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Design of an Adenosine Analogue that Selectively Improves the Affinity of a Mutant U1A Protein for RNA

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Abstract: The RNA recognition motif (RRM), one of the most common RNA binding domains, contains three highly conserved aromatic amino acids that participate in stacking interactions with RNA bases. We have investigated the contribution of these highly conserved aromatic amino acids to the affinity of the complex formed between the N-terminal RRM of the U1A protein and stem loop 2 of U1 snRNA. Previously, we found that substitution of one of these conserved aromatic amino acids, Phe56, with Ala resulted in a large destabilization of the complex. Here, we have modified A6, the base in stem loop 2 RNA that stacks with Phe56, to compensate for a portion of the destabilization caused by the Phe56Ala mutation. We have designed two modified adenosines, A-3CPh and A-4CPh, in which a phenyl group is linked to the adenosine such that it may replace the phenyl group that is eliminated by the Phe56Ala mutation in the complex. We have found that incorporation of A-3CPh into stem loop 2 RNA stabilizes the complex formed with Phe56Ala by 0.6 kcal/mol, while incorporation of A-4CPh into stem loop 2 RNA stabilizes this complex by 1.8 kcal/mol. Either base modification destabilizes the wild-type complex by 0.8−0.9 kcal/mol. Experiments with other U1A mutant proteins suggest that the stabilization of the complex between the Phe56Ala U1A protein and stem loop 2 RNA is due to a specific interaction between the Phe56Ala U1A protein and A6-4CPh stem loop 2 RNA.

Introduction

The RNA recognition motif (RRM), also known as the ribonucleoprotein (RNP) domain or the RNA binding domain (RBD), is one of the largest families of RNA binding domains and is found in proteins that participate in almost every step of gene expression.[1−5] The target sites of RRMs are single-stranded RNA oligonucleotides that vary in sequence, structure, and flexibility. The RRM is comprised of an antiparallel β-sheet flanked by two α-helices.[6] The most conserved amino acids of the RRM that contact RNA are found in the central two strands of the β-sheet and contribute primarily to the nonspecific recognition of RNA.[3] Target site specificity is provided by the variable regions of the RRM and the cooperation of multiple RRMs in the same protein.[7] The modularity of the RRM enables selective binding of diverse single-stranded target sites and makes the RRM one of the most general RNA-binding scaffolds.

An understanding of the recognition principles that enable nonspecific and specific target site recognition in RRM−RNA complexes is important to describe the biological processes that involve RRM−RNA complexes, to develop small molecules that can specifically modulate RRM−RNA binding, and to modify or redesign RRM−RNA complexes rationally. An effective method for discovering and testing recognition and design principles in biomolecular complexes is to recover the binding energy lost from an initial destabilizing modification by redesigning either component of the complex.[8−14] Recently, this approach has been used to engineer protein−ligand complexes in order to probe and control biological pathways selectively.[15−18] In this paper, we describe the design of a modified RNA nucleoside to compensate for the destabilization caused by the

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elimination of a highly conserved aromatic amino acid from an RRM−RNA complex.

There are three highly conserved stacking interactions between aromatic amino acids and RNA bases in RRM−RNA complexes.[3,19−26] Because all of the nucleic acid bases can participate in stacking interactions, and because these interactions are highly conserved, stacking interactions are likely to contribute significantly to nonspecific RNA binding by the RRM. Stacking interactions between aromatic amino acid side chains and nucleic acid bases are more common in the recognition of nonhelical nucleic acids than helical nucleic acids. For example, stacking interactions are found in complexes formed by other RNA binding proteins, single-stranded DNA binding proteins, and DNA repair proteins.[27−39] In these complexes, Phe participates more often in stacking interactions than Tyr or Trp.[40,41] Although Phe, Trp, and Tyr stack with all four bases, there is a preference for Phe to stack with A. In particular, the stacking interaction between Phe and A is more common than any other stacking interaction in RRM−RNA complexes.[41] Because stacking interactions are highly conserved in RRM−RNA complexes, their modification may reveal general recognition principles for the formation of RRM−RNA complexes.

We have investigated RNA recognition by the N-terminal RRM of the U1A protein as a model for RRM−RNA complexes. The U1A protein is a spliceosomal protein that binds to stem loop 2 of U1 snRNA with high affinity and specificity.[42,43] Although the U1A protein contains two RRMs, only the N-terminal RRM binds to RNA.[44,45] Structures of the free N-terminal RRM and the complex formed with stem loop 2 RNA have been determined by NMR and X-ray crystallography.[19,46] The N-terminal RRM of the U1A protein contains two of the three conserved aromatic amino acids found in RRMs, Phe56 and Tyr13. In the X-ray crystal structure, Phe56 stacks with A6 and C7, and Tyr13 stacks with C5 of stem loop 2 RNA (Figure 1).[19] Previously, we found that Phe56 is essential for the stability of the U1A protein−RNA complex (Figure 2).[47,48] Mutation of Phe56 to any other aromatic amino acid did not destabilize the complex significantly, but mutation to either Leu or Ala resulted in a large decrease in binding free energy. It is unlikely that loss of the stacking interaction alone is responsible for the large decrease in binding affinity observed upon substitution of Phe56 with Leu or Ala. These mutations may change the binding free energy by altering both direct interactions and cooperative networks of interactions involving Phe56 in both the free U1A proteins and the complexes formed with stem loop 2 RNA.

In this paper, we describe experiments in which the RNA target site is altered to compensate for the loss of binding affinity caused by the substitution of Ala for Phe56. We have developed the modified adenosines, A-3CPh and A-4CPh (Figure 3), which possess a tethered phenyl group that can fill the cavity left by the Phe56Ala mutation. We predicted that these modifications would stabilize the complex with the Phe56Ala U1A protein

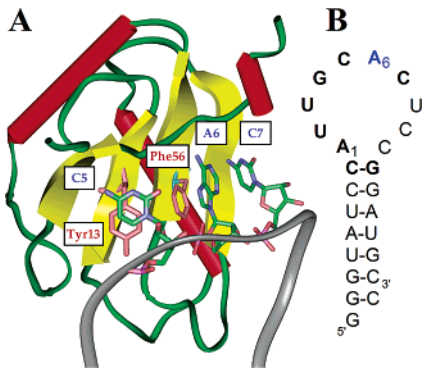

Figure 1. (A) Crystal structure of the complex formed between the N-terminal RRM of the U1A protein and stem loop 2 RNA.[19] Only part of stem loop 2 RNA is shown. The stacking interactions among Phe56, A6, and C7 and between Tyr13 and C5 of stem loop 2 RNA are shown. (B) Secondary structure of stem loop 2 RNA used in these experiments. The nucleotides that form the binding site for the U1A protein are shown in boldface.

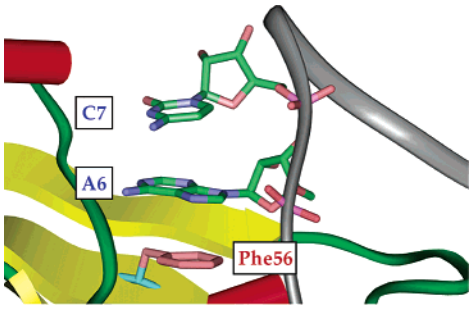

Figure 2. Close-up of the stacking interactions between Phe56 of the U1A protein and A6 and C7 of stem loop 2 RNA.[19]

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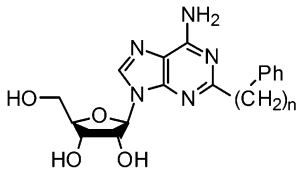

n=3 A-3CPh
n=4 A-4CPh

Figure 3. Designed adenosine analogues.

by interacting favorably with amino acids in the cavity and by helping to prevent structural changes in the complex that occur as a result of the Phe56Ala mutation. We find that the complex formed with the Phe56Ala U1A protein is stabilized by both adenosine modifications, while the complex formed with the wild-type protein is destabilized. Thus, a residue involved in the conserved stacking interaction can be rationally modulated to change the relative binding affinities of the wild-type and mutant proteins.

