**On the conformational stability of the smallest
RNA kissing complexes maintained through two G·C base pairs**

Wally Chu, Akila Weerasekera, and Chul-Hyun Kim

Abstract

Two identical 5'GACG3' tetra-loop motifs with different stem sequences (called H2 and H3) are found in the 5' end region of Moloney Murine Leukemia Virus (MMLV) genomic RNA. They play important roles in RNA dimerization and encapsidation through two identical tetra-loops (5'GACG3') forming a loop-to-loop kissing complex, the smallest RNA kissing complex ever found in nature. We examined the effects of a loop-closing base pair as well as a stem sequence on the conformational stability of the kissing complex. UV melting analysis and gel electrophoresis were performed on eight RNA sequences mimicking the H2 and H3 hairpin tetra-loops with variation in loop-closing base pairs. Our results show that changing the loop closing base pair from the wildtype (5'A·U3' for H3, 5'U·A3' for H2) to 5'G·C3'/5'C·G3' has significant effect on the stability of the kissing complexes: the substitution to 5'C·G3' significantly decreases both thermal and mechanical stability, while switching to the 5'G·C3' significantly increases the mechanical stability only. The kissing complexes with the wildtype loop-closing base pairs (5'A·U3' for H3 and 5'U·A3' for H2) show different stability when attached to a different stem sequence (H2 stem vs. H3 stem). This suggests that not only the loop-closing base pair itself, but also the stem sequence, affects the conformational stability of the RNA kissing complex.

Key words: RNA structure, UV melting analysis, kissing complex, Moloney Murine Leukemia Virus, Dimerization, Encapsidation

Introduction

Some RNA molecules show kissing interactions through base pairings between the complementary sequences of two hairpin loops, often reinforced by tertiary contacts from their neighboring bases [1]. Some were found in the same RNA molecules, forming intramolecular kissing complexes [2,3]. Others were formed between two separate RNA molecules, resulting in intermolecular kissing complexes [4,5]. RNA kissing complexes play important roles in RNA dimerization and packaging processes in retroviruses [4,5]. A highly conserved 5'GACG3' tetra-loop motif found in the genomic RNA of Moloney Murine Leukemia Virus (MMLV) forms a homo-dimeric kissing complex through two intermolecular G-C base pairs [6] (Fig. 1(a)). This is the smallest kissing complex ever found in nature. MMLV is a retrovirus that has been extensively studied for its mechanism of viral assembly and packaging [7-9] and serves as a model system for Human Immunodeficiency Virus (HIV) because of its similarities in RNA packaging mechanism.

Like other retroviruses, the genomic RNA of MMLV exists as a dimer of two identical (+)-strand RNAs. The RNA dimerization happens through non-covalent interactions at the 5' ends of two identical RNAs [10, 11]. It is a prerequisite to RNA packaging, the process of proper entry of viral RNAs into each viral capsid. At the 5' end region of the RNA, two highly conserved 5'GACG3' tetra-loop motifs (Fig. 1(b), H2 and

H3 wildtype) are involved in both RNA dimerization and packaging processes [12-18]. An NMR structure of an 18-mer RNA mimicking the H3 5'GACG3' hairpin tetra-loop motifs (H3-WT) revealed the formation of a kissing complex through two G-C Watson-Crick base pairs only (Fig. 1(a)) [6]. The existence of these kissing complexes was confirmed in a larger RNA construct that contains the whole 5' end region which is responsible for RNA dimerization and packaging [19, 20]. In this study, we have measured the thermodynamic parameters of these smallest RNA kissing complexes and investigated the effects of loop-closing base pairs as well as stem sequences on their conformational stability. Using the *in vitro* transcription method [21, 22], we synthesized eight RNA sequences mimicking the hairpin tetra-loop motifs of H2 and H3 with different loop-closing base pairs (Fig. 1(b)). UV melting analysis and polyacrylamide gel electrophoresis experiments were conducted on them.

Materials & Methods

Preparation of RNA constructs. Eight RNA sequences were synthesized *in vitro* using T7 RNA polymerase [21,22]. They were purified through the processes described previously [6,23]. All RNA samples for both UV melting and native gel electrophoresis were in a sodium phosphate buffer (pH = 6.5) with 50 mM NaCl and 0.1 mM EDTA.

