J|A|C|S
ARTICLES
Published on Web 08/13/2002

Long RNA Dangling End Has Large Energetic Contribution to Duplex Stability

Tatsuo Ohmichi,[†] Shu-ichi Nakano,[†] Daisuke Miyoshi,[‡] and Naoki Sugimoto*[,†,‡]

Contribution from the High Technology Research Center and Department of Chemistry, Faculty of Science and Engineering, Konan University, 8-9-1 Okamoto, Higashinada-ku, Kobe 658-8501, Japan

Received January 8, 2002

Abstract: Long terminal unpaired nucleotides known as dangling ends play interesting roles in biological systems. Previous studies, however, only dealt with the energy contributions of single dangling bases. The energy contributions of long dangling ends on the stability of duplexes have not been systematically studied. We now report a quantitative increase in stability of RNA−RNA and DNA−DNA duplexes containing a long dangling end. We found a larger enhancement of the stability by the long RNA dangling end of the RNA−RNA duplex than has been observed for the DNA duplexes. It is also found that structural stabilizations by long dangling ends seem to originate from the single-stranded stacking interactions of nucleotides. These results indicate that RNA stability can be achieved by increasing the length of the dangling end. The thermodynamic parameters of the long dangling ends are useful for designing ribozymes and antisense oligonucleotides, and for the prediction of the RNA secondary structure like the pseudoknot.

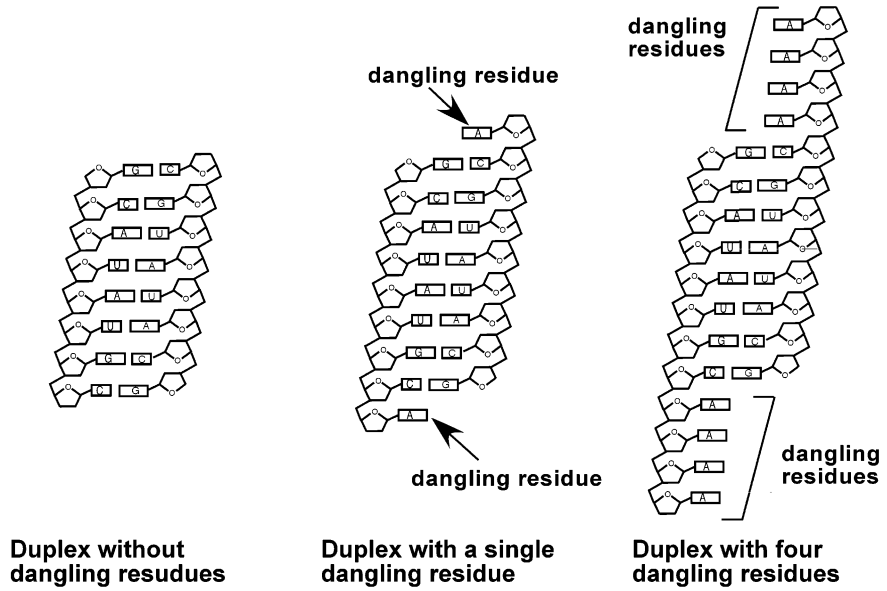
Introduction

Non-Watson−Crick base pairs in RNA and DNA play important roles as catalytic and tertiary contact domains.[1] Their structures and thermodynamic properties have been investigated in an effort to understand the biological function associated with these unpaired regions.[2−7] Terminal unpaired nucleotides also known as dangling ends may play interesting roles in biological systems. Dangling nucleotides on RNA and DNA duplexes can stabilize a helical structure through stacking of adjacent base pairs. For example, the dangling nucleotides represented by 5′ACCA3′ at the 3′ terminus of tRNA stabilize the cloverleaf structure of tRNA,[8] and the interaction between mRNA and tRNA is stabilized by the dangling ends adjacent to the codon−anticodon pair.[9,10] It has also been reported that a dangling nucleotide at the 3′ end of a pseudoknot RNA stabilizes the stem structure.[11] Moreover, recent studies of RNAi (RNA interference) have shown that 2−3 nt dangling ends are important for the RNAi functionality.[12,13] In the case of RNAi, short double-stranded RNAs (dsRNA) are formed with the 2−3 nt RNA dangling ends after cleavage with RNase III.[14] Although the 2−3 nt RNA dangling end results after treatment with RNase III, a short dsRNA not possessing a 2−3 nt RNA dangling end displays no gene silencing activity.[12,13] These observations clearly show that long dangling residues have the requisite energy contributions needed to stabilize the helix, thus resulting in the observed biological activities. However, previous studies only dealt with the energy contributions of single dangling bases.[15−18] The systematic energy contributions of long dangling ends, which consist of many dangling bases, have hitherto not been reported (Scheme 1). Here, we report a quantitative increase in the stability of an RNA−RNA duplex containing a long RNA dangling end. We found that the stabilizing effect of dangling ends on RNA duplexes is much larger than that on DNA duplexes.

