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PUM2, a novel murine Puf protein, and its consensus RNA-binding site

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ABSTRACT

Members of the Puf family of RNA-binding proteins from *Drosophila*, *Caenorhabditis elegans*, and *Dictyostelium* are known to function as translational repressors. To identify mammalian proteins that might regulate posttranscriptional gene expression, we have characterized a novel murine Puf protein, PUM2. *Pum2* transcripts were expressed in all murine tissues examined, suggesting the gene influences processes common to many cell types. Like all Puf family members, PUM2 contains a C-terminal RNA-binding domain related to the *Drosophila* Pumilio homology domain (PUM-HD). Two features found in the amino-terminus of PUM2, regions rich in serine and glutamine/alanine-rich regions, were also identified in most Puf family members. RNA sequences capable of binding with high affinity (6.5 nM) to a 48-kDa recombinant protein containing the PUM2 PUM-HD were isolated by using an iterative amplification–selection protocol (SELEX). The consensus sequence [UGUANAUARNNNNBBBBSCCS] of the PUM2 binding element (PBE) is related to, but distinct from, the 3′ end of the *Drosophila* Nanos response element. The characterization of PUM2 and potential RNA-binding site will assist efforts to assess the extent and mechanism by which mammalian genes are regulated at a posttranscriptional level.

Keywords: Nanos response element; Pumilio; RNA–protein interactions; translational control

INTRODUCTION

RNA-binding proteins are emerging as important positive and negative posttranscriptional regulators of cellular gene expression. Processes regulated by RBPs include mRNA processing, stability, localization, and translation. The importance of posttranscriptional gene regulation is highlighted in *Drosophila*, where a cascade of regulatory RBPs is vital for the development of the germline and the embryonic axis (Lasko, 1999). In mammals, RBPs influence such processes as cell cycle control (Luscher et al., 1985; Wang et al., 1996, 2000; Antic & Keene, 1997), neuronal differentiation (Behar et al., 1995; Antic et al., 1999; Blichenberg et al., 1999), cytokine expression (Shaw & Kamen, 1986; Bohjanen et al., 1991; Kern et al., 1997; Atasoy et al., 1998), iron metabolism (reviewed in Klausner et al., 1993; Henderson, 1996), DNA recombination, and genome maintenance (Hicks et al., 2000). In many cases, posttranscriptional regulation requires *cis*-acting sequences located in either the 3′ or 5′ untranslated region (UTR) of the transcript.

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Members of the Puf family of RBPs function as repressors of translation when bound to the 3′ UTR of selected transcripts. The *Drosophila* Pumilio Puf protein is required for proper abdominal segment formation in the early syncitial blastoderm. Pumilio binds maternally supplied *hunchback* (*hb*) transcripts in a sequence-specific manner on a pair of Nanos response elements (NREs). Pumilio recruits brain tumor (BRAT) and posterior-localized Nanos proteins forming a quaternary complex that represses *hb* translation (Sonoda & Wharton, 1999, 2001). The resulting gradient of Hb protein helps form the anterior–posterior axis (Lehmann & Volhard, 1987; Murata & Wharton, 1995; Wharton et al., 1998; Parisi & Lin, 2000). Maternal Pumilio is also required for G2 arrest and migration of germline progenitor cells from the posterior pole of the blastoderm to the developing gonad (Asaoka-Taguchi et al., 1999; Parisi & Lin, 1999). Later germline development requires zygotically expressed Pumilio protein, particularly to maintain germline stem cells (Lin & Spradling, 1997; Forbes & Lehmann, 1998). Although all downstream targets of Pumilio involved in pole cell migration and stem cell maintenance have not been identified, cyclin B is a likely mediator of G2 arrest. Translation of cyclin B is normally repressed in pole cells, and a regulatory sequence has been mapped to an NRE-like

element located in the 3′ UTR. Mutations in either Pumilio or the regulatory element result in misexpression of cyclin B and premature mitosis (Dalby & Glover, 1992; Asaoka-Taguchi et al., 1999). Orthologous Pum and Nanos proteins also interact with the cyclin B 3′ UTR in *Xenopus* oocytes (Nakahata et al., 2001), together with the CPE-binding protein (CPEB; de Moor & Richter, 1999; Groisman et al., 2000; Tay et al., 2000), suggesting that at least some puf protein functions are evolutionarily conserved.

Puf proteins in *Caenorhabditis elegans*, *Dictyostelium*, and *Saccharomyces cerevisiae* have also been described that influence the expression of a variety of target transcripts. Repression of fem-3 translation by the *C. elegans* FBF Puf protein inhibits spermatogenesis, allowing oogenesis to proceed in the developing hermaphrodite. *Cis*-acting regulatory signals recognized by FBF are located in the 3′ UTR of fem-3 transcripts (Zhang et al., 1997). In *Dictyostelium*, PufA binds the 3′ UTR of *pkaC* mRNA, inhibiting its translation during normal growth. Repression of PufA under starvation conditions induces PKA-C expression and promotes fruiting body development (Souza et al., 1999). Finally, the *S. cerevisiae* puf proteins Mpt5 and Puf3p participate in the posttranscriptional regulation of HO and *COX17*, respectively (Olivas & Parker, 2000; Tadauchi et al., 2001).

Puf proteins are characterized by a relatively large and highly conserved RNA-binding domain, called the Pum-homology domain (PUM-HD). The PUM-HD domain consists of eight copies of a 36 amino acid motif located in the C terminal region of the protein (Barker et al., 1992; Macdonald, 1992; Zamore et al., 1997). Each Pum repeat consists of a triple helix bundle, and together, the eight repeats align to form a curved superhelical structure with distinct concave and convex surfaces (Edwards et al., 2001; Wang et al., 2001). Mutational studies suggest that the RNA molecule is bound by the inner concave surface, whereas cofactors such as Nanos and BRAT are bound to the outer convex surface.

The functions of nearly all Puf family members characterized to date are consistent with a role for Puf proteins in regulating the translation of specific transcripts. Therefore, the identification of additional PUM-HD-containing proteins and their targets is expected to reveal new protein–RNA interactions important for the posttranscriptional regulation of specific genes. At least two human genes encoding PUM-HD-containing proteins are known to exist. However, neither the functions nor the target transcripts have been determined for these vertebrate Puf proteins. With the aim of identifying RBPs with gene-specific regulatory functions, we have cloned a novel murine Puf gene designated *PUM2* (Pumilio-like 2). We have identified new protein sequence motifs outside of the PUM-HD that are common in many of the Puf family members and have characterized the RNA-binding properties of the PUM2 PUM-HD. Using an iterative PCR method (systematic evolution of ligands by exponential enrichment; SELEX), we have identified a high affinity PUM2-binding site, essential information for the identification of PUM2 target RNAs. The site we define, the first such site described for a mammalian Puf protein, is related to but distinct from the targets of invertebrate Puf proteins. Our data therefore help define the properties of the mammalian Puf family, a family that is expected to include proteins involved in regulating gene expression.