UV Melting Analysis. UV absorbance of the RNA at 260 nm was measured every fifteen seconds using the kinetics mode of a Genesys 2 UV/Vis Spectrometer (Thermoelectron), while increasing the temperature from 10 °C to 90 °C at the rate of 0.7 °C /min, using a NESLAB RTE 7 refrigerated/heated bath (Thermoelectron). The melting data were

processed and further analyzed using Kaleidagraph version 4.5 (Synergy Software).

Polyacrylamide gel electrophoresis. All eight RNA sequences were run on 15% polyacrylamide native gel at 4 °C at 50-100 V for 6 to 12 hours as described before [23]. They were also run on 20% denaturing gel (7M Urea) at 150 V under the same condition.

Results

H3-WT kissing complex is thermodynamically more stable than H2-WT

We performed UV melting analysis [24] on both H2-WT and H3-WT. The UV melting plot of H2-WT showed two melting transitions – a major transition at a melting temperature, $T_{m1} = 66.5$ °C and a minor transition at a much lower melting temperature, T_{m2} (Fig. 2 (a)). T_{m2} increases as RNA concentration increases (24 °C for 4 μ M and 43 °C for 180 μ M), indicating that the minor transition involves a bi-molecular interaction [24], which is likely the dissociation of a kissing complex into two monomeric hairpin tetra-loops (Fig. 1(a)). A similar trend was observed in H3-WT (data not shown): a concentration-independent major transition at a higher melting temperature ($T_{m1} = 83.5$ °C) and a concentration-dependent minor transition at a lower melting temperature ($T_{m2} = 34.0$ °C for 4 μ M, and 51.0 °C for 180 μ M). T_m values of the major transitions for both H2 and H3 RNA constructs were independent of their RNA concentrations, suggesting that the transitions represent the subsequent unfolding of two monomeric hairpin tetra-loops after the dissociation of the kissing complex. T_m of the major transition for H2-WT (66.5 °C) is much lower than H3-WT (83.5 °C), suggesting that H2-WT hairpin tetra-

loop is thermodynamically less stable than H3-WT hairpin tetra-loop, which is expected given that the stem of H3-WT has one additional base pair and is more G-C rich.

The melting temperatures of the minor transitions (T_{m2}) were obtained at various RNA concentrations for both RNA constructs. Plots of $1/T_{m2}$ vs. a natural logarithm function of total RNA concentration were produced. As shown in Fig. 2(b), the plot for H2-WT fit well to the van't Hoff equation [25], $1/T_m = (\Delta S^\circ)/(\Delta H^\circ) + [(R)/(\Delta H^\circ)]\ln(C_T)$, where C_T is total RNA concentration, R is gas constant, T_m is a melting temperature for a bimolecular interaction. This confirmed the bimolecular nature of the kissing interactions. From the plot, we calculated ΔH° and ΔS° , and consequently ΔG° using the well-known thermodynamic relation, $\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$. As shown in Table 1, ΔG° for the formation of the kissing complex was about -6.9 kcal/mol for H3-WT and -6.1 kcal/mol for H2-WT. These free energy values are significantly higher than the ΔG° for the formation of two consecutive C-G/G-C base pairs in a linear double helix strand, flanked with a G-C or C-G base pair at both ends, calculated by nearest neighbor hypothesis (5'GCGC3'/3'CGCG5', $\Delta G^\circ = -4.36$ kcal/mol) [26-28]. The buttressing interactions by an adenine base in the 5'GACG3' tetra-loop may explain their unusual stability [6]. Also, the kissing complex of H3-WT is more stable than that of H2-WT by about 1.0 kcal/mol in ΔG° . Though ΔH° values are similar, the ΔS° value is slightly more negative for H2-WT, suggesting that the shorter length of H2 stem may lead to larger entropic loss during the formation of a kissing complex.

