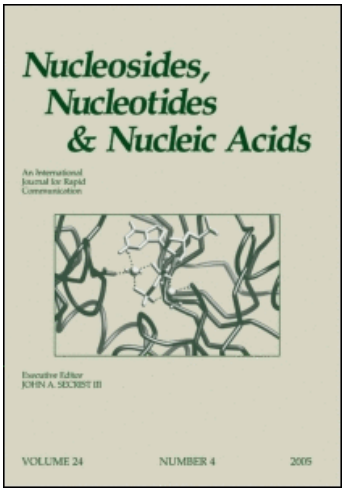

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Tailoring the Peptide-Binding Specificity Of An RNA by Combinations of Specificity-Altering Mutations

Maki Sugaya[a]; Futoshi Nishimura[b]; Akira Katoh[a]; Kazuo Harada[b]
[a] Department of Materials and Life Science, Faculty of Science and Technology, Seikei University, Tokyo, Japan [b] Department of Life Sciences, Tokyo Gakugei University, Tokyo, Japan

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TAILORING THE PEPTIDE-BINDING SPECIFICITY OF AN RNA BY COMBINATIONS OF SPECIFICITY-ALTERING MUTATIONS

Maki Sugaya,[1] Futoshi Nishimura,[2] Akira Katoh,[1] and Kazuo Harada[2]

[1]*Department of Materials and Life Science, Faculty of Science and Technology, Seikei University, Musashino, Tokyo, Japan*
[2]*Department of Life Sciences, Tokyo Gakugei University, Koganei, Tokyo, Japan*

□ *In this study, the ability to tailor the peptide-binding specificity of an RNA was investigated. First, variants of the Rev-response element (RRE) RNA with different specificities toward the natural binding partner, Rev, and two RRE-binding aptamers, the RSG-1.2 and the Kl peptides, were identified. Next, hybrid RRE mutants with combinations of two sets of specificity-altering substitutions were tested for peptide-binding specificity. It was shown that in most cases the results of the combination of individual mutations were of an additive nature, therefore providing a way to manipulate the peptide-binding specificity of an RNA in a predictable manner.*

Keywords HIV RRE RNA; arginine-rich peptide; bacterial reporter system; peptide-binding specificity; rational design

INTRODUCTION

Biochemical and structural studies on the interaction of arginine-rich peptides and their RNA sites has considerably increased our understanding of RNA-polypeptide interactions.[1,2] In particular, the interaction of the HIV Rev peptide and the RRE RNA has been extensively studied. The Rev peptide, a short peptide corresponding to the RNA-binding domain of the HIV Rev protein (Figure 1B), has been shown to bind to an internal loop region of the RRE consisting of U45 to C51 and U66 to A75 of the RRE (Figure 1A).[3–5] In vitro selection experiments suggested the presence of a non-canonical G48-G71 base pair in the internal loop that can be replaced by an isostructural A48-A71 base pair (Figure 1A).[6,7] The NMR structure

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Address correspondence to Kazuo Harada, Department of Life Sciences, Tokyo Gakugei University, Nukuikita-machi 4-1-1, Koganei, Tokyo 184-8501, Japan. E-mail: harada@u-gakugei.ac.jp

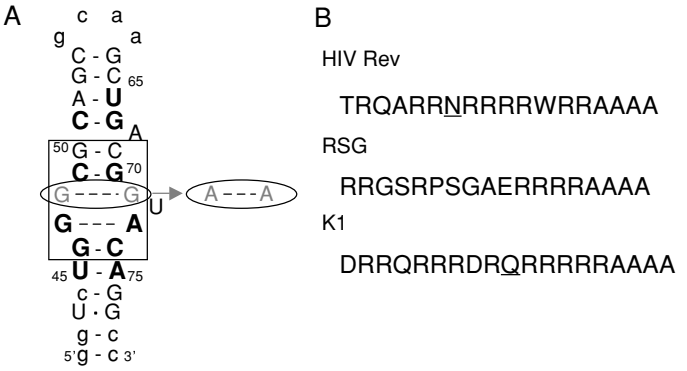

FIGURE 1 (A) The secondary structure of the RRE with nucleotides important for Rev-binding are shown in bold and those important for RSG-binding are boxed. The G-G base pair that may covary to an isostructural A-A base pair, and which is important for Kl-binding is shown in gray. (B) The amino acid sequences of the HIV Rev peptide and two selected RRE-binding peptides, RSG and Kl. The critical aspargine residue of Rev and the conserved glutamine residue of Kl are underlined.

