Engineering RNA sequence specificity of Pumilio repeats

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Puf proteins bind RNA sequence specifically and regulate translation and stability of target mRNAs. A ''code'' for RNA recognition has been deduced from crystal structures of the Puf protein, human Pumilio1, where each of eight repeats binds an RNA base via a combination of three side chains at conserved positions. Here, we report the creation of seven soluble mutant proteins with predictably altered sequence specificity, including one that binds tightly to adenosine-uracil-rich element RNA. These data show that Pumilio1 can be used as a scaffold to engineer RNA-binding proteins with designed sequence specificity.

protein design | Puf proteins | protein–RNA interaction | adenosine-uracil-rich elements

Specific nucleotide sequences in mRNA molecules, often in noncoding sequences, regulate processes such as turnover, translation, localization, and splicing that are necessary for producing correct protein products in the right amounts at the right times and places. Proteins that bind to the specific RNA sequences often direct these posttranscriptional regulatory events, which are important during both embryonic development and cellular homeostasis. Two examples are the regulatory roles of sequences in the 3′ UTRs of mRNAs and near alternative splice sites in pre-mRNAs. Expression of TNFα is regulated by an adenosine-uracil-rich element (ARE) in the 3′ UTR of its mRNA, a common motif that confers instability on the message (1, 2). This sequence is recognized by the tristetraprolin (TTP) family of proteins, which initiates degradation of the ARE-containing mRNA (3). Overproduction of TNFα, as in the absence of TTP, results in inflammatory disorders such as rheumatoid arthritis, cachexia, and autoimmunity (4). Alternative splicing events are directed by specific exon-intron junction motifs and positive and negative regulatory sequences within the exons or introns. Mutations in these sequences can result in splicing defects that lead to diseases such as cancer, spinal muscular atrophy, and cystic fibrosis (5).

Because of the importance of RNA regulatory sequences in human health and disease, RNA-binding proteins with designed specificity could be important as tools for understanding processes directed by specific RNA sequences or perhaps as therapeutic agents. For example, an RNA-binding protein with specificity for a splicing regulatory sequence could induce a preferred alternatively spliced product by blocking an alternative splicing event. Or an RNA-binding protein with specificity for a particular RNA could be tracked by fusing the RNA-binding domain with a green fluorescent protein partner. Other work on designing RNA-binding proteins has focused on folded RNA targets such as the HIV-1 Rev response element RNA by using zinc fingers or arginine-rich motif peptides and relied generally on screening of proteins with selected randomized positions or building a chimeric molecule (6–12). Our previous studies of the Puf family protein, human Pumilio1, a sequence-specific RNA-binding protein, suggested that it could be used as a scaffold for engineering linear sequence-specific RNA-binding proteins by site-directed mutagenesis (13).

Puf family RNA-binding proteins regulate the expression of their target mRNAs by binding to specific sequences in the 3′ UTR. A common function of Puf proteins is regulating germ-line stem cell development, perhaps their ancestral function (14). *Drosophila melanogaster* Pumilio (DmPUM), the founding member of the Puf family, was noted first for regulating abdominal development by repressing the translation of maternal *hunchback* (hb^{mat}) mRNA (15–17) and is also important for regulating germ-line stem cell development (18–20), anterior patterning (21), and neuronal function (22–25). DmPUM represses hb^{mat} expression by binding to tandem sequence motifs, Nanos response elements (NREs), in the hb^{mat} 3′ UTR (26). A UGU triplet is important for DmPUM binding and for translational regulation in other organisms (27–36).