* To whom correspondence should be addressed. Phone: +81-78-435-2497. Fax: +81-78-435-2539. E-mail: sugimoto@konan-u.ac.jp.
[†] High Technology Research Center, Konan University.
[‡] Department of Chemistry, Konan University.

(1) (a) Hertel, K. J.; Stage-Zimmermann, T. K.; Ammons, G.; Uhlenbeck, O. C. *Biochemistry* **1998**, *37*, 16983−16988. (b) Wu, M.; Tinoco, I., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 11555−11560. (c) Testa, S. M.; Gryaznov, S. M.; Turner, D. H. *Biochemistry* **1998**, *37*, 9379−9385. (d) Ohmichi, T.; Kool, E. T. *Nucleic Acids Res.* **2000**, *28*, 776−783.
(2) Xia, T.; SantaLucia, J., Jr.; Burkard, M. E.; Kierzek, R.; Schroeder, S. J.; Jiao, X.; Cox, C.; Turner, D. H. *Biochemistry* **1998**, *37*, 14719−14735.
(3) SantaLucia, J., Jr.; Allawi, H. T.; Seneviratne, P. A. *Biochemistry* **1996**, *35*, 3555−3562.
(4) Sugimoto, N.; Nakano, S.; Yoneyama, M.; Honda, K. *Nucleic Acids Res.* **1996**, *24*, 4501−4505.
(5) Sugimoto, N.; Nakano, S.; Katoh, M.; Matsumura, A.; Nakamuta, H.; Ohmichi, T.; Yoneyama, M.; Sasaki, M. *Biochemistry* **1995**, *34*, 11211−11216.
(6) Ohmichi, T.; Nakamuta, H.; Yasuda, K.; Sugimoto, N. *J. Am. Chem. Soc.* **2000**, *122*, 11286−11294.
(7) Sugimoto, N.; Nakano, M.; Nakano, S. *Biochemistry* **2000**, *39*, 11270−11281.
(8) Limmer, S.; Hofmann, H. P.; Ott, G.; Sprinzl, M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 6199−6202.
(9) Yoon, K.; Turner, D. H.; Tinoco, I., Jr.; von der Haar, F.; Cramer, F. *Nucleic Acids Res.* **1976**, *3*, 2233−2241.
(10) Ayer, D.; Yarus, M. *Science* **1986**, *231*, 393−395.
(11) Du, Z.; Giedroc, D. P.; Hoffman, D. W. *Biochemistry* **1996**, *35*, 4187−4198.
(12) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494−498.
(13) Elbashir, S. M.; Lendeckel, W.; Tuschl, T. *Genes Dev.* **2000**, *15*, 188−200.
(14) Bernstein, E.; Caudy, A. A.; Hammond, S. M.; Hannon, G. J. *Nature* **2001**, *409*, 363−366.
(15) Sugimoto, N.; Kierzek, R.; Turner, D. H. *Biochemistry* **1987**, *26*, 4554−4558.
(16) Bommarito, A.; Peyret, N.; SantaLucia, J., Jr. *Nucleic Acids Res.* **2000**, *28*, 1929−1934.
(17) Barnes, T. W.; Turner, D. H. *J. Am. Chem. Soc.* **2001**, *123*, 4107−4118.
(18) Guckian, K. M.; Schweitzer, B. A.; Ren, R. X.-F.; Sheils, C. J.; Tahmassebi, D. C.; Kool, E. T. *J. Am. Chem. Soc.* **2000**, *122*, 2213−2222.

Scheme 1



Materials and Methods

Material Preparations. All DNA and RNA oligonucleotides were synthesized on solid supports using standard β-cyanoethyl phosphoramidite methods on an Applied Biosystems (ABI) model 391 DNA/RNA synthesizer. The synthesized DNA oligonucleotides containing 5′-end dimethoxytrityl (DMT) groups were removed from the solid support, and base blocking groups were removed by treatment with concentrated 25% ammonia at 55 °C for 3 h. After being dried in a vacuum, the DNA oligonucleotides were passed through a Poly-Pak cartridge (Glen Research Co., Ltd.) with 2% trifluoroacetic acid (TFA) to remove 5′-end DMT groups. The synthesized RNA oligonucleotides without 5′-end DMT groups were removed from the solid support, and base blocking groups were removed by treatment with concentrated 25% ammonia in ethanol (3:1, v/v) at 55 °C for 3 h. After being dried in a vacuum, the 2′-silyl protective groups were removed by resuspending the pellet in 50 equiv of tetrabutylammonium fluoride (TBAF) per silyl, and the mixtures were incubated overnight in the dark at room temperature.[19] After deblocking operations, the DNA and RNA oligonucleotides were desalted through a C-18 Sep-Pak cartridge column (Waters). The DNA and RNA oligonucleotides were purified by reverse-phase high performance liquid chromatography (HPLC) on a TSKgel Oligo DNA RP column (Tosoh) with a linear gradient of 0−50% MeOH/H_2O containing triethylammonium acetate (TEAA, pH 7.0). The final purities of the DNA and RNA oligonucleotides were confirmed to be >99% by HPLC. The purified DNA and RNA oligonucleotides were desalted again with a C-18 Sep-Pak cartridge before use.

