Patterns and plasticity in RNA-protein interactions enable recruitment of multiple proteins through a single site


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mRNA control hinges on the specificity and affinity of proteins for their RNA binding sites. Regulatory proteins must bind their own sites and reject even closely related noncognate sites. In the PUF [Pumilio and fem-3 binding factor (FBF)] family of RNA binding proteins, individual proteins discriminate differences in the length and sequence of binding sites, allowing each PUF to bind a distinct battery of mRNAs. Here, we show that despite these differences, the pattern of RNA interactions is conserved among PUF proteins: the two ends of the PUF protein make critical contacts with the two ends of the RNA sites. Despite this conserved “two-handed” pattern of recognition, the RNA sequence is flexible. Among the binding sites of yeast Pufo4p, RNA sequence dictates the pattern in which RNA bases are flipped away from the binding surface of the protein. Small differences in RNA sequence allow new modes of control, recruiting Pufo5p in addition to Pufo4p to a single site. This embedded information adds a new layer of biological meaning to the connections between RNA targets and PUF proteins.

mRNA turnover | RNA regulation | translation | 3′UTR elements

Proteins bind specific mRNAs to regulate their stability, translation, and localization. Individual proteins bind and control batteries of functionally related mRNAs. Each regulatory protein must interact tightly with cognate sites and reject even closely related sequences. This specificity underlies coordinate control and regulatory networks.

The PUF [Pumilio and fem-3 binding factor (FBF)] family of proteins is exemplary. These proteins bind elements in 3′ untranslated regions (3′UTRs), termed PUF binding elements (PBEs) (1, 2). PUFs commonly repress translation or enhance mRNA decay (3–8) but also can activate and localize mRNAs (7, 9–14). The six PUF proteins of Saccharomyces cerevisiae control distinct sets of RNAs that comprise distinct functional groups and bind ~850 mRNAs, or 10–15% of the mRNA species in that organism (15, 16), Caenorhabditis elegans, Drosophila, and human PUF proteins interact with a similarly large number of mRNAs (17–21). PUFs are important in stem cell control, learning, pattern formation, cell fate determination, and cell cycle control (22, 23). Understanding how PUFs acquire their specificity for groups of mRNAs is a critical step in discerning their roles in these events.

The RNA binding domain (RBD) of PUF proteins comprises eight ~40-aa PUF repeats that form a crescent (24–26). RNA binds to the inner concave surface, with the N terminus of the protein bound to the 3′ end of the RNA (27). PBEs of biological targets of PUF proteins contain a 5′ UGU sequence (2, 8, 15–18, 26, 28). Each PUF repeat consists of three α-helices and contains three critical amino acid side chains that can contact RNA. Two so-called “edge-on” residues make hydrogen bonds and Van der Waals contacts with RNA bases; another, called a “stacking” residue, forms planar stacking interactions. The edge-on and stacking contacts determine the base specificity of a PUF repeat (27).

Human Pumilio exemplifies the simplest condition in which each of the eight PUF repeats contacts a single base (27). Other PUFs require “extra” bases relative to human Pumilio; these are accommodated by “base flipping” (29–31). These bases do not contact the edge-on or stacking side chain but flip away from the protein instead.

We sought to probe several PUF/RNA complexes to identify those features of the interaction that were general, as well as those that were idiosyncratic. We examined three PUF proteins, altering edge-on and stacking residues. Our results support a “two-handed” model in which interactions at the ends of the complex are critical. They reveal unexpected plasticity in the PUF-RNA interactions achieved via alternative base flipping patterns and add a previously undescribed layer of biological meaning in the signals that govern mRNA regulatory control.

Results

Systematic Mutagenesis Reveals a Common Pattern of Recognition.

To probe the basis of PUF specificity, we systematically mutated residues in C. elegans FBF-2 and S. cerevisiae Pufo4p and Pufo5p. In each PUF repeat, we converted stacking or edge-on residues to alanine. We reasoned that this strategy would reveal if these residues contributed to RNA binding and test the accuracy of the known crystal structures in a cellular context.

We assessed binding in the yeast three-hybrid system (32, 33) (Fig. 1). A PUF protein fused to the GAL4 activation domain was expressed in cells carrying a hybrid RNA with the PBE of interest. Binding of the PUF and RNA triggers LacZ expression, which correlates with the affinity of the interaction (34). Analysis of the three PUF/RNA complexes follows.