The loop-closing base pair affects the stability of the kissing complex

We performed UV melting experiments on the H2 and H3 mutants at different RNA concentrations and plotted $1/T_m$ vs. total RNA concentration. Fig. 3(a) shows an example of H2-AU mutant in which T_{m2} for the minor transitions increases with increasing RNA concentration. The plot was consistent with the expected linear plot from van't Hoff's relation (Fig. 3 (b)). Using this method, thermodynamic parameters for three H2 mutants were calculated (Table 1). ΔG° of H2-AU is similar to that of H2-WT with increase in the kissing interaction (more negative ΔH°) and decrease in the conformational flexibility (more negative ΔS°) compared to H2-WT, indicating little change in the thermal stability of its kissing complex. H2-CG produced the kissing complex with the lowest thermal stability (the least negative ΔG°), with decrease in both kissing interactions (less negative ΔH°) and conformational flexibility (more negative ΔS°) compared to H2-WT. H2-GC shows the strongest kissing interactions (the most negative value of ΔH°), yet due to a larger entropic loss by the kissing interactions (more negative ΔS°), the thermodynamic stability of the kissing complex (ΔG°) was little changed from the wildtype.

Similar results were obtained for H3 mutants (Table 1). H3-UA and H3-GC form kissing complexes similar in their thermal stability to H3-WT (similar in ΔG°), while the kissing complex of H3-CG is much less stable than the other three. ΔG° of H3-UA is similar to that of H3-WT with increase in the kissing interaction (more negative ΔH°) and decrease in the conformational stability (more negative ΔS°) compared to H3-WT. H3-CG produced the kissing complex with the lowest thermal stability (the least negative ΔG°), with a slight increase in the kissing interaction (more negative ΔH°) and a

significant decrease in conformational flexibility (more negative ΔS°). H3-GC shows the strongest kissing interactions (the most negative value of ΔH°), yet due to a larger entropic loss (more negative ΔS°), the thermodynamic stability of the kissing complex (ΔG°) was little changed from the wildtype.

Noting that both H3-WT /H2-AU and H3-UA/H2-WT have the same loop-closing base pair (5'A•U3' or 5'U•A3'), but with different stem sequences, we investigated the effect of the stem sequence on the conformational stability of the kissing complex. In both cases, the kissing complex with H3 stem shows slightly higher thermal stability than the ones with H2 stem (Table 1). However, the stem sequences affect different thermodynamic parameters for different loop-closing base pairs. For the 5'A•U3' base pair, the lower entropic loss with H3 stem (less negative ΔS°) was a determining factor for differences in their thermal stabilities while for the 5'U•A3', it was the increase in the kissing interactions with H3 stem (more negative ΔH°). The effect of the stem was not clear in the cases of the 5'G•C3' and 5'C•G3' base pairs. In particular, for 5'C•G3', little difference in thermal stability was observed between H2 stem and H3 stem. Though similar in ΔG° , H3-CG shows more negative values for both ΔH° and ΔS° compared to H2-CG, suggesting that the presence of H3 stem increases the strength of the kissing interactions (more negative ΔH°) while decreasing its conformational flexibility (more negative ΔS°).

The loop-closing base pair affects the mechanical resilience of the kissing complexes

A recent single-molecule pulling study showed that the H3 kissing complex has unusual mechanical stability against pulling force [29]. Since native gel electrophoresis is another way of exploring mechanical resilience [30], we ran all eight RNA constructs on a native gel. As controls, we used two monomeric hairpin loops similar in size to H2 and H3 constructs: H2-GUCG (a monomeric hairpin tetra-loop, 5'GUCG3' with H2 stem) and sgRNA (an 18-nt long hairpin hexa-loop (six nucleotides-long loop) mimicking a promoter sequence of subgenomic RNA of Brome Mosaic Virus [BMV, 31]). As shown in Fig. 4 (a), H2 constructs were run along with H2-GUCG as a control, while H3 constructs were with BMV sgRNA as a control. Both H3-WT and H2-WT ran at a significantly slower speed with more diffuse bands compared to the monomeric control RNAs, indicating that the kissing complexes largely maintained their conformations against mechanical disturbance during native gel electrophoresis, which is consistent with a recent single molecule pulling study [29]. H2-AU runs at a similar speed to H2-WT, suggesting little change in its mechanical resilience. Both ran slower than the monomeric control, which is expected given that the control RNA (H2-GUCG) does not form a kissing complex [6]. Both showed more diffuse bands than H2-GUCG, suggesting the existence of a wide range of kissing conformations, possibly caused by variation in the joining angle between two tetra-loop motifs, and/or dissociation of the kissing complexes at various time points during their run. H2-GC showed the slowest migration rate with the most diffused band, revealing its strongest mechanical resilience among all H2 constructs. H2-CG showed the least diffused band with the fastest migration rate close to the monomeric control RNA, suggesting that its kissing complexes may have been