Single-strand concentrations of the DNA and RNA oligonucleotide were determined by measuring the absorbance at 260 or 280 nm at high temperature. Single-strand extinction coefficients were calculated from mononucleotide and dinucleotide data using a nearest-neighbor approximation.[20]

UV Melting Measurements. Absorbance measurements in the UV region were made on Hitachi U-3200 and U-3210 spectrophotometers. Melting curves (absorbance vs temperature curves) were measured at 260 nm with these spectrophotometers connected to a Hitachi SPR-7 or SPR-10 thermoprogrammer. The conditions of the samples and buffer were the same as those used for the CD measurements. The heating rate was 0.5 or 1.0 °C min^{-1}. Water condensation on the cuvette exterior at the low-temperature range was avoided by flushing with a constant stream of dry N_2 gas. Prior to the experiment, the buffer was degassed by heating to 90 °C for 10 min.

Determination of Thermodynamic Parameters for Duplex Formations. All melting curves were fitted with a curve fitting procedure to obtain three thermodynamic parameters, the enthalpy ($\Delta H°$), entropy ($\Delta S°$), and free-energy changes ($\Delta G°_{37}$) at 37 °C, for the formation of the nucleic acid duplex as described elsewhere.[2,7,21,22] This method provides an estimation of the thermodynamic values from the shape of each melting curve. To increase the accuracy of these parameters, we also evaluated them from plots of T_m^{-1} versus $\ln(C_t)$, where T_m is the melting temperature, and C_t is the total strand concentration, respectively. Using the slope and vertical axis intercept, we analyzed the thermodynamic parameters according to eqs 1 and 2

$$T_m^{-1} = R \times \ln(C_t)/\Delta H° + \Delta S°/\Delta H° \quad (1)$$

$$\Delta G°_{37} = \Delta H° - 310.15 \times \Delta S° \quad (2)$$

where R is the gas constant.

Estimated errors for the thermodynamic values ($\sigma_{\Delta H°}$, $\sigma_{\Delta S°}$, and $\sigma_{\Delta G°_{37}}$) derived from a curve fitting procedure were calculated from the standard deviations among data points of each melting curve measured at different C_t's. Those for $\Delta H°$ and $\Delta S°$ ($\sigma_{\Delta H°}$, $\sigma_{\Delta S°}$) from T_m^{-1} versus $\ln(C_t)$ plots were estimated from the linearity of the plots, and those for $\Delta G°_{37}$ ($\sigma_{\Delta G°_{37}}$) were calculated using eq 3

$$(\sigma_{\Delta G°_{37}})^2 = (\sigma_{\Delta H°})^2 + (310.15)^2(\sigma_{\Delta S°})^2 - 2 \times 310.15 \times (R_{\Delta H°,\Delta S°}) \times \sigma_{\Delta H°} \times \sigma_{\Delta S°} \quad (3)$$

where $R_{\Delta H°,\Delta S°}$ is the correlation coefficient between $\Delta H°$ and $\Delta S°$.[3,4] The final thermodynamic parameters were evaluated from the average values obtained from curve fitting and T_m^{-1} versus $\ln(C_t)$ plots.

Results and Discussion

Effects of the Dangling Length on RNA Duplex Stability. Figure 1 shows the CD spectrum of r(AUGCAU), r(AUG-

(19) Okumoto, Y.; Ohmichi, T.; Sugimoto, N. *Biochemistry* **2002**, *41*, 2769−2773.
(20) Richards, E. G. In *Handbook of Biochemistry and Molecular Biology: Nucleic Acids*, 3rd ed.; Fasman, G. D., Ed.; CRC Press: Cleveland, OH, 1975; Vol. I, p 597.
(21) Bevington, P. R. *Data Reduction and Error Analysis for the Physical Science*, 2nd ed.; McGraw-Hill: New York, 1968.
(22) Sugimoto, N.; Wu, P.; Hara, H.; Kawamoto, Y. *Biochemistry* **2001**, *40*, 9396−9405.

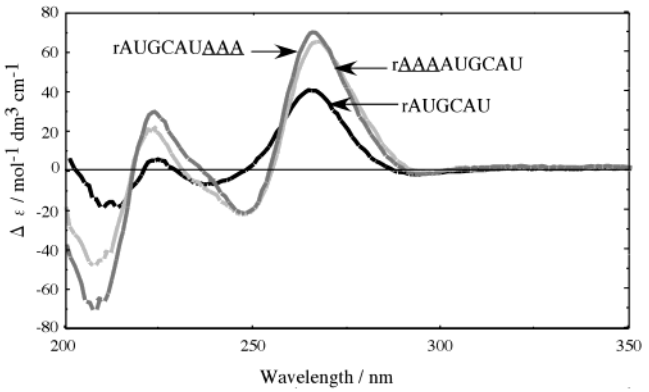


Figure 1. rAUGCAU, rAUGCAUAAA, and rAAAAUGCAUAAA at about a 10 μM total strand concentration. All spectra were measured in 1 M NaCl buffer (pH 7.0) at 5 °C.