FBF-2-gld-1 FBEa complex. Most stacking and edge-on residues in FBF-2 were required for binding of the gld-1 FBEa sequence (FBE), because alanine substitutions reduced β-galactosidase (β-gal) activity to background levels (Fig. 2A). However, alanine substitutions in the stacking residues of repeats 1 and 5, as well as in the edge-on residues of repeats 1, 4, 5 and 8, allowed 10% or more β-gal activity. These data are consistent with interactions in the crystal structures of FBF-2/RNA complexes (30).

In the FBF-2 crystal structures, the edge-on residues in repeat 8 contact the U1 RNA base, yet their replacement with alanines did not disrupt binding. However, a lysine (K557) in a downstream helix also appears to contact U1. Mutation of this lysine (K557A), combined with repeat 8 edge-on alanine mutations, reduced activity to 3% of WT; K557A alone had no effect (Fig. S1).

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Data deposition: The crystallography, atomic coordinates, and structure factors reported in the paper have been deposited in the Protein Data Bank, wwwpdb.org (PDB ID code 4D25).

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Puf4p interacts with yeast RNA. Puf4p differs from FBF-2 in specificity and base flipping pattern (29). Our Puf4p mutagenesis showed that the majority of stacking and edge-on residues were required for Puf4p to bind to the HO Puf4 binding site (4BE) (Fig. 2D). Mutations in the stacking residues of repeats 5 and 7 and the edge-on residues of repeat 4 allowed >10% reporter activity; these residues do not contact the RNA in the determined structure (29).

We next prepared mutations in the 4BE in which bases were individually altered to each of the other three identities. Bases 5 and 7, which allowed >10% β-gal activity to two or more alternate bases, were less constrained than bases 3 and 4; bases 6 and 8 were intermediate (Fig. 2F). U3 and A4 are part of the core 5' UGUGU sequence conserved in PUF protein target RNAs, and they, along with U8, lie across from stacking and edge-on residues necessary for binding. In contrast, U5 and U7 are flipped away from the protein consistent with the toleration of substitutions at these positions (Fig. 2F). The flexibility of base 6 is more enigmatic, because the amino acid residues opposite were important for tight binding. We suspect this reflects plasticity in the pattern of flipping, as we later discuss.

To quantify the effects of key mutations, we purified bacterially expressed, GST–FBF-2 proteins, including WT and the four mutants in repeats 4 and 5. RNA binding was assessed using gel shift assays (Fig. S2). The observed affinities correlated with the data obtained in the three-hybrid system, in that substitution of nucleotide substitution data are from a study by Bernstein et al. (49). The 10% cutoff is arbitrary and corresponds to a four- to fivefold effect on the Kd (34).

Fig. 2. Systematic mutagenesis reveals a common pattern of recognition. Yeast three-hybrid binding of FBF-2 (A), Puf4p (D), and Puf3p (G) stacking and edge-on alanine mutant proteins to cognate sequences. The β-gal levels were normalized to that of WT proteins bound to their cognate PUF sites (FBF: UGUGGCAUA, 4BE: UGUAAUUAU, and 3BE: UGUAAAUAU). (B) Quantitation of three independent EMSA experiments (Fig. S2) for FBF-2 WT and repeat 4 and 5 alanine mutants. Yeast three-hybrid binding of single nucleotide substitutions in the 4BE (E) and the 3BE (H). The β-gal levels were normalized to binding of the WT 4BE or 3BE sequence. The identity of the base present in each mutant is indicated immediately below the bars; the identity in WT is indicated below that. Amino acid side chains and bases in the FBF-2/4BE (P), Puf4p/4BE (F), and Puf3p/3BE (I) complexes are derived from the crystal structures (29, 30, 35). Green amino acid chains allowed >10% β-gal when mutated to alanine, and red side chains allowed <10% when mutated to alanine. Green RNA nucleotides allowed ≥10% to two or more alternate bases, red nucleotides allowed <10% to two or more identities, and black RNA nucleotides were not tested. FBE nucleotide substitution data are from a study by Bernstein et al. (49). The 10% cutoff is arbitrary and corresponds to a four- to fivefold effect on the Kd (34).

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**Puf3p/Cox17 site B complex.** The core binding site of Puf3p consists of eight bases, which bind without flipping. Most Puf3p amino acid substitutions diminished β-gal levels to less than 10% of WT Puf3p bound to the Cox17 site B sequence (3BE). However, substitutions of the stacking residues in repeats 1, 5, 7, and 8, as well as of the edge-on residues in repeats 4 and 5, still allowed >10% activity (Fig. 2G). Most of these results are consistent with the two structures of Puf3p bound to different RNAs (35): Repeat 7 does not make a stacking interaction with the RNA, and repeat 4 can bind different bases. The plasticity of repeat 5 was surprising, because this repeat in most PUF proteins recognizes a purine.