of the Rev-RRE complex later confirmed the formation of the G48-G71 base-pair as well as a G47-A73 base pair that binds to a critical asparagine residue of the Rev peptide.[8]

Several peptides that bind to a similar region of the RRE, but with higher affinities and using a different binding strategy as the Rev peptide have been identified from random peptide libraries.[9] The RSG-1.2 peptide, which binds to the RRE several times more strongly than the Rev peptide, was selected from a relatively simple random library consisting of the three amino acids, arginine (R), serine (S), and glycine (G), followed by mutagenesis and reselection (Figure 1B).[10,11] While the helical Rev peptide binds along the major groove of the RRE in a manner similar to the binding of α-helices to the major groove of double-stranded DNA,[8] the RSG-1.2 peptide was found to bind to a similar region of the RRE in an unstructured-turn-helix conformation with the helix axis of the peptide almost perpendicular to that of the RRE.[12,13] In another selection of RRE-binding peptides from a complex library consisting of a polyarginine doped by codon-based mutagenesis using 12 amino acids, a putative α-helical high-affinity RRE-binding peptide, the Kl peptide, was identified (Figure 1B).[14] The Kl peptide contained a conserved glutamine residue that may bind to the G47-A73 base pair of the RRE in a manner analogous to the asparagine residue of the Rev peptide.

On the other hand, we have previously shown that single nucleotide substitutions or similarly small changes in the HIV Rev-response element (RRE) RNA sequence that result in well-defined secondary structural changes can dramatically alter the specificity of the RRE toward the Rev and RSG-1.2 peptides.[15] When the RRE nucleotides important for RSG-1.2-binding were determined, a considerable difference was observed compared to those

of the Rev peptide as illustrated in Figure 1A. Strikingly, several nucleotide substitutions in the upper stem region of the RRE, where the nucleotide requirement for Rev- and RSG-binding differed, were found to switch the peptide-binding specificity of the RRE from a bifunctional Rev- and RSG-1.2-binding mode to either a Rev-specific or a RSG-1.2-specific mode.

In this study, we expanded our studies on the peptide-binding specificity of RRE mutants to include the Kl peptide, and found that RRE mutants could be classified into a number of groups depending on their specificity toward the Rev, RSG-1.2, and Kl peptides. We then examined the peptide-binding specificity of hybrid RNAs containing combinations of these specificity-altering base substitutions. It was shown that in most cases the effect of the individual base substitutions were of an additive nature, therefore providing a way to manipulate the peptide-binding specificity of an RNA in a predictable manner.

RESULTS AND DISCUSSION

Determination of Nucleotide Requirements for the Binding of the RRE to the Kl Peptide

In this study, in order to expanded our studies on the peptide-binding specificity of RRE mutants to include the Kl peptide in addition to the Rev and RSG-1.2 peptides, we first determined the nucleotide requirements for the binding of RRE to the Kl peptide. This was carried out by selecting for Kl-binding RRE variants from a doped RRE library using a bacterial reporter system (Figure 2) and comparing the selected sequences shown

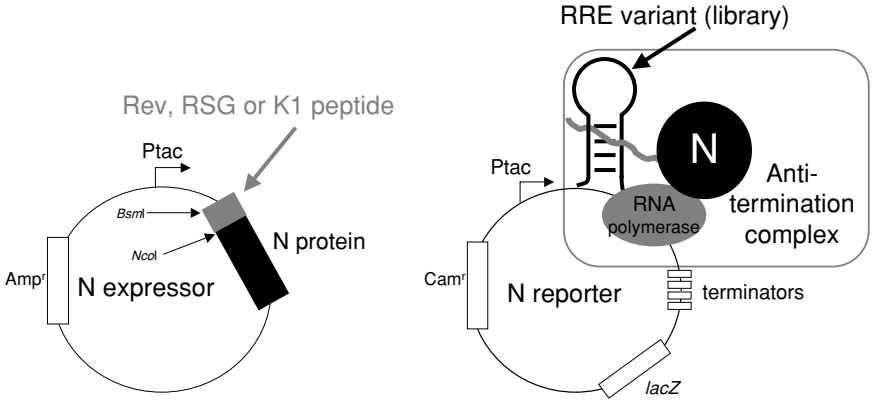

FIGURE 2 The bacterial two-plasmid reporter system for measuring transcriptional antitermination by heterologous RNA-binding polypeptides fused to the λ N protein. The RNA-binding peptide, in this case the Rev, RSG-1.2, or Kl peptide, fused to the N protein, is expressed under the control of a tac promoter from a pBR plasmid. The reporter plasmid contains the LacZ gene, also under the control of the tac promoter, so that binding of the Rev, RSG-1.2, or Kl peptide to the RNA site of interest, in this case RRE variants of a doped RRE library, results in reporter gene expression.