Results

Design of Adenosines with Tethered Phenyl Groups. We chose 2-(4-phenylbutyl)adenosine and 2-(3-phenylpropyl)adenosine as our target molecules (Figure 3). Experimental work on stacking interactions with small molecules has shown that stacking interactions between purines and aromatic rings can occur with linkers as short as three or four methylene groups, although a "herringbone" orientation between the rings can be observed with the four-methylene-group linker.[49−51] We performed B3LYP/6-31G ab initio geometry optimization calculations with Gaussian 98[52] on adenosines linked to phenyl with propyl and butyl groups. As expected, the adenine and the propyl-linked phenyl group exhibited a parallel stacked orientation, while the adenine and the phenyl group linked with the butyl group exhibited a "herringbone" orientation. The phenyl groups were linked to the C2 position of adenine to minimize disruption of the hydrogen bonding network between A6 and the U1A protein.[19]

Preparation of the Free Nucleoside. 2-(3-Phenylpropyl)-adenosine (**2**) was prepared in two steps from 2-iodoadenosine (Scheme 1). A Heck coupling of 2-iodobenzene with allylbenzene led to the formation of compound **1**. The Heck reaction gave the highest yield when carried out in a sealed tube with 1 equiv of palladium and 2 equiv of tri-*o*-tolylphosphine. Subsequent hydrogenation with 10% palladium on carbon under hydrogen gave the propyl-linked product **2**. 2-(4-Phenylbutyl)-adenosine (**4**) was also prepared in two steps from 2-iodobenzene. A Sonogashira coupling between 2-iodoadenosine and 4-phenyl-1-butyne formed 2-(4-phenyl-1-butyn-1-yl)adenosine (**3**), according to the procedures of Abiru et al.[53] Complete reduction of the alkyne by 10% palladium on carbon under hydrogen formed the butyl-linked product **4**.

Protection of Modified Adenosines for Solid-Phase RNA Synthesis. The modified adenosines **2** and **4** were appropriately protected for solid-phase RNA synthesis (Scheme 2). Initially dimethylformamidine (DMF) was chosen as the 6-NH_2 protecting group because removal of this group from the RNA oligonucleotide is facile.[54,55] However, the DMF group was not stable under the conditions used to introduce the *tert*-butyldimethylsilyl (TBDMS) group onto the 2′-OH, described later. Therefore, a benzoyl protection was used for the 6-NH_2 of both modified adenosines. The 5′-OH was then protected as the dimethoxytrityl (DMT) ether to form compounds **7** and **8**.[56] The 2′-OH of compound **7** was silylated under standard conditions with *tert*-butyldimethylsilyl chloride in THF in the presence of $AgNO_3$.[57] Modest selectivity for 2′-*O*-silylation (**9**, 42%) over 3′-*O*-silylation (**11**, 28%) was observed.

Compound **8** was not silylated under standard conditions. Piccirilli and co-workers reported the silylation of highly hindered 2′-hydroxyl groups with either *tert*-BuMgCl or KH with 18-crown-6.[58] We observed no silylation products from the reaction of *tert*-BuMgCl with either TBDMSCl or TBDMSOTf. However, silylation was achieved using KH/18-crown-6 and TBDMSCl. The reaction mixture, including KH and 18-crown-6, was cooled to 0 °C before compound **8** was added. The mixture was then cooled to −78 °C, and the TBDMSCl solution was added dropwise. At −78 °C, a mixture of 2′,3′-*O*-disilyl, 2′-*O*-silyl, and 3′-*O*-silyl nucleosides were observed initially. The 2′,3′-*O*-disilyl product quickly converted to the 2′-*O*- or 3′-*O*-silyl products. The reaction was quenched, and the silyl derivatives were purified immediately to afford the 2′-*O*-isomer (**10**, 30%) and the 3′-*O*-isomer (**12**, 30%) as white solids. Both isomers are stable in the solid phase and can be stored at −20 °C under N_2 for weeks without isomerization.

The 2′-*O*- and 3′-*O*-silylated products were identified by ^{1}H,^{1}H NOE difference spectroscopy and ^{1}H,^{1}H correlated NMR spectroscopy (^{1}H,^{1}H COSY). Irradiation of H(1′) of the 2′-*O*-silyl isomers of 2-(3-phenylpropyl)adenosine (**9**) and 2-(4-phenylbutyl)adenosine (**10**) gave no NOE on the 3′-OH, while irradiation of H(4′) gave an NOE of 4.9% for **9** and 4.0% for **10** on the 3′-OH. Irradiation of H(1′) gave an NOE of 4.8% on the 2′-OH for the 3′-*O*-silyl isomers of 2-(3-phenyl-1-proplyl)-adenosine (**11**) and 2-(4-phenylbutyl)adenosine (**12**), while irradiation of H(4′) resulted in no NOE on the 2′-OH. The expected coupling between the 3′-H and the 3′-OH was observed in the ^{1}H,^{1}H COSY spectra of the 2′-*O*-silyl isomers of 2-(3-phenylpropyl)adenosine (**9**) and 2-(4-phenylbutyl)adenosine (**10**).

The 3′-phosphoramidites of both modified adenosines (**13** and **14**) were prepared under standard conditions and isolated in

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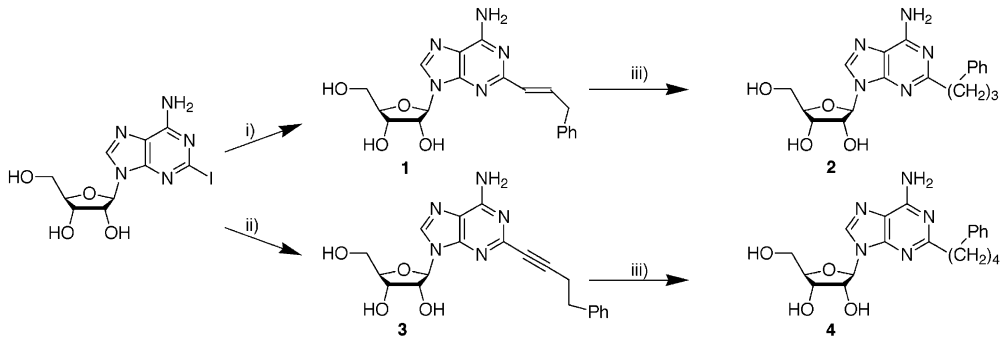
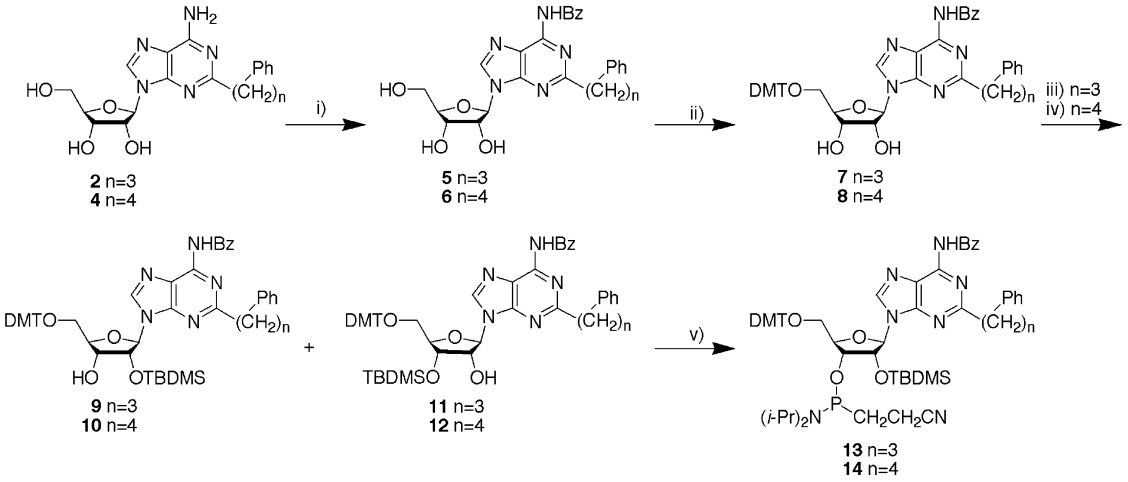
Scheme 1 [a]



