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Published on Web 03/04/2006

An Efficient Thermally Induced RNA Conformational Switch as a Framework for the Functionalization of RNA Nanostructures

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Abstract: RNA offers a variety of interactions and dynamic conformational switches not available with DNA that may be exploited for the construction of nanomolecular structures. Here, we show how the RNA loop−loop, or "kissing", interaction can be used to construct specific circular RNA arrangements that are capable of thermal isomerization to alternative structures. We also show how this thermally induced structural rearrangement can be used to unmask a functional RNA structure, in this case, a peptide-binding RNA structure, the Rev-response element (RRE) of HIV, thereby acting as a functional peptide-binding switch. The relative ease with which the RRE could be engineered into the RNA substrates suggested that a variety of functional RNA structures may be introduced. In addition, the structural rearrangement was extremely efficient, showing that the "kissing" complexes described in this study may provide a useful framework for the construction of functional RNA-based nanostructures, as well as aid in our understanding of the way RNA functions in biological systems.

Introduction

Nucleic acid base complementarity has been shown to be a powerful means by which to construct specific nanomolecular structures. DNA has been preferred because of chemical and enzymatic stability, facile synthesis, and well-developed procedures for manipulation and modification.[1,2] On the other hand, RNA offers a greater variety of interactions as well as dynamic conformational switches not available with DNA that may be exploited.[3−5] Here, we show how an RNA tertiary interaction can be used to construct specific RNA structures that are capable of isomerization to an alternative structure, thereby acting as a functional switch.

RNA structure bears similarities to that of protein in that it is hierarchical in nature. In the case of RNA, the primary nucleotide sequence folds into secondary structural elements, which in turn interact with each other through tertiary interactions to form the tertiary structure. Structural studies have revealed a wide variety of tertiary interactions unique to RNA that involve non-Watson−Crick base-pair and base-triple interactions as well as interactions involving ribose and phosphate moieties.[3−5] On the other hand, one distinct feature of RNA folding when compared to protein folding is that individually prepared secondary structural domains of an RNA can self-assemble to reconstitute a functional structure.[6] This makes RNA an attractive material for constructing artificial self-assemblies. For example, the tetraloop−tetraloop receptor interaction, a frequently observed RNA tertiary interaction, has been used to construct one-dimensional RNA self-assemblies[7] and to design novel self-folding RNAs.[8] However, the repertoire of tetraloop−tetraloop receptor interactions are somewhat limited, and it would be useful to be have additional interactions that can be utilized for the construction of complex assemblies.

The loop−loop, or "kissing", interaction is an RNA tertiary interaction that has been observed in RNA folding,[3−5] antisense RNA control,[9,10] and the dimerization of retroviral genomic RNA.[11−13] The dimerization initiation site (DIS) of the human immunodeficiency virus (HIV) forms a kissing dimer (K) mediated by the annealing of a self-complementary hexanucleotide (5′-GCGCGC-3′) in the loop, which is thought to be the first step in viral RNA dimerization (Figure 1A). Since the specificity of the interaction is dictated by complementary base-pairing between the two hairpin loops, the loop−loop interaction appeared to be an ideal framework for generating specific loop−loop interactions that can be used to construct specific structures. Indeed, we previously showed that RNA building blocks containing two DIS-based stem−loops connected by short

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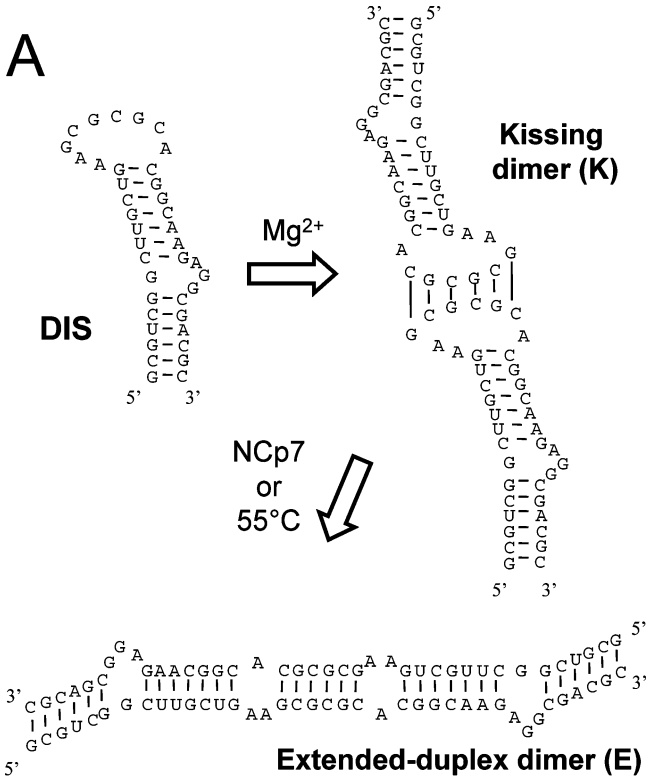