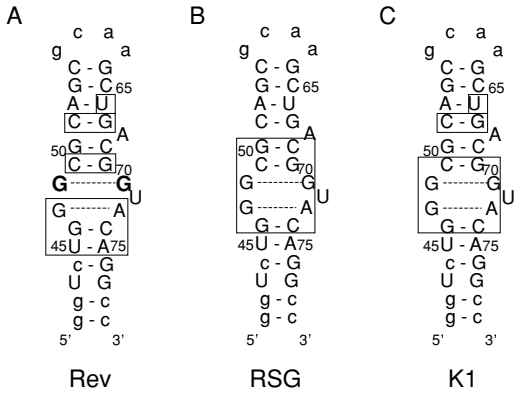

FIGURE 3 A comparison of RRE nucleotides important for Rev, RSG, and Kl peptide binding. Nucleotides within the RRE that are important for binding to the Rev (A), RSG (B), and Kl (C) peptides are boxed. The G-G base-pair highlighted in bold can be replaced by an A-A base-pair for Rev and RSG-binding.

in Table 1.[15] The differences in the nucleotide requirements of the RRE toward the Rev, RSG, and Kl peptides are summarized in Figure 3.

When the clones with Kl- and Rev-binding activities similar to those of the wildtype RRE were compared (Table 1, upper 22 clones), the nucleotide requirements for Kl-binding were found to be similar to those of the Rev peptide, except that the G-G base pair did not covary to an A-A base pair as in the case of the Rev and RSG peptides (Figure 1A). This suggested that the Kl peptide may be directly contacting the G-G base pair, unlike in the case of the Rev and RSG peptides, where the G-G to A-A base pair substitution is tolerated.[6,7,15] Indeed, clone **Gl***, a G-G to A-A variant identified in a previous study,[15] was found to have considerably lower activity toward the Kl peptide (Table 1). Next, when RRE variants with reduced activity toward the Rev peptide were compared (clones 18, 19, and 21), unlike in the case of Rev-binding, the U45-A75 base-pair was not conserved. In order to confirm the importance of this base pair for Rev-binding, and not for Kl-binding, a number of previously identified RRE-variants with substitutions at these positions were tested for Rev- and Kl-binding. As a result, a Kl-specific RRE variant, **G16***, derived from clone **G16** (Table 1) with a single nucleotide substitution (A75G) was identified.

The Specificity of RRE Variants Toward the Rev, RSG-1.2, and Kl Peptides

Various Rev- and/or RSG-specific RRE variants **Gl***, **G9**, **G30** and **R26** that were identified in a previous study, as well as the Kl-specific RRE variant **G16*** identified in this study were tested for their ability to bind to the Rev, RSG, and Kl peptides using the bacterial reporter system (Figure 2),[10]

TABLE 1 Kl-binding RRE variants and their antitermination activity against Kl, Rev, and BIV Tat peptides