Puf family proteins contain RNA-binding domains, known as the Pumilio homology domain (PUM-HD) or Puf domain. The PUM-HD comprises eight sequence repeats, called PUM repeats, and flanking N- and C-terminal regions (16, 17, 28, 37). Crystal structures of the fly PUM-HD (38) and *Homo sapiens* Pumilio 1 homology domain (HsPUM1-HD) (39) revealed eight α-helical structural modules packed together into a curved shape. Crystal structures of HsPUM1-HD in complex with high-affinity RNA ligands corresponding to the fly NRE sequence showed that each of the eight PUM modules interacts with an RNA base. Each RNA base is recognized by three well conserved amino acids at specific positions in the second of three α helices in each repeat of HsPUM1-HD. Two amino acid side chains make hydrogen bond or van der Waals contacts with the Watson–Crick edge of an RNA base and the third amino acid side chain makes stacking interactions with the aromatic rings of the RNA bases (Fig. 1*a*). The Watson–Crick edge of the RNA base is recognized by a specific combination of amino acid side chains: glutamate and serine recognize guanine; glutamine and cysteine/serine recognize adenine; and glutamine and asparagine recognize uracil (Fig. 1*b*; ref. 13). The third amino acid side chain is generally sandwiched between two bases, except between bases 2 and 3 and 5 and 6. Guided by this recognition code, we report here the production of site-directed mutants of HsPUM1-HD with altered RNA sequence specificity.

Results

Engineering Guanine to Uracil Specificity. To change the sequence specificity of a PUM repeat from G to U, we mutated Glu-1083, Ser-1079, and Asn-1080 in repeat 7 of HsPUM1-HD to glutamine, asparagine, and tyrosine, respectively, to match the combination of amino acid side chains seen in repeats 2, 6, and 8 that bind to uracil. With this combination of mutations in the coding sequence, we were not able to produce soluble protein. We experienced the same difficulty with another combination of three mutations (see *Materials and Methods*).

Owing to this technical difficulty and the possibility that the stacking interactions are not as critical for sequence specificity,

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The authors declare no conflict of interest.

Abbreviations: ARE, adenosine-uracil-rich element; NRE, Nanos response element; HsPUM1-HD, *Homo sapiens* Pumilio 1 homology domain.

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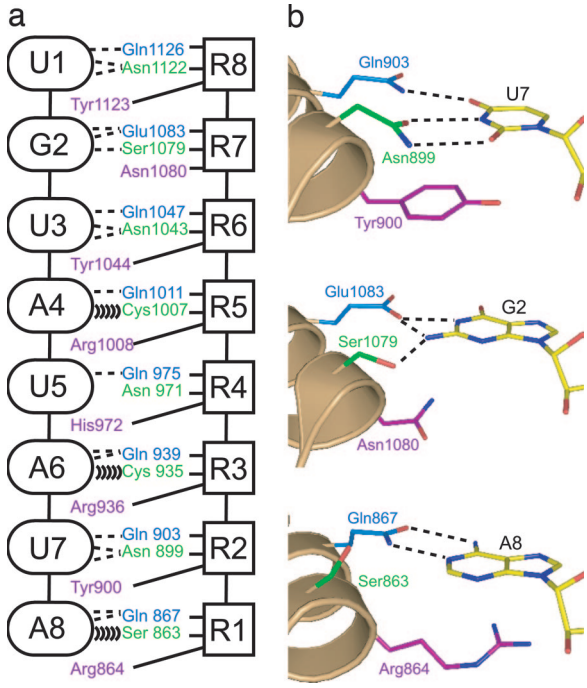

Fig. 1. Recognition of RNA by HsPUM1-HD. (*a*) Schematic representation of RNA:protein interaction between HsPUM1-HD and NRE RNA (13). Protein repeats are indicated by squares and RNA bases by ovals (dashed lines, hydrogen bonds; parentheses, van der Waals contacts). Blue, green, and purple side chains, respectively, are in equivalent positions in each repeat. (*b*) Interaction of HsPUM1-HD with a uracil (*Top*), guanine (*Middle*), or adenine (*Bottom*). The RNA and side chains that contact the RNA are shown in stick representation (dark blue, nitrogen; red, oxygen; yellow, light blue, green, or purple, carbon). PDB ID code is 1M8Y.