[a] (i) allylbenzene, $Pd(OAc)_2$, $P(o\text{-tolyl})_3$, Et_3N, CH_3CN. (ii) 4-phenyl-1-butyne, CuI, $(Ph_3P)_2PdCl_2$, Et_3N, DMF. (iii) Pd/C, H_2, EtOH.

Scheme 2 [b]



[b] (i) TMSCl, PhCOCl, pyridine. (ii) DMTCl, pyridine. (iii) TBDMSCl, $AgNO_3$, THF. (iv) KH, 18-crown-6, TBDMSCl, THF. (v) $(i\text{-Pr})_2NP(OCH_2CH_2CN)Cl$, collidine, *N*-methylimidazole, THF.

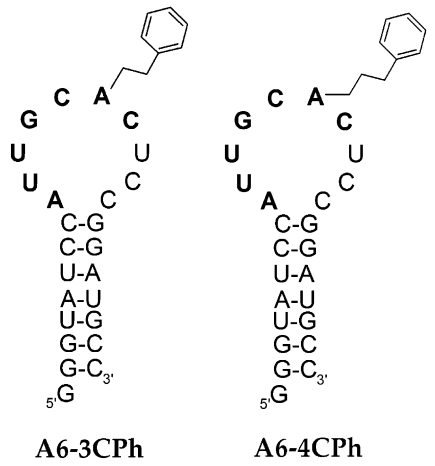


Figure 4. Secondary structure of stem loop 2 RNAs containing the designed adenosine analogues.

62−68% yield.[59] Two peaks in the ^{31}P NMR spectrum that correspond to a pair of diastereomers with chemical shifts of approximately 150 ppm were observed.

Synthesis and Characterization of RNA. A6 was substituted with 2-(4-phenylbutyl)adenosine (A6-4CPh) and with 2-(3-phenylpropyl)adenosine (A6-3CPh) in stem loop 2 RNA (Figure 4). Coupling yields of the modified nucleotides were between 50% and 90%. The coupling reactions were not optimized, but sufficient material was obtained to perform the experiments described in this paper. The resulting RNA was deprotected with NH_4OH/EtOH (3:1) at 55 °C for 12 h, followed by treatment with a solution of TEA/3HF. The RNA was purified by denaturing polyacrylamide gel electrophoresis. To confirm that the RNA was fully deprotected and that the modified bases were incorporated into the RNA, the molecular weights of the RNA oligonucleotides were determined by MALDI mass spectrometry and correct composition was confirmed by enzymatic hydrolysis.[60]

The effect of the tethered phenyl groups on the stability of stem loop 2 RNA was evaluated by melting curves obtained by UV and CD spectroscopies. The UV and CD melting profiles of the modified stem loop 2 RNAs and the wild-type stem loop 2 RNA were similar, and the calculated melting temperatures (T_m) were within experimental error. In addition, the CD spectra of the modified and wild-type stem loop 2 RNAs were similar. These data suggest that the modification of A6 does not significantly alter the structure or the stability of stem loop 2 RNA.

Equilibrium Binding of U1A Proteins to A6-3CPh and A6-4CPh Stem Loop 2 RNAs. The affinities of the two modified stem loop 2 RNAs for the wild-type U1A protein were measured by gel mobility shift assays (Figure 5 and Table 1).[48] Examples of plots illustrating the fraction RNA bound as a

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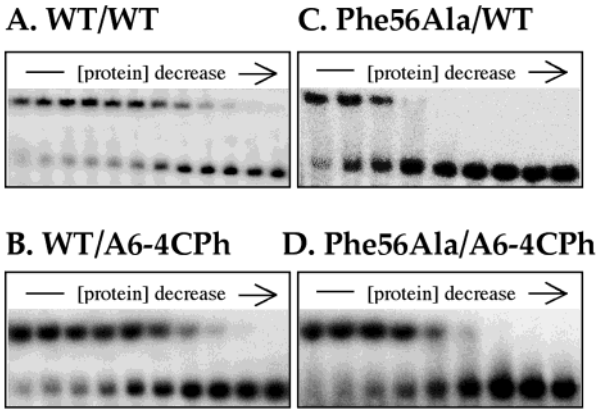

Figure 5. Examples of gel mobility shift analyses of the wild-type and Phe56Ala U1A proteins binding to wild-type and A6-4CPh stem loop 2 RNAs. In each gel, the slower moving band is the complex and the faster moving band is the free RNA.

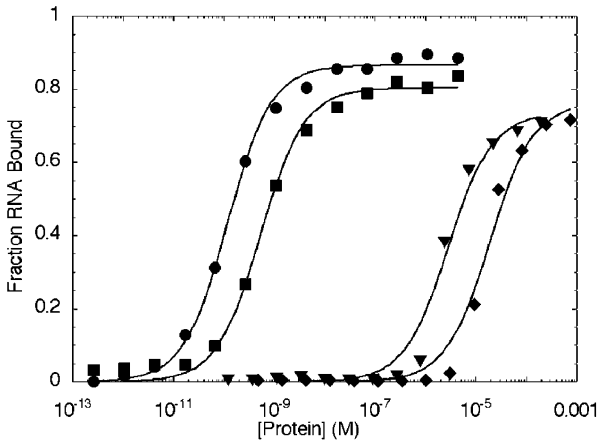

Figure 6. Plots illustrating the fraction RNA bound as a function of protein concentration: wild-type U1A protein/wild-type stem loop 2 RNA complex (●), wild-type U1A protein/A6-4CPh stem loop 2 RNA complex (■), Phe56Ala U1A protein/wild-type stem loop 2 RNA (◆), Phe56Ala U1A protein/A6-4CPh stem loop 2 RNA complex (▼).

function of protein concentration are shown in Figure 6. The A6-3CPh and A6-4CPh stem loop 2 RNAs bound the U1A protein with 0.9 (± 0.3) and 0.8 (± 0.2) kcal/mol less binding energy, respectively, than the wild-type stem loop 2 RNA (Table 2). To investigate whether the appended phenyl groups restore binding affinity lost upon substitution of Phe56 with Ala, we measured the affinity of the Phe56Ala U1A protein for A6-3CPh and A6-4CPh stem loop 2 RNAs (Table 1). Both adenosine substitutions increased binding affinity, but greater stabilization was observed for A6-4CPh, 1.8 (± 0.4) kcal/mol (Table 2). Previously, we reported the binding of a series of U1A proteins mutated at Phe56 to stem loop 2 RNA.[47,48] We measured the binding affinity of the Phe56Trp and Phe56Leu U1A proteins for A6-3CPh and A6-4CPh stem loop 2 RNAs to probe whether the amino acid at position 56 affects the binding of the U1A protein to the modified RNAs. The affinity of the Phe56Trp U1A protein for the wild-type stem loop 2 RNA was nearly equivalent to that of the wild-type protein, but the affinity of the Phe56Leu U1A protein for the wild-type stem loop 2 RNA was 4.3 kcal/mol less than that of the wild-type protein. The substitution of A-3CPh or A-4CPh for A6 destabilized the Phe56Trp U1A protein−stem loop 2 RNA complex by 0.5 (± 0.4) and 0.8 (± 0.3) kcal/mol, respectively. This destabilization is within experimental error of that observed when A-3CPh or A-4CPh was substituted for A6 in the wild-type complex (Table 2). In contrast, the incorporation of either A-3CPh or A-4CPh in stem loop 2 RNA improved binding of the Phe56Leu U1A protein by 0.4 (± 0.2) and 0.3 (± 0.2) kcal/mol, respectively.