Clone	doped region[b]	Colony color assay (X-gal)[a]		
		Kl	Rev	Tat
RRE	GGUCUGGGGCGCAGCGCAAGCUGACGGUACAGGCC	6	3	0
40	GGUCUGGGGCGCA**A**CG**A**AAG**U**UGACGGUACAGGCC	6	4	0
3	GGUCUGGGGCGCAG**U**GC**U**AGCUGACGGUACAGGCC			
36	GGUCUGGGGCGCA**A**CGCAAG**U**UGACGGUACAGGCC			
49	GGUCUGGGGCGCA**C**CGCA**C**G**G**UGACGG**A**ACAGGCC	6	4	0
1	GGUCUGGGGCGCAGC**U**CAAGCUGACGG**A**ACAGGCC			
8	GGUCUGGGGCGCAGCGCA**U**GCUGACGG**A**ACAGGCC			
32	GGUCUGGGGCGCAGC**U**CA**U**GCUGACGGUACAGGCC	6	4	0
41	GGUCUGGGGCGCAGC**UG**AAGCUGACGGUACAGGCC			
43	GGUCUGGGGCGCAGCGC**UC**GCUGACGGUACAGGCC			
12	GGUCUGGGGCGCAGCGC**C**AGCUGACGGUACAGGCC	6	4	0
42	GGUCUGGGGCGCAGCGCA**U**GCUGACGGUACAGGCC			
46	GGUCUGGGGCGCAGCGC**G**AGCUGACGGUACAGGCC			
25	GGUCUGGGGCGCAGCGC**C**A**U**CUGACGGUACAGGCC	6	3	0
7	GGUCUGGGGCGCAGCGCA**G**GCUGU**C**GGUACAGGCC	6	3	0
4	GGUCUGGGGCGCAG**U**GCAA**U**CUGACGGUACAGGCC	6	4	0
9	GGUCUGGGGCGCAGCGCA**CA**CUGACGGUACAGGCC	6	4	0
2	GGUCUGGGGCGCAG**U**GCAAGCUGACGGUACAGGCC			
10	GGUCUGGGGCGCAG**A**G**GT**A**U**CUGACGGUACAGGCC	6	3	0
5	GGUCUGGGGCGCAGC**A**CAA**U**CUGACGGUACAGGCC	6	3	0
31	GGUCUGGGGCGCAGCGCAA**A**CUGACGGUACAGGCC			
20	GGUCUGGGGCGCAGCGC**C**AGCU**CG**CGGUACAGGCC	6	3	0
48	GGUCUGGGGCGCAG**U**G**G**AAGCUG**CA**GGUACAGGCC	6	4	0
18	GGUC**G**GGGGCGCAGCGCA**C**GCUGA**G**GGUAC**C**GGCC	6	1	0
15	GGUCUGGGGCGCAG**UU**C**U**AGCUGA**U**GGUACAGGCC	5	4	0
14	GGUCUGGGGCGCAGCGC**C**AGCUG**UU**GGUACAGGCC	5	2	0
19	GGUC**G**GGGGCGCAGCG**A**AAGCUGACGGUAC**U**GGCC	6	2	0
16	GGUCUGGGGCGC**U**GCGCAAGCUGACGGUACAGGCC	5	3	0
21	GGUC**G**GGGGCGCAGCGCAAGCUGACGGUAC**U**GGCC	5	0	0
17	GGUCUGGGGCGCAGCG**U**AAG**G**UGACGGUACAGGCC	5	5	0
6	GGUCUGGGGCGCAG**U**GCA**U**GCUG**U**CGG**G**ACAGGCC	4	2	0
G1*	GGUCUGG**A**CGCAGCGCAAGCUGACG**AC**ACAGGCC	1	5	-
G16	GGUCUGGGGCGCAG**U**GCA**U**GCUGACGGUAC**G**GGCC	0	6	ND

[a]X-gal colony color assays were performed with the Rev, Kl, and BIV Tat (negative control) peptides as described.[14] Numbers represent +'s used to score blue color intensity.

[b]The nucleotide sequence of the RRE used as a starting point for doped library design with the nucleotides known to be important for Rev-binding are shadowed. The nucleotide sequences of the selected RRE variants are shown with those differing from the wildtype RRE in bold and underlined.