we mutated only Glu-1083 and Ser-1079 in repeat 7 of HsPUM1-HD to glutamine and asparagine, respectively. We then analyzed the binding specificity of this mutant protein, MUT7-2, by determining the equilibrium binding constants for this mutant protein and a correspondingly mutated RNA, G2U, or a "wild-type" NRE RNA. As predicted, MUT7-2 bound the cognate G2U RNA 25-fold more tightly than wild-type RNA (Table 1 and Fig. 2*a*). Wild-type protein prefers G to U at this position by 123-fold (Table 1). Competition experiments confirmed the G-to-U sequence specificity change of MUT7-2. MUT7-2 bound cognate G2U RNA 9-fold more tightly than wild-type RNA, whereas wild-type protein bound 14-fold more tightly to wild-type RNA than to mutant G2U RNA (Fig. 3*a*). As we had seen previously, competition assays appear to underestimate the difference in affinity (13).

We had shown previously that the sequence specificity of repeat 6 could be changed from U to G by mutation of its RNA base recognition residues (13). We therefore combined those mutations with the mutations for MUT7-2 to create a protein (MUT6-2/7-2: Q1047E/N1043S/E1083Q/S1079N) that recognizes UUG in place of the characteristic UGU triplet typically found in Puf protein target RNAs. In direct binding assays, MUT6-2/7-2 binds its cognate RNA, GU23UG, 34-fold more tightly than wild-type RNA, whereas wild-type protein binds wild-type RNA 3,300-fold more tightly than mutant GU23UG RNA (Table 1 and Fig. 2*b*). In other words, wild-type protein binds mutant GU23UG RNA weakly (K_D, 1,600 nM), but mutation of four amino acid side chains increased the affinity ≈90-fold (K_D, 18 nM). Competition experiments confirmed the sequence specificity change of MUT6-2/7-2 (Fig. 3*b*).

Engineering Adenine to Guanine Specificity. To change the sequence specificity of a repeat from A to G, we mutated Gln-867 of repeat 1 to glutamate (MUT1–1); the second base recognition residue remained a serine. In direct binding assays, MUT1-1 binds its cognate RNA, A8G, 146-fold more tightly than wild-type RNA, whereas wild-type protein binds wild-type RNA 3.3-fold more tightly than mutant A8G RNA (Table 1 and Fig. 2*c*). We performed a similar experiment with repeat 3 and mutated Gln-939 and Cys-935 to glutamate and serine, respectively (MUT3-2). MUT3-2 binds its cognate RNA, A6G, 55-fold more tightly than wild-type RNA and wild-type protein binds wild-type RNA 1.5-fold more tightly than mutant A6G RNA (Table 1 and Fig. 2*d*). The modest difference in binding affinity of the wild-type protein when a G is substituted for A in the RNA sequence suggests that repeats with glutamine and cysteine/serine at the base recognition positions bind relatively well to either A or G. This result is consistent with the presence of a G at positions 3 and 5 of *in vivo* RNA targets of other Puf proteins, despite the conservation of the base recognition residues (13). However, a glutamate in place of glutamine influences specificity for G. This result is consistent with the observation that glutamate is present only in repeat 7 of Puf proteins, the only repeat to recognize G, and with *ab initio* calculations of side chain:base interactions that suggest glutamate strongly favors interaction with G (40).

To investigate the importance of the glutamate residue in recognition of G, we examined the binding of wild type protein to RNAs with either G (wild type), U (G2U), or A (G2A) at position 2. In direct binding assays, the wild type protein is highly selective for G at position 2, binding 123-fold or 33-fold less well to G2U or G2A RNAs, respectively (Table 1). We then mutated Glu-1083 to glutamine (MUT7-1) and tested the binding of the mutant protein to wild type, G2U, and G2A RNAs. In direct binding assays, MUT7-1 binds wild type (K_D, 7.7 nM) and G2U (K_D, 5.7 nM) RNAs with similar affinity. Therefore, glutamate appears to be important for selectivity of G vs. U. MUT7-1 binds G2A RNA with lower affinity (K_D, 35 nM) than to wild-type RNA. This result is surprising, because we would have predicted that MUT7-1, with glutamine and serine, would prefer A to G. However, repeat 7 is unusual, lacking a residue that can form a stacking interaction with the base; the asparagine side chain present is not long enough to form this interaction. Perhaps the absence of a stacking interaction influences the ability to recognize an A at this site, and the special nature of repeat 7 may be important for the high conservation of the UGU triplet in target sequences recognized by Puf proteins.