Discussion

The modified adenosines A-3CPh and A-4CPh were designed to stabilize the complex formed with the Phe56Ala U1A protein but not the wild-type complex. In fact, the wild-type complex is destabilized by both adenosine modifications. This destabilization could result from free energy changes in either the free RNA or the complex. Although the thermodynamic melts of A6-4CPh and A6-3CPh stem loop 2 RNAs showed that their stability was not affected by the modified adenosines, these experiments primarily probe duplex stability and may not have revealed changes in the stability or dynamics of the loop that could affect binding to the U1A protein. In the complex with the wild-type U1A protein, the adenine modifications may disturb the structure surrounding A6 and any interactions that depend on this structure. One possibility is that the hydrogen bonding network formed between A6 and the U1A protein is destabilized, even though the phenyl groups have been linked to C2 of A6 in order to minimize disruption of these hydrogen bonds. Our previous work has shown that binding is sensitive to conservative modifications of both the RNA and the protein at this position, which suggests that any changes in the geometry of the interaction between A6 and amino acids in the U1A protein caused by the base modifications will destabilize the complex.[47,48,61] As a result of the sensitivity of the complex to modifications of A6, the wild-type U1A protein is selective for the wild-type stem loop 2 RNA over A6-3CPh or A6-4CPh stem loop 2 RNAs.

In contrast to the wild-type protein, the Phe56Ala U1A protein binds with higher affinity to A6-3CPh and A6-4CPh stem loop 2 RNAs than to the wild-type stem loop 2 RNA. A6-3CPh and A6-4CPh could improve the binding affinity of the Phe56Ala U1A protein for stem loop 2 RNA by stabilizing the complex or destabilizing the free RNA. However, any changes in the free energy of the free RNA will also affect binding of the wild-type U1A protein. Therefore, changes in the free energies of the complexes must be responsible for the observed destabilization of the wild-type complex and stabilization of the Phe56Ala U1A protein−stem loop 2 RNA complex by the A-4CPh and A-3CPh substitutions. A-3CPh and A-4CPh could stabilize the complex between the Phe56Ala U1A protein and stem loop 2 RNA by participating in favorable interactions in the cavity formed by the Phe56Ala mutation and by minimizing structural changes that disrupt other binding interactions in the complex. Binding would also be favored by placement of the hydrophobic phenyl group and linker in the cavity formed by the Phe56Ala mutation. These mechanisms of stabilization depend on the complexes formed with Phe56Ala and wild-type U1A proteins having similar structures. Although we do not know the structure of the complex between the Phe56Ala U1A protein and stem loop 2 RNA, molecular dynamics simulations and binding experiments with stem loop 2 RNAs containing modified adenosines at position 6 have suggested that large structural

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Table 1. Binding Affinities of Wild-Type and Mutant U1A Proteins for Wild-Type and Modified Stem Loop 2 RNAs

		RNA		
protein		wild-type	A6-3CPh	A6-4CPh
wild-type	K_d (M)	$2.1\ (\pm 0.8) \times 10^{-10}$	$8.9\ (\pm 5.4) \times 10^{-10}$	$8.2\ (\pm 4.7) \times 10^{-10}$
	$\Delta G°$ (kcal/mol)[a]	-13.2 ± 0.2	-12.3 ± 0.3	-12.4 ± 0.3
Phe56Trp	K_d (M)	$6.3\ (\pm 3.2) \times 10^{-10}$	$1.7\ (\pm 0.8) \times 10^{-9}$	$2.5\ (\pm 1.4) \times 10^{-9}$
	$\Delta G°$ (kcal/mol)[a]	-12.5 ± 0.3	-12.0 ± 0.3	-11.7 ± 0.5
Phe56Leu	K_d (M)	$3.2\ (\pm 0.9) \times 10^{-7}$	$1.5\ (\pm 0.5) \times 10^{-7}$	$1.9\ (\pm 1.1) \times 10^{-7}$
	$\Delta G°$ (kcal/mol)[a]	-8.9 ± 0.2	-9.3 ± 0.2	-9.2 ± 0.3
Phe56Ala	K_d (M)	$2.4\ (\pm 2.1) \times 10^{-5}$	$8.2\ (\pm 5.0) \times 10^{-6}$	$1.2\ (\pm 0.7) \times 10^{-6}$
	$\Delta G°$ (kcal/mol)[a]	-6.3 ± 0.5	-6.9 ± 0.3	-8.1 ± 0.4

[a] $\Delta G°$ is the free energy of association of the complex.

Table 2. Comparison of the Destabilization Energy ($\Delta\Delta G°$) Resulting from Each Phenyl-Tethered Adenine in Complexes Formed with the Wild-Type and Mutant U1A Proteins

	RNA	
	A6-3CPh	A6-4CPh
protein	$\Delta\Delta G°$ (kcal/mol)[a,b]	$\Delta\Delta G°$ (kcal/mol)[a,b]
wild-type	0.9 ± 0.3	0.8 ± 0.2
Phe56Trp	0.5 ± 0.4	0.8 ± 0.3
Phe56Leu	-0.4 ± 0.2	-0.3 ± 0.2
Phe56Ala	-0.6 ± 0.2	-1.8 ± 0.4

[a] $\Delta\Delta G°$ is the difference in binding free energies between the complex indicated in the table and the complex formed with the wild-type stem loop 2 RNA. [b] The individual $\Delta\Delta G°$ values were calculated from binding assays performed simultaneously with the same set of protein dilutions for the two RNAs being compared.

changes of the complex do not occur upon substitution of Phe56 with Ala.[48,62]

If the increase in stabilization of the complex formed between the Phe56Ala U1A protein and stem loop 2 RNA is due to a specific, favorable interaction between A-3CPh or A-4CPh and the Phe56Ala U1A protein, then the incorporation of A-3CPh and A-4CPh into the A6 position of stem loop 2 RNA should not improve the binding affinity of other U1A proteins mutated at position 56, such as the Phe56Trp and Phe56Leu U1A proteins. As expected, the substitution of A-3CPh and A-4CPh for A6 destabilizes the complexes formed between stem loop 2 RNA and the Phe56Trp and wild-type U1A proteins nearly equally. In contrast, the complex between the Phe56Leu U1A protein and stem loop 2 RNA was stabilized by both adenosine substitutions. However, the increase in binding affinity observed when A-4CPh was incorporated into the complex formed with the Phe56Ala U1A protein was significantly greater than the increase in binding affinity observed when either modified adenosine was incorporated into the complex formed with the Phe56Leu U1A protein. These results suggest that recognition of A6-4CPh stem loop 2 RNA by the Phe56Ala U1A protein is distinct from the recognition of A6-3CPh stem loop 2 RNA by the Phe56Ala U1A protein or the recognition of either modified stem loop 2 RNA by the Phe56Leu U1A protein.