RESULTS

Cloning and expression of murine *Pum2*

Due to the large and highly conserved PUM-HD that constitutes the RNA-binding domain of Puf proteins, expressed sequence tags (ESTs) encoding potential Puf proteins could be identified by conceptual translation. The *Drosophila* Pumilio sequence was compared to the EST databases by using the BlastX program (Gish & States, 1993) and matched several ESTs. These included at least two murine Puf transcripts that are similar to the human genes KIAA0099 and KIAA0235, identified by large-scale cDNA sequencing projects (Nagase et al., 1995, 1996). Using one of these ESTs (AA473499) as a probe, *Pum2* was cloned from an E8.5–E9.0 mouse embryonic cDNA library. Five positive clones were isolated that contained overlapping sequences. The largest clone contained an open reading frame encoding a 1066 amino acid protein (Fig. 1A) within a 3569 nt insert (GenBank accession number AY027917). Stop codons are present in all reading frames upstream of the initial Met codon and downstream of the termination codon, suggesting that the entire coding sequence has been cloned. However, the 3′ UTR is probably incomplete, as a polyadenylation signal was not identified.

Like other Puf family members, PUM2 contains a PUM-HD, the putative RNA-binding domain. This region of the murine sequence appears to be highly conserved across species, sharing 79% sequence identity with *Drosophila* Pumilio and 59% and 28% identity with *Dictyostelium* PufA and *C. elegans* FBF-2, respectively (Fig. 1B). In addition, the PUM2 PUM-HD is 98% and 90% identical to the corresponding regions in the human KIAA0235 and KIAA0099 proteins, respectively. The repeats, as aligned by amino acid sequence (Zamore et al., 1997) in Figure 1B, are localized in a slightly different register in the three-dimensional structure of the protein (Edwards et al., 2001; Wang et al., 2001).

Previous studies have failed to identify structural similarities among Puf proteins outside of the Pum-HD. However, our analysis of mouse PUM2 revealed two striking features: (1) a region between amino acids 259

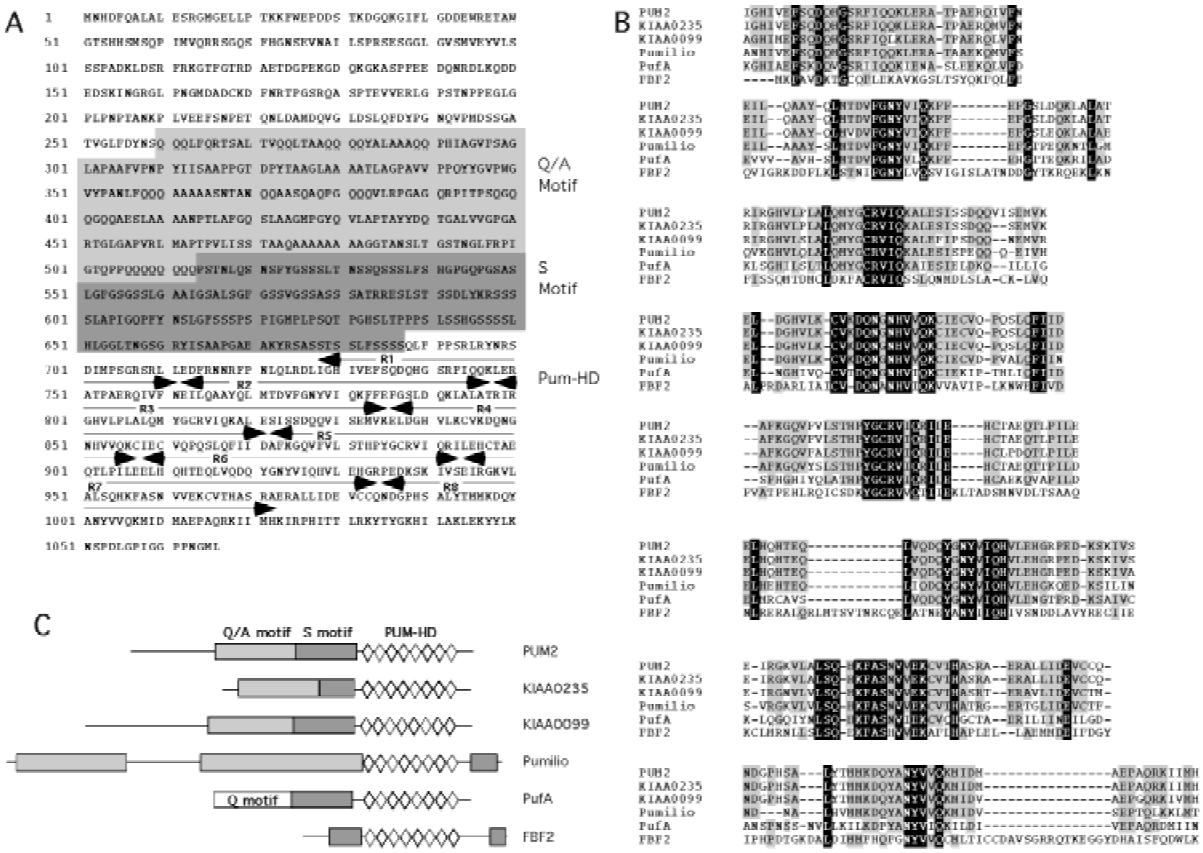


FIGURE 1. Sequence and structures of PUM2 and other Puf proteins. **A**: Deduced amino acid sequence of PUM2. Eight repeats (R1–R8) that comprise the Pumilio homology domain (Pum-HD) are indicated by arrows. Regions rich in glutamine and alanine (Q/A Motif, light gray box) and rich in serine (S-Motif, dark gray box) are shaded. **B**: Amino acid sequence alignment of Pum-HDs found in PUM2, human KIAA0235 (Nagase et al., 1996; accession no. BAA19665) and KIAA0099 (Nagase et al., 1995; accession no. BAA07895), *Drosophila* Pumilio (Barker et al., 1992; accession no. AAB59189), *Dictyostelium* PufA (Souza et al., 1999; accession no. AAD39751), and *C. elegans* FBF2 (Zhang et al., 1997; accession no. Q09312). Black shading indicates residues conserved in all Puf proteins, and gray shading is for residues conserved in a majority of proteins. The alignment was produced using the ClustalW program (Thompson et al., 1994). **C**: Domain structure of PUM2 and other Puf family members showing the eight repeats (◇) of the Pum-HD and the glutamine/alanine-rich motifs (light gray) and serine-rich (dark gray) motifs. The white box in PufA represents a region rich solely in glutamine.

and 513 that is rich in glutamine and alanine (15% and 21%, respectively, with several stretches of polyglutamine and polyalanine), and (2) a serine-rich region (over 30%) between amino acids 514 and 687 (Fig. 1A). We found similar structures in other Puf family members (Fig. 1C). All Puf proteins contained serine-rich regions (S motif) consisting of at least 25% serines in a 50 amino acid region. A glutamine/arginine (QA) motif in which these amino acids comprised at least 30% of a 50-residue stretch was absent only from FBF-2 and PufA, but the amino terminus of PufA contains 32% glutamine.

Northern blot analysis of total RNA from various adult tissues and embryonic stem (ES) cells was used to determine the size and distribution of *Pum2* transcripts (Fig. 2). In all tissues examined, two transcripts were observed of approximately 4 and 6 kb in length. Whereas *Pum2* was expressed in all tissues, levels varied as compared to β actin transcripts, with the highest levels expressed in the brain.