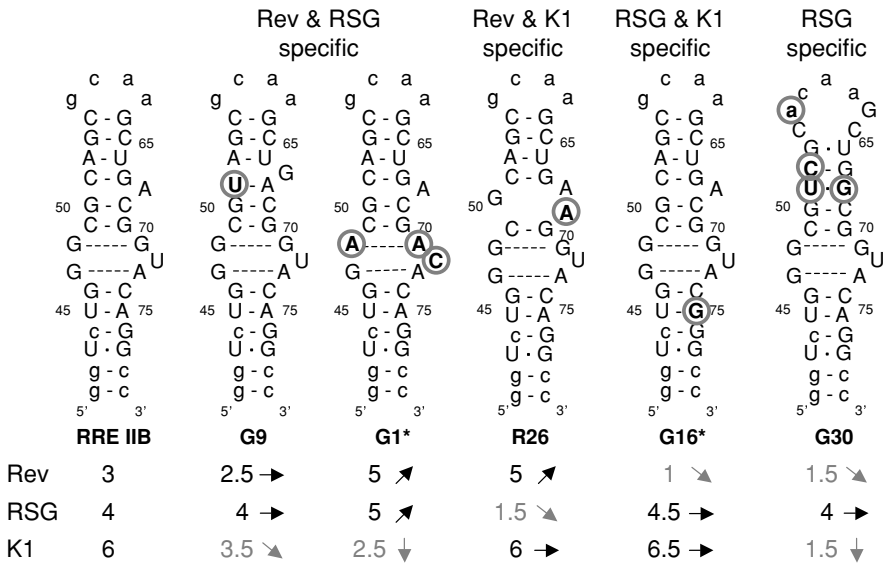

FIGURE 4 The predicted secondary structures of the RRE and RRE mutants with distinct peptide-binding specificities and their in vivo peptide-binding activities. The numbers represent the intensity of colony color scored using the LacZ reporter system. The arrows indicate the increase or decrease of in vivo activity of the mutant RRE relative to the wildtype RRE, where horizontal arrows represent no change (±0.5 units), diagonal arrows represent significant differences of up to 3 units, and vertical arrows indicate large changes of greater than 3 units. Significant decreases in β-galactosidase expression are indicated in gray, and lead to four different classes of peptide-binding specificities as indicated.

and the results are summarized in Figure 4. In the bacterial reporter system, antitermination complex formation mediated by the RNA-peptide interaction results in β-galactosidase expression. Although the orientation of the RNA-peptide complex appears to influence the stability of the antitermination complex and the resulting antitermination activity, when comparing related sets of RNA-peptide complexes, β-galactosidase expression scored by intensity of colony color on tryptone/agar plates containing 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (Xgal) has been shown to correspond to binding affinity. Those colonies with blue color similar to that of the wildtype RRE and Rev peptide (Kd = 60 nM), the RSG peptide (Kd = 12 nM), and the Kl peptide (Kd = 0.5 nM) scored as 3+, 4+, and 6+, respectively, with one plus unit generally corresponding to 3- to 10-fold differences in binding affinity.[16]

The base substitutions in clone **G9** and **Gl*** lead to loss of binding to, for the most part, only the Kl peptide, while those in **R26** and **G16*** resulted in loss of binding to the RSG and Rev peptides, respectively, and those in **G30** lead to the loss of binding to both Rev and Kl peptides. As a result, the mutants could be classified into a total of four peptide-specificity groups, the Rev- & RSG-specific **G9** and **Gl***, the Rev- & Kl-specific **R26**, the RSG-1.2- & Kl-specific **G16***, and the RSG-specific **G30.** In the case of mutant **Gl*** and

R26, an increase in the binding toward the Rev peptide, and in the case of **Gl***, an increase was also observed for the RSG-1.2 peptide.

The Peptide-Binding Specificity of Hybrid RRE Mutants with Combinations of Specificity-Altering Substitutions

To examine whether the base substitutions in clones **G9**, **Gl***, **R26**, **G16***, and **G30** could be combined to create new specificities, for example a Rev-specific clone or a Kl-specific clone, in a predictable manner, hybrid RRE mutants with all possible combinations of two sets of substitutions were tested for peptide-binding specificity using the bacterial reporter system. Of the ten combination of two sets of mutations, the **G9** and **G30** mutations overlapped and could not be combined, leaving nine combinations that could be tested.

Antitermination activities of the nine possible hybrid RREs against the Rev, RSG-1.2, and Kl peptides were determined, and the results are shown in Table 2. In the **G9/R26** and **R26/G30** hybrids, the pairs of base substitutions both resulted in distict secondary structural changes in the upper stem

TABLE 2 In vivo peptide-binding activities of hybrid RREs containing combinations of specificity-altering base substitutions[a]