quickly dissociated into monomeric hairpin tetra-loops during its run. Both H2-CG and H2-GUCG showed two discrete bands, implicating the presence of a minor conformation, which requires further study. A similar trend was observed in H3 constructs. Though the migration rate of H3-WT was a little slower than H3-UA, both bands were similarly diffused. H3-GC ran at the slowest migration speed with the most diffused band, while H3-CG showed the fastest migration speed with the least diffused band, which was closest to BMV sgRNA, its monomeric control. Due to its larger loop (hexa-loop), sgRNA was expected to run at a slower migration rate than the monomeric hairpin tetra-loops of H3 constructs. Similar to H2-CG, both H3-CG and sgRNA showed a minor band, suggesting the possible existence of a minor conformation. As shown in Fig. 4 (b), on a 20% denaturing gel, all eight RNA constructs ran at the expected speed based on their molecular weights, which confirmed that the observed differences in migration speed and band pattern on a native gel originated from differences in their RNA conformations, not from degradation of the RNA samples. Therefore, the multiple bands observed in H3-CG, H2-CG, and sg RNA were not the results from degradation of the RNA samples, but from the existence of minor conformations. In fact, secondary structure prediction suggests that BMV sgRNA may form more than one conformation. In conclusion, a kissing complex with a 5'G•C3' loop-closing base pair retains the strongest mechanical resilience while the kissing complex with 5'C•G3' is the most fragile. In addition, for each of three loop-closing base pairs, 5'A•U3', 5'U•A3' 5'G•C3', the pairs with H3 stem showed slower migration rates with more diffused bands than the ones with H2 stem, probably more than the expected difference in migration rate due to

difference in their size (H3 stem has seven base pairs while H2 stem has six). This suggests that H3 stem might provide stronger mechanical resilience than H2 stem. For the case of 5'CG3', the trend was not clear – the difference in their migration rate may be just due to the difference in their size, indicating that their kissing complexes are very fragile for both H2 and H3 stems and may quickly lose its conformation during the run on a native gel.

Discussion

Recent single molecule pulling studies on the kissing complex of MMLV confirmed its unusual mechanical stability [29, 32,33], and the importance of the stacking interactions from a 5' adenine in the tetra-loop [34]. The observed mechanical resilience of the RNA kissing complex is presumed to be relevant to its biological function. In this study, we have investigated the conformational stability of this kissing complex with different loop-closing base pairs and stem sequences against both thermal and mechanical disturbances.

First, we found that H2-WT also forms intermolecular loop-to-loop kissing complexes similar to H3-WT discovered in the previous study [6]. UV melting plots on H2-WT showed a concentration-dependent minor transition while native gel electrophoresis showed a diffused band with a slower migration rate than its monomeric counterpart. These results are consistent with the previous structural studies on the 5' end region of MMLV genomic RNA [6,19,20].

Second, all eight H2 and H3 RNA constructs form kissing complexes, suggesting that the propensity of them forming a kissing complex, primarily lies in the unique conformation of their 5'GACG3' tetra-loops. This explains why the 5'GACG3' tetra-loop motif is highly conserved among murine type C retroviruses [18].