PUM2 binds an RNA sequence similar to but distinct from the Pumilio response element

To determine the RNA-binding properties of PUM2, we first attempted to purify full-length recombinant PUM2 protein expressed in *Escherichia coli*. However, the full-length protein proved to be insoluble, and we were unable to purify sufficient amounts for analysis. We subsequently expressed only the PUM2 PUM-HD as a 48-kDa 6xHis-tagged fusion protein. It seemed likely that this protein would retain the RNA-binding properties of the full-length protein, as full-length Pumilio protein and the Pumilio PUM-HD bind RNA with similar specificity and affinity (Zamore et al., 1997, 1999).

Like Pumilio, the PUM-HD of human KIAA0099 can bind the NRE (Zamore et al., 1997). Because the PUM-HD regions of PUM2 and KIAA0099 are 90% identical, we expected that PUM2HD would also bind the NRE, and this possibility was examined by gel mobility shift assay. Two RNA–protein complexes were

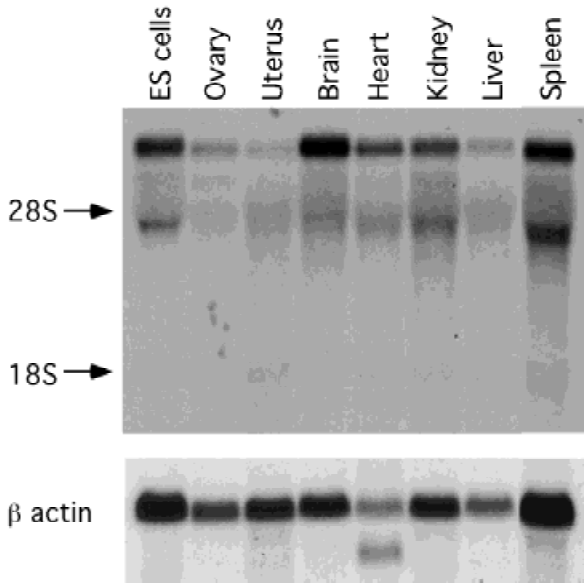


FIGURE 2. Distribution of *Pum2* transcripts in different mouse tissues. Northern blot analysis of RNAs from various mouse tissues and the D3 ES cell line following hybridization to a ^{32}P-labeled 2.2-kb *Pum2* cDNA fragment (top panel) and to a β actin probe (bottom panel).

resolved on a native gel following incubation with ^{32}P-labeled NRE and increasing concentrations of PUM2HD (Fig. 3A). In contrast, when the NRE was replaced by an unrelated sequence of similar size, corresponding to the multiple cloning site of pBluescript KS, RNA–protein complexes were observed only at the highest protein concentration (Fig. 3A). Excess cold NRE, or poly(U), but not poly(A) or poly(C), were able to compete for binding (Fig. 3B). The NRE was not expected to represent an optimal PUM2-binding site. However, the interaction between PUM2HD and the NRE demonstrated that the recombinant protein has RNA-binding activity and provided a useful baseline for judging the relative specificity and affinity of interactions with other RNA sequences.

To identify a consensus RNA-binding site for murine PUM2, two independent SELEX experiments were performed. SELEX selects for oligonucleotides that preferentially bind proteins, starting from a pool of random sequences (Tuerk & Gold, 1990; Fitzwater & Polisky, 1996). For the first experiment, the design of the starting oligonucleotide pool was based on known features of the *Drosophila* NRE. In addition, random sequences

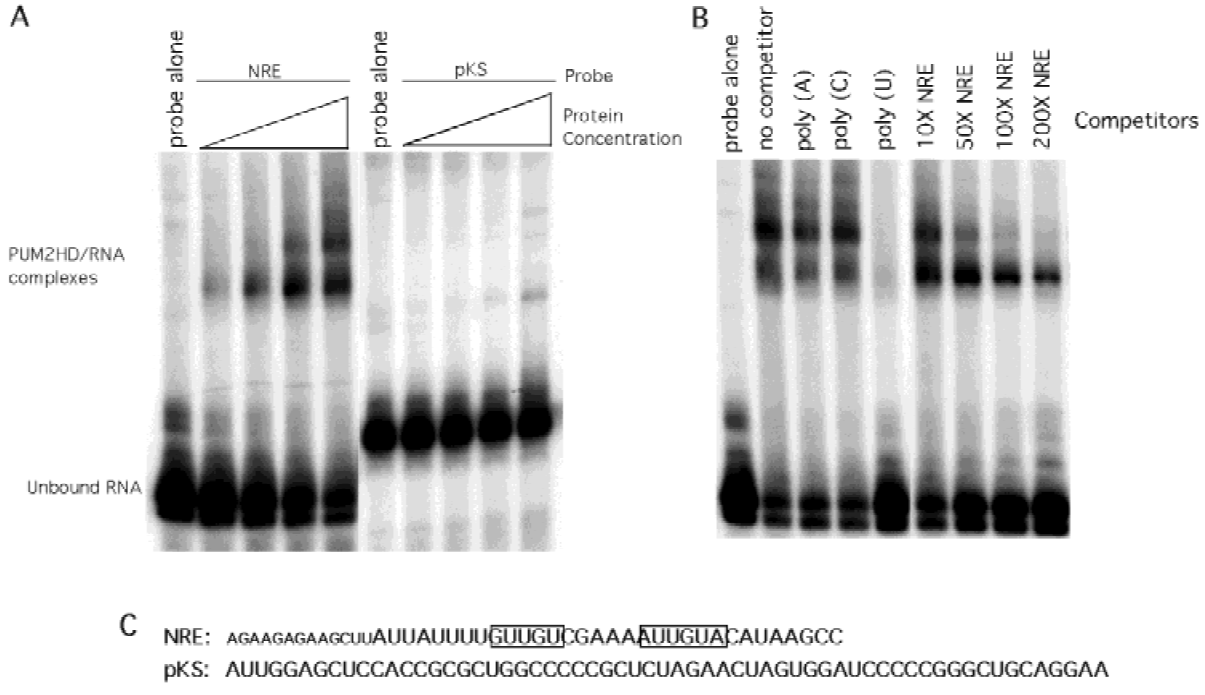


FIGURE 3. The PUM2 PUM-HD binds the *Drosophila* Nanos response element. **A**: A gel mobility shift assay generated by using 30 fmol of a ^{32}P-UTP-labeled RNA containing either the Nanos response element or a control sequence transcribed from the multiple cloning site of pBluescript (pKS) and 5 nM, 10 nM, 25 nM, and 50 nM of PUM2HD. **B**: A gel shift assay was performed using 30 fmol ^{32}P-UTP-labeled NRE and 100 nM PUM2HD in the presence of excess RNA competitors. In lanes 3, 4, and 5, 200 ng of the indicated polyribonucleotide oligomer were added to the binding reaction. In lanes 6–9, increasing amounts of cold NRE were added to the reactions as specific competitors. **C**: Riboprobe sequences. Capital letters denote the NRE sequence with boxes indicating the interrupted 11-bp motif that is conserved between the two NREs present in *hunchback* transcripts.

used in SELEX must be 20 nt or less, to ensure that all possible templates are represented in the initial pool. Because the NRE is 32 nt, oligonucleotides were synthesized in which 20 nt of random sequence were flanked by the first 7 and last 10 nt of the NRE. These flanking sequences are less conserved among *Drosophila* species and contribute less to Pumilio binding than the central region that contains a repeated 11-nt motif (shown in boxes in Fig. 3B; Murata & Wharton, 1995; Wharton et al., 1998; Wharton & Struhl, 1991). The oligonucleotides also contained a terminal T7 RNA polymerase promoter.