		Rev / RSG	Rev / K1	RSG / K1	RSG
	Clone # (RRE) Rev 3 RSG 4 K1 6	G1* ↗ 5 (+2) ↗ 5 (+1) ↓ 2.5 (-3.5)	R26 ↗ 5 (+2) ↘ 1.5 (-2.5) → 6 (-)	G16* ↘ 1 (-2) → 4.5 (+0.5) → 6.5 (+0.5)	G30 ↘ 1.5 (-1.5) → 4 (-) ↓ 1.5 (-4.5)
Rev / RSG	G9 → 2.5 (-0.5) → 4 (-) ↘ 3.5 (-2.5)	↗ 4 (+1) → 4.5 (+0.5) ↓ 0.5 (-5.5)	↘ 1.5 ↓ 1 ↓ 1.5	↘ 2 (-1) → 4 (-) ↘ 3 (-3)	-
	G1* ↗ 5 (+2) ↗ 5 (+1) ↓ 2.5 (-3.5)		↘ 1 ↓ 1 ↓ 1	↗ 4 (+1) ↗ 7 (+3) ↘ 3 (-3)	↘ 1 ↘ 2 ↓ 1
Rev / K1	R26 ↗ 5 (+2) ↘ 1.5 (-2.5) → 6 (-)			↘ 2 (-1) ↓ 0.5 (-2.5) → 6 (-)	↘ 1.5 ↓ 0.5 ↓ 0
RSG / K1	G16* ↘ 1 (-2) → 4.5 (+0.5) → 6.5 (+0.5)				↘ 0.5 (-2.5) → 4 (-) ↓ 1.5 (-4.5)

[a]Combinations that lead to loss of activity against all three peptides are shown with a white background. Those combinations that lead to hybrid RREs with new binding specificities are shown with the gray background. The arrows indicate the increase or decrease of in vivo activity of the mutant and hybrid RREs relative to the wildtype RRE, where horizontal arrows represent no change (±0.5 units), diagonal arrows represent significant differences of up to 3 units, and vertical arrows indicate large changes of greater than 3 units. The first values represent the intensity of colony color scored using the LacZ reporter system, and the values in parenthesis represent the change in intensity compared to wildtype RRE.

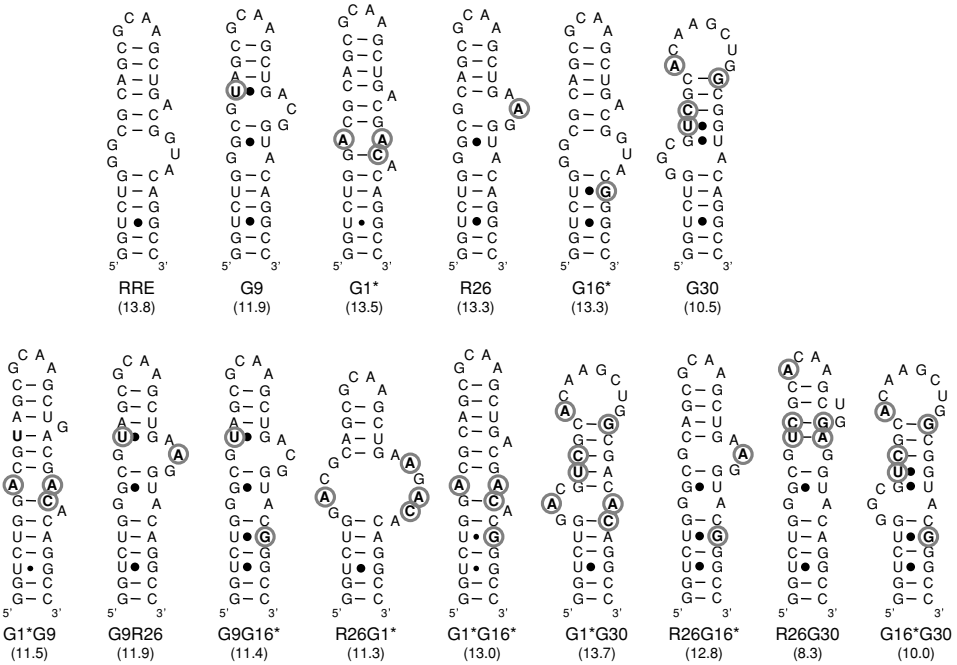

FIGURE 5 The lowest free-energy structures of mutant and hybrid RREs and their calculated free energy ($-\Delta G^{\circ}_{37}$) as determined by MulFold version 2.

regions and would be expected to alter or destabilize the conformation of the other. Therefore, not surprisingly, an almost complete loss of binding to all three peptides was observed. It should be added, however, that the **R26/G30** hybrid would be expected to have no activity against all three peptides if the peptide-binding specificities of the two sets of substitutions are assumed to be additive.