Favorable interactions between the hydrophobic tethered phenyl groups and Leu may contribute to the greater affinity of A6-3CPh and A6-4CPh stem loop 2 RNAs for the Phe56Leu U1A protein. Previously, we substituted 4-methylindole for A6 in stem loop 2 RNA and measured the ability of the wild-type and Phe56 mutant U1A proteins to bind to this modified stem loop 2 RNA.[61] In these experiments, the affinity of the wild-type U1A protein for stem loop 2 RNA was decreased by 2 kcal/mol by the substitution of 4-methylindole for A6. Phe56Leu and Phe56Ala U1A proteins bound with 1.8 and 1.0 kcal/mol higher affinity, respectively, to the stem loop 2 RNA containing 4-methylindole than to the wild-type stem loop 2 RNA. These experiments suggested that the hydrophobicity of 4-methylindole contributed to the energetics of the interaction of stem loop 2 RNA with the U1A protein and that this effect was most important for complexes formed with the Phe56Leu U1A protein. In contrast, we find that the incorporation of A-4CPh into stem loop 2 RNA is most stabilizing for the complex formed with the Phe56Ala U1A protein, not the Phe56Leu U1A protein. These data support the proposal that the favorable interaction of the Phe56Ala U1A protein with A6-4CPh stem loop 2 RNA is due to a specific interaction of this modified adenosine with the Phe56Ala U1A protein.

In conclusion, we have developed a novel modified adenosine, A-4CPh, that improves the binding affinity of Phe56Ala U1A protein for stem loop 2 RNA, but destabilizes the complex formed with the wild-type protein. It is unlikely that the improvement in binding is due to a nonspecific hydrophobic effect because incorporation of A-C3Ph into stem loop 2 RNA does not lead to as great an improvement in binding affinity and the complex formed between the Phe56Leu U1A protein and stem loop 2 RNA is not as stabilized by either base substitution. When the experiments described in this paper and our previously reported results are taken together,[61] we have developed two methods to specifically improve the stability of complexes of stem loop 2 RNA with individual U1A mutant proteins, while destabilizing the complex with the wild-type U1A protein. Substitution of A-4CPh for A6 in stem loop 2 RNA stabilizes the complex with the Phe56Ala U1A protein, while substitution of 4-methylindole for A6 in stem loop 2 RNA stabilizes the complex with the Phe56Leu U1A protein. These results show that the RNA base can be modified so that the relative ability of wild-type and mutant proteins to recognize RNA is altered. Since Phe-A stacking interactions are highly conserved in RRM−RNA complexes and are also found in many other protein−single-stranded nucleic acid complexes,[27−39,41] the adenosine analogues A-4CPh and 4-methylindole may also be used to alter the recognition interfaces of other protein−nucleic acid complexes. More generally, these experiments demonstrate that protein−RNA interfaces can be reengineered to alter specificity,[14] which may enable the extension of the powerful applications of protein−ligand reengineering[15−18] to protein−RNA complexes.

Experimental Procedures

General. Commercial solvents and reagents were used as received unless otherwise noted. Before use, acetonitrile and pyridine were

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distilled from calcium hydride, THF was distilled from sodium and benzophenone, and DMF was distilled from CaO and stored over activated 4 Å molecular sieves. Flash chromatography was carried out with Silicycle Ultrapure silica gel 60 (230−400 mesh). Prep TLC was carried out on glass backed 1000 μm 60 Å silica gel with an F254 indicator (Analtech). Analytical TLC was carried out on glass backed 250 μm silica Gel GF (Analtech). Mass spectra were performed by the Mass Spectrometry Facility at the University of Illinois at Urbana-Champaign. ^{1}H NMR, ^{31}P{^{1}H}, COSY, and NOE difference spectra were obtained on 300 and 500 MHz spectrometers. ^{1}H NMR chemical shifts are reported in δ (ppm) in reference to residual proton signals in the deuterated solvent. ^{31}P{^{1}H} chemical shifts are reported in δ (ppm) relative to an external standard of 85% H_3PO_4. All exchangeable protons were detected by the addition of D_2O. 2-Iodoadenosine and *2*-(4-phenyl-1-butyn-1-yl)adenosine (**3**) were prepared according to literature procedures.[53,63−68] The synthesis and purification of the N-terminal RRM of the U1A protein, amino acids 2−102, and the Phe56Ala, Phe56Leu, and Phe56Trp U1A mutant proteins has been reported previously.[48]

2-(Allylbenzene)adenosine (1). A solution of 2-iodoadenosine (0.243 g, 0.62 mmol), palladium (II) acetate (0.142 g, 0.63 mmol), tri-*o*-tolyphosphine (0.387 g, 1.27 mmol), triethylamine (0.24 mL, 1.7 mmol), and allylbenzene (0.80 mL, 6.0 mmol) in freshly distilled acetonitrile (10 mL) was placed in a dry sealed tube and purged with N_2 for 5 min. The reaction mixture was stirred in an 80 °C oil bath overnight. After TLC showed that the 2-iodoadenosine was completely consumed, the mixture was cooled to room temperature, filtered through a Celite pad, washed with CH_2Cl_2 (200 mL), and concentrated under reduced pressure. The residue was purified by preparatory TLC (10% MeOH/$CHCl_3$) to give compound **1** as a light yellow solid (0.092 g, 53%). ^{1}H NMR (DMSO-d_6) (mixture of two isomers) δ 8.29 and 8.30 (s, 1H, H8), 7.22−7.43 (m, 8H, *H*C═CHCH$_2$, Ph, NH$_2$), 6.53 (m, 1H, HC═C*H*CH$_2$), 5.88 (d, J = 6.7 Hz, 1H, 1′-H), 5.63 (dd, J = 7.7, 4.1 Hz, 1H, 5′-OH), 5.45 (m, 1H, 2′-OH), 5.21 (m, 1H, 3′-OH), 4.66 (m, 1H, 2′-H), 4.15 (m, 1H, 3′-H), 3.98 (m, 1H, 4′-H), 3.54−3.72 (m, 4H, 5′-H, 5″-H, CH$_2$Ph). MS(ES) calcd for $C_{19}H_{21}N_5O_4$ [MH$^+$], 384.16; found, 384.13.

General Procedure for Hydrogenation. A suspension of compound **1** or **3** (approximately 1 mmol) and 10% Pd/C (0.120 g) in EtOH (50 mL) was stirred under H_2 at atmospheric pressure and room temperature for 24−48 h until the starting material was completely hydrogenated by NMR. The reaction mixture was filtered through a Celite pad and washed with EtOH (100 mL). The filtrate and washings were concentrated to dryness under reduced pressure and purified by flash chromatography (10% MeOH/$CHCl_3$).

2-(3-Phenylpropyl)adenosine (2). Hydrogenation of compound **1** (0.752 g, 1.96 mmol) gave a yellow foam (0.642 g, 85%). ^{1}H NMR (DMSO-d_6) δ 8.22 (s, 1H, H8), 7.15−7.30 (m, 7H, Ph, NH$_2$), 5.84 (d, J = 6.3 Hz, 1H, 1′-H), 5.64 (dd, J = 7.8, 3.9 Hz, 1H, 5′-OH), 5.41 (d, J = 6.3 Hz, 1H, 2′-OH), 5.20 (d, J = 4.2 Hz, 1H, 3′-OH), 4.64 (app dd, J = 11.5, 5.8 Hz, 1H, 2′-H), 4.13 (m, 1H, 3′-H), 3.97 (m, 1H, 4′-H), 3.51−3.68 (m, 2H, 5′-H, 5″-H), 2.58−2.67 (m, 4H, CH$_2$Ph, CH$_2$A), 1.99 (m, 2H, CH$_2$). HRMS(FAB) calcd for $C_{19}H_{23}N_5O_4$ [MH$^+$], 386.182 830; found, 386.182 900.