The original pool of synthetic oligonucleotides was amplified by PCR and transcribed by RNA polymerase. The resulting RNA templates were incubated with 1 μM of PUM2HD (which decreased to 100 nM by cycle 10), and protein–RNA complexes were recovered by Ni-affinity chromatography. The RNA was converted to cDNA, amplified by PCR, and transcribed to provide templates for the next round of SELEX. Aliquots of the PCR product were cloned following 0, 7, and 10 rounds of selection, and random clones were sequenced.

From the analysis of the unselected clones, it is clear that sequences in the original pool were biased, such that 14/20 clones were composed of at least 50% adenosine (Fig. 4). Nevertheless, all but one of the clones derived from selected ligands contained the sequence UGUA, which is also found in the 11-nt NRE motif (Fig. 4). Moreover, by the 10th round, many of the selected ligands had lost nucleotides from the original region of random sequence, and in some cases, only the UGUA sequence remained. In contrast, all clones from the initial pool had full-length inserts, indicating that the shorter templates had been selected during the experiment. These results indicate that the UGUA sequence is an important feature of the PUM2-binding site.

Given the obvious sequence bias in the starting pool for the first SELEX experiment, and the loss of nucle-

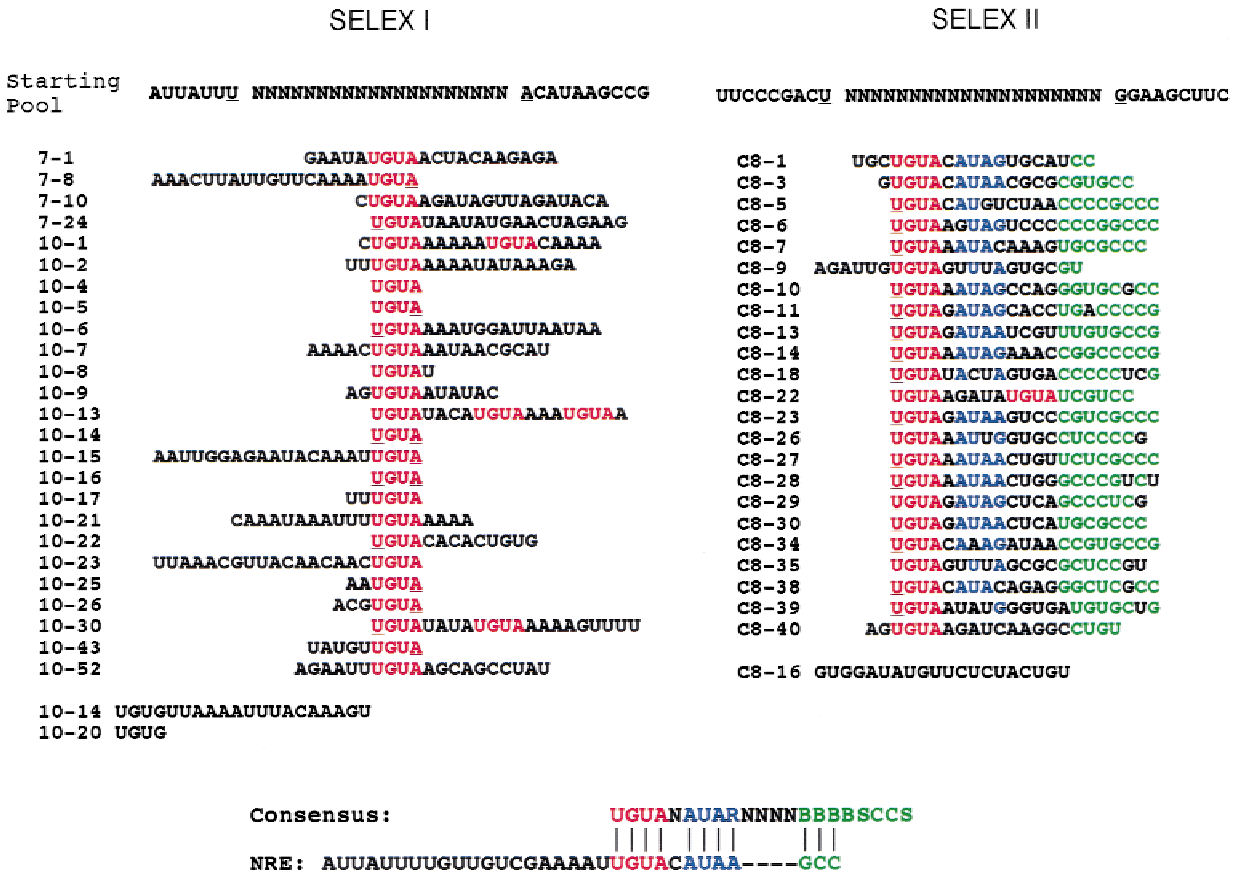


FIGURE 4. Identification of a consensus PUM2 RNA-binding site. The sequence of the initial pool of random oligonucleotides is shown for each SELEX experiment, including both the random (N) and flanking nucleotides. The flanking sequence in the SELEX I pool represents portions of the NRE sequence (see Fig. 3C) that by themselves are necessary but are not sufficient for Pumilio binding. Sequences of individual selected clones are shown. Clones were produced and sequenced following rounds 7 and 10 for SELEX I, and round 8 for SELEX II and are aligned below each starting sequence. Nucleotides derived from either the 5′ or 3′ flanking region are underlined; all other nucleotides were selected from the random sequence. The sequence UGUA (shown in red) was present in nearly all clones. Shown in blue and green letters is the AUAR element and the GC-rich region, respectively, that were identified in SELEX II. The RNA-binding site for PUM2 is shown at the bottom with its alignment to the NRE. The IUB code is used for mixed base sites (R= A + G, B = G + C + U, S = G + C).

otides from the randomized region in selected clones, a second SELEX experiment was performed. In this experiment, the region of random 20-mers was flanked only by sequences required for PCR amplification and reverse transcription. Moreover, full-length PCR products were gel purified after alternate rounds of SELEX to counteract the apparent preferential amplification of smaller PCR products.

Analysis of 20 unselected clones did not reveal any inherent bias in the sequence of the starting oligonucleotide pool (data not shown). Moreover, clones recovered after eight rounds of selection contained a larger and more complex consensus sequence than was observed in the first experiment (Fig. 4). All clones contained a UGUA sequence, usually located at the extreme 5′ end. Most clones also contained an AUAR element followed by a 3′ GC-rich region. Overall, the consensus sequence resembles the 3′ half of the NRE (Fig. 4), which contains an AUAR element located 1 nt from the UGUA sequence followed by a GC region. However, the spacing of the different elements is not conserved.

