 3' double overhang on the stability of duplex formation. The model improves the prediction of free energy and melting temperature when tested against six sequences with different core duplexes.

Keywords: thermodynamics; 3' dangling end; stability

INTRODUCTION

Single 3' dangling nucleotides have been shown to significantly increase the stability of an RNA duplex (Freier et al. 1983, 1986; Petersheim and Turner 1983; Sugimoto et al. 1987). The geometry of the RNA helix allows for the 3' dangling nucleotide to form stacking interactions with neighboring bases in the helix. For certain 3' overhanging bases, the stabilization is almost equivalent to that observed by some nearest-neighbor interactions. These studies also conclude that 3' dangling purine bases result in higher stabilizations of the core duplex than pyrimidine base overhangs. In this paper, we investigate the stabilization contribution of doubly dangling nucleotides on the 3' end of an RNA core duplex. These results may contribute to further understanding of base stacking interactions and improve predictions of RNA secondary structures.

Double ribonucleotide dangling 3' ends are one of the defining characteristics of small interfering RNAs (siRNAs), which are major components of the RNA interference

(RNAi) pathway, an endogenous post-transcriptional gene silencing mechanism (Fire et al. 1998; Elbashir et al. 2001). Long dsRNAs expressed by viruses, transposons, or endogenous genes within eukaryotic organisms result in siRNAs (Fire et al. 1998; Zamore et al. 2000). A ribonuclease III type enzyme, known as Dicer, converts these long dsRNAs into smaller ~21-nucleotide-long oligonucleotides or siRNAs (Bernstein et al. 2001). The structure of siRNAs is a 19-nt base-paired helix containing two unpaired dangling nucleotides at the 3' end of both strands in the duplex (Elbashir et al. 2001).

One of the two strands of the duplex is assembled into the active RNAi complex and the other is functionally inactive (Khvorova et al. 2003; Schwarz et al. 2003). The selection of the active strand is related to the difference in thermodynamic stability of the two ends of the duplex (Khvorova et al. 2003; Schwarz et al. 2003). In these studies, either the thermodynamic contribution of the 3' dangling nucleotides to the stability of the duplex was ignored (Khvorova et al. 2003) or held constant as two deoxythymidine residues (Schwarz et al. 2003). The results of this study indicate that the second 3' dangling nucleotide significantly affects the stability of the helix and, therefore, should be considered when determining the stability of an RNA helix or helix end.

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RESULTS

To determine the role of the 3' double terminal overhangs on the stability of RNA duplexes, a set of 3' double terminal overhangs were prepared and the thermodynamics of duplex formation measured by optical melting. The GGCC core duplex was chosen because the stability of all the single 3' terminal overhangs have been previously determined (Freier et al. 1983). The measured thermodynamic parameters for all 16 3' double terminal overhangs are presented in Table 1. Thermodynamic parameters were determined using both the melt curve analysis and the T_M dependence models. Data from both models agreed within 10% for all sequences, consistent with the two-state model. Table 1 shows that oligomers with 3' double overhangs, with a purine as the first overhanging nucleotide, are more stable than those containing pyrimidine first dangling nucleotides in the order A>G>U>C, from most to least stable. This trend is similar to that observed for single 3' overhangs (Freier et al. 1983).

In order to determine the stability contributed to the oligomer by the second 3' dangling nucleotide, the differences in the thermodynamic values between the sequences studied and the corresponding sequence containing only the first overhanging nucleotide were also determined for all of the oligomers studied using equations similar to Equation 1.

$$\Delta G_{37}^{\circ} 3'XY = \frac{1}{2}(\Delta G_{37}^{\circ} (GGCCXY)_2 - \Delta G_{37}^{\circ} (GGCCX)_2) \quad (1)$$

The summary of these results is presented in Table 2.