Figure 1. (A) Proposed two-step dimerization of the dimerizaion initiation site (DIS) of the HIV genome RNA. (B) Discrimination of the loop−loop complex or "kissing" form (K) and the thermodynamically stable extended duplex (E) by PAGE using gels containing and not containing Mg^{2+}.

linkers can be assembled into specific one- and two-dimensional structures by using a combination of several designed non-self-complementary loop sequences.[14] This approach has subsequently been used to construct two-dimensional arrays in a controlled manner.[15]

On the other hand, the DIS kissing complex has been shown to isomerize in vitro to a thermodynamically stable extended-duplex dimer (E) in the presence of the HIV nucleocapsid protein (NCp7) or by thermal treatment (55 °C).[11,16,17] The kissing complex (K) and the stable complex (E) can be discriminated by native polyacrylamide gel electrophoresis (PAGE), since the stable form can be observed on both Tris-borate (TB) gels containing Mg^{2+} (TBM gels) and gels lacking Mg^{2+} (TBE gels), while the relatively unstable kissing form can only be observed on the TBM gel (Figure 1B).

Here, in an attempt to determine whether RNA assemblies constructed in this way could undergo the structural isomerization illustrated in Figure 1A, the thermal isomerization of circular and linear RNA assemblies was studied. It was found that circular assemblies isomerize more efficiently than their linear counterparts, presumably due to cooperative melting of stems upon isomerization of the circular assemblies. We also demonstrate how the thermal isomerization can be used as a functional switch to unmask a peptide-binding RNA site.

Materials and Methods

Materials. RNA substrates were transcribed in vitro using T7 RNA polymerase and DNA templates that had been prepared from synthetic DNAs as described previously.[14] DNA templates for RNA substrates **1**−**8** were prepared by annealing two oligonucleotides complementary at the 3′-end and by second-strand synthesis using Taq polymerase. The sequence of the oligonucleotides can be found in the Supporting Information. The secondary structures of RNA substrates **1**−**8** are shown in Figure 2.

Assembly of Circular and Noncircular RNA Assemblies I−IV and Their Thermally Induced Isomerization. Specific combinations (**I**−**IV**) of RNA substrates **1**−**4** (24 pmol total) (Figure 3A) in H_2O (6 μL) were heated at 95 °C for 5 min and immediately cooled on ice. To this solution was added 2 × PN buffer to give a final concentration of 10 mM sodium phosphate (pH 7.0) and 50 mM NaCl (1 × PN buffer), and the mixtures were incubated at the indicated temperature for 30 min. In the case of slow-cooled samples, the RNA mixtures (**I**−**IV**) (24 pmol total) in 1 × PN buffer (12 μL) were heated at 95 °C for 5 min and slowly cooled to room temperature over a period of up to 2 h. To these solutions was added one-fourth the volume (4 μL) of loading buffer containing glycerol, the solution was divided into two aliquots, and the aliquots were analyzed separately by electrophoresis on nondenaturing 8% polyacrylamide gels (acrylamide:bisacrylamide = 30:1) in TBM buffer (89 mM Tris, 89 mM boric acid, 0.1 mM $MgCl_2$) and TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA) at 23 °C. RNA bands were visualized by staining with ethidium bromide and irradiation using a UV transilluminator (Figure 3B,C).

