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

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Edited by Marlene Belfort, University at Albany, State University of New York, Albany, NY, and approved February 24, 2012 (received for review January 11, 2012)

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 Puf4p, 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 Puf5p in addition to Puf4p 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* Puf4p and Puf3p. 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 the *gld-1* FBEa sequence (*FBE*), because alanine substitutions reduced β-galactosidase (β-gal) activity to background levels (Fig. 2*A*). 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).

Author contributions: C.T.V., D.F.P., C.Q., Z.T.C., T.M.T.H., and M.W. designed research; C.T.V., D.F.P., and C.Q. performed research; C.T.V., D.F.P., C.Q., Z.T.C., T.M.T.H., and M.W. analyzed data; and C.T.V., D.F.P., T.M.T.H., and M.W. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1200521109/-/DCSupplemental.

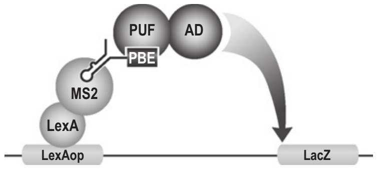
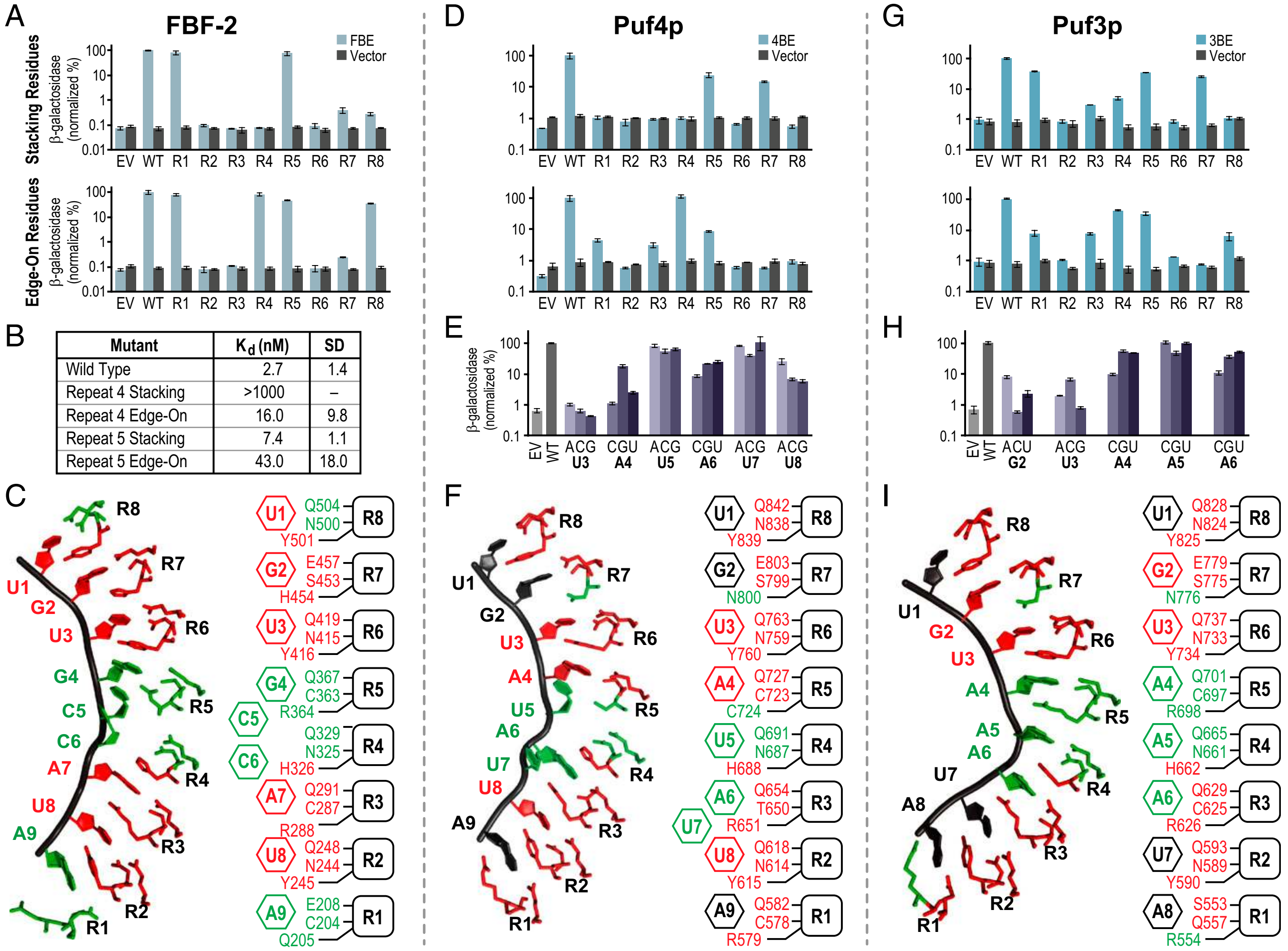