The influence of the second 3' dangling nucleotide on the stability of the duplex can be grouped into three categories based upon the identity of two nucleotides in the overhang. If the first 3' nucleotide is a pyrimidine, no additional stabilization is observed upon the addition of the second 3' dangling nucleotide. When the first nucleotide is a purine, the stacking of the second nucleotide makes a significant contribution to the stability of the duplex. Interestingly, sequences that contain a 3' double nucleotide dangling end with a 5' pur-pyr 3' have greater stability (0.6 kcal/mol on average) than those sequences containing an overhang with 5' pur-pur 3' (0.4 kcal/mol on average).

The results in Table 2 suggest that the influence of the second 3' dangling end is dependent upon the nature (purine vs. pyrimidine) of both the first and second base. The results were combined to develop a simple model to predict the influence of 3' double overhangs on the stability of RNA duplexes. The model is presented in Table 3. If the first 3' terminal mismatch is a pyrimidine, no additional stabilization is necessary. When the first 3' nucleotide is a purine followed by a second 3' terminal pyrimidine, the helix is stabilized by -0.6 kcal/mol. When the 3' double terminal

TABLE 1. Thermodynamic parameters for duplex formation^a

Sequences	Average of curve fits				T_M^{-1} vs. log C_1 plots			
	$-\Delta H^{\circ}$ (kcal/mol)	$-\Delta S^{\circ}$ (eu)	$-\Delta G_{37}^{\circ}$ (kcal/mol)	T_M^b (°C)	$-\Delta H^{\circ}$ (kcal/mol)	$-\Delta S^{\circ}$ (eu)	$-\Delta G_{37}^{\circ}$ (kcal/mol)	T_M^b (°C)
(GGCCAA) ₂	54.4 ± 4.3	143.4 ± 14.1	9.9 ± 0.2	63.3	47.7 ± 3.6	123.1 ± 12.0	9.5 ± 0.2	63.9
(GGCCAG) ₂	52.6 ± 6.2	138.8 ± 18.7	9.6 ± 0.4	61.8	56.4 ± 6.5	150.1 ± 19.5	9.8 ± 0.4	61.5
(GGCCAC) ₂	54.4 ± 3.3	143.2 ± 10.3	10.0 ± 0.2	64.0	56.7 ± 2.0	149.9 ± 6.0	10.2 ± 0.1	63.8
(GGCCAU) ₂	55.3 ± 4.8	146.4 ± 14.5	9.9 ± 0.4	62.7	60.6 ± 3.7	162.2 ± 11.2	10.3 ± 0.3	62.4
(GGCCAp) ₂ ^c	45.5	118	8.8	58.0				
(GGCCGA) ₂	53.3 ± 1.7	141.4 ± 5.4	9.5 ± 0.1	60.7	54.4 ± 1.3	144.6 ± 4.0	9.5 ± 0.1	60.6
(GGCCGG) ₂	52.7 ± 2.3	139.0 ± 7.1	9.6 ± 0.2	61.7	52.2 ± 1.8	137.6 ± 5.6	9.5 ± 0.1	61.6
(GGCCGC) ₂	56.3 ± 2.6	148.9 ± 8.0	10.1 ± 0.1	63.6	58.0 ± 1.0	154.2 ± 3.1	10.2 ± 0.1	63.4
(GGCCGU) ₂	56.1 ± 4.0	148.6 ± 12.1	10.0 ± 0.3	62.9	59.0 ± 3.7	157.5 ± 11.3	10.2 ± 0.3	62.7
(GGCCGp) ₂ ^c	43.5	112	8.8	57.5				
(GGCCUA) ₂	46.3 ± 4.1	123.6 ± 12.8	8.0 ± 0.2	53.3	45.1 ± 1.0	120.1 ± 3.2	7.9 ± 0.1	52.9
(GGCCUG) ₂	47.7 ± 3.5	127.8 ± 11.0	8.0 ± 0.1	53.0	50.1 ± 2.4	135.7 ± 7.7	8.1 ± 0.1	52.5
(GGCCUC) ₂	47.0 ± 2.7	125.9 ± 8.1	8.0 ± 0.2	53.0	50.5 ± 1.7	136.7 ± 5.4	8.1 ± 0.1	52.7
(GGCCUU) ₂	48.5 ± 3.1	130.3 ± 9.6	8.1 ± 0.2	53.0	48.0 ± 1.3	128.9 ± 3.9	8.0 ± 0.1	52.8
(GGCCUp) ₂ ^c	43.6	115	7.8	51.5				
(GGCCCA) ₂	39.4 ± 3.7	105.7 ± 11.7	6.7 ± 0.3	45.0	42.0 ± 5.3	113.9 ± 16.9	6.7 ± 0.3	44.6
(GGCCCG) ₂	41.4 ± 2.7	111.0 ± 8.8	7.0 ± 0.2	47.5	49.5 ± 3.2	136.3 ± 10.3	7.2 ± 0.1	46.9
(GGCCCC) ₂	41.2 ± 4.2	110.3 ± 13.2	7.0 ± 0.2	47.0	49.4 ± 1.9	136.4 ± 5.9	7.1 ± 0.1	46.4
(GGCCCU) ₂	44.3 ± 3.0	118.1 ± 9.6	7.7 ± 0.2	51.7	43.5 ± 3.1	116.0 ± 9.6	7.6 ± 0.1	50.9
(GGCCCP) ₂ ^c	37.2	97	7.0	47.5				