Assembly of Masked RRE-Containing RNAs and Their Isomerization and Peptide-Binding Properties. Mixtures of RNA substrates **5** and **6**, or **7** and **8**, (16 pmol total) in H_2O (8 μL) were heated at 95 °C for 5 min and immediately cooled on ice. To this solution was added 2 × PN buffer (8 μL), and the mixture was incubated at 37 or 65 °C for 30 min. Slow-cooled samples were prepared in 1 × PN buffer as described above. To these solutions were added 4 × peptide-binding buffer [7 μL, 40 mM HEPES−KOH (pH 7.5), 400 mM KCl, 4 mM $MgCl_2$, 2 mM EDTA (pH 8.0), 4 mM DTT, 80 μg/mL *Escherichia coli* tRNA, 40% glycerol] and the appropriate peptide (5 μL, 16 pmol), and the mixtures were incubated on ice for 10 min and analyzed on TBM and TBE gels as described above (Figure 4B,C). The effect of the peptide on the thermal isomerization was studied by adding 4 × peptide-binding buffer and the appropriate peptide prior to incubation at 37 or 65 °C (Figure 5B).

Results and Discussion

Thermally Induced Structural Isomerization of Dimeric RNA Assemblies. To determine whether RNA assemblies constructed in a previous study would undergo isomerization to the thermodynamically stable conformations, combinations (**I**−**IV**) of RNA building blocks **1**−**4** were incubated at various temperatures and analyzed by PAGE (Figure 3). An important feature of the RNA substrates used in the present study is the presence of the asymmetric four nucleotide loops in the stems

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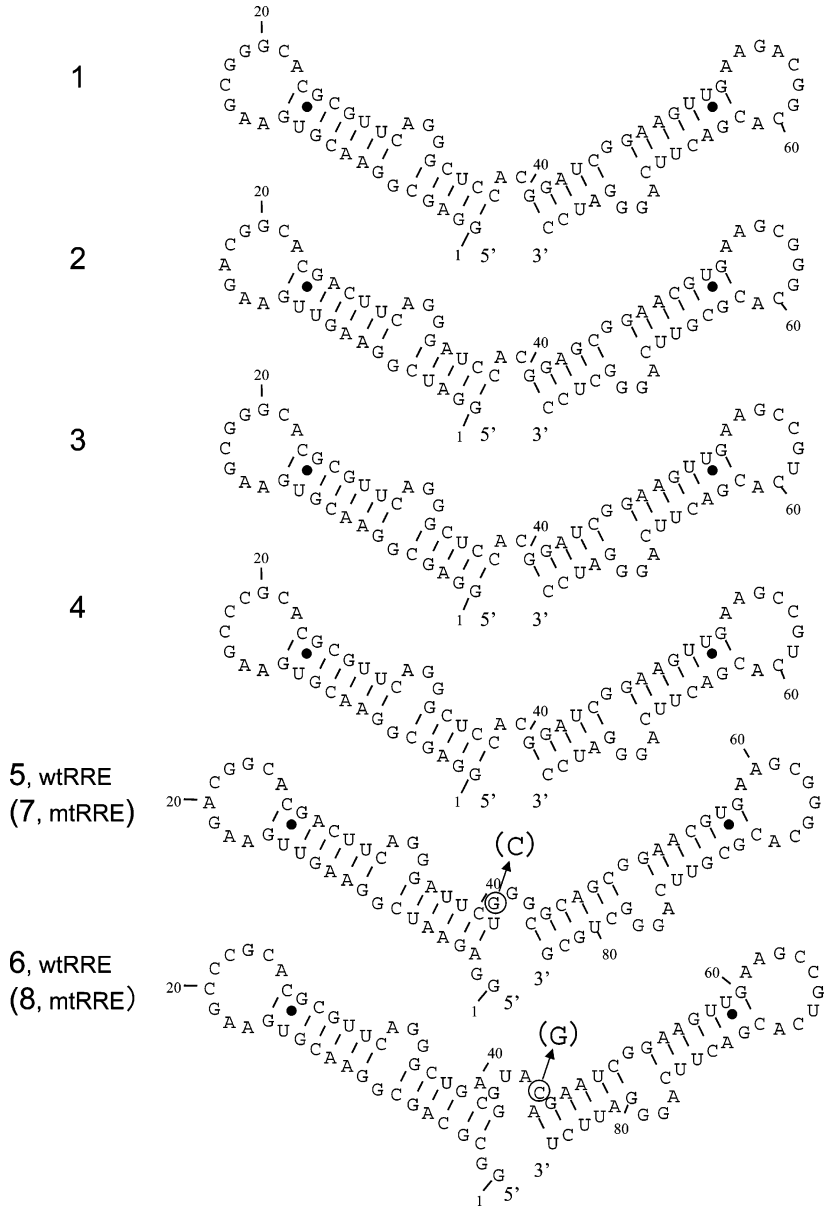

Figure 2. Secondary structures of RNA substrates (**1**−**8**) used in this study.

which facilitate melting of the stems upon isomerization (Figure 2). In addition, the sequence of the stems were designed to avoid incorrect intra- and intermolecular annealing.