The results from SELEX II provided additional insights on the results of the first SELEX experiment. Thus, in 11 of the 27 ligands analyzed from SELEX I, the UGUA sequence was positioned at the 3′ end of the variable region, and in 9, the last A was derived from the flanking sequence (Fig. 4). This creates a UGUA element positioned 1 nt from an AUAR sequence, the latter being provided by the flanking sequence. Two additional clones, 7-10 and 10-7, contained an AUAR derived from the variable sequence.

Gel shift assays were performed to determine the binding constants for interactions between the PUM2HD and the NRE and C8-30 sequences (Fig. 5). C8-30 was analyzed because it provided the best match to the consensus SELEX II binding site. For comparison, we also analyzed a human PUMHD (HsPUMHD), expressed in *E. coli* as a GST fusion protein (Zamore et al., 1997), and PUM2HDΔR5 (a mutant PUM2HD which lacks repeat 5, amino acids 871–907). As estimated from the disappearance of free RNA, the PUM2HD bound the C8-30 and NRE sequences with K_ds of 6.5 and 8.5 nM, respectively, whereas the K_ds of PUMHD for the same sequences were 19 and 54 nM, respectively. Removal of repeat 5 in PUM2HDΔR5 reduced but did not abolish binding to either the C8-30 (K_d = 10 nM) or NRE (K_d = 36 nM) sequences.

Drosophila Pumilio binds the NRE as a monomer and produces a single band in gel shift assays, although additional complexes may form at higher protein concentrations (>100 nM; Zamore et al., 1999). However, two complexes were produced by interactions between the PUM2HD and C8-30 and between the PUM2HD and NRE at protein concentrations above 25 nM. This raises the possibility that PUM2HD binds C8-30 as a multimer. To examine this issue, gel shift assays were performed in which the 48-kDa 6xHis-tagged PUM2HD and a 67-kDa GST-tagged PUM2HD (Fig. 6) were used either individually or in combination. Whereas both proteins individually produced two complexes with distinct mobilities, no additional bands that would suggest mixed complexes were formed by combining the two proteins.

To compare the relative affinities of PUM2HD for different sequences, a competitive gel shift assay was performed (Fig. 7A), using C8-30 as the ^{32}P-labeled probe. As before, two RNA–protein complexes were observed in the absence of competitors; however, an additional lower molecular weight band was also observed in the samples containing probe alone. This band became more pronounced in the presence of competitor RNAs for unknown reasons. RNAs transcribed from C0-1, C10-5, C8-22, C8-39, C8-30 (Fig. 4), and the NRE (Fig. 7B) were used as competitors. C0-1, a sequence from the initial SELEX II pool, failed to compete for PUM2HD binding, confirming that PUM2HD binding is sequence specific. C10-5, a clone identified in SELEX I, contains only the UGUA site and the flanking NRE sequences, and competes with efficiency similar to the NRE. C8-22 and C8-39, isolated in the second SELEX experiment, lack the AUAR element; however, C8-22 contains two UGUA sites. These probes, along with C8-30, appear to have the highest affinity for PUM2, as they are the best competitors for PUM2 binding. The concentrations of competitor necessary to inhibit 50% of the binding activity are shown for quantitative comparison (Fig. 7B).

DISCUSSION

Identification of regulatory RBPs and their target RNAs is expected to clarify the extent and mechanism by which mammalian gene expression is controlled at a posttranscriptional level. Here we report on the characterization of a novel murine Puf protein, PUM2. Similar to other Puf family members, PUM2 contains a highly conserved RNA-binding domain, the PUM-HD, located in the C-terminal region. We also identified two N-terminal serine- and glutamine/alanine-rich regions of PUM2 that are shared by most Puf family members, but have not been previously described. Although these features appear to be conserved, their functional significance is unclear. They do not appear to be required for RNA binding, as purified PUM-HDs of KIAA0099 (Zamore et al., 1997), Pumilio (Murata & Wharton, 1995; Wharton et al., 1998), and PUM2 (reported here) can bind RNA in a sequence-specific manner with high affinity. Moreover, they do not appear to be required for interactions with known protein partners such as Nanos and BRAT (Sonoda & Wharton, 1999, 2001; Edwards et al., 2001). Interestingly, the Nanos protein also contains a glutamine/alanine motif between amino acids 111 and 205, raising the possibility that additional unknown factors may bridge the two proteins or regulate