Engineering Adenine to Uracil Specificity. To change the sequence specificity of a repeat from A to U, we mutated Cys-935 of repeat 3 to asparagine (MUT3-1); the second base recognition residue remained a glutamine. In direct binding assays, MUT3-1 bound to its cognate RNA, A6U, 6-fold more tightly than to wild-type RNA, whereas wild-type protein binds wild-type RNA 17-fold more tightly than to mutant A6U RNA (Table 1 and Fig. 2*e*).

Engineering an ARE Binding Pumilio1. AREs found in the 3′ UTRs of a class of mRNAs are well known determinants of RNA instability (1, 2). By sequence alignment, we noticed that we could alter the sequence specificity of HsPUM1-HD to recognize an ARE RNA by introducing two sets of mutations, MUT7-2 and MUT3-1 (E1083Q/S1079N/C935N). We created this protein and found that MUT7-2/3-1 binds its cognate ARE RNA with essentially the same affinity (K_D, 0.60 nM) as wild-type protein to wild-type RNA (Table 1 and Fig. 2*f*). MUT7-2/3-1 binds ARE RNA 467-fold more tightly than wild-type RNA, whereas wild-type protein binds wild-type RNA 23-fold more tightly than ARE RNA (Table 1). Furthermore, MUT7-2/3-1 binds ARE RNA ≈20-fold more tightly than wild-type

Table 1. Binding of wild-type and mutant HsPUM1-HD proteins to wild-type and mutant RNAs

Protein	RNA	RNA sequence*	K_D, nM	Trials	K_{rel}†
		87654321			
WT protein	NRE1–19 (WT RNA)	CCAGAAUUGUAUAUAUUCG	0.48 ± 0.21	6	1
WT protein	G2U	CCAGAAUU**U**UAUAUAUUCG	59 ± 12	3	123
WT protein	G2A	CCAGAAUU**A**UAUAUAUUCG	16 ± 3.9	4	33
WT protein	GU23UG	CCAGAAUU**UG**AUAUAUUCG	1,600 ± 420	3	3,300
WT protein	A8G	CCAGAAUUGUAUAU**G**UUCG	1.6 ± 0.15	3	3.3
WT protein	A6G	CCAGAAUUGUAU**G**UAUUCG	0.72 ± 0.11	3	1.5
WT protein	A6U	CCAGAAUUGUAU**U**UAUUCG	8.0 ± 3.1	3	17
WT protein	ARE	**UU**A**UUUA**U**U**UAU**U**UAUU	11 ± 3.4	4	23
G → U: MUT7-2 (S1079N/E1083Q)					
MUT7-2	G2U	CCAGAAUU**U**UAUAUAUUCG	6.0 ± 1.3	3	1
MUT7-2	WT RNA	CCAGAAUUGUAUAUAUUCG	150 ± 28	3	25
U → G, G → U: MUT6-2/7-2 (N1043S/Q1047E/S1079N/E1083Q)					
MUT6-2/7-2	GU23UG	CCAGAAUU**UG**AUAUAUUCG	18 ± 6.7	5	1
MUT6-2/7-2	WT RNA	CCAGAAUUGUAUAUAUUCG	610 ± 78	3	34
A → G: MUT1-1 (Q867E)					
MUT1-1	A8G	CCAGAAUUGUAUAU**G**UUCG	1.3 ± 0.38	5	1
MUT1-1	WT RNA	CCAGAAUUGUAUAUAUUCG	190 ± 42	4	146
A → G: MUT3-2 (C935S/Q939E)					
MUT3-2	A6G	CCAGAAUUGUAU**G**UAUUCG	0.051 ± 0.004	5	1
MUT3-2	WT RNA	CCAGAAUUGUAUAUAUUCG	2.8 ± 0.36	4	55
MUT7-1 (E1083Q)					
MUT7-1	G2U	CCAGAAUU**U**UAUAUAUUCG	5.7 ± 0.55	4	1
MUT7-1	G2A	CCAGAAUU**A**UAUAUAUUCG	35 ± 5.4	3	6.1
MUT7-1	WT RNA	CCAGAAUUGUAUAUAUUCG	7.7 ± 1.1	3	1.4
A → U: MUT3-1 (C935N)					
MUT3-1	A6U	CCAGAAUUGUAU**U**UAUUCG	0.50 ± 0.099	4	1
MUT3-1	WT RNA	CCAGAAUUGUAUAUAUUCG	2.8 ± 1.3	3	5.6
G → U, A → U: MUT7-2/3-1 (C935N/S1079N/E1083Q)					
MUT7-2/3-1	ARE	**UU**A**UUUA**U**U**UAU**U**UAUU	0.60 ± 0.26	6	1
MUT7-2/3-1	WT RNA	CCAGAAUUGUAUAUAUUCG	280 ± 49	4	467

