MOLECULAR BASIS OF RNA RECOGNITION BY THE EMBRYONIC POLARITY DETERMINANT MEX-5*

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Running Title: MEX-5 RNA recognition

Embryonic development requires maternal proteins and RNA. In Caenorhabditis elegans, a gradient of CCCH tandem zinc finger (TZF) proteins coordinates axis polarization and germline differentiation. These proteins govern expression from maternal mRNAs by an unknown mechanism. Here we show that the TZF protein MEX-5, a primary anterior determinant, is an RNA-binding protein that recognizes linear RNA sequences with high affinity but low specificity. The minimal binding site is a tract of six or more uridines within a nine to thirteen nucleotide window. This sequence is remarkably abundant in the 3'-untranslated region of C. elegans transcripts, demonstrating that MEX-5 alone cannot specify mRNA target selection. In contrast, human TZF homologs tristetraprolin (TTP) and ERF-2 bind with high specificity to UUAUUUAUU elements. We show that mutation of a single amino acid in each MEX-5 zinc finger confers TTP-like specificity to this protein. We propose that divergence of this discriminator residue modulates the RNA-binding specificity in this protein class. This residue is variable in nematode TZF proteins, but is invariant in other metazoans. Therefore, the divergence of TZF proteins and their critical role in early development is likely a nematode-specific adaptation.

Embryogenesis is the process by which a fertilized oocyte transforms into a multicellular organism. Although the zygote contains all of the information required for development, zygotic DNA alone is not sufficient to drive patterning. Somatic cell nuclear transfer experiments, like those used to clone Dolly the sheep, demonstrate that maternal factors present in the oocyte cytoplasm are needed for the initiation of development (1). These maternal factors are proteins and quiescent mRNAs (2); they coordinate early development prior to the onset of zygotic transcription.

In the nematode worm Caenorhabditis elegans, polarization of the body axes occurs after fertilization and requires several highly conserved maternal factors termed PAR proteins (3-10). Prior to fertilization, these proteins are uniformly distributed in the cytoplasm. Once the sperm penetrates the oocyte, they localize to opposing cortical domains in a process that requires microtubules derived from the asters of the sperm pronucleus. The PAR network coordinates asymmetric translation of several cell signaling proteins (11,12) (glp-1, apx-1, mom-2, and mom-5) and transcription factors (13) (skn-1, pal-1, and pop-1) encoded by maternal mRNAs present throughout the 1-cell embryo. The PAR proteins are thought to locally deactivate maternal RNA-binding proteins thereby modulating the stability or translation efficiency of maternal mRNAs.

Consistent with this hypothesis, posterior localization of PAR-1 promotes anterior localization of two putative RNA-binding proteins, MEX-5 and MEX-6 (14,15). Though these proteins are 70% identical, their contributions to development only partially overlap. Disruption of mex-5 causes embryonic death with a terminal phenotype that includes proliferation of muscle
(MEX = Muscle Excess). In contrast, deletion of mex-6 does not affect viability. Two major roles in development have been attributed to MEX-5. First, it controls segregation of the germ line from the soma by activating zif-1, which promotes anterior turnover of three germ line-specific maternal proteins (POS-1, PIE-1, and MEX-1) (16). The overall result of this pathway is a gradient of MEX-5/6 from anterior to posterior and an opposing gradient of POS-1, PIE-1, and MEX-1 (Fig. 1A). Second, MEX-5 plays a relatively uncharacterized role in maintaining PAR polarity via a feedback loop with PAR-1 (15). It is not yet clear if the two roles are linked at the molecular level. Additionally, there may be other roles for MEX-5 that have not yet been described. For example, residual posterior MEX-5 accumulates on the posterior centrosome and in P-granules, RNA-rich bodies that segregate with and determine the germ line lineage (14,15). The functional ramifications of this localization are not known.