CAUAAA), and r(AAAAUGCAU) in 1 M NaCl buffer (pH 7.0) at 5 °C (underline indicates dangling nucleotides). It is known that a CD spectrum of an RNA−RNA duplex has a positive peak around 270 nm and a relatively weak and negative peak around 235 nm, indicating a normal A-form structure.[23] All CD spectra of the RNA−RNA helixes with and without dangling nucleotides have a positive peak around 270 nm and an intense negative peak around 240 nm (Figure 1). Our result indicates that the structure of an RNA duplex with a long dangling end is the A-form conformation. Thus, the insertion of long dangling end nucleotides into the core helix at 5′ and 3′ does not alter the A-form conformation.

UV melting curves are used to measure the thermodynamic stability of the RNA−RNA helix with long dangling nucleotides (Figure 2a). We measured melting transition (T_m) as a function of duplex concentration and calculated thermodynamic parameters by plotting T_m^{-1} versus log(C_t) (Figure 2b). All experiments were done at 1 M NaCl, although the condition is not a typical physiological condition, because previous thermodynamic studies for single dangling nucleotides were done at 1 M NaCl so that the same salt condition is useful for comparison of our new thermodynamic data for the long dangling end with the previous folding. Also, although the low NaCl condition is physiological, the concentration of $MgCl_2$ is on the micromolar order. It is known that the predicted RNA folding in 1 M NaCl is similar to the ones predicted in 6 mM $MgCl_2$.[24] Thus, the 1 M NaCl condition is not so different of a condition from the real physiological condition to predict RNA folding. The thermodynamic effects of one to four dangling adenines on r(GCAUAUGC) and r(AUGCAU) were measured. All RNA−RNA helixes with dangling nucleotides showed a two-state transition in 1 M NaCl. Differences of less than 10% were observed between parameters as determined by T_m^{-1} versus log(C_t) plots and curve fittings. Final thermodynamic parameters were determined from average values obtained from curve fittings and T_m^{-1} versus log(C_t) plots. The results of the thermodynamic measurements for RNA−RNA duplexes containing from one to four dangling nucleotides (pH 7.0 and 1 M NaCl) are presented in Table 1.

The free-energy changes at 37 °C upon addition of the dangling ends was also calculated (Table 1). The stabilization

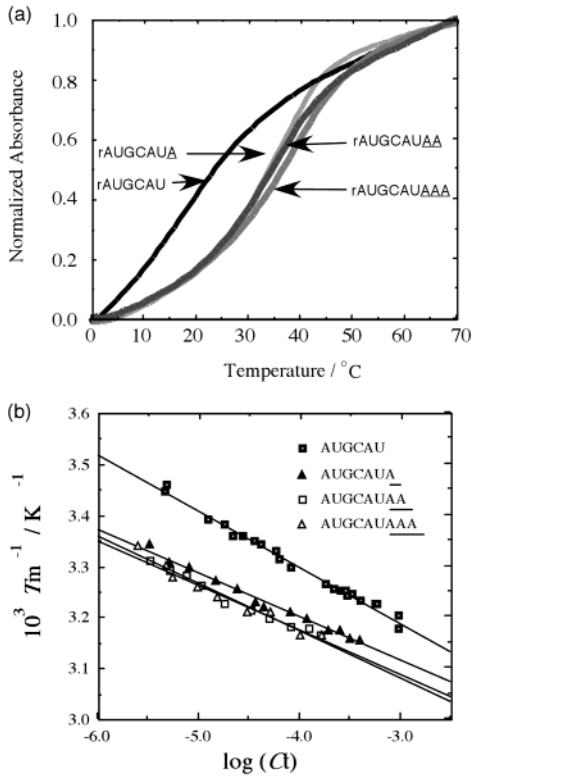


Figure 2. (a) Normalized melting curves and (b) T_m^{-1} versus log(C_t) plots of rAUGCAU, rAUGCAUA, rAUGCAUAA, and rAUGCAUAAA. All melting curves were measured at about a 10 μM total strand concentration in 1 M NaCl buffer (pH 7.0).