*RNA bases recognized by the eight PUM repeats are underlined and the numbers above represent the PUM repeat that recognizes that base. Boldface letters note positions that are changed relative to wild-type NRE RNA.

†K_{rel} represents either the affinity of wild-type protein for the mutant RNA relative to the affinity of wild-type protein for wild-type RNA or the affinity of mutant protein for wild-type RNA relative to the affinity of mutant protein for its cognate RNA.

protein binds to ARE RNA (Table 1). Competition experiments confirmed this sequence specificity change (Fig. 3*c*).

Discussion

In this study, we demonstrate that HsPUM1-HD can be engineered to bind to different RNA targets by site-directed mutagenesis of side chains that make specific interactions with RNA bases. The seven mutant HsPUM1-HD proteins represent sequence specificity changes of RNA bases from G to U, U to G, A to G, and A to U. We find that mutagenesis of the two side chains that contact the Watson–Crick edge of the base is sufficient to alter sequence specificity. Thus, the identity of the amino acid side chains making stacking interactions with the RNA bases does not appear to be important for specificity. This result is consistent with the ability of Opperman *et al.* (41) to use the Pumilio RNA recognition rules to examine *Caenorhabditis elegans* Puf protein specificity by mutating only the side chains in specific repeats that recognize the Watson–Crick edges of the corresponding bases.

In many cases, we were able to design mutant proteins that bind to their cognate RNAs as tightly as the wild-type protein binds to the NRE target sequence (MUT1-1, MUT3-2, MUT3-1, and MUT7-2/3-1). In other cases, however, the mutant proteins bound less tightly to their cognate RNAs (MUT7-2 and MUT6-2/7-2). For example, when the G2U mutation is introduced into the wild-type RNA, the binding affinity of the wild-type protein is reduced dramatically (K_D, 0.48 nM for wild-type RNA vs. 59 nM, G2U mutant RNA). Introducing the E1083Q/S1079N mutations recovers a portion of this binding affinity (K_D, 6.0 nM for MUT7-2 and G2U RNA), although the recovery of binding affinity remains ≈10-fold lower than that between wild-type protein and wild-type RNA. We cannot yet explain why all of the mutant proteins did not recover fully the binding affinity. We do note that in the cases where the mutants bind more weakly, repeat 7 was mutated, and the special nature of that repeat may make it more sensitive to mutations. We also note that making two nucleotide mutations in the RNA does not always affect wild-type protein binding additively. For the GU23UG RNA, the combination of the G2U and U3G mutations appears additive. The G2U mutation reduces affinity 123-fold (Table 1) and the U3G mutation reduces affinity 30-fold (13), whereas the GU23UG mutations reduce affinity ≈3,000-fold. However, for the ARE RNA, wild-type protein binding affinity for the G2U mutant is 123-fold weaker and for the A6U mutant is 17-fold weaker, but the combination of the two in the ARE RNA is only 23-fold weaker. These results suggest that the sequence-specific recognition of RNA by Pumilio proteins may be more complex than the two base-interacting residues. Understanding these additional factors may assist the design of proteins with multiple mutant repeats while retaining subnanomolar binding affinity. Nevertheless, in the experiment presented here, the K_D of all mutants was at least 20 nM, and the K_D of the MUT7-2/3-1