2-(4-Phenylbutyl)adenosine (4). Hydrogenation of compound **3** (0.425 g, 1.07 mmol) gave a white foam (0.340 g, 79%). ^{1}H NMR (DMSO-d_6) δ 8.23 (s, 1H, H8), 7.12−7.28 (m, 7H, Ph, NH$_2$), 5.83 (d, J = 6.7 Hz, 1H, 1′-H), 5.72 (dd, J = 8.1, 3.9 Hz, 1H, 5′-OH), 5.40 (d, J = 6.3 Hz, 1H, 2′-OH), 5.18 (d, J = 4.3 Hz, 1H, 3′-OH), 4.64 (app dd, J = 11.5, 6.5 Hz, 1H, 2′-H), 4.13 (m, 1H, 3′-H), 3.97 (m, 1H, 4′-H), 3.51−3.69 (m, 2H, 5′-H, 5″-H), 2.66 (t, J = 8.2 Hz, 2H, CH$_2$A), 2.62 (t, J = 7.6 Hz, 2H, CH$_2$Ph), 1.71 (m, 2H, C*H*$_2$CH$_2$A), 1.59 (m, 2H, C*H*$_2$CH$_2$Ph); HRMS(FAB) calcd for $C_{20}H_{25}N_5O_4$ [MH$^+$], 400.198 480; found, 400.198 400.

General Procedure for Benzoylation of 6-NH$_2$. Free nucleoside **2** or **4** (approximately 1.2 mmol) was dried by coevaporation with 3 × 6 mL of dry pyridine and then suspended in dry pyridine (9.1 mL). To the suspension was added trimethylsilyl chloride (1.17 mL, 9.22 mmol). After the mixture was stirred for 2 h at room temperature, it was cooled to 0 °C and benzoyl chloride (0.42 mL, 3.6 mmol) was added dropwise over 10 min. After the addition was complete, the mixture was stirred at 0 °C for 5 min, warmed to room temperature, and stirred for an additional 2 h. The reaction was quenched by the addition of water (3 mL) at 0 °C. The reaction mixture was stirred for 5 min at room temperature, and then concentrated aqueous ammonia (3 mL) was added. After the mixture was stirred for 15 min, it was poured into water (50 mL) and extracted with CH_2Cl_2 (5 × 15 mL). The organic phase was dried over Na_2SO_4, filtered, and concentrated under reduced pressure. The residue was purified by flash chromatography using a step gradient from 100% EtOAc to 10% MeOH/EtOAc.

***N*^{6}-Benzoyl-2-(3-phenylpropyl)adenosine (5).** Reaction of compound **2** (0.424 g, 1.10 mmol) gave a white foam (0.365 g, 68%). ^{1}H NMR (DMSO-d_6) δ 11.16 (s, 1H, NHCO), 8.64 (s, 1H, H8), 8.04 (d, J = 7.1 Hz, 2H, PhCO), 7.51−7.65 (m, 3H, PhCO), 7.15−7.31 (m, 5H, Ph), 6.03 (d, J = 6.1 Hz, 1H, 1′-H), 5.56 (d, J = 6.1 Hz, 1H, 2′-OH), 5.28 (d, J = 4.7 Hz, 1H, 3′-OH), 5.20 (m, 1H, 5′-OH), 4.70 (app dd, J = 11.3, 6.9 Hz, 1H, 2-′H), 4.21 (m, 1H, 3′-H), 4.01 (m, 1H, 4′-H), 3.57−3.75 (m, 2H, 5′-H, 5″-H), 2.91 (t, J = 7.7 Hz, 2H, CH$_2$A), 2.68 (t, J = 7.6 Hz, 2H, CH$_2$Ph), 2.09 (m, 2H, CH$_2$). HRMS(FAB) calcd for $C_{26}H_{27}N_5O_5$ [MH$^+$], 490.209 044; found, 490.208 900.

***N*^{6}-Benzoyl-2-(4-phenylbutyl)adenosine (6).** Reaction of compound **4** (0.485 g, 1.21 mmol) gave a light yellow foam (0.510 g, 83%). ^{1}H NMR (DMSO-d_6) δ 11.16 (s, 1H, NHCO); 8.61 (s, 1H, H8), 8.03 (d, J = 7.6 Hz, 2H, PhCO), 7.52−7.67 (m, 3H, PhCO), 7.13−7.29 (m, 5H, Ph), 5.99 (d, J = 6.4 Hz, 1H, 1′-H), 5.54 (d, J = 6.1 Hz, 1H, 2′-OH), 5.27 (d, J = 4.7 Hz, 1H, 3′-OH), 5.20 (m, 1H, 5′-OH), 4.69 (m, 1H, 2′-H), 4.19 (m, 1H, 3′-H), 3.98 (m, 1H, 4′H), 3.53−3.71 (m, 2H, 5′-H, 5″-H), 2.92 (t, J = 7.4 Hz, 2H, CH$_2$A), 2.63 (t, J = 7.4 Hz, 2H, CH$_2$Ph), 1.81 (m, 2H, C*H*$_2$CH$_2$A), 1.64 (m, 2H, C*H*$_2$CH$_2$Ph). HRMS(FAB) calcd for $C_{27}H_{29}N_5O_5$ [MH$^+$], 504.224 694; found, 504.224 800.

General Procedure for DMT Protection of 5′-OH. Compound **5** or **6** (approximately 1 mmol) was dried by coevaporation with 3 × 5 mL of dry pyridine and suspended in dry pyridine (4.7 mL). To the suspension was added 4,4′-dimethoxytrityl chloride (0.378 g, 1.11 mmol). The mixture was stirred under N_2 atmosphere overnight. After TLC (25% hexanes/EtOAc) showed the reaction was complete, it was quenched by the addition of MeOH (3 mL) and stirred for 1 h. The reaction mixture was then concentrated to dryness under reduced pressure, coevaporated with toluene (5 mL) to remove pyridine, and dissolved in CH_2Cl_2 (50 mL). The organic phase was washed with 5% $NaHCO_3$ and saturated NaCl. It was then dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by flash chromatography. The residue was loaded onto the column with 100% EtOAc and eluted with a step gradient of 100% hexanes, 50% hexanes/EtOAc, 100% EtOAc, and 10% MeOH/EtOAc.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{6}-benzoyl-2-(3-phenylpropyl)adenosine (7). Reaction of compound **5** (0.279 g, 0.57 mmol) gave a yellow foam (0.384 g, 85%). ^{1}H NMR ($CDCl_3$) δ 8.78 (s, 1H, NHCO), 8.24 (s, 1H, H8), 8.02 (d, 2H, J = 7.8 Hz, PhCO), 7.51−7.62 (m, 3H, PhCO), 6.74−7.31 (m, 18H, Ph), 6.29 (broad s, 1H, OH), 5.98 (d, J = 6.0 Hz, 1H, 1′-H), 4.80 (m, 1H, 2′-H), 4.48 (m, 1H, 3′-H), 4.41 (m, 1H, 4′-H), 3.76 (s, 6H, OCH$_3$), 3.24−3.47 (m, 2H, 5′-H, 5″-H), 3.05 (t, J = 7.5 Hz, 2H, CH$_2$A, overlapping with a singlet, 1H, OH), 2.74 (t, J = 7.5

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Hz, 2H, CH_2Ph), 2.21 (m, 2H, CH_2). HRMS(FAB) calcd for $C_{47}H_{45}N_5O_7$ [MH^+], 792.339 724; found, 792.339 900.