Fig. 1. Diagram of the yeast three-hybrid assay. Light gray circles represent LexA/MS2 fusion protein to tether RNA to promoter via a LexA binding site; dark gray circles represent PUF/AD fusion to activate transcription of the LacZ reporter gene. PBE (black box) indicates the PBE in the MS2 loop containing RNA. AD, *GAL4* activation domain.

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 the stacking residue in repeat 4 was severely deleterious (Fig. 2*B*).

Puf4p/HO Puf4 binding site complex. 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. 2*D*). 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. 2*E*). U3 and A4 are part of the core 5′ UGUR 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. 2*F*). 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.



Mutant	K_d (nM)	SD
Wild Type	2.7	1.4
Repeat 4 Stacking	>1000	–
Repeat 4 Edge-On	16.0	9.8
Repeat 5 Stacking	7.4	1.1
Repeat 5 Edge-On	43.0	18.0

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 (*FBE*: UGUGCCAUA, *4BE*: UGUAUAUUA, and *3BE*: UGUAAAUA). (*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/*FBE* (*C*), 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 K_d (34).

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% that of WT Puf3p bound to the *Cox17* site B sequence (*3BE*). However, substitutions of the stacking residues in repeats 1, 5, and 7, as well as of the edge-on residues in repeats 4 and 5, still allowed >10% activity (Fig. 2*G*). 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 U3 in the conserved UGU sequence were the most constrained. At positions 4–6, at least two bases allowed >10% of WT levels (Fig. 2*H*). A4 and A5 lie across from noncritical stacking and edge-on residues (Fig. 2*I*). 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. 2 *C*, *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. 3*A*). 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/*FBE* 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/*FBE* (Fig. 3*B*). 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. 3*C*). 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 (UGUAUCAUA) by X-ray crystallography (Fig. 4*A* 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 C_α 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. 4*B*). This is similar to the FBF-2/*FBE* structure (Fig. 4*C*) but different from that of Puf4p and the canonical *4BE*, where U7 is flipped and A6 stacks between R651 and H688 (Fig. 4*D*). 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 UGUANAAUA 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%; $P = 7 \times 10^{-7}$ by Fisher's exact test; Fig. 5*A*). 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%; $P = 9 \times 10^{-8}$). 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. 5*B*). 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-