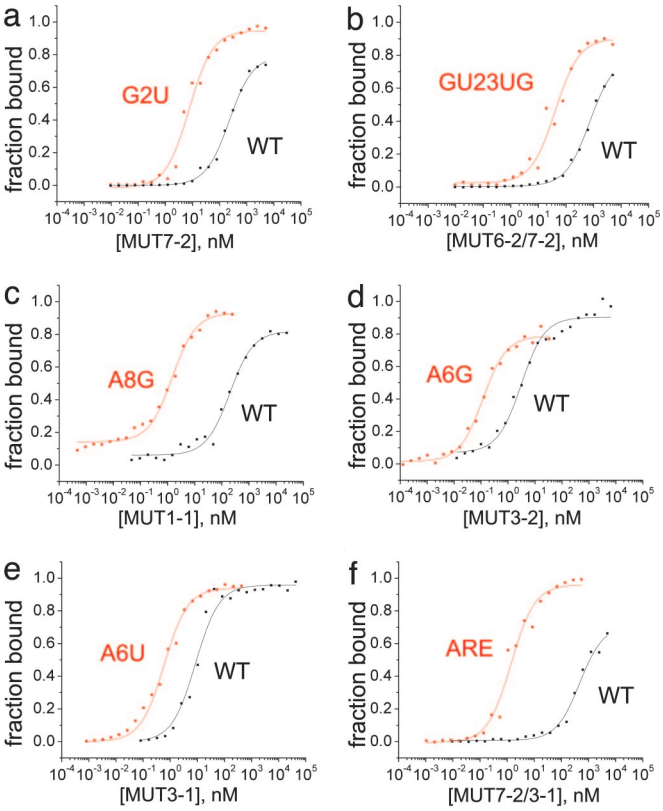

Fig. 2. Analysis of RNA binding for mutant HsPUM1-HD proteins. Representative analyses of equilibrium binding data for MUT7-2 protein and G2U RNA (*a*), MUT6-2/7-2 protein and GU23UG RNA (*b*), MUT1-1 protein and A8G RNA (*c*), MUT3-2 protein and A6G RNA (*d*), MUT3-1 protein and A6U RNA (*e*), and MUT7-2/3-1 protein and ARE RNA (*f*). Binding to cognate RNA is red and to wild-type RNA is black. These analyses assume one HsPUM1-HD binds to one RNA molecule.