Mixtures of pairs of RNA substrates (Figure 3A, combinations **I**−**IV)** were denatured at 95 °C and quick-cooled to ensure formation of the stem−loop structures, followed by incubation at 37, 46, 55, and 65 °C. The samples were analyzed by electrophoresis on TBM (0.1 mM Mg^{2+}) and TBE (no Mg^{2+}) gels at 23 °C and visualized by ethidium bromide (Figure 3B,C). It was expected that both the kissing complex and the thermodynamically stable extended complex would be observed on the TBM gel, while only the stable form would observed on the TBE gel.[11,16,17] A sample that was slow-cooled (S) following denaturation was prepared to indicate the position of the thermodynamically stable extended complex (lanes 5, 10, 15, and 20).

When both loops were designed to form the loop−loop complex (**III**), the presence of dimeric and higher (tetrameric, hexameric, etc.) assemblies was observed on the TBM gel at low incubation temperatures (Figure 3B, lane 11). On the basis of previous studies, we concluded that these low mobility species were circular tetrameric, hexameric, and higher kissing complexes.[14] Upon incubation of the RNAs at higher temperatures, these low mobility species appeared to be converted to dimeric species (Figure 3B, lanes 12−14), suggesting that the structural isomerization had occurred. Indeed, on the TBE gel, where the thermodynamically stable extended forms were expected to be predominantly observed as low mobility species, a gradual increase in the amount of the dimeric species was observed as the incubation temperature was raised (Figure 3C, lanes 11−14). Together, this suggested that the thermal isomerization to the stable conformation had occurred, accompanied by the conversion of dimeric and higher order kissing complexes to the stable dimeric form.

Next, when only one loop was allowed to "kiss" (Figure 3A, combination **II**), resulting in a linear arrangement, on the TBM gel, dimeric species were observed regardless of incubation temperature as expected (Figure 3B, lanes 6−9), while on the