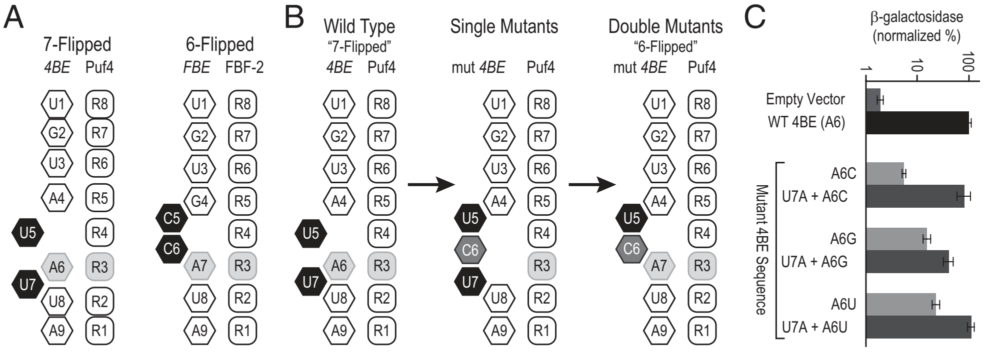


Fig. 3. Alternate binding pattern is dictated by the RNA sequence. (*A*) Known flipping conformations. Black indicates flipped bases, and light gray indicates repeat 3 and contacting adenosine. (*Left*) Seven-flipped pattern exemplified by Puf4p bound to a canonical *4BE*. (*Right*) Six-flipped pattern exemplified by FBF-2 bound to *FBE*. (*B*) Predicted flipping in mutated binding sites; shading is as in *A*. (*Left*) Seven-flipping, as occurs in canonical *4BE*; U5 and U7 are flipped. (*Center*) Mutation in the sixth position of the RNA is predicted to decrease the interaction with repeat 3, and thus to reduce binding. The A6C mutation is depicted in dark gray. U5, C6, and U7 are all predicted to be flipped. (*Right*) Double mutant (A6C U7A) carrying the alteration in position 6 plus a change to adenosine in position 7 (U7A) is predicted to restore binding. (*C*) Binding of single and double mutants. Binding of WT Puf4p to the indicated mutant RNAs was assayed using the yeast three-hybrid system. The β-gal levels were normalized to that of WT Puf4p bound to the canonical Puf4p binding element, *4BE*.

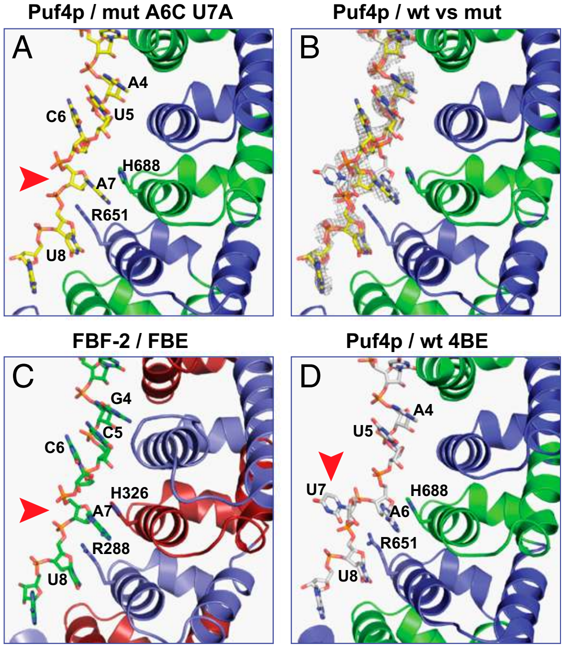


Fig. 4. Structure of Puf4p bound in a six-flipped conformation. (*A*) Crystal structure of Puf4p bound to the A6C U7A mutant *4BE* sequence. Puf4p is shown as a ribbon diagram with repeats colored alternately blue and green, and the mutant RNA is shown as a stick model (yellow represents carbon, blue represents nitrogen, red represents oxygen, and orange represents phosphorus). The red arrow indicates base 7 bound by Puf4p repeat 3. (*B*) Ribbon diagram of Puf4p with superposition of WT (7-flipped, white) and A6C U7A mutant (6-flipped, yellow) RNAs. A composite simulated annealing omit map of the Puf4p/mutant RNA structure contoured at 1.0 σ is shown superimposed with the RNA. (C) Six-flipped conformation of FBF-2 complexes. The crystal structure of FBF-2 is bound to *gld-1 FBEa* RNA (6-flipped). FBF-2 repeats are colored alternately blue and red, and the RNA is shown as a stick model colored as in *A* but with green carbon atoms. The red arrow indicates base 7 bound by FBF-2 repeat 3. (*D*) Seven-flipped conformation of Puf4p bound to a canonical *4BE*. The *4BE* RNA is white. The red arrow indicates base 7 that is flipped away from the binding surface of Puf4p.

tained the six-flipped sequence, whereas 11% of mRNAs bound only by Puf4p contained the six-flipped sequence ($P = 1 \times 10^{-5}$). 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 (*5BE*) and *3BE* were used as cognate sites to normalize the data to maximum activity for Puf5p and Puf3p, respectively.