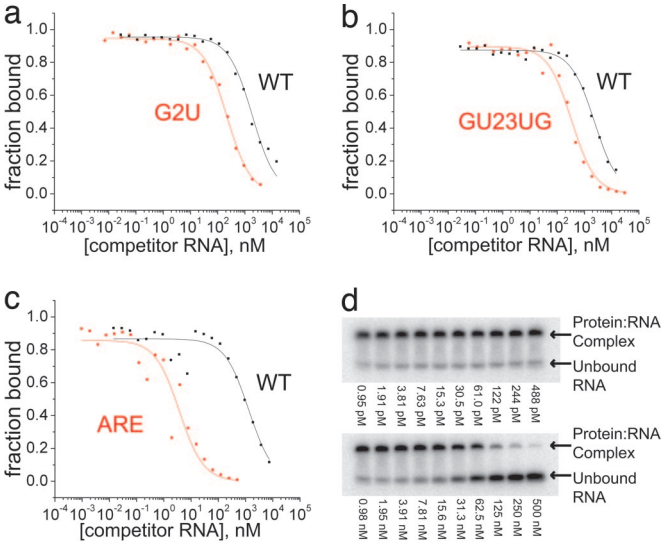

Fig. 3. Analysis of competitive RNA binding for mutant HsPUM1-HD proteins. Representative analyses of equilibrium-binding competition data for MUT7-2 protein (*a*), MUT6-2/7-2 protein (*b*), and MUT7-2/3-1 protein (*c*). Competition of binding of mutant protein to cognate mutant RNA with unlabeled wild-type RNA is black and with unlabeled cognate RNA is red. (*d*) A representative EMSA of an equilibrium-binding competition for MUT7-2 protein. Labeled cognate mutant RNA was competed with unlabeled cognate mutant RNA. The concentrations of unlabeled competitor RNA are indicated under each lane in the gel.

protein for ARE RNA was subnanomolar, making it likely that the RNA sequence specificity code can be used to generate high affinity, high selectivity RNA-binding proteins that recognize specific sequences.

Although the three-dimensional structures of many RNA-binding proteins in complex with RNA targets have been determined, none of these is quite as dramatic as Pumilio in its dependence on base-specific interactions and the clarity of a recognition code. Thus, it provides a unique scaffold to rationally design sequence-specific RNA-binding proteins. The work described here demonstrates that soluble proteins with altered sequence specificity can be produced by site-directed mutagenesis by using the HsPUM-HD as a scaffold.

We envision these proteins could be used to modulate gene expression by competing for regulatory RNA-binding sites or delivering effector molecules that repress or induce translation to specific RNA targets. In addition, a designed protein could be used to track specific RNA molecules in cells by tethering the PUM-HD to a sensor molecule (e.g., a fluorescent tag). Some of these processes also could be performed by antisense oligonucleotides or RNAi technology. The use of a protein as the targeting molecule provides additional flexibility in allowing fusion of a second protein functional domain while maintaining tissue-specific expression with appropriate promoters.

Materials and Methods

Preparation of HsPUM1-HD Mutant Proteins. Site-directed mutagenesis reactions of the pTYB3 plasmid encoding HsPUM1-HD (39) were performed by using the QuikChange II XL Site-Directed Mutagenesis Kit or QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Wild-type and mutant HsPUM1-HD proteins were expressed in *Escherichia coli* strain BL21(DE3) and purified as described in ref. 39. No other proteins were observed when purified proteins were examined by SDS/PAGE and Coomassie blue staining. Purified proteins were concentrated to 2–6 mg/ml in 10 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM DTT, and 0.1-ml aliquots were flash-frozen in liquid nitrogen and stored at −80°C for further analysis. We were unable to express soluble proteins when we mutated the third RNA interacting residue that forms a stacking interaction with the base in repeats 7 (S1079N/N1080Y/E1083Q or N1043S/Y1044N/Q1047E/S1079N/N1080Y/E1083Q) and 3 (C935N/R936Y).