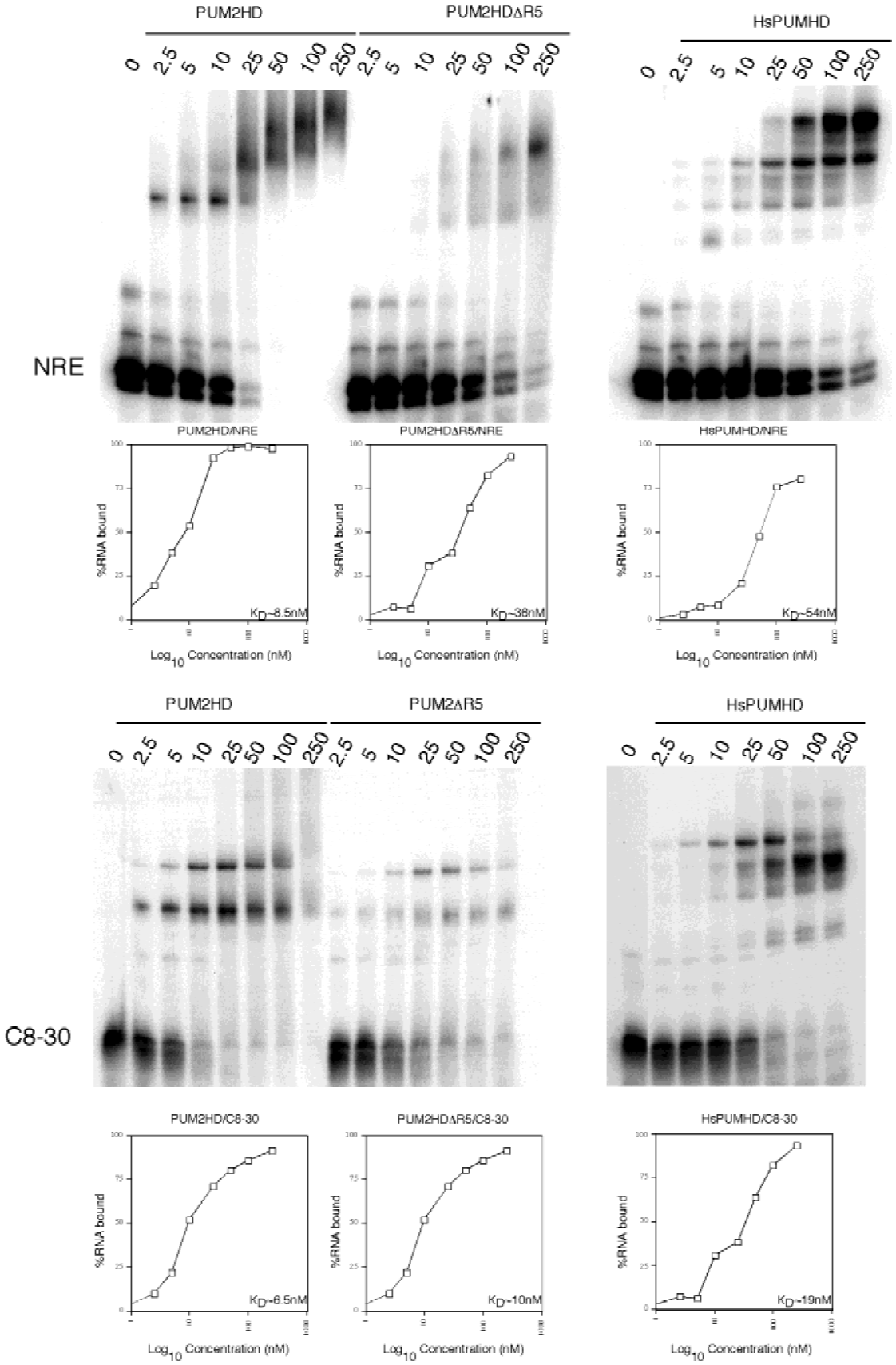


FIGURE 5. Binding of PUM2HD, PUM2HDΔR5, HsPUMHD to the C8-30 and NRE sequences. A gel mobility shift assay was performed using 15 fmol ^{32}P-UTP-labeled C8-30 or NRE probes. The proteins and probes used for each assay are indicated. The binding constant was established based on the disappearance of free RNA as measured by phosphorimager densitometry.

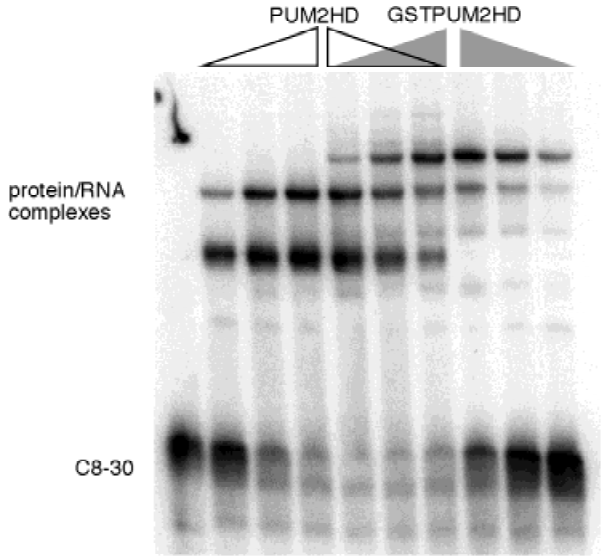


FIGURE 6. Comparative binding of the 48-kDa His6-tagged PUM2HD and the 67-kDa GST-tagged PUM2HD to the C8-30 sequence. The binding pattern of each individual protein was determined by incubating 5, 10, and 15 nM of His6-tagged PUM2HD (lanes 2–4) or GST-tagged PUM2HD (lanes 10–8) with 15 fmol ^{32}P-UTP-labeled C8-30, followed by native gel electrophoresis. The analysis of both proteins in combination (lanes 5–7) involved mixing 5, 10, and 15 nM of GST-tagged PUM2HD together with 15, 10, and 5 nM of His6-tagged PUM2HD, respectively.

their activity. The serine-rich motif in the Puf proteins may act as a negative element, as its removal from FBF enhances interactions between FBF and NOS-3, as measured in a yeast two-hybrid assay (Kraemer et al., 1999).

Invertebrate Puf genes exhibit temporal and spatially restricted expression patterns. Pumilio transcripts are found throughout the *Drosophila* syncitial blastoderm, but are later restricted to the developing germline (Barker et al., 1992; Macdonald, 1992). In *C. elegans*, Fbf is expressed solely in the developing gonad (Zhang et al., 1997). PufA is expressed only when *Dictyostelium* are in the growth phase of their development (Souza et al., 1999). In contrast, *Pum2* transcripts are expressed in all adult tissues examined (Fig. 2). Similar findings have been reported for the human genes KIAA0099 and KIAA0235 (Kikuno et al., 2000). The widespread expression of *Pum2* transcripts suggests that it may regulate processes common to many cell types.

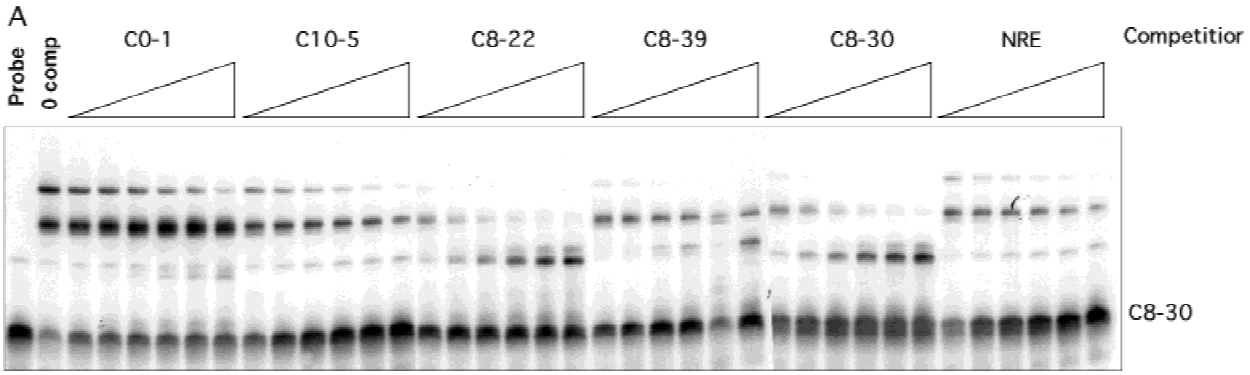
From SELEX experiments using the PUM2 PUM-HD, we identified a consensus high affinity binding sequence, the PUM2 binding element (Fig. 4). The PBE shares features with the *Drosophila* NRE, but has several distinct differences. The UGUA sequence appears to be the single most important determinant of PUM2 binding, as it was present in nearly all selected templates from two independent SELEX experiments. The UGUA sequence is also part of the conserved interrupted 11-nt motif in the *Drosophila* NRE, a target sequence that the murine PUM2 PUM-HD can also bind. Notably, mutations in the UGUA sequence abolish Pumilio binding in vitro, and have the most severe effects in vivo as compared to mutations elsewhere in the NRE (Murata & Wharton, 1995; Wharton et al., 1998; Sonoda & Wharton, 1999).

In addition to UGUA, the PBE consensus contains two other motifs, an AUAR sequence and a GC rich region. Similar elements are also found in the 3′ half of the NRE, but are spaced differently. In both the NRE and the PBE, the UGUA and AUAR sequence are separated by a single nucleotide. However, in the PBE, the GC-rich region is separated from the AUAR sequence rather than directly following it, as is found in the NRE. As assessed by mobility shift binding assays, the apparent affinity of murine PUM2HD (K_d = 6.5 nM) and human PUM-HD (K_d = 8.5 nM) for the SELEX winner sequence, C30-8, was greater than for the NRE. However, the gel shift analysis was complicated by the formation of multiple, shifted complexes. This undermines the accurate determination of a binding constant, as binding can only be inferred from the loss of free RNA. A more accurate assessment of relative affinity was provided by competition experiments (Fig. 7). Again, the PUM2HD bound with higher affinity to PBE-related sequences than to the NRE or less related sequences.