Puf4p bound to all possible six-flipped sequences at levels near that of its canonical seven-flipped site, the *4BE* (Figs. 3*C* and 5*C*). 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. Puf3p 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 *puf4*Δ *puf5*Δ yeast (Fig. 6*A*). *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. 6*A*) 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. 6*B*). 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. 6*C*). 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

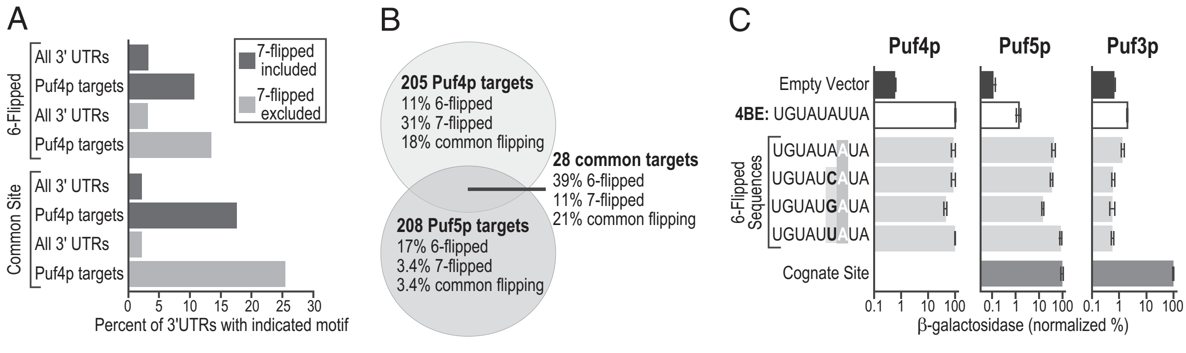


Fig. 5. Endogenous Puf4p targets contain six-flipped sites. (*A*) Enrichment of six-flipped sites in RNAs associated with Puf4p. The six-flipped site is enriched in Puf4p targets relative to its abundance among all 3′UTRs. Black bars represent an analysis of elements in the total population of RNA sequences, and gray bars represent an analysis of elements in the population of sequences from which seven-flipped sites have been removed. The common sequence UGUANAAUA, which could potentially adopt either flipping pattern, was scored separately from the six-flipped and seven-flipped patterns. Immunopurification data are from a study by Gerber et al. (15). (*B*) Enrichment of the six-flipped site among Puf5p targets and Puf4p/Puf5p common targets (15).The percentage of 3′UTRs containing a six-flipped or seven-flipped site is indicated. Values do not add up to 100%, implying that other binding elements may exist or that some associations in the RNA-binding protein immunopurification-microarray (RIP-chip) were indirect. (*C*) Puf4p and Puf5p both bind six-flipped sequences. Binding of Puf4p, Puf5p, and Puf3p to each RNA was assayed using the yeast three-hybrid system, normalized to each PUF bound to its cognate binding site (Puf4p: *4BE*–UGUAUAUUA, Puf5p: *5BE*–UGUAUGUAAU, and Puf3p: *3BE*-UGUAAAUA*)*. (*Left*) Sequences of the six-flipped sequences analyzed (gray shading indicates region of interest in the RNA, and white shading indicates adenosine at position 7).