To test whether repeat 5 binding was relatively less important for Puf3p specificity, we analyzed RNA base substitutions at central positions in the 3BE. As expected, G2 and U5 in the conserved UGU sequence were the most constrained. At positions 4–6, at least two bases allowed >10% of WT levels (Fig. 2H). A4 and A5 lie across from noncritical stacking and edge-on residues (Fig. 2I). A6 of 3BE is located across from critical residues yet shows little constraint.

**Summary.** Each PUF protein yielded a similar binding pattern in which the central PUF repeats were less stringently required than those at the ends of the structure. Bases opposite amino acid residues that were less critical and all flipped bases generally were flexible in identity (Fig. 2C, F, and I). The data suggest a “two-handed” mode of recognition, in which interactions at the two ends of the complex are the most critical.

**Alternate Binding Pattern Is Dictated by the RNA Sequence.** FBF-2 and Puf4p bind nine base RNA sequences using different flipping patterns (Fig. 3A). In the canonical Puf4p/4BE complex, the base at position 7 is flipped away from the protein and an adenosine at position 6 binds repeat 3. This pattern, which we call “seven-flipped,” is the only one described for Puf4p. In the FBF-2/4BE complex, the base at position 6 flips and bases 4–6 stack. We call this pattern “six-flipped.” In both cases, base 5 also flips and stacks with base 4.

We reasoned that the preference of Puf4p repeat 3 for an adenosine may dictate the flipping pattern of Puf4p targets. Thus, for the seven-flipped pattern, an adenosine at the sixth RNA position is bound by repeat 3 and base 7 is excluded from binding and flipped. However, if an adenosine is at the seventh position and absent from the sixth, as in a canonical FBE, A7 might bind repeat 3 and base 6 would flip, yielding a pattern like FBF-2/4BE (Fig. 3B). This model predicts that the flipping pattern could be altered experimentally by manipulating the RNA sequence.

To test this hypothesis, we designed mutations in the canonical 4BE. We compared binding of Puf4p to single A6 mutants with that of the same mutations in combination with U7A. All single mutations of A6 weakened binding (Fig. 3C). In each position 6 background, an adenosine (U7A) restored binding to nearly WT levels. The effect was most striking with A6C U7A, because the A6C single mutation reduced binding the most. These data support the view that Puf4p can bind in either a six-flipped or seven-flipped mode, as dictated by the RNA.

**Structure of Puf4p Bound in a Six-Flipped Conformation.** To test our model directly, we determined the structure of Puf4p in complex with the A6C U7A mutant 4BE RNA (UGUAUAUA) by X-ray crystallography (Fig. 4A and Table S1; PDB ID code 4DZS). The Puf4p protein structure showed little change vs. that bound to WT 4BE RNA (29) (PDB ID code 3BX2) (rmsd of 0.62 Å over 328 Cα atoms). RNA bases 1–3 and base 5 in these two structures superimposed well with small shifts in bases 4, 8, and 9. As predicted, bases 6 and 7 adopted different positions. A composite omit map of the A6C U7A complex revealed that bases 5 and 6 (U5 and C6) were stacked directly and flipped away from the RNA binding surface of the protein; base 7 directly contacted repeat 3 and stacked between R651 and H688 (Fig. 4B). This is similar to the FBF-2/4BE structure (Fig. 4C) but different from that of Puf4p and the canonical 4BE, where U7 is flipped and A6 stacks between R651 and H688 (Fig. 4D). Thus, Puf4p can bind RNA in the six-flipped conformation, supporting the hypothesis that the RNA sequence dictates the pattern of binding and emphasizing plasticity in the Puf4p complexes.

**Endogenous Puf4p Targets Contain Six-Flipped Sites.** We tested whether six-flipped sites were enriched among endogenous mRNAs physically associated with Puf4p (15). The two types of sites can be described as UGUANNAUA (6-flipped) and UGUANANUA (7-flipped). The sequence UGUANAUUA falls into both types; we scored such overlapping sequences separately.

The six-flipped sites were enriched in Puf4p target mRNAs vs. all 3′ UTRs throughout the transcriptome (11% vs. 3%; $P = 7 \times 10^{-10}$ by Fisher’s exact test; Fig. 5A). Twenty-two target mRNAs with six-flipped sites in their 3′ UTRs were identified (Table S2). The six-flipped site was even more enriched among those mRNAs that did not possess a seven-flipped site (13.5% vs. 3%; $P = 9 \times 10^{-12}$). The overlapping sequence also was enriched among Puf4p targets. These findings support the conclusion that Puf4p binds mRNAs in vivo through both six-flipped and seven-flipped sites.