A mutant PUM2HD protein (PUM2HDΔR5) that lacked repeat 5 of the homology domain also bound to the PBE and NRE with apparent K_ds of 10 and 36 nM, respectively. This was unexpected, as deletion analysis of *Drosophila* Pumilio suggests that all eight repeats and the conserved flanking are required for RNA binding (Zamore et al., 1997). However, it appears that the exact excision of repeat 5 may maintain the concave structure (Edwards et al., 2001; Wang et al., 2001) of the PUM RNA-binding site.

Alignment of the PBE and NRE reveals that the homology between the two binding sites is confined to the 3′ half of the NRE. This difference in overall structure between the PBE and NRE is interesting and raises the possibility that in vivo RNA targets of PUM2 may contain as yet unidentified elements 5′ to the UGUA core sequence. Although our results cannot exclude this possibility, it is clear that sequences 3′ to the UGUA element have a greater impact on binding affinity, given that only a few ligands were selected that did not place the UGUA element at the 5′ end (Fig. 4). This preference for the 3′ half site was previously observed in studies of the human protein KIAA0099 binding to the NRE, where removal of sequences 5′ to the UGUA had little effect on binding (Zamore et al., 1997). This is in contrast to the Pumilio, which exhibits high affinity binding only with the entire NRE, although weak binding to the half site can be observed with purified protein (Wharton et al., 1998). From this result, we believe that should in vivo PUM2 targets contain motifs 5′ to the UGUA core sequence, these motifs are unlikely to be related

A



B

Name	Sequence	IC50
C0-1	UUCCCGACUAUAACGAGGUCAUUCUGCCUGGAAGCUUC	>1.5 uM
C10-5	AUUAUUUUGUAACAUAAGCCG	75-150 nM
C8-22	UUCCCGACUUGUAAGAUAUGUAUCGUCCGGAAGCUUC	<75 nM
C8-39	UCCCGACUUGUAAUAUGGGUGAUGUGCUGGGAAGCUUC	<75 nM
C8-30	UUCCCGACUGUAGAUAACUCAUGCGCCCGGAAGCUUC	<75 nM
NRE	AUUAUUUUGUUGUCGAAAAUUGUACAUAAGCC	75-150 nM

FIGURE 7. Competitive gel shift assay of PUM2HD-RNA interactions. **A**: A competitive gel mobility shift assay was performed using 15 fmol ^{32}P-UTP-labeled C8-30 as a probe together with 5 nM PUM2HD protein, and several selected and unselected RNAs at concentrations of 75, 150, 300, 600, 900, and 1,500 nM. The competitor and probe sequences were from SELEX I (C10-5) and SELEX II (C8-22, C8-39, and C8-30), in addition to the Pumilio binding site, NRE. A random RNA (C0-1) was used as a control. **B**: Competitor RNA sequences. The concentration of competitor RNA required to reduce the amount of the shifted band by 50% (IC_{50}) was determined by phosphorimager densitometry.

to the NRE sequence or to have significant effects on the affinity.

Puf proteins are gene-specific RBPs, and like many RBPs that regulate protein expression, they identify their targets through elements located in the 3′ UTR. As a Puf family member, murine PUM2 is also likely to be a regulatory RBP, interacting with its RNA partners through the 3′ UTR. Determining the consensus binding site, described here, will aid in the identification of PUM2 targets. Even though it contains several degenerate nucleotides, the PBE that we have characterized is large enough to be used in database searches to identify target transcripts, particularly if combined with a requirement for the PBE site to be located within a 3′ UTR. As the human and mouse genome sequences are completed, it will become possible to identify all genes that contain a PBE in their 3′ UTR and systematically examine these transcripts for translational regulation by PUM2 or related mouse and human proteins.

MATERIALS AND METHODS

Isolation and sequence analysis of *Pum2* cDNA clones

A 0.8-kb *Sal*I/*Sal*I fragment from EST AA473499, identified by conceptual translation as containing several Puf repeats, was used to screen an 8.5–9.0 dpc mouse embryonic cDNA Lambda Zap II library (gift from Kathy Mahon). Five positive clones were identified out of a total of 10^6 plaques. cDNA inserts were excised and rescued as pBluescript phagemids using ExAssist helper phage (Stratagene). The largest and most complete cDNA was sequenced using the BigDye Terminator Cycle Sequencing Kit (ABI) and analyzed on a Prism 377 automated sequencer. Templates were prepared by subcloning fragments into pBluescript and sequenced with T7 and T3 primers. Custom internal oligonucleotides (Gibco-BRL) were used to fill in any gaps. Sequences were compiled and analyzed using Sequencher 3.0 software (Gene Codes Corporation). The gene was designated *Pum2* (Pumilio-like 2) based on the recommendation of the mouse and human nomenclature committees (L. Maltais, pers. comm.). Alignment of the *Pum2* sequence with other Puf family members was performed by using the ClustalW algorithm (Thompson et al., 1994).

Northern blot analysis

Total RNA was isolated from adult C57BL/6J mice and 129Sv embryonic stem cells using TRIzol (Gibco-BRL) according to the manufacturer's instructions. Approximately 10 μg of RNA were denatured, separated on a 1.2% agarose/5% formaldehyde gel, and transferred to Hybond-N membrane (Amersham). *Pum2* transcripts were detected using a cDNA probe, corresponding to nt 2297–3569, that was amplified using T3 primer and Puf Fw primer, 5′-TGGTTCCAGATTCATAC AGC-3′ and labeled with [α^{32}P]-dCTP.

Protein purification

Due to the insolubility of full-length recombinant PUM2 protein, all binding studies were performed using only the RNA-binding portion of PUM2. His6-tagged PUM2HD was created by cloning a *Bam*HI fragment of the *Pum2* cDNA, encompassing the PUM-HD (amino acids 645–1066), into the *Bam*HI site of pET 28A (Novagen). PUM2HDΔR5 was constructed by inserting a *Cla*I site at position 2802 of the His6-tagged PUM2HD by PCR, which was subsequently used to remove nt 2694–2801 as a *Cla*I fragment. The deletion maintains the PUM2 open reading frame. To purify the protein, an overnight culture of PUM2HD in BL21 DE3 cells was diluted 1:20 in LB media and grown at 37 °C until an OD_{600} of 0.5 was reached. Cells were induced with 0.1 mM IPTG and allowed to grow for an additional 4 h. Cells were collected and frozen at −80 °C overnight. Cells were then thawed on ice for 15 min and resuspended in lysis buffer (50 mM NaH2PO4, pH 8.0, 300 mM NaCl) containing 10 mM imidazole. Lysozyme was added to a final concentration of 1 mg/mL and allowed to digest for 30 min on ice, followed by sonication. Following centrifugation, soluble protein was recovered in the supernatant and mixed with 1/10 vol NiNTA resin for 1.5 h at 4 °C. The NiNTA resin was washed once in lysis buffer containing 25 mM imidazole and again in lysis buffer containing 50 mM imidazole, and the protein was eluted using 200 mM imidazole. HsPUMHD (D5-9) was provided by Philip Zamore and the protein was purified as described (Zamore et al., 1997). For GSTPUM2HD, the *Bam*HI fragment of the *Pum2* cDNA was cloned into pGEX-2T vector (Pharmacia Biotech) and purified using the same conditions as were used for HsPUMHD. All proteins were dialyzed in 20 mM HEPES, pH 7.4, 150 mM KCl, 0.1 mM EDTA, 2 mM DTT, 20% glycerol, and 0.02% Tween 20. Protein concentration was determined using the Bio-Rad protein assay.