Of the remaining seven hybrid RREs, the peptide-binding specificities of the five hybrids highlighted in gray in Table 2 appear to be of an additive nature, with the sum of the specificity change of the individual sets of substitutions agreeing well with the experimental values. For example, combination of the **G9** substitution, where the change in antitermination activity against the Rev, RSG, and Kl peptides was (−0.5, 0, −2.5), and the **Gl*** substitution, which was (2, 1, −3.5), resulted in a hybrid RRE **G9/G1*** with a specificity change of (1, 0.5, −5.5), which is similar to the calculated specificity of (1.5, 1, −6). Since both the **G9** and **Gl*** substitutions were Rev & RSG-specific, combination of the two resulted in a hybrid RRE with reduced Kl-binding, and as a consequence, improved Rev- and RSG-1.2-specificity. A similar additivity was observed for the remaining four hybrids **G9/G16***, **G1*/G16***, **R26/G16***, and **G16*/G30**, and the difference between the calculated values and the experimental values were almost all within 1 colony color unit. The only exceptions were the 1.5 colony color

unit differences observed for the Rev-binding activity of **G9/G16*** and the RSG-binding activity of **G1*/G16***. In the case of **G9/G16***, this appeared to be because the negative effect of **G16*** on Rev-binding was not dominant, whereas in the case of **G1*/G16***, the positive effect that the **Gl*** mutation has against the RSG-1.2 peptide appeared to be dominant over the negative effect of **G16***. It is particularly worth noting that the combination of the Rev & Kl-specific mutation of **R26** with the RSG-1.2- & Kl-specific mutation of **G16*** resulted in the creation of a novel highly Kl-specific hybrid RRE. In addition, combination of the RSG-1.2- & Kl-specific mutation of **G16*** with the RSG-1.2-specific mutation of **G30** resulted in a decrease in Rev- and Kl-binding to yield a more specific RSG-1.2-binding hybrid RRE.

The low-binding affinity of the **G1*/G30** toward all three peptides may be due to the formation of a stable alternative secondary structure as predicted by an RNA folding algorithm, MulFold version 2.0. The majority of the hybrid RREs including those that exhibited the predicted activities such as the **G1*/G9** hybrid folded into an overall secondary structure similar to the wildtype RRE, and appeared to require minimum rearrangement in the internal loop region to form the peptide-binding site. On the other hand, the **G1*/G30** hybrid folded into a considerably different secondary structure with an alternative upper stem- loop that is expected to be difficult to rearrange to form the peptide-binding site. The **G1*/R26** hybrid, which was anticipated to yield a new Rev-specific hybrid, did not show activity toward all three peptides, suggesting that the two mutations, although separated by one base pair, are altering the conformation of the other mutants.

CONCLUSIONS

In conclusion, the results show that the peptide-binding specificity of an RNA can be manipulated in a semi-rational manner by combinations of specificity-altering base substitutions, provided that the pairs of substitutions are sufficiently separated in space and do not result in the formation of stable alternative structures. A similar strategy is also likely to be applicable to aptamers so that their ligand-binding specificity may be modified to create novel binding specificities or fine-tuned by reducing nonspecific binding. In general, the effects of the individual substitutions were of an additive nature except for the positive effect of the **Gl*** mutation against the Rev and RSG-1.2 peptides, which was dominant over the negative effect of the second mutation. While further structural studies will be necessary to understand the exact nature of the changes in specificity observed in this study, the results demonstrate the adaptability and modularity of RNA structure in creating new binding specificities, which is in contrast to the case of peptides, where a switch in RNA-binding specificity has been shown to require several amino acid substitutions to support.[17] This difference may be attributed to the ability that a given RNA has to adopt a multitude

of relatively stable structures predominantly through alternate Watson-Crick base pairing patterns, while retaining sufficient flexibility to adapt these structures to different binding partners.[18] This multidimensionality of conformational space that RNA has for peptide binding was likely to have been utilized in the evolution of RNA-protein interactions, and an understanding of this mechanism is expected to provide insights on such processes as the emergence of novel RNA viruses and drug resistance.

EXPERIMENTAL

In Vivo Selection of Kl Peptide-Binding RRE Variants

A DNA cassette prepared in a previous study encoding a library of RRE variants, in which a 26 nucleotide region that included U45-C54 and G64-A75 of the wildtype sequence and a GCAA tetraloop cap (Figure 1A) was doped at a frequency of 24% per residue, was used.[15] This level of doping was expected to result in an average of 5–6 substitutions per sequence, and a representation of most of the RRE variants with 2 substitutions, including the G-G to A-A substitution observed in previous in vitro selections[6,7] when 10^5 sequences were screened.