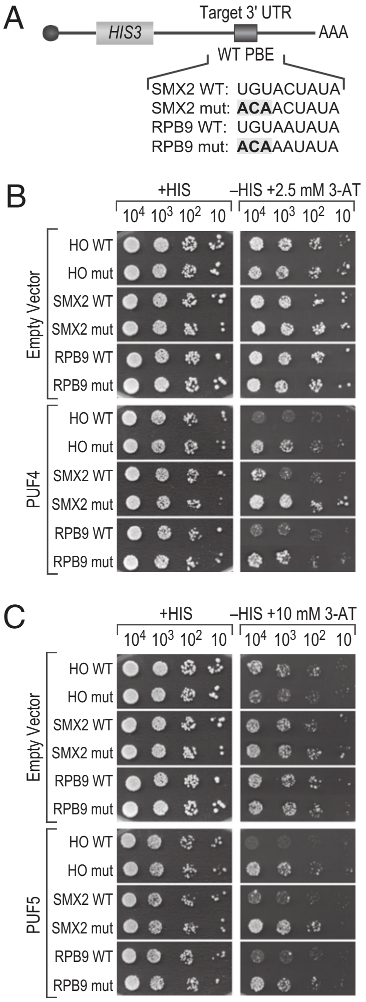


Fig. 6. Both Puf4p and Puf5p repress endogenous 3′UTRs with six-flipped sequences in vivo. (*A*) Reporter RNAs were used in the assays. Each mRNA was generated from a plasmid expressed in yeast. The *HIS3* ORF is followed by the 3′UTR sequences of *SMX2* and *RPB9* mRNAs, as indicated. Both WT and mutant (mut) forms of the binding elements were analyzed (their sequences are provided). The UGU trinucleotide was mutated to ACA to test PUF specificity (bold with gray highlight). (*B*) Repression by Puf4p. Full-length Puf4p was expressed in a W303 *puf4Δ puf5Δ* strain. Yeast were serially diluted (to ~10^4, 10^3, 10^2, and 10 cells) and spotted onto media with histidine (control) or without histidine plus 3-amino-1,2,4-triazole (3-AT) as indicated. Repression of reporter RNAs was measured by suppression of growth on selective media. The *HO* 3′UTR, used as a control, contained both Puf4 and Puf5 WT binding sites, whereas the *HO* mut had both sites destroyed. (*C*) Repression by Puf5p as in *B* but with Puf5p instead of Puf4p.

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_2Cys_6 DNA-binding proteins Gal4p and Ppr1p bind as homodimers to two sites separated by a spacer, with each monomer reading 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. 5*C* 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 targets 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*-α) *mpt5*::Kan^R *puf4*::*TRP1* as described (4).

Yeast Three-Hybrid Plasmids and Assays. pACT2 FBF-2 was as described (47). Puf4p (amino acids 536–888), Puf3p (amino acids 511–879), and Puf5p (amino acids 28–860) were cloned into pGADT7. Amino acids mutated to alanine by site-directed mutagenesis are 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 assayed 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 purifications, EMSA, and filter binding experiments were performed essentially as described (47, 49). Minor changes are described in *SI Materials and Methods*.

X-Ray Crystallography. The RBD of Puf4p was expressed and purified using a protocol similar to that described previously (29). Details are provided 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. *HIS3-HO* 3′UTR reporter plasmids were modified based on the work of Hook et al. (34) (*SI Materials and Methods*). The p415-glyceraldehyde-3-phosphate dehydrogenase (GPD) Puf4p is as described (3). Full-length Puf5 was cloned into the p415-GPD vector. Repression assays were performed in the W303 *puf4*Δ *puf5*Δ yeast strain but were as described otherwise (3).

ACKNOWLEDGMENTS. We thank Dr. Lars Pedersen and the staff at the Southeast Regional Collaborative Access Team beamline for help with X-ray data collection and Dr. Vanderploeg for help with figures. Data were collected at Southeast Regional Collaborative Access Team 22-ID beamline at the Advanced Photon Source, Argonne National Laboratory. Supporting institutions may be found at www.ser-cat.org/members.html. This work was supported by Grants GM50942 and GM095169 from the National Institutes of Health (to M.W. and Z.T.C. respectively), by a grant from the Intramural Research Program of the National Institute of Environmental Health Sciences (to T.M.T.H.), and by National Institutes of Health/National Institute of General Medical Sciences Grant 5T32GM08349 (to D.F.P.). Use of the Advanced Photon Source was supported by the US Department of Energy, Office of Science, Office of Basic Energy Sciences, under Contract W-31-109-Eng-38.

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