5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{6}-benzoyl-2-(4-phenylbutyl)adenosine (8). Reaction of compound **6** (0.510 g, 1 mmol) gave a yellow foam (0.638 g, 78%). ^{1}H NMR (DMSO-d_6) δ 11.15 (s, 1H, NHCO), 8.50 (s, 1H, H8), 8.03 (d, J = 7.7 Hz, 2H, PhCO), 7.51−7.65 (m, 3H, PhCO), 6.77−7.38 (m, 18H, Ph), 6.05 (d, J = 4.9 Hz, 1H, 1′-H), 5.61 (d, J = 5.4 Hz, 1H, OH), 5.29 (d, J = 5.3 Hz, 1H, OH), 4.78 (m, 1H, 2′-H), 4.33 (m, 1H, 3′-H), 4.10 (m, 1H, 4′-H), 3.70 (s, 3H, OCH_3), 3.69 (s, 3H, OCH_3), 3.21−3.34 (m, 2H, 5′-H, 5″-H), 2.78 (t, J = 7.5 Hz, 2H, CH_2A), 2.56 (t, J = 7.5 Hz, 2H, CH_2Ph), 1.67 (m, 2H, CH_2CH$_2$A), 1.58 (m, 2H, CH_2CH$_2$Ph). HRMS(FAB) calcd for $C_{48}H_{47}N_5O_7$ [MH^+], 806.355 374; found, 806.355 700.

Procedure for the Silylation of 5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{6}-benzoyl-2-(3-phenylpropyl)adenosine. To a suspension of compound **7** (0.079 g, 0.10 mmol) in dry pyridine (1 mL) was added $AgNO_3$ (0.026 g, 0.15 mmol). After the $AgNO_3$ completely dissolved, a solution of *tert*-butyldimethylsilyl chloride (0.017 g, 0.14 mmol) in dry THF (1 mL) was added dropwise. The flask was covered with aluminum foil, and the reaction was stirred under N_2 overnight. The reaction mixture was diluted with CH_2Cl_2 (2 mL), filtered to remove AgCl, and washed with 5% $NaHCO_3$ (3 mL). The aqueous layer was extracted with CH_2Cl_2 (3 × 3 mL), and the combined CH_2Cl_2 extractions were washed with water (2 × 3 mL) and saturated NaCl solution (3 × 3 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The resulting sticky residue (∼1 mL) was coevaporated with toluene (2 × 4 mL) in vacuo to remove residual pyridine. The mixture was separated by prep TLC (1:1 EtOAc/hexanes) to give the 2′-*O*-silyl (**9**, R_f = 0.44, 0.038 g, 42%) and the 3′-*O*-silyl (**11**, R_f = 0.23, 0.026 g, 28%) products as white solids.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-*N*^{6}-benzoyl-2-(3-phenylpropyl)adenosine (9). ^{1}H NMR (DMSO-d_6) δ 11.14 (s, 1H, NHCO), 8.49 (s, 1H, H8), 8.02 (d, J = 7.2 Hz, 2H, PhCO), 7.50−7.65 (m, 3H, PhCO), 6.78−7.41 (m, 18H, Ph), 6.05 (d, J = 4.8 Hz, 1H, 1′H), 5.21 (d, J = 6.0 Hz, 1H, 3′OH), 4.93 (app t, J = 4.9 Hz, 1H, 2′H), 4.27 (app dd, J = 10.6, 4.9 Hz, 1H, 3′H), 4.15 (app dd, J = 9.2, 4.6 Hz, 1H, 4′H), 3.703 (s, 3H, OCH_3), 3.700 (s, 3H, OCH_3), 3.31−3.40 (m, 2H, 5′-H, 5″-H, overlapping with broad water peak), 2.75 (m, 2H, CH_2A), 2.56 (t, J = 7.6 Hz, 2H, CH_2Ph), 1.95 (m, 2H, CH_2), 0.76 (s, 9H, *t*-Bu), −0.02 (s, 3H, SiMe), −0.11 (s, 3H, SiMe). HRMS-(FAB) calcd for $C_{53}H_{59}N_5O_7Si$ [MH^+], 906.426 203; found, 906.426 000.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-*N*^{6}-benzoyl-2-(3-phenylpropyl)adenosine (11). ^{1}H NMR ($CDCl_3$) δ 8.76 (s, 1H, NHCO), 8.22 (s, 1H, H8), 8.02 (d, J = 7.1 Hz, 2H, PhCO), 7.50−7.60 (m, 3H, PhCO), 6.76−7.40 (m, 18H, Ph), 6.06 (d, J = 4.4 Hz, 1H, 1′H), 4.72 (app dd, J = 10.1, 5.2 Hz, 1H, 2′H), 4.62 (app t, J = 4.9 Hz, 1H, 3′H), 4.20 (m, 1H, 4′H), 3.77 (s, 6H, OCH_3), 3.27−3.53 (m, 3H, 5′-H, 5″-H and 2′OH), 2.97 (t, J = 7.7 Hz, 2H, CH_2A), 2.69 (t, J = 7.6 Hz, 2H, CH_2Ph), 2.13 (m, 2H, CH_2), 0.89 (s, 9H, *t*-Bu), 0.087 (s, 3H, SiMe), 0.006 (s, 3H, SiMe).

Procedure for the Silylation of 5′-*O*-(4,4′-Dimethoxytrityl)-*N*^{6}-benzoyl-2-(4-phenylbutyl)adenosine. KH in oil was transferred to a preweighed flask under N_2, washed with hexanes (3 × 2 mL), and dried in vacuo. The flask was filled with N_2 and weighed again to determine the weight of KH (0.063 g, 1.575 mmol). To the flask was added THF (0.2 mL) and 18-crown-6 (0.060 g, 0.24 mmol). After the 18-crown-6 dissolved, the mixture was cooled to 0 °C, and compound **8** (0.136 g, 0.17 mmol) in THF (1 mL) was added dropwise. After gas evolution stopped, the mixture was cooled to −78 °C and *tert*-butyldimethylsilyl chloride (0.032 g, 0.21 mmol) in THF (0.8 mL) was added dropwise. The reaction was monitored by TLC (1:1 EtOAc/hexanes) and was quenched by the addition of water (2 mL) at 0 °C. The mixture was extracted with CH_2Cl_2 (6 × 7 mL). The combined organic layers were dried over Na_2SO_4 and evaporated to dryness in vacuo. The mixture was separated by prep TLC (1:1 EtOAc/hexanes) to give both 2′-*O*-silyl (**10**, R_f =0.47, 0.047 g, 30%) and 3′-*O*-silyl (**12**, R_f = 0.28, 0.047 g, 30%) products as white solids.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-*N*^{6}-benzoyl-2-(4-phenylbutyl)adenosine (10). ^{1}H NMR (DMSO-d_6) δ 11.13 (s, 1H, NHCO), 8.48 (s, 1H, H8), 8.02 (d, J = 7.2 Hz, 2H, PhCO), 7.51−7.66 (m, 3H, PhCO), 6.80−7.40 (m, 18H, Ph), 6.03 (d, J = 4.8 Hz, 1H, 1′H), 5.21 (d, J = 5.7 Hz, 1H, 3′OH), 4.93 (app t, J = 4.9 Hz, 1H, 2′H), 4.26 (app dd, J = 10.5, 5.1 Hz, 1H, 3′H), 4.12 (app dd, J = 8.1, 3.9 Hz, 1H, 4′H), 3.703 (s, 3H, OCH_3), 3.699 (s, 3H, OCH_3), 3.21−3.40 (m, 2H, 5′-H, 5″-H, overlapping with broad water peak), 2.73 (m, 2H, CH_2A), 2.53 (m, 2H, CH_2Ph), 1.52−1.69 (m, 4H, CH_2CH$_2$Ph, CH_2CH$_2$A), 0.75 (s, 9H, *t*-Bu), −0.05 (s, 3H, SiMe), −0.15 (s, 3H, SiMe). HRMS(FAB) calcd for $C_{54}H_{61}N_5O_7Si$ [MH^+], 920.441 853; found, 920.442 200.