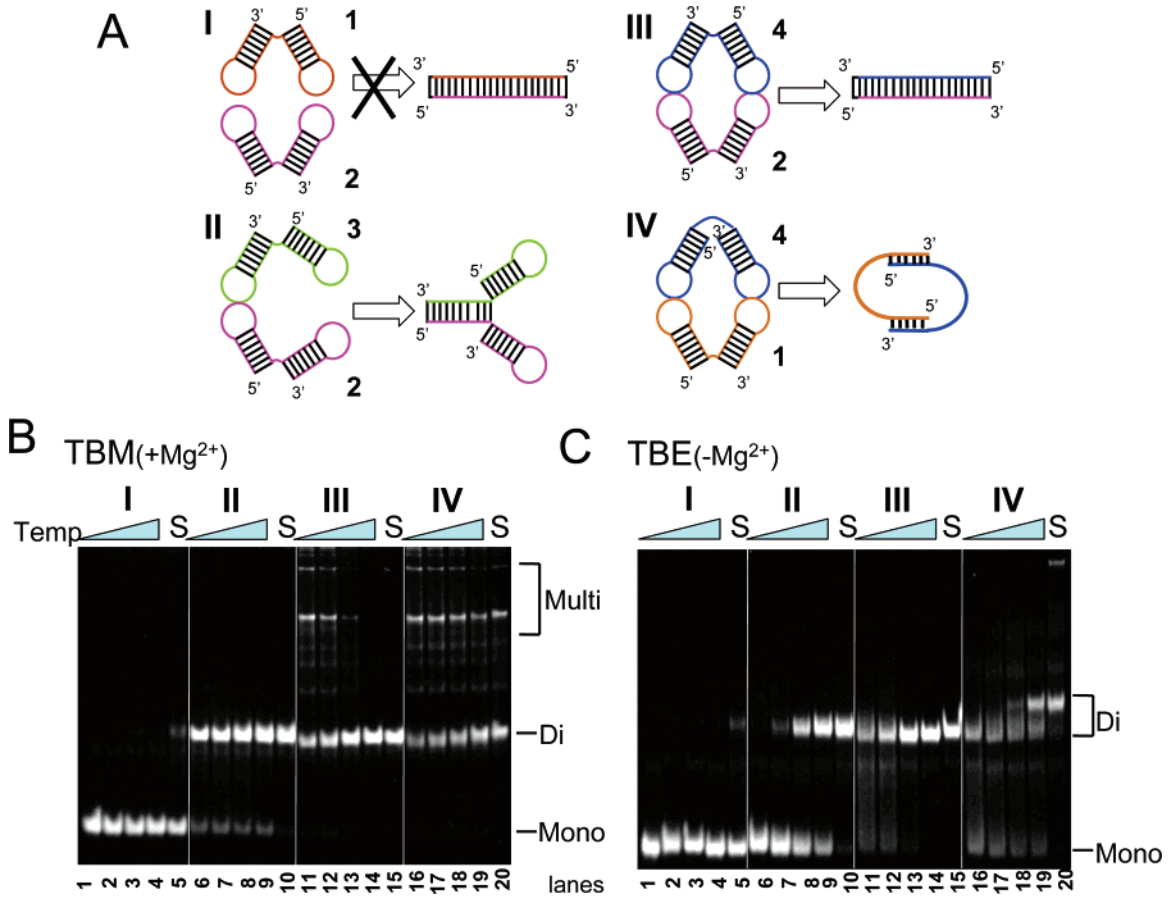

Figure 3. Thermal isomerization of dimeric RNA assemblies. (A) Schematic illustration of the proposed structural isomerizations for the pairs of RNA substrates **I** (**1** + **2**), **II** (**2** + **3**), **III** (**2** + **4**), and **IV** (**1** + **4**). (B) Analysis of reactions of **I**−**IV** containing 1 μM (24 pmol total) of each RNA substrate in PN buffer [10 mM sodium phosphate (pH 7.0), 50 mM NaCl] at 37, 46, 55, and 65 °C (lanes 1−4, 6−9, 11−14, and 16−19) and those slow-cooled (S) by PAGE on TBM (+Mg^{2+}) gels. (C) Analysis of reactions in part B by PAGE on TBE (−Mg^{2+}) gels.

TBE gel, a gradual increase in the dimeric species, presumably the stable complex, was observed at higher incubation temperatures (Figure 3C, lanes 6−9). Interestingly, the transition of this linear kissing complex to the stable complex appeared to require a higher incubation temperature (>65 °C) compared to the circular arrangement of combination **III**, which was complete at 55 °C. The midpoint of the transition for the circular arrangement could be estimated to be roughly 40 °C (Figure 3C, lanes 11−14), while that of the linear arrangement was between 55 and 60 °C (Figure 3C, lanes 6−9). This suggested that, in the circular kissing arrangement (**III**), the isomerization was promoted by cooperative melting of the two kissing complexes.

In contrast, stable complex formation was not observed for combination **I**, where kissing hairpins are not available to trigger isomerization (Figure 3B,C, lanes 1−4). On the other hand, a circular arrangement **IV** with the 5′ to 3′ direction reverse that of combination **III** also appeared to undergo the kissing to stable transition, but was inefficient, presumably due to topological constraints (Figure 3B,C, lanes 16−19).

An RNA Conformational Switch for Peptide Binding. To show how the structural isomerization described above may be used to unmask a functional RNA structure, we designed a peptide-binding RNA switch that could be turned on by thermal treatment. Specifically, sequences corresponding to the Rev-response element (RRE) RNA of HIV, the RNA-binding site of the HIV Rev peptide, were introduced into the linker region of the RNA building blocks **5**−**8** so that the RRE secondary structure was formed upon thermal isomerization to the extended conformation (Figure 4A). We chose the RRE because the peptide-binding region is relatively small, consisting of an internal loop structure that forms canonical G−A and G−G base-pairs and the surrounding region, and because the mode of binding to the HIV Rev peptide is well-characterized, so that mutant RREs that do not bind to the Rev peptide are known.[18] In addition, an RRE-binding aptamer, the RSG-1.2 peptide, which binds 6 to 7 times more strongly to the RRE than the Rev peptide is known, allowing a comparison of binding affinity on the efficiency of isomerization.[19] The BIV Tat peptide,[20] which does not bind to the RRE, was used as a negative control.