EMSAs. RNA oligonucleotides were obtained from Dharmacon, Inc. (Lafayette, CO) and radiolabeled at the 5′ end by using [γ-^{32}P]ATP (PerkinElmer Life Sciences, Wellesley, MA) and T4 polynucleotide kinase (New England BioLabs, Ipswich, MA) by following manufacturer directions. The radiolabeled RNAs were purified on 20% polyacrylamide gels (Invitrogen, Carlsbad, CA) run with 1× Tris-Borate-EDTA (TBE) (89 mM Tris/89 mM boric acid/2.5 mM EDTA, pH 8.3) buffer at room temperature. Binding reactions included ≈15 pM radiolabeled RNA and varying concentrations of protein (Figs. 2 and 3) incubated in binding buffer [10 mM Hepes, pH 7.4/50 mM KCl/1 mM EDTA/0.01% (vol/vol) Tween-20/0.1 mg/ml BSA/1 mM DTT]. Binding reactions were incubated for 1–2 h at room temperature and immediately analyzed by electrophoresis on 6% nondenaturing polyacrylamide gels (Invitrogen) run in 0.5× TBE at a constant 100 V for 20 min at 4°C. Gels were dried and exposed to storage phosphor screens (GE Healthcare, Piscataway, NJ) for 5–10 days, scanned with a Typhoon 8600 Imager (GE Healthcare), and analyzed with Image Quant 5.2 software (GE Healthcare). The analyzed data were fit to the equation:

$$\text{fraction bound} = \frac{B_{\max}[protein]}{K_D + [protein]}, \quad [1]$$

using Origin 7.5 software (OriginLab, Northampton, MA).

Dissociation constants were adjusted based on the percentage of active protein in each preparation [WT, 33%; MUT7-2, 46%; MUT6-2/7-2, 73%; MUT1–1, 80%; MUT3–2, 86% (wild-type RNA experiments)/47% (mutant RNA experiments); MUT7–1, 70%; MUT3-1, 61%; MUT7-2/3-1, 46%]. To determine the percentage of active wild-type protein, three mixtures of wild-type protein and wild-type RNA were prepared by using a fixed concentration of RNA (10.8 μM) but three different concentrations of protein (1.5 μM, 6 μM, or 10.8 μM). The samples were incubated at room temperature for 30 min, then loaded onto a Superdex 200 10/300 GL (1.0 cm $\times$ 30.0 cm) size exclusion column (GE Healthcare) and eluted with 10 mM Tris·HCl, pH 7.4/150 mM NaCl/5 mM DTT monitoring absorbance at 254 nm. Each sample gave an absorbance peak for unbound RNA at an elution volume (Ve) of 13.3 ml and a peak for the protein:RNA complex at Ve of 10.6 ml. The peak height for unbound RNA decreased with increasing protein amount as expected. The area under the peaks for unbound RNAs and bound RNA were integrated by using the Unicorn software (GE Healthcare), and the fraction of unbound RNA was calculated as [unbound/(bound + unbound)]. The fraction of unbound RNA (y axis) was plotted against the protein concentration (x axis). The data were fit to a straight line by linear regression ($R^2 = 0.982$) with a predicted y intercept of 0.92, consistent with an expected value of 1. Extrapolating the straight line to the x axis (unbound RNA = 0) gave the concentration of protein necessary to saturate the fixed concentration of RNA. The molar ratio of the fixed amount of RNA to the extrapolated protein amount gave the percentage of active wild-type protein. The percentages of active protein for the mutant proteins were determined likewise. In each case, the three data points for each mutant protein fit well to straight lines by linear regression with a y intercept (b) near 1: MUT 7-2, $R^2 = 0.988$, $b = 0.94$; MUT6-2/7-2, $R^2 = 0.999$, $b = 1.01$; MUT1-1, $R^2 = 0.990$, $b = 1.02$; MUT3-2, $R^2 = 0.999$, $b = 1.04$; MUT7-1, $R^2 = 0.951$, $b = 0.94$; MUT3-1, $R^2 = 0.994$, $b = 1.00$; and MUT7-2/3-1, $R^2 = 0.996$, $b = 0.99$.

The RNA oligonucleotides used in previous studies included sequences from both the Box A and Box B sequences of NRE RNA. For these studies, we have designed shorter oligonucleotides that contain only Box B sequences. HsPUM1-HD binds to the NRE1–19 wild-type RNA used in this study ≈9-fold more weakly than to the NRE34 wild-type RNA used in the previous study, but the interpretation of results is clearer without the presence of the weak secondary binding site in the Box A sequence.

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