due to the dangling end was calculated using $\Delta\Delta G^\circ_{37} = \{\Delta G^\circ_{37(\text{duplex with dangling ends})} - \Delta G^\circ_{37(\text{core duplex})}\}/2$. The $\Delta\Delta G^\circ_{37}$ values for a single dangling adenine at the 3′ terminus were −1.5 (5′G/CA3′) and −0.6 kcal mol^{-1} (5′A/UA3′) per dangling end. These values are similar to those previously reported for 5′G/CA3′ and 5′A/UA3′ (−1.7 and −0.7 kcal mol^{-1}, respectively).[15,25] Moreover, the $\Delta\Delta G^\circ_{37}$values for a single adenine dangling end at the 5′ terminus were −0.3 (5′AG/C3′) and −0.3 (5′AA/U3′) kcal mol^{-1} per dangling end, which are similar to the reported values of −0.2 (5′AG/C3′) and −0.3 (5′AA/U3′) kcal mol^{-1}.[15,25] The results also show that 3′ dangling ends are energetically favored over 5′ dangling ends, as previously reported. These results confirm our choice of nucleic acid sequences as being suitable for the investigation of the effects of dangling length on duplex stability.

Interestingly, the results indicate that the RNA duplex was considerably stabilized by increasing the number of the dangling adenines. Although the calculated free-energy changes were largest with the addition of the first dangling adenine, duplexes containing four adenines showed much higher stability than those containing a single adenine. The $\Delta\Delta G^\circ_{37}$ values for single, two, three, and four dangling adenines at the 3′ terminus of r(GCAUAUGC) were −1.5 (A_1), −1.9 (A_2), −2.4 (A_3), and −2.5 (A_4) kcal mol^{-1}. Also, even if the 3′ terminal base pair is AU, the stabilization increased with dangling end. The $\Delta\Delta G^\circ_{37}$ values for single, two, three, and four dangling adenines at the 3′ terminus of r(AUGCAU) were −0.6 (A_1), −0.9 (A_2), −1.2 (A_3), and −1.1 (A_4) kcal mol^{-1}. Moreover, when the long

(23) Saenger, W. *Principles of Nucleic Acid Structure*; Springer-Verlag: New York, 1984.
(24) Sugimoto, N.; Sasaki, M.; Tanaka, A. *Supramol. Chem.* **1993**, *2*, 99−102.
(25) Burkard, E. M.; Kierzek, R.; Turner, D. H. *J. Mol. Biol.* **1999**, *290*, 967−982.

Table 1. Thermodynamic Parameters for Self-Complementary RNA Duplex Formation Measured in 1 M NaCl[a]

sequence	$\Delta H°$ kcal mol^{-1}	$\Delta S°$ cal mol^{-1} K^{-1}	$\Delta G°_{37}$ kcal mol^{-1}	T_m[b] °C	$\Delta\Delta G°_{37}$[c] kcal mol^{-1}
Core I					
rGCAUAUGC	−70.6	−197	−9.3	54.0	
3′ dangling ends					
rGCAUAUGCA	−72.7	−195	−12.2	67.4	−1.5
rGCAUAUGCAA	−84.1	−229	−13.2	67.5	−1.9
rGCAUAUGCAAA	−91.5	−250	−14.1	68.3	−2.4
rGCAUAUGCAAAA	−94.5	−259	−14.3	68.3	−2.5
5′ dangling ends					
rAGCAUAUGC	−71.4	−198	−9.9	56.3	−0.3
rAAGCAUAUGC	−81.0	−238	−10.3	55.7	−0.5
rAAAGCAUAUGC	−87.1	−246	−10.8	56.4	−0.8
rAAAAGCAUAUGC	−96.8	−276	−11.1	55.6	−0.9
Core II					
rAUGCAU	−47.4	−138	−4.8	31.1	
3′ dangling ends					
rAUGCAUA	−49.3	−140	−6.0	38.9	−0.6
rAUGCAUAA	−53.8	−152	−6.6	42.7	−0.9
rAUGCAUAAA	−55.8	−157	−7.1	45.2	−1.2
rAUGCAUAAAA	−57.9	−164	−6.9	43.8	−1.1
5′ dangling ends					
rAAUGCAU	−49.6	−143	−5.3	34.7	−0.3
rAAAUGCAU	−54.1	−156	−5.7	37.3	−0.5
rAAAAUGCAU	−55.0	−158	−6.0	38.7	−0.6
rAAAAAUGCAU	−55.1	−156	−6.8	43.4	−1.0

[a] All experiments were done in a buffer containing 1 M NaCl, 10 mM Na_2HPO_4, and 1 mM Na_2EDTA (pH 7.0). Thermodynamic parameters are evaluated from the average values obtained from curve fitting and T_m^{-1} versus log(Ct) plots. Estimated errors are ±4% in $\Delta H°$, ±4% in $\Delta S°$, and ±8% in $\Delta G°_{37}$. [b] Melting temperatures are calculated at a total strand concentration of 100 μM. [c] $\Delta\Delta G°_{37} = \{\Delta G°_{37(\text{duplex with dangling ends})} - \Delta G°_{37(\text{core duplex})}\}/2$.