RNA substrates containing sequences corresponding to the wild-type (WT) RRE (**5** and **6**) or a mutant (MT) RRE with a G−C base-pair flipped to a C−G base-pair that abolishes binding to the Rev or RSG-1.2 peptides (**7** and **8**) were prepared. The pairs of RNAs were folded by the quick-cool method and incubated at either 37 or 65 °C, after which the appropriate peptide was added. The mixtures were analyzed on a TBM gel (0.005 mM Mg^{2+}, Figure 4B) and a TBE gel (no Mg^{2+}, Figure

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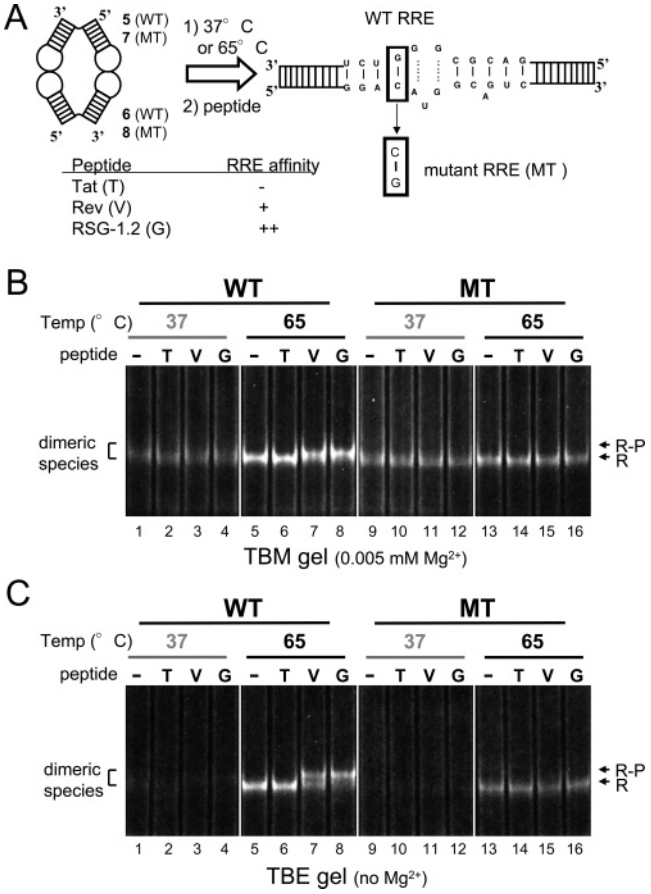

Figure 4. Thermally induced RNA conformational switch for peptide-binding. (A) Proposed thermal isomerization and formation of wild-type (WT) and mutant (MT) RREs. (B) Analysis of the reactions of WT- and MT-RRE containing RNAs (16 pmol, 0.6 μM) incubated at 37 or 65 °C, followed by the addition of no peptide (−), Tat (T), Rev (V), or RSG-1.2 (G) peptide (16 pmol, 0.6 μM) by PAGE on TBM (0.005 mM Mg^{2+}) and TBE gels (no Mg^{2+}). (C) Analysis of reactions in part B by PAGE on TBE (−Mg^{2+}) gels.

4C). In the TBM gel, where both the loop−loop complex and the thermodynamically stable extended form can be observed, dimeric species were observed for both the WT and MT RNA regardless of the incubation temperature (Figure 4B). On the other hand, on the TBE gel, dimeric species were observed only when the RNA substrates were incubated at 65 °C, indicating that the isomerization to the extended form had occurred (Figure 4C, lanes 5−8 and 13−16). As expected, peptide-binding to the RNA was observed only for the RNA substrate bearing the WT RRE sequence, as indicated by the mobility shift of the dimeric species only when either the Rev (V) or the RSG-1.2 (G) peptide was present (Figure 4C, lanes 7 and 8). In addition, the extent of gel shift was found to correspond to the RRE-binding affinity of the peptide, where, in the case of the RSG-1.2 peptide, a nearly complete shift to the slowly migrating RNA−peptide complex was observed, while a considerable amount of the faster migrating free RNA remained in the case of the Rev peptide.