Third, changing the wild type loop-closing base pair (5'U•A3' for H2 and 5'A•U3' for H3) to 5'G•C3' or 5'C•G3' significantly affects the stability of their kissing complexes. This is consistent with previous studies that demonstrated the significant contributions of the loop-closing base pair to the thermal stability of loop-loop kissing interactions in various biological systems such as the kissing complex of TAR-aptamer of HIV [35-37], and the RNA I-RNA II complex of ColE1 plasmid [38, 39]. Both H2-CG and H3-CG showed drastic decrease in both thermal and mechanical stability of their kissing complexes, suggesting that the change of 5'A•U3'/5'U•A3' to 5'C•G3' may partially disrupt even the conformation of the 5'GACG3' tetra-loop, making it less optimized for loop-to-loop kissing interactions. Changing the loop-closing base pair to 5'G•C3' noticeably increases the mechanical resilience of the kissing complex, though its thermal stability remains similar to the wildtype. The 5'G•C3' loop-closing base pair increases the strength for the kissing interactions compared to the wildtype, evidenced by more negative ΔH° . However, this increase is offset by the increase in entropic loss (more negative ΔS°), resulting in similar ΔG° to the wildtype. Given that the mutants with 5'G•C3' showed the most negative ΔH° for both H2 and H3, its slowest migration rate on a native gel at 4 °C may be explained by the dominant role of ΔH° in determining the conformational stability of the kissing complex at a low temperature. As mechanical

resilience might be an important property for their biological roles in RNA dimerization and encapsidation processes, we speculate that both H2 and H3 5'GACG3' tetra-loop motifs might have evolved to have a 5'U•A3' or 5'A•U3' as their loop-closing base pairs for optimal mechanical stability that ensures the timely association and dissociation of their kissing complexes in response to external force.

Fourth, comparison of ΔG° at 37 °C between the tetra-loops with H2 and the tetra-loops with H3 stems revealed that the kissing complexes with H3 stems are consistently more stable than the ones with H2 stems for the wild type loop-closing base pairs, 5'U•A3' or 5'A•U3'. This suggests that the characteristics of the stem sequence also affect the conformational stability of the kissing complex. Future studies should include in-vivo experiments investigating the effects of the loop-closing base pairs of the H2 and H3 hairpin tetra-loop motifs on the viability of MMLV during its life cycle.

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Legends for figures

Fig. 1 (a) The schematic representation of the formation of the homo-dimeric kissing complex from two monomeric RNA hairpin tetra-loop motifs (H3) through two intermolecular G·C base pairs from their 5'GACG3'tetra-loops. **(b)** RNA sequences and expected secondary structures of H3 wildtype (18 nt long), H2 wildtype (16 nt long), and their related mutants.

Fig. 2 (a) UV melting plots of H2-WT at two different RNA concentrations. The y-axis is the first derivative of the absorbance of the RNA at 260 nm with respect to temperature ($\frac{dA_{260}}{dT}$). **(b)** A plot of the inverse of the melting temperature of the dissociation of a kissing complex ($\frac{1}{T_{m2}}$) in the unit of absolute temperature (K), versus a natural logarithm

function of the molar concentration of the total RNA ($\ln(C)$) for H2-WT RNA. The data points fit well with a linear plot in which its slope and y-axis value provide the estimation for the change of enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) during the formation of the kissing complexes.

Fig. 3 (a) Melting plots of H2-AU at different RNA concentrations. The y-axis is the first derivative of the absorbance of the RNA sample at 260 nm with respect to temperature, ($\frac{dA_{260}}{dT}$). **(b)** The plots of the inverse of the melting temperature of the dissociation of a kissing complex ($\frac{1}{T_{m2}}$), versus natural logarithm function of the molar concentration of

the total RNA ($\ln(C)$) for H2-AU. The data fit well with a linear plot in which its slope and its y-axis value provide the estimation for the change of enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) during the formation of the kissing complexes.

Fig. 4 (a) 15% native gel electrophoresis at 4 °C. From the left, 1.H2-WT 2.H2-AU, 3.H2-GC, 4.H2-CG, 5.H2-GUCG (control) 6.H3-WT, 7.H3-UA 8.H3-GC, 9.H3-CG 10. Brome Mosaic Virus (BMV) subgenomic RNA (sgRNA) (control). **(b)** 20% denaturing gel for H2 & H3 constructs: 1. BMV sg RNA, 2.H2-GUCG, 3.H2-AU, 4.H2-GC, 5.H2-CG 6.H3-WT, 7.H3-UA, 8.H3-GC, 9.H3-CG. Insets; expected secondary structures of

H2-GUCG and BMV sgRNA. The possible GC base pair in the hexa-loop of BMV sg RNA was noted by a dashed line between two nucleotides. The RNA samples ran from the top to the bottom for all three gels.