MEX-5, MEX-6, POS-1, PIE-1, and MEX-1 are all CCCH-type tandem zinc finger proteins (Fig. 1B, hereafter TZF). This class is typified by tristetraprolin (TTP), a mammalian protein involved in regulating inflammation response by destabilizing TNF-α transcripts (17-20). The expression pattern of several key maternal transcripts is perturbed in TZF mutants leading to the hypothesis that they directly regulate maternal mRNA stability or translation efficiency (14,21-24). If so, then the network of maternal RNA regulation in the embryo may be governed by differences in the RNA-binding specificity of each protein. Consistent with this model, TTP is an exquisitely specific RNA-binding protein; it recognizes nonameric UUAUUUAUU sequences present in the 3'-untranslated region (UTR) of its targets (19,20,25,26). An NMR structure of the mammalian TTP homolog ERF-2 (also known as Tis11D) demonstrates that each finger individually recognizes a UAUU repeat (27). In contrast, an in vitro interaction between any of the TZF proteins from C. elegans and RNA has not been demonstrated, and as such their mRNA target specificity has not been explored.

MEX-5 and MEX-6 diverge from TTP in a few notable ways (Fig. 1B): (i) nine amino acids rather than eight separate the first two cysteines in each zinc finger, (ii) the spacing between fingers is lengthened, and (iii) several highly conserved amino acids that contribute to RNA-binding in mammalian TZF proteins are not conserved in MEX-5 and MEX-6. These differences could impact the ability of MEX-5 and MEX-6 to bind to RNA. Moreover, MEX-5 has been shown to interact with ZIF-1 protein in a yeast two-hybrid assay (16), suggesting that it may not regulate this factor at the RNA level. We set out to describe the RNA-binding properties of MEX-5 in order to probe its role in patterning the anterior-posterior axis.

EXPERIMENTAL METHODS

Protein expression constructs: Fragments of mex-5 and mex-6 containing the TZF domain (amino acids 236-350 and 250-400, respectively) were amplified from ORFeome clones (Open Biosystems) and sub-cloned into the vector pMal-c (NEB). Mutations of pMal-MEX-5(236-350) were prepared by site-directed mutagenesis using quickchange (Stratagene).

Purification of recombinant proteins: TZF domains from MEX-5, MEX-6, and mutants thereof were expressed and purified from E. coli JM109 cells as C-terminal fusions to maltose binding protein. Liquid cultures grown at 37 deg. C were induced in mid log phase with 0.1 mM IPTG. Zinc acetate was added to a final concentration of 100 μM at the time of induction. Harvested cells were resuspended in lysis buffer (50 mM Tris pH 8.8, 200 mM NaCl, 2 mM DTT, EDTA free protease inhibitor tablet (Roche), 100 μM Zn(OAc)\(_2\)) and lysed by sonication. Soluble protein was purified over an amylose column (NEB). Fractions containing the fusion protein were pooled and dialyzed into Q buffer (50 mM Tris, pH 8.8, 20 mM NaCl, 2 mM DTT, and 100 μM Zn(OAc)\(_2\)) and then further purified over a Hi-trap Q HP column (GE Healthcare). Final purification was achieved by combining fractions containing the protein and dialyzing them into S buffer (50 mM MOPS, pH 6.0, 20 mM NaCl, 2 mM DTT, 100 μM Zn(OAc)\(_2\)) before running them over a Hi-trap S HP column (GE Healthcare). Care was taken to minimize the amount of time the protein was exposed to pH 6.0 buffer. Pure fractions as determined by
coomassie-stained SDS-PAGE were combined and dialyzed into storage buffer (20 mM Tris, pH 8.0, 20 mM NaCl, 100 μM Zn(OAc)$_2$, 2 mM DTT). After dialysis, the protein concentration was determined using Beer’s law by measuring absorbance at 280 nm and a calculated extinction coefficient determined using the ProtParam server (28). The protein was concentrated to approximately 50 μM before storage at 4 deg. C.

Analytical Gel Filtration Chromatography: A Superdex 200 10/300 GL column (10mm x 300 mm, GE Healthcare) was used to determine the apparent molecular weight of MBP-MEX-5 (236-350) and its mutants. The column was equilibrated on an AKTA FPLC with two column volumes of filtration buffer (50 mM Tris, pH 8.0, 300 mM NaCl) at a flow rate of 0.