^aSolutions are 1.0 M NaCl, 10 mM sodium cacodylate, 0.5 mM EDTA at pH 7.

^bCalculated at 10^{-4} M oligomer concentration.

^cFreier et al. 1983.

TABLE 2. Stabilization by addition of second 3' dangling end in 1 M NaCl

$\Delta\Delta G_{37}^{\circ}$ (kcal/mol) values for addition of second 3' overhang.				
GGCCXZ ^a				
Z	A	G	U	C
X				
A	-0.4	-0.4	-0.6	-0.6
G	-0.4	-0.4	-0.6	-0.7
U	-0.1	-0.1	-0.1	-0.1
C	+0.2	-0.1	-0.2	-0.0
$\Delta\Delta H^{\circ}$ (kcal/mol) values for addition of second 3' overhang.				
GGCCXZ ^a				
Z	A	G	U	C
X				
A	-2.8	-4.5	-6.2	-5.0
G	-5.2	-4.5	-7.0	-6.8
U	-1.0	-2.6	-2.3	-2.6
C	-1.7	-4.1	-3.3	-4.0
$\Delta\Delta S^{\circ}$ (eu) values for addition of second 3' overhang.				
GGCCXZ ^a				
Z	A	G	U	C
X				
A	-7.6	-13.2	-18.2	-14.3
G	-15.5	-13.1	-20.5	-19.8
U	-3.4	-8.4	-7.3	-8.2
C	-6.4	-13.3	-10.0	-13.2

^aValues calculated as described in text.

overhang is two purine residues, the stabilization of the helix is -0.4 kcal/mol.

To test the generality of conclusions from this work, thermodynamic parameters were also measured for six test sequences with 3' double overhangs but different core sequences. The measured and predicted (both with and without the influence of the second 3' dangling end) thermodynamic values for the six sequences are presented in Table 4.

DISCUSSION

3' Double dangling ends generally increase the stability of RNA duplexes relative to duplexes with only a single 3' dangling nucleotide. As shown in Table 1, addition of the second 3' dangling end increases the T_M of the helix by 0°C–6.1°C for a 10⁻⁴ M solution. The increase in T_M is less than that observed for the addition of the first 3' dangling nucleotide, which increases the T_M by 7°C–24°C (Freier et al. 1983). The additional stabilization afforded by the second 3' dangling nucleotide is sequence dependent. The T_M for helices, where the first dangling nucleotide is a purine, is on average 4.2°C higher upon the addition of the second dangling nucleotide. For helices, where the first dangling nucleotide was a pyrimidine, the average increase in T_M was only 1.2°C.