RNA transcripts

The DNA template for the NRE RNA was constructed by annealing complimentary oligonucleotides (NRE antisense 5′-AATTCGGCTTATGTACAATTTTCGACAACAAAATAATA-3′, NRE sense 5′-AGCTTATTATTTTGTTGTCGAAAATTGTA CATAAGCCG-3′) that contained the published NRE sequence (Murata & Wharton, 1995) and incorporated an *Eco*RI site and a *Hin*dIII site. The annealed oligonucleotides were then cloned into pDP18 (Ambion). PBluescript was used as the template for pKS RNA. Linearized template was prepared in both cases by digesting with *Eco*RI. Templates for rC0-1, rC10-5, rC8-22, rC8-39, and rC8-30 were prepared by the Milligan technique (Milligan et al., 1987). In short, a single oligonucleotide was prepared for each probe that contained the antisense sequence that was determined in the SELEX experiments and the T7 binding sequence (C0-1Rv 5′-GAAGCTTCCAGGCAGAATGACCTCGTTATAGTCGGGAAT TCGGATCCCTATAGTGAGTCG-3′, C10-5 5′-CGGCTTATG TTACAAAATAATCCCTATAGTGAGTCG-3′, C8-22 5′-GAAG CTTCCGGGACGATACATATCTTACAAGTCGGGAATTCGG ATCCCTATAGTGAGTCG, C8-30 5′-GAAGCTTCCGGGGC GCCATGAGTTATCTACAGTCGGGAATTCGGA TCCCTATA GTGAGTCG-3′, C8-39 5′-GAAGCTTCCCAGCACATCACC CATATTACAGTCGGGAATTCGGATCCCTATAGTGAGTCG-3′). The oligonucleotides were annealed to a complementary T7 sequence (5′-TAATACGACTCACATATAGGG-3′) prior to transcription. The templates were transcribed using T7 polymerase (Boehringer Mannheim) according to the manufacturer's suggestion. Either 1 μg (NRE and pKS) or 50 ng (C0-1, C10-5, C8-22, C8-39, and C8-30) of template was used in each reaction. For the radiolabeled RNAs, the final concentration of ATP, CTP, and GTP was 333 μM, the final concentration of rUTP was 33 μM, and 120 μCi of [α^{32}P]-UTP was included in the reaction. RNA transcripts were purified by denaturing PAGE.

Mobility shift assay

Binding reactions were performed as described in Zamore et al. (1997). Purified proteins were diluted in reaction buffer (RB: 10 mM HEPES, 1 mM EDTA, 50 mM KCl, 2 mM DTT, 0.1 mg/mL BSA, and 0.02% Tween-20). Binding reactions included either 100 fmol or 15 fmol radiolabeled RNA that was denatured at 65 °C for 10 min prior to addition to the reaction. When used, competitors were mixed with the radiolabeled probe before addition to the reaction. The reactions were incubated at room temperature for 25 min, and immediately analyzed by electrophoresis at 250 V for 3 h through a 6% (acrylamide:bisacrylamide 29:1) native gel in 0.5× TBE at 4 °C. Gels were prerun at 250 V for 3 h prior to loading. The amount of free and bound RNA was determined using a Phosphorimager (Fuji) and Imagegauge software.

In vitro selection

SELEX procedures were performed as described (Tuerk, 1990; Fitzwater & Polisky, 1996; Perez, 1997). The DNA oligonucleotides for synthesis of the starting pool were as follows: SELEX I: 5′-GCGTCTCGAGAAGCTTCCGGCTTAT GT$(N)_{20}$AAATAATAGTCGGGAATTCGGATCCCTATAGTGA GTCGATTA-3′; SELEX II: 5′-GCGTCTCGAGAAGCTTCC $(N)_{20}$AGTCGGGAATTCGGATCCCTATAGTGAGTCGTATTA-3′. DNA templates were amplified in a 10-mL PCR reaction containing 208 ng of starting oligonucleotides, 0.2 μM T7 primer (5′-TAATACGACTCACTATAGGGATCCGAATTCCCG ACT 3′), 0.2 μM RT primer (5′ GCGTCTCGAGAAGCTTCC-3′), 0.2 mM dNTPs, 3.0 mM $MgCl_2$, and 100 U Taq polymerase (Perkin Elmer). After five cycles of amplification (96 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s) and a final extension period of 5 min at 72 °C, the DNA was precipitated with 0.1 vol 3 M NaOac, pH 5.2, and 2.5 vol of EtOH. Full-length templates were PAGE (12% native gel run in 1× TBE) purified for both primary PCR reactions, and for rounds 2, 4, 6, and 8 for the second SELEX experiment to prevent truncation. The initial RNA pool was transcribed from 1 μg of purified template using 120 U of T7 polymerase in a 250-μL reaction containing buffer components, 0.3 mM NTPs, and 36 U of RNAguard (Pharmacia). For subsequent rounds, the RNA was transcribed from one-fifth of the PCR reaction in a 100-μL reaction containing 40 U T7 polymerase. Following 2 h of incubation at 37 °C, the reaction was treated with 1 U of DNase (Gibco-BRL) for 30 min at 37 °C and inactivated by the addition of EDTA to a final concentration of 0.5 mM. RNA was isolated by phenol-chloroform extraction and ethanol precipitation. The precipitated RNA was resuspended in 100 μL of 1× RB.

To prevent selection of RNAs that bind NiNTA resin, transcripts for each round were first precleared in NiNTA resin (25 μL of a 1:1 RB:resin slurry) that had been washed three times in 1× RB. Following centrifugation, the precleared RNA was mixed with PUM2HD and incubated for 30 min at room temperature. NiNTA resin (25 μL of a 1:1 RB:resin slurry) was then added and incubated at 4 °C for 30 min to purify the protein–RNA complexes. Following three washes in 1× RB, the purified protein–RNA complexes were incubated with 5 μg of proteinase K (Sigma) and 100 μL of 1× proteinase K buffer (200 mM Tris, pH 7.5, 25 mM EDTA, 300 mM NaCl, and 2% SDS) for 30 min at 55 °C. Bound RNA was isolated by phenol-chloroform extraction and ethanol precipitation. Protein concentrations used in the subsequent selection rounds were as follows: 1 μM for rounds C1 and C2; 500 nM for rounds C3, C4, and C5; 250 nM for rounds C6, C7, and C8; and 100 nM for round C9. The extracted RNA was reverse transcribed using Superscript II (Gibco-BRL) according to the manufacturer's protocol in the presence of 1.75 μM RT primer. The resulting single-strand DNAs were amplified in a 300-μL PCR reaction and one-fifth of the amplified product was used for the next round.

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