Next, to investigate the effect of the peptide on the efficiency of the isomerization, RNA substrates containing the WT RRE (**5** and **6**) or the MT RRE (**7** and **8**) were folded by the quick-cool method, incubated in the presence of the appropriate peptide at 37, 46, 55, and 65 °C and analyzed on a TBE gel (no Mg^{2+},

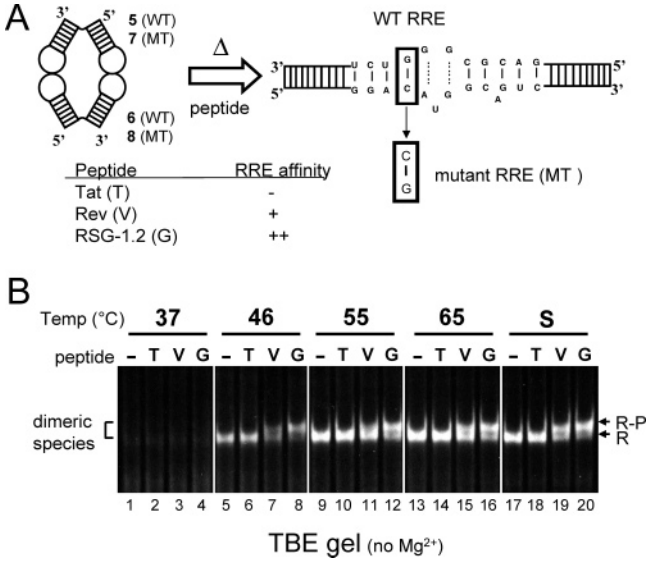

Figure 5. Thermally induced RNA conformational switching in the presence of peptide. (A) Proposed thermal isomerization and formation of wild-type (WT) and mutant (MT) RREs in the presence of peptide. (B) PAGE analysis of the thermal isomerization of WT-RRE containing RNAs in the presence of peptide on a TBE gel.

Figure 5B). A slow-cooled sample (S) was included as a control (Figure 5B, lanes 17−20). The isomerization appeared to be nearly complete at 55 °C (Figure 5B, lanes 9−12) as indicated by the similar pattern of band shifting compared to the slow-cooled sample. However, the extent of the isomerization increased proportionally regardless of the peptide present, indicating that the peptide did not have a significant effect on the rate of isomerization.

Conclusions

The above results show how specific RNA assemblies constructed using kissing interactions can be isomerized to alternative structures by thermal treatment. We also showed how this structural rearrangement could be used to unmask a functional RNA structure, in this case, a peptide-binding RNA structure. While Schroeder and co-workers have utilized the isomerization of kissing complexes to construct RNAs that form ribozymes upon isomerization, RNA substrates with self-complementary loop sequences were used, necessitating radio-labeling to visualize the substrate of interest. Furthermore, the conversion itself was rather inefficient.[21] We believe that the approach presented in this study provides a general framework for the construction of functional RNA-based nanostructures, since the isomerization reactions of the circular arrangements were particularly efficient and were complete at approximately 40 °C, presumably due to cooperative melting of the stems. The temperature of isomerization may also be adjusted, in principle, by changing the length of the RNA stems. In addition, the functional switch may be triggered thermally, as in this study, or by an RNA chaperone protein such as the HIV NCp7. The relative ease with which the peptide-binding RNA structure could be introduced into the RNA substrates also suggests that the circular RNA assemblies used in this study may provide a robust framework into which a variety of functional domains may be introduced, providing a different means to control RNA

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structure and function that may complement known ligand-induced RNA switches in RNA nanotechnology. Although the isomerization is irreversible and cannot be used as a simple on−off switch, the process can be recycled by heat denaturation and quick-cooling. In addition, since the process described resembles the dynamic structural conversion of RNA components in complex RNA−protein complexes such as the spliceosome,[22] the design of model systems such as those presented here may aid in our understanding of the way RNA functions in biological systems.

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Acknowledgment. We thank Colin Smith for critical reading of the manuscript. This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture to K.H. (No. 16510082).

Supporting Information Available: Sequences of the oligodeoxynucleotides used for the construction of double-stranded templates for transcription. This material is available free of charge via the Internet at http://pubs.acs.org.

JA0572093