The error in ΔG_{37}° value is $\sim \pm 0.20$ kcal/mol, done by averaging the standard errors for free energy values from both the melt curve analysis and the temperature dependence model. The $\Delta\Delta G_{37}^{\circ}$ values obtained from sequences with the 3' double dangling ends containing a pyrimidine (U or C) as the first dangling nucleotide are all within the calculated range of error independent of the identity of the second dangling nucleotide, indicating that, in this case, the additional nucleotide does not contribute to the stability of the duplex. The $\Delta\Delta G_{37}^{\circ}$ values for sequences with double 3' dangling ends containing a purine as the first overhanging nucleotide are greater than the calculated error.

The influence of the second nucleotide of the 3' double terminal overhang is significantly different than that observed for the influence by the single 3' terminal overhangs. For the single 3' terminal overhangs, the stabilization is very sequence dependent (Freier et al. 1983, 1986; Petersheim and Turner 1983; Sugimoto et al. 1987; Fire et al. 1998); 3' terminal purine nucleotides (1.1 kcal/mol on average) contribute nearly twice the stabilization as 3' terminal pyrimidines (0.5 kcal/mol on average). The identity of the nearest neighbor (the closing base pair) also influences the stabilization contribution of the 3' dangling end, on average, 0.5 kcal/mol, for bases adjacent to an AU (A/U or U/A) terminal end and 1.1 kcal/mole for bases adjacent to a GC terminal end (Freier et al. 1983, 1986; Petersheim and Turner 1983; Sugimoto et al. 1987; Fire et al. 1998).

For 3' double nucleotide dangling ends, no additional helix stabilization is observed when a second dangling nucleotide is adjacent to a 3' dangling pyrimidine residue (Tables 2, 3). This result may be due to the lower stacking stability of pyrimidine bases relative to purine bases (Turner 2000 and references cited therein). This is particularly true in the sequences studied here, as the core duplex has two C residues adjacent to the 3' dangling end. It is not obvious why the stacking of a pyrimidine residue (as the 3' double overhang) increases the stability of the duplex more than a purine residue when the first nucleotide of the 3' double overhang is a purine. We are currently using molecular modeling to understand the trends observed here.

To test the model developed, six additional duplex sequences containing 3' double dangling ends (with different core sequences) were studied (Table 4). The measured and predicted thermodynamic values for duplex formation are listed in Table 4. The predicted values were determined

TABLE 3. Average stabilization by addition of second 3' dangling end in 1 M NaCl

Sequence of 3' double dangling ends	$-\Delta\Delta G_{37}^{\circ}$ kcal/mol	$-\Delta\Delta H^{\circ}$ kcal/mol	$-\Delta\Delta S^{\circ}$ eu
5'-pyr-X	0.0	0.0	0.0
5'-pur-pyr	-0.6	-6.2	-18.2
5'-pur-pur	-0.4	-4.3	-12.4

TABLE 4. Measured and predicted thermodynamic parameters for test sequence duplex formation^a

Sequences	Average of curve fits				T_M^{-1} vs log C_1 plots			
	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G_{37}^\circ$ (kcal/mol)	T_M^b (°C)	$-\Delta H^\circ$ (kcal/mol)	$-\Delta S^\circ$ (eu)	$-\Delta G_{37}^\circ$ (kcal/mol)	T_M^b (°C)
(UGCGCACC) ₂	53.5 (52.0)	141.8 (138.6)	9.6 (9.0)	61.3 (58.3)	54.6	145.1	9.6	60.9
(UGCGCAAC) ₂	60.8 (60.0)	163.0 (162.8)	10.3 (9.6)	62.4 (58.2)	68.9	187.1	10.8	62.2
(UGCGCAAG) ₂	59.7 (60.0)	160.0 (162.8)	10.0 (9.6)	61.5 (58.2)	61.4	165.2	10.2	61.4
(AGCGCUGG) ₂	58.5 (61.9)	157.2 (169.4)	9.8 (9.3)	60.3 (56.6)	66.0	179.9	10.1	59.7
(CCGGAC) ₂	50.0 (46.8)	136.1 (128.5)	7.8 (6.3)	50.7 (45.6)	50.30	137.2	7.8	50.4
(CCGGCA) ₂	39.4 (40.9)	109.7 (115.0)	5.4 (5.2)	34.8 (33.7)	38.4	106.5	5.3	34.3