**Plasticity Enables Dual Control.** Re-examining the list of mRNAs with putative six-flipped sites (15), we observed that many were immunopurified with Puf5p as well as with Puf4p (Fig. 5B). Eleven (50%) of 22 Puf4p targets with six-flipped sites were enriched in both Puf4p and Puf5p immunopurifications. Of the 28 mRNAs that were bound by both proteins (15), 39% con-
tained the six-flipped sequence, whereas 11% of mRNAs bound only by Puf4p contained the six-flipped sequence (P = 1 × 10^{-5}). Similarly, six-flipped RNAs were enriched in Puf4p/Puf5p targets compared with those bound only by Puf5p (39% vs. 17%; P = 0.002). In principle, dual enrichment could be attributable to the presence of two PUF sites or to binding of both Puf4p and Puf5p to a single PBE.

To test whether a single PBE could bind Puf4p and Puf5p, we examined binding of Puf4p and Puf5p to mutant RNA sequences, using Puf3p as a control. The HO Puf5 binding site (3BE) and 3BE were used as cognate sites to normalize the data to maximum activity for Puf5p and Puf5p, respectively.

Puf4p bound to all possible six-flipped sequences at levels near that of its canonical seven-flipped site, the 4BE (Figs. 3C and 5C). Strikingly, Puf5p also bound well to the six-flipped sequences, yielding β-gal levels at least two orders of magnitude above background levels. A single nucleotide change in the 4BE (U7A) increased activity ~40-fold. Puf5p did not bind above background, suggesting that binding to these elements was confined to the Puf4p and Puf5p pair.

To test whether six-flipped sites were repressed by both Puf4p and Puf5p in vivo, we examined repression of HIS3 reporter mRNAs bearing 3’UTRs derived from SMX2 and RPB9 mRNAs by individually expressing Puf4p or Puf5p in puf4Δ puf5Δ yeast (Fig. 6A). SMX2 and RPB9 mRNAs were identified in our computational analysis of putative Puf4p targets with six-flipped sites (Table S2). Repression of the HIS3 reporter prevents growth under selective conditions (4, 5, 36). As controls, we analyzed mutant reporters (“mut” in Fig. 6A) in which the UGU of each binding element was converted to ACA. Repression was compared with that of a reporter carrying the 3’UTR of HO mRNA, a well-characterized target with both Puf4 and Puf5 sites. Expression of Puf4p repressed both the SMX2 and RPB9 reporters under selective conditions to levels comparable to the HO 3’UTR (Fig. 6B). Repression was dependent on the six-flipped PBEs. Puf5p repressed the same reporter mRNAs, yielding data very similar to Puf4p and, again, required the PBEs (Fig. 6C). We conclude that six-flipped sites are bound and repressed in vivo by both Puf4p and Puf5p.

Discussion

The binding patterns of PUF proteins to their RNA targets are similar, despite divergence in their RNA specificity. However, recognition is unexpectedly flexible: The precise RNA sequence dictates whether one or two PUFs can bind, and so defines patterns of regulation in vivo.

Mutagenesis of the interface of FBF-2, Puf4p, and Puf3p complexes suggests a two-handed model for PUF-RNA interactions. In this model, the two ends of the protein’s RBD “grasp” the two ends
of the RNA binding site. One “hand” relies on repeats 6, 7, and 8, and it contacts the 5′ UGU; the other relies in repeats 1, 2, and 3, and it contacts all or part of the 3′ AUA. The atomic interactions involved are nearly invariant, although the spacing of the elements and the requirement for extra bases vary with the protein.

The two-handed model bears comparison to certain transcription factors. For example, the Zn$_2$Cys$_6$ DNA-binding proteins Gal4p and Prp1p bind as homodimers to two sites separated by a spacer, with each monomer reaching one-half of the site (37). The architecture of PUF complexes is similar, although the two regions reside in a single polypeptide. In both cases, two separate sets of interactions must both occur and be separated by spacer nucleotides of appropriate length.

PUF proteins can be engineered to bind new RNA sequences (38). By linking a modified PUF to an effector domain, regulation can be targeted to specific mRNAs (39–41). The plasticity of interactions reported here raises considerations for such studies: Flexibility may cause off-target effects. Thus, specificity must be examined broadly, as in the design of zinc-finger proteins (42, 43).