5′-*O*-(4,4′-Dimethoxytrityl)-3′-*O*-(*tert*-butyldimethylsilyl)-*N*^{6}-benzoyl-2-(4-phenylbutyl)adenosine (12). ^{1}H NMR (DMSO-d_6) δ 11.14 (s, 1H, NHCO), 8.55 (s, 1H, H8), 8.02 (d, J = 7.3 Hz, 2H, PhCO), 7.51−7.66 (m, 3H, PhCO), 6.75−7.35 (m, 18H, Ph), 5.98 (d, J = 4.7 Hz, 1H, 1′H), 5.46 (d, J = 6.2 Hz, 1H, 3′OH), 4.84 (app dd, J = 10.8, 5.3 Hz, 1H, 2′H), 4.55 (app t, J = 4.8 Hz, 1H, 3′H), 4.02 (app dd, J = 9.6, 4.7 Hz, 1H, 4′H), 3.69 (s, 3H, OCH_3), 3.68 (s, 3H, OCH_3), 3.20−3.40 (m, 2H, 5′-H, 5″-H), 2.75 (m, 2H, CH_2A), 2.53 (m, 2H, CH_2Ph), 1.52−1.69 (m, 4H, CH_2CH$_2$Ph, CH_2CH$_2$A), 0.83 (s, 9H, *t*-Bu), 0.071 (s, 3H, SiMe), 0.027 (s, 3H, SiMe).

General Procedure for the Synthesis of Phosphoramidites 13 and 14. To a suspension of compound **9** or **10** (1 equiv) in dry THF was added collidine (8 equiv). After the mixture was cooled on ice, *N*-methylimidazole (0.5 equiv) was added, followed by 2-cyanoethyl *N*,*N*-diisopropylchlorophosphoramidite (8 equiv) dropwise. After the mixture was warmed to room temperature and stirred for 1 h, the reaction was complete as shown by TLC (10% ether/CH_2Cl_2). After the mixture was cooled on ice, collidine (0.2 mL) and MeOH (0.2 mL) were added to consume the excess phosphorylating reagent and the solvent was removed in vacuo. The residue was dissolved in CH_2Cl_2 (8 mL), washed with 5% $NaHCO_3$ (2 × 4 mL), and saturated NaCl (2 × 4 mL). The combined aqueous phases were extracted with CH_2Cl_2 (4 × 4 mL). The combined CH_2Cl_2 phases were dried over Na_2SO_4, filtered, and concentrated to dryness under reduced pressure. The residue was purified using prep TLC in 10% ether/CH_2Cl_2.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-*N*^{6}-benzoyl-2-(3-phenylpropyl)adenosine 3′-*N*,*N*-Diisopropyl(cyanoethyl)-phosphoramidite (13). Compound **9** (0.151 g, 0.167 mmol) reacted to give a white foam (0.116 g, 63%). ^{31}P NMR (DMSO-d_6) δ 151.246, 149.901.

5′-*O*-(4,4′-Dimethoxytrityl)-2′-*O*-(*tert*-butyldimethylsilyl)-*N*^{6}-benzoyl-2-(4-phenylbutyl)adenosine 3′-*N*,*N*-Diisopropyl(cyanoethyl)-phosphoramidite (14). Compound **10** (0.124 mg, 0.135 mmol) reacted to give a white foam (0.104 g, 69%). ^{31}P NMR (DMSO-d_6) δ 151.957, 150.550.

Synthesis and Purification of RNA. RNA sequences were synthesized on a 1 μmol scale with an Applied Biosystems ABI 394 DNA/RNA synthesizer using standard protocols. Coupling yields of the modified nucleotides were ∼50−90% determined by colorimetric quantitation of the trityl fractions. All reaction columns and chemicals were purchased from Glen Research. RNAs were cleaved and deprotected with ethanolic ammonia (3:1 NH_4OH/EtOH solution, 500 μL) at 55 °C for 12 h. After the beads cooled to room temperature, they were washed with ethanolic ammonia (4 × 250 μL) and the combined ethanolic ammonia fractions were concentrated to dryness in vacuo. The TBDMS protecting groups were removed in neat TEA/3HF solution (250 μL) at room temperature for approximately 12 h, and the reaction was quenched by water (250 μL). The RNA was precipitated sequentially with *n*-butanol and ethanol and was then purified on a 20% denaturing polyacrylamide gel [20% acrylamide, 20:1 mono/bisacrylamide, 7 M urea in TBE (89 mM Tris-borate, 2 mM EDTA), 15 cm × 40 cm × 0.75 mm, 3 h at 50 W]. The desired band was

excised from the gel, extracted with TE buffer (10 mM Tris, 1 mM EDTA pH7.4), dialyzed against TE buffer, concentrated to dryness by speed-vac, and desalted by ethanol precipitation. The concentration of the RNA was determined by UV at 260 nm. Correct composition was confirmed by MALDI mass spectrometry and enzymatic hydrolysis.[60]

RNA Melting Experiments. CD spectra were recorded on a Jasco J-810 CD spectrometer using 20 μM RNA in buffer containing 250 mM NaCl, 20 mM sodium cacodylate, pH 6.5, 0.5 mM EDTA, and 1 mM $MgCl_2$. CD melting curves were obtained by heating at a rate of 0.5 °C/min or 1 °C/min using 0.2 cm path length cells, monitored at 211 and 260 nm between 24 and 90 °C. UV melting experiments were performed on a Beckman DU 650 UV spectrometer using 4 μM RNA in the same buffer as that used in the CD melting experiments. UV melting curves were obtained by heating at a rate of 1 °C/min using 1 cm path length cells, monitored at 260 and 280 nm between 30 and 90 °C.

Gel Mobility Shift Assays. The equilibrium binding of stem loop 2 RNA to the U1A protein was monitored by electrophoretic gel mobility shift assays. ^{32}P-labeled stem loop 2 RNA (0.033 nM) was incubated with competitor tRNA (1 mg/mL) and varying amounts of U1A protein for 20 min at room temperature in a buffer containing 10 mM Tris-HCl, pH 7.4, 0.5% Triton X-100, 1 mM EDTA, and 250 mM NaCl. After addition of glycerol to a final concentration of 5%, the bound and free RNA were separated using an 8% polyacrylamide gel (80:1 acrylamide/bisacrylamide, 18 cm × 16 cm × 1.5 mm) in 100mM Tris-borate pH 8.3, 1mM EDTA, 0.1% Triton X-100 for 35 min at 350 V. The temperature of the gel was maintained at 25 °C by a circulating water bath. Gels were visualized on a Molecular Dynamics Storm 840 phosphorimager. Fraction RNA bound versus protein concentration was plotted and curves were fitted to the equation:

$$\text{fraction bound} = 1/(1 + K_d/[P])$$

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Supporting Information Available: ^{1}H NMR spectra for all new compounds, mass spectra, denaturing polyacrylamide gels of A6-3CPh and A6-4CPh RNA, and the HPLC elution profile of the enzymatic hydrolysis reaction of A6-4CPh RNA. This material is available free of charge via the Internet at http://pubs.acs.org.

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