Table 2. Thermodynamic Parameters for Self-Complementary DNA Duplex Formation Measured in 1 M NaCl[a]

sequence	$\Delta H°$ kcal mol^{-1}	$\Delta S°$ cal mol^{-1} K^{-1}	$\Delta G°_{37}$ kcal mol^{-1}	T_m[b] °C	$\Delta\Delta G°_{37}$[c] kcal mol^{-1}
Core III					
dGCATATGC	−55.8	−155	−7.8	49.0	
3′ dangling ends					
dGCATATGCA	−58.8	−162	−8.7	53.7	−0.5
dGCATATGCAA	−60.1	−166	−8.7	53.5	−0.5
dGCATATGCAAA	−62.4	−172	−9.0	54.3	−0.6
dGCATATGCAAAA	−65.5	−182	−9.0	53.4	−0.6
5′ dangling ends					
dAGCATATGC	−56.1	−154	−8.4	52.8	−0.3
dAAGCATATGC	−56.3	−154	−8.5	53.3	−0.4
dAAAGCATATGC	−56.1	−152	−8.9	55.9	−0.6
dAAAAGCATATGC	−57.3	−156	−8.9	55.3	−0.6
Core IV					
dATGCGCAT	−62.0	−171	−9.3	54.4	
3′ dangling ends					
dATGCGCATA	−63.1	−171	−10.0	60.2	−0.4
dATGCGCATAA	−64.0	−173	−10.3	61.1	−0.5
dATGCGCATAAA	−65.9	−180	−10.1	59.3	−0.4
dATGCGCATAAAA	−66.7	−183	−9.8	57.4	−0.4
5′ dangling ends					
dAATGCGCAT	−64.6	−175	−10.2	61.0	−0.5
dAAATGCGCAT	−65.0	−176	−10.3	61.0	−0.5
dAAAATGCGCAT	−65.5	−178	−10.4	61.3	−0.6
dAAAAATGCGCAT	−69.9	−192	−10.4	59.4	−0.6

[a] All experiments were done in a buffer containing 1 M NaCl, 10 mM Na_2HPO_4, and 1 mM Na_2EDTA (pH 7.0). Thermodynamic parameters are evaluated from the average values obtained from curve fitting and T_m^{-1} versus log(Ct) plots. Estimated errors are ±4% in $\Delta H°$, ±4% in $\Delta S°$, and ±8% in $\Delta G°_{37}$. [b] Melting temperatures are calculated at a total strand concentration of 100 μM. [c] $\Delta\Delta G°_{37} = \{\Delta G°_{37(\text{duplex with dangling ends})} - \Delta G°_{37(\text{core duplex})}\}/2$.

dangling end was at the 5′ terminal of the RNA duplex, the influence of the fourth nucleotide was observed. The $\Delta\Delta G°_{37}$ values for single, two, three, and four dangling adenines at the 5′ AU terminus were −0.3 (A_1), −0.5 (A_2), −0.6 (A_3), and −1.0 (A_4) kcal mol^{-1}. The contribution of the single-stranded ACCA to the stability of the aminoacyl stem of tRNA was previously investigated in 0.1 M NaCl.[8] A 7-bp RNA duplex with the single-strand ACCA 3′ terminus (r(UAGCUCCACCA)/r(GGGGCUA): underline notes dangling nucleotides) derived from the aminoacyl stem of *E. coli* tRNA(Ala) was stabilized by the ACCA dangling end. The $\Delta\Delta G°_{37}$ values for single, two, three, and four dangling adenines at the 3′ terminus were −0.8 (A), −0.9 (AC), −1.2 (ACC), and −1.3 (ACCA) kcal mol^{-1}. Although the largest contribution to the stability gain due to the ACCA end is provided by the first dangling 3′ nucleotide, the influence of even the fourth nucleotide is measurable such as seen in our result at low salt concentration. The agreement between our results and previous results would indicate that RNA stability can be achieved by increasing the length of the dangling end.