^aSolutions are 1.0 M NaCl, 10 mM sodium cacodylate, 0.5 mM EDTA at pH 7.^bCalculated at 10⁻⁴ M oligomer concentration. Values in parentheses are predicted: top row is the predicted values for single 3' terminal overhang duplexes and bottom row is the predicted values as described in the text for duplexes with 3' double overhangs.

with and without the inclusion of the influence of the second 3' terminal overhang to determine whether its inclusion would improve the predictions. Note that for two of these sequences, where the first 3' overhang is a pyrimidine, the predicted values are the same. In general, the enthalpy and entropy for the duplexes, with the first 3' overhang a purine, were predicted more accurately without the inclusion of the addition of the second 3' dangling end. However, both the free energy at 37°C (on average by 0.5 kcal/mol) and T_M (on average by 2°C) were more accurately predicted by the inclusion of the contribution of the second dangling end. Therefore, inclusion of the stabilization of a duplex due to the second nucleotide on a 3' double dangling end improves the prediction of duplex stability. The model predicts the stability of sequences with terminal GC base pairs more accurately than for those with terminal AU base pairs. We are currently investigating the influence of 3' double terminal overhangs on the stability of sequences with terminal AU base pairs.

In their thermodynamic analysis of siRNAs, Khvorova et al. (2003), found the average 5' terminal free energy for functional and nonfunctional siRNAs to differ by ~2.3 kcal/mol. Mutation of a terminal CG base pair to an AU base pair also dramatically alters the functional asymmetry of siRNA duplexes (Schwarz et al. 2003). The difference in energy between a terminal CG pair (adjacent to an AU base pair) and the terminal AU pair is ~1.8 kcal/mol (Xia et al. 1998). The energy differences observed by altering a terminal base pair is similar to the differences in energy that can be contributed by the 3' terminal double overhangs. For

example, a 3' C residue on a CG base pair adds 0.8 kcal/mol to the stability of a duplex end (no additional stabilization is contributed by the second nucleotide), while a 3' G with a second dangling pyrimidine would contribute 2.3 kcal/mol to the stability of the duplex end. Thus, the 3' terminal double overhangs can significantly alter the stability of the duplex ends and therefore influence strand selection by the RISC complex.

MATERIALS AND METHODS

RNA synthesis and purification

All oligomers were synthesized on CPG solid supports (Applied Biosystems 392 DNA/RNA Synthesizer) with phosphoramidites with the 2' hydroxyl protected as the *tert*-butyl dimethylsilyl ether from Glen Research. Oligomers underwent ammonia and fluoride deprotection, and crude sample was purified using preparative TLC (n-propanol:ammonium hydroxide:water, 55:35:10) and Sep-Pak C18 (Waters) chromatography. Sample purity was determined through analytical TLC, and was >95%.

Melting curve and data analysis

Optical melting experiments were performed using a Beckman DU 640 Spectrophotometer and High Performance Temperature Controller at 280 nm. Absorbance changes for oligomers in 1 M NaCl melt buffer (1 M NaCl, 0.01 M cacodylic acid, 0.001 M ethylenediamine tetraacetic acid, pH 7.0) were recorded as function of temperature from 95°C to 10°C at a rate of 1°C/min as described previously (Serra et al. 1994). The experiment was repeated at 10

varying sample concentrations to give at least a 50-fold concentration range (10 μ M–1 mM) for each sample. Absorbance versus temperature profiles were fit to a two-state model with sloping base lines using a nonlinear least squares program (McDowell and Turner 1996). Thermodynamics parameters for the oligomers were determined from both the average of the individual melt curves and plots of the reciprocal melting temperature (T_M^{-1}) versus $\ln(C_i)$ for self-complementary sequences.

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