Our data establish that Puf4p accommodates both “six-flipping” and “seven-flipping” patterns. These two flipping patterns embed biological information: Six-flipping specifies that either Puf4p or Puf5p can bind, whereas seven-flipping specifies that only Puf4p can do so (Figs. 5C and 6 B and C).

Alternate binding modes have implications for patterns of control. Simple alterations in binding sites can expand control of particular mRNAs, as did the U7A mutation in the 4BE that allowed Puf5p binding. More dramatically, changes in a protein’s preferences among alternative sites would alter regulation globally, gaining or losing sets of targets. After PUF gene duplication, evolution may lead to changes in RNA specificity. Extant yeast PUFs may capture proteins at two points in this evolutionary path. Puf1p and Puf2p are closely related and likely arose through duplication; 90% of the 40 mRNAs bound to Puf1p are also bound to Puf2p (15). Puf4p and Puf5p are more distantly related, and a much smaller fraction of their mRNA targets overlaps, likely attributable to diversification of the proteins’ binding preferences.

The structures and sequences in the RNA and protein that dictate alternative sites are only partially understood. Base-flipping often requires that the RNA nucleotides stack on adjacent bases or that amino acid side chains interact with the phosphate backbone. As a result, sequence context can influence binding (31). Puf4p binds to RNAs in six-flipped and seven-flipped modes with little apparent conformational change in the protein. Future determination of Puf5p structures and further work on its sequence specificity are needed to understand how it binds multiple sites.

We suggest that the information embedded in Puf4p binding sites is important in vivo. Repression by Puf4p is dependent on deadenylation, whereas repression by Puf5p is not (3–5). Thus, the information embedded in the binding element specifies which form of repression occurs. Similarly, because PUF proteins appear to vary in abundance with growth conditions (15, 44), the precise sequence in the 3′ UTRs of their target mRNAs may dictate how the target mRNA responds in different conditions. A six-flipped site, which binds Puf5p, will continue to be controlled even under conditions in which Puf4p is down-regulated. Interactions between Puf5p and components of signaling pathways suggest it may respond to signals that Puf4p does not (45, 46).

PUF protein networks hinge on the specificity of PUF-RNA interactions. Our results reveal conserved features of the PUF-RNA interface, and demonstrate that more information is embedded in the RNA element than has been appreciated. This adds previously undescribed biological meaning to the nature of the RNA binding sites.

### Materials and Methods

#### Yeast Strains

All yeast three-hybrid assays were performed using the YBZ-1 strain (34). Repression assays (Fig. 6) were done using strain W303 (MAT-a mpts3::Kan-puf4::TRP1 as described (4)).

#### Yeast Three-Hybrid Plasmids and Assays

pACT2 FBF-2 was as described (47). Puf4p (amino acids 536–888), Puf5p (amino acids 511–879), and Puf5p (amino acids 28–860) were cloned into pGADT7. Amino acids were mutated to alanine by site-directed mutagenesis as indicated in Table S3. Mutant RNA sequences were expressed using the p3HR2 vector as in the study by Stumpf et al. (48) and their binding assay as described (32, 34). Within an experiment, the relative β-gal levels for each protein were normalized to that of the protein binding to its canonical WT site (Puf3p to 3BE, Puf4p to 4BE, Puf5p to 5BE, and FBF-2 to FBE), which was set to 100%. Values represent an average of three biological replicates, and error bars display the SD.

#### Protein Purifications, EMSA, and Filter Binding

WT and mutant FBF-2 purification, EMSA, and filter binding experiments were performed essentially as described (47, 49). Minor changes are described in SI Materials and Methods.
Motif Enrichment. *S. cerevisiae* 3′ UTR lengths were taken from Nagalakshmi et al. (50); when no experimental 3′ end was given, 3′ UTRs were defined as 200 nucleotides past the stop codon. The 3′ UTR sequences were extracted from release 64 of the S288C reference genome (Saccharomyces Genome Database project; http://downloads.yeastgenome.org/). The 224 PuF5p target mRNAs include duplicate 3′ UTRs, which we only counted once, resulting in 208 unique targets. Gene duplicates were not treated differently genome-wide.

**Repression Assays.** HS3-HO 3′ UTR reporter plasmids were modified based on the work of Hook et al. (34) (SI Materials and Methods). The p415-glycerolaldohex-3-phosphate dehydrogenase (GPD) PuF5p is as described (3). Full-length PuF5 was cloned into the p415-GPD vector. Repression assays were performed in the W303 p415 PuF5 yeast strain but were as described otherwise (3).

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