Effects of the Dangling Length on DNA Duplex Stability. The effects of the length of dangling adenine tracts on DNA duplex stability were investigated to compare a RNA−RNA duplex with a DNA−DNA duplex. The thermodynamic effects of one to four dangling adenines on d(GCATATGC) and d(ATGCGCAT) were measured. The free-energy changes at 37 °C upon addition of the dangling ends were also calculated using the same method as for the RNA−RNA duplex. The $\Delta\Delta G°_{37}$ values for a single dangling adenine at the 3′ terminus were −0.5 (5′G/CA3′) and −0.4 kcal mol^{-1} (5′A/TA3′) per dangling end. All thermodynamic measurements of DNA sequences with a single dangling end have been reported.[16] Our results are not so different from those previously reported for 5′G/CA3′ and 5′A/TA3′ (−0.8 and −0.5 kcal mol^{-1}, respectively).[16] The $\Delta\Delta G°_{37}$ values for a single adenine dangling end at the 5′ terminus were −0.3 (5′AG/C3′) and −0.5 (5′AA/U3′) kcal mol^{-1} per dangling end, which are also similar to the reported values of −0.6 (5′AG/C3′) and −0.5 (5′AA/U3′) kcal mol^{-1}.[16] Thus, our DNA sequences are suitable for the investigation of the effects of dangling length on duplex stability as well as the RNA−RNA duplex. The results also show that the effect of dangling ends on the stability for the DNA duplexes are not the same as those observed for RNA duplexes. Previous studies for a single dangling nucleotide indicate that DNA with 5′ dangling ends are more or equally as stable as their RNA counterparts, while RNA motifs with 3′ dangling ends are more or equally as stable as their DNA counterparts.[16,25] Our data are in agreement with the previously observed tendency and also support the relationship between the geometric difference and the difference in stability of 3′ versus 5′ RNA dangling ends. For A-form RNA structures, 3′ nucleotides are well stacked on top of the adjacent base pair, whereas 5′ dangling ends are more poorly displayed. For B-form DNA, however, dangling ends show efficient geometric intrastrand stacking. The thermodynamic differences between the DNA−DNA duplex and RNA−RNA duplex are due to the structural properties described above.

The comparison with the effect of a long dangling end in a RNA helix interestingly shows that the effect of dangling adenine tracts on DNA duplex stability is not as marked (Table 2). Although the presence of a single adenine (dA_1) increased the stability of the DNA duplex, the presence of longer adenine

tracts did not significantly alter the calculated free energies. Lengthening the dangling adenines from one to four enhanced the RNA duplex stability by 1.0−0.5 kcal mol^{-1}; however, those in DNA less affected the duplex stability ($\Delta\Delta G^\circ_{37} \geq -0.2$ kcal mol^{-1}). The thermodynamic effect of four dangling nucleotides at the 5′ end on DNA hairpins has been studied, and the dangling sequence-dependent stability has been reported.[26] Four dangling adenines next to an adjacent 5′G/C3′ base pair stabilized the hairpin by ∼1 kcal mol^{-1} at 37 °C; that is not so different from our result that d(AAAAGCATATGC) with four dangling adenines was stabilized by 0.6 kcal mol^{-1} more than on d(GCATATGC). Thus, the data show that the length of the dangling adenine appeared to have less effect on DNA duplex stability. Previous studies have shown that single-strand stacking of ApA is similar to that of dApA.[27] The ΔH° values of ApA and dApA are −7.2 and −7.3 kcal/mol, respectively. Table 2 shows that as the length of the dangling residues on DNA helix increased, the enthalpy change became negatively larger. It was, however, completely compensated by entropy changes to give a smaller free-energy increment. Thus, the difference in the effect of the long dangling between duplexes of varying dangling adenine length on RNA and DNA might be due to differences in the translational entropy contributed by the binding or release of water molecules and ions, and in the conformational entropy.

Relationship between Free-Energy Increments and the Length of the Dangling End. The largest free-energy changes induced by the presence of a single dangling adenine were observed for r(GCAUAUGC) ($\Delta\Delta G^\circ_{37} = -1.5$ kcal mol^{-1} per dangling end). Using $\Delta\Delta\Delta G^\circ_{37(2)} = \{\Delta\Delta G^\circ_{37(\text{duplex with two dangling residues})} - \Delta\Delta G^\circ_{37(\text{duplex with single dangling residue})}\}$ and $\Delta\Delta\Delta G^\circ_{37(3)} = \{\Delta\Delta G^\circ_{37(\text{duplex with three dangling residues})} - \Delta\Delta G^\circ_{37(\text{duplex with two dangling residues})}\}$ to calculate the free-energy change induced by the addition of the second and third dangling adenines, respectively, we obtained $\Delta\Delta\Delta G^\circ_{37(2)}$ and $\Delta\Delta\Delta G^\circ_{37(3)}$ values of −0.4 and −0.5 kcal mol^{-1}, respectively. Moreover, the 3′ long dangling ends are energetically favored over 5′ dangling long ends, such as is seen in the case of a single dangling end. Figure 3 indicates the relationship between the free-energy increment by a single dangling adenine and enhanced stability by increasing the number of the dangling adenines on RNA duplexes. The concomitant change in free energy induced by a single dangling adenine, and the enhanced stability of RNA duplexes induced by increasing the number of dangling adenines, suggests that the changes in free energy associated with the presence of long dangling ends are proportional to the strength of the stacking interactions between the first dangling residue and an adjacent base pair. Structural stabilizations by long dangling ends are thought to originate from single-stranded stacking interactions of nucleotides. When the first dangling residue stacks favorably on an adjacent base pair, the second dangling nucleotide would then prefer to stack on the first dangling nucleotide. Because the stacking interactions between single-stranded residues are not very stable, it is reasonable to suppose that free-energy changes become smaller for distal dangling residues. With the relationship illustrated in Figure 3, it is possible to speculate free-energy increments for long dangling ends on RNAs.