Legends for Table

Table 1. Calculated thermodynamic parameters of H2-WT, H3-WT and their mutants with different loop-closing base pairs. At least, three independently measured UV melting data were used for the calculation. All of theoretical T_m values (the last column) were calculated based on the thermodynamic parameters for each RNA construct, and were consistent to the experimental values.

	ΔH° (kcal/ mol)	ΔS° (cal/mol)	ΔG° (T=37 °C) (kcal/mol)	Theoretical T_{m2} (°C) ([RNA] = 180 μ M)
H2-WT	-31.8 $\pm$ 1.9	-82.8 $\pm$ 5.9	-6.1 $\pm$ 0.1	45.0
H2-AU	-33.7 $\pm$ 2.9	-89.5 $\pm$ 9.8	-5.9 $\pm$ 0.1	42.9
H2-GC	-36.2 $\pm$ 2.3	-96.7 $\pm$ 7.3	-6.2 $\pm$ 0.02	44.9
H2-CG	-30.7 $\pm$ 2.1	-85.2 $\pm$ 6.7	-4.3 $\pm$ 0.03	26.8
H3-WT	-31.3 $\pm$ 1.6	-78.9 $\pm$ 4.9	-6.9 $\pm$ 0.2	52.8
H3-UA	-33.2 $\pm$ 4.3	-85.4 $\pm$ 13.2	-6.6 $\pm$ 0.3	50.6
H3-GC	-37.0 $\pm$ 1.7	-98.0 $\pm$ 5.1	-6.6 $\pm$ 0.1	48.2
H3-CG	-34.1 $\pm$ 1.2	-96.0 $\pm$ 3.9	-4.3 $\pm$ 0.05	28.7

Table 1. Calculated thermodynamic parameters of H2-WT, H3-WT and their mutants with different loop-closing base pairs. At least, three independently measured UV melting data were used for the calculation. All of theoretical T_m values (the last column) were calculated based on the thermodynamic parameters for each RNA construct, and were consistent to the experimental values.

Fig. 1 (a) The schematic representation of the formation of the homo-dimeric kissing complex from two monomeric RNA hairpin tetra-loop motifs (H3) through two intermolecular G•C base pairs from their 5'GACG3'tetra-loops. **(b)** RNA sequences and expected secondary structures of H3 wildtype (18 nt long), H2 wildtype (16 nt long), and their related mutants.

(a)

(b)

Fig. 2 (a) UV melting plots of H2-WT at two different RNA concentrations. The y-axis is the first derivative of the absorbance of the RNA at 260 nm with respect to temperature ($^{\circ}\text{C}$). **(b)** A plot of the inverse of the melting temperature of the dissociation of a kissing complex ($^{\circ}\text{C}$) in the unit of absolute temperature (K), versus a natural logarithm function of the molar concentration of the total RNA ($\ln(C)$) for H2-WT RNA. The data points fit well with a linear plot in which its slope and y-axis value provide the estimation for the change of enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) during the formation of the kissing complexes.

(a)

(b)

Fig. 3 (a) Melting plots of H2-AU at different RNA concentrations. The y-axis is the first derivative of the absorbance of the RNA sample at 260 nm with respect to temperature, $(\frac{dA_{260}}{dT})$. **(b)** The plots of the inverse of the melting temperature of the dissociation of a kissing complex $(\frac{1}{T_{m2}})$, versus natural logarithm function of the molar concentration of the total RNA $(\ln(C))$ for H2-AU. The data fit well with a linear plot in which its slope and its y-axis value provide the estimation for the change of enthalpy (ΔH), entropy (ΔS), and free energy (ΔG) during the formation of the kissing complexes.

(a)

(b)

Fig. 4 (a) 15% native gel electrophoresis at 4 °C. From the left, 1.H2-WT 2.H2-AU, 3.H2-GC, 4.H2-CG, 5.H2-GUCG (control) 6.H3-WT, 7.H3-UA 8.H3-GC, 9.H3-CG 10. Brome Mosaic Virus (BMV) subgenomic RNA (sgRNA) (control). **(b)** 20% denaturing gel for H2 constructs: 1. BMV sg RNA, 2.H2-