In the primary selection, this doped library DNA was introduced into the pACK plasmid carrying the NPT II reporter and transformed into Kl peptide expressor cells (N567/pBR Kl) (Figure 2).[14] Of the estimated 1.8×10^6 transformants, 4.2% survival was observed on tryptone plates containing kanamycin (5 μg/ml). In a secondary screen to eliminate reporter plasmid-related false positives, the PCR-amplified library insert was reintroduced into the pAC plasmid carrying the LacZ reporter, retransformed into N567/pBR Kl cells, and 40.2% of the colonies exhibited blue color on plates containing X-gal. Reporter gene expression was scored by intensity of colony color,[11,16] with those colonies with blue color similar to that of the wildtype RRE and Kl peptide scored as 6+, and those similar to wildtype RRE and Rev peptide scored as 3+. Fifty-nine colonies with blue colors scored as 4+ to 6+ were picked, plasmid isolated, and the library portion of the pAC plasmid was sequenced. After excluding overlapping clones, a total of 30 unique clones were obtained. Representative clones were tested for antitermination activity against the Kl and Rev peptides, as well as the BIV Tat peptide (Table 1). None of the clones showed antitermination activity in BIV Tat expressor cells, indicating that false positives had been eliminated.[11]

The Preparation of Expression Plasmids for RRE Variants and in vivo Peptide-Binding Analysis

The double-stranded DNA insert encoding the RRE variant sequence was prepared by annealing two oligonucleotides complementary at the

3′-ends, and second strand synthesis using Taq polymerase. The oligonucleotides used are listed in Supplementary Table 1. A solution containing the corresponding sense oligodeoxynucleotide (8.8 μM), and antisense oligodeoxynucleotide (8.8 μM), 0.3 mM dNTP, 4.5 mM $MgCl_2$, 1 × PCR buffer, and Taq polymerase was heated at 90°C for 5 minutes, slow cooled to room temperature, then incubated at 72°C for 10 minutes. The double stranded DNA was digested with PstI and BamHI, and introduced into the PstI and BamHI sites of pAC plasmid using T4 DNA ligase. The ligation mixtures were used to transform N567 cells, and cells were spread onto tryptone plates containing ampicillin and tetracycline. Several colonies were picked, pAC plasmid isolated, and the region coding the RRE variant was sequenced on an ABI 3130 sequencer using BigDye Terminator v3.1 Cycle sequencing Kit (ABI). Antitermination activity of each expression plasmid was carried out using the colony colour assay using tryptone plates containing X-gal (80 μg/ml).

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SUPPLEMENTAL TABLE 1 Oligonucleotides used to construct expressor plasmids encoding RRE variants

G9/G30-sense	5′-gggCTGCAGGTCGACGCTCTTAAAAATTAAGGTCTGGGCGT-3′;
G16*/R26-sense	5′-gggCTGCAGGTCGACGCTCTTAAAAATTAAGGTCTGGGCGCAGCGCAAGCTG-3′
G9-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCTGTACCGTCAGCTTGCGCTACGCCCAGACCTTAA-3′
G30-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCTGTACCGCCAGCTTGTGCGACGCCCAGACCTTAA-3′
G16*-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCCGTACCGTCAGCTTGCGCTGCGC-3′
R26-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCTGTACCTTCAGCTTGCGCTGCGC-3′
G1*-sense	5′-gggCTGCAGGTCGACGCTCTTAAAAATTAAGGTCTGGACG-3′
G1*/G9-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCTGTGTCGTCAGCTTGCGCTACGTCCAGACCTTAA-3′
G1*/G16-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCCGTGTCGTCAGCTTGCGCTGCGTCCAGACCTTAA-3′
G1*/G30-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCTGTGTCGCCAGCTTGTGCGACGTCCAGACCTTAA-3′
G1*/R26-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCTGTGTCTTCAGCTTGCGCTGCGTCCAGACCTTAA-3′
G9/G16*-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCCGTACCGTCAGCTTGCGCTACGCCCAGACCTTAA-3′
G9/R26-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCTGTACCTTCAGCTTGCGCTACGCCCAGACCTTAA-3′
G16*/G30-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCCGTACCGCCAGCTTGTGCGACGCCCAGACCTTAA-3′
G16*/R26-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCCGTACCTTCAGCTTGCGCTGCGC-3′
R26/G30-anti	5′-cggGGATCCCTGCTTTGAATGCTGGCCTGTACCTCCAGCTTGTGCGACGCCCAGACCTTAA-3′