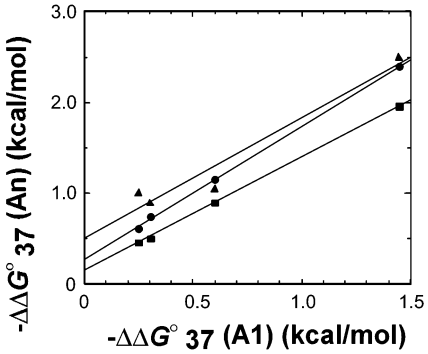


Figure 3. Relationship between the free-energy increment by a single dangling adenine, $\Delta\Delta G^\circ_{37}(A_1)$ and enhanced stability by increasing the number of dangling adenines on the RNA duplexes. The free-energy increments for one adenine, $\Delta\Delta G^\circ_{37}(A_1)$, are plotted versus those for two (■), three (●), and four (▲) as represented by $\Delta\Delta G^\circ_{37}(A_n)$, where n is the number of dangling adenines. These plots are fitted with $\Delta\Delta G^\circ_{37}(A_2) = 1.25\cdot\Delta\Delta G^\circ_{37}(A_1) - 0.13$, $r^2 = 1.00$, $\Delta\Delta G^\circ_{37}(A_3) = 1.47\cdot\Delta\Delta G^\circ_{37}(A_1) - 0.27$, $r^2 = 0.99$, and $\Delta\Delta G^\circ_{37}(A_4) = 1.33\cdot\Delta\Delta G^\circ_{37}(A_1) - 0.50$, $r^2 = 0.95$.

Finally, the long dangling ends are observed in some biological systems. The studies detailing the effect of dangling ends on the efficiency of RNAi clearly show that gene silencing efficiency is affected by the length of the dangling end.[28] Basically, the double-stranded RNA (dsRNA) with longer dangling nucleotides more than 3 nts has less activities for gene regulation.[28] Even if dsRNA with dangling ends representing the same region of the target mRNA are used, the gene silencing efficiency of the dsRNA containing four dangling residues was lower than the corresponding dsRNA containing two or three dangling residues (unpublished data). Our results indicate that four dangling residues are much more effective at stabilizing the helix than two or three dangling residues. The dsRNA containing dangling residues may work as a guide sequence to bind the complementary mRNA in the RNAi pathway. A dsRNA containing long dangling ends may bind less to its cognate mRNA because of the intrinsic high stability of the long dangling ends. Also, the stable dangling end was structured by the stacking interaction. The stable long dangling end might induce an unsuitable structure for recognition by the protein that works in the RNAi pathway. This consideration would be reasonable because it is known that if molecular binding depends on many weak contacts, the small difference in the structure induces the specificity for the recognition.[29] Moreover, the determination of the thermodynamic properties of long dangling ends might prove useful in the design of hybridization probes such as ribozymes and antisense oligonucleotides, and in the prediction of RNA secondary structure.[2,30] For example, to predict and estimate the activity and rate-limiting steps of a ribozyme reaction, information concerning the stability of the ribozyme−substrate complex is necessary to calculate the dissociation rate constant.[31] Because the binding of the ribozyme to the substrate produces long dangling ends, our parameters concerning long dangling ends are helpful in estimating and predicting ribozyme activity. The stability of the pseudoknot

(26) Doktycz, M. J.; Paner, T. M.; Amaratunga, M.; Benight, A. S. *Biopolymers* **1990**, *30*, 829−845.
(27) Olsthoorn, S. M.; Bostelaar, L. J.; DeRooij, J. F. M.; Van Boom, J. H.; Altona, C. *Eur. J. Biochem.* **1981**, *115*, 309−321.
(28) Elbashir, S. M.; Martinez, J.; Patkaniowska, A.; Lendeckel, W.; Tuschl, T. *EMBO J.* **2001**, *20*, 6877−6888.
(29) Herschlag, D. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 6921−6925.
(30) Zuker, M. *Science* **1989**, *244*, 48−52.
(31) Stage-Zimmermann, T. K.; Uhlenbeck, O. C. *RNA* **1998**, *8*, 875−889.

in gene 32 mRNA encoded by the 28-nucleotide core sequence is significantly influenced by the number and nature of the immediately adjacent "single-stranded" 5′ and/or 3′ nucleotides appended to the core structure. This suggests that the long dangling ends stabilize the pseudoknot RNA.[32] Thus, our results also indicate that RNA stability may be achieved by increasing the length of the dangling end.

Acknowledgment. We thank M. Nakano and M. Yoneyama for helpful works and comments. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports, and Culture, Japan, to N.S.

JA0255406

(32) Qiu, H.; Kaluarachchi, K.; Du, Z.; Hoffman, D. W.; Giedroc, D. P. *Biochemistry* **1996**, *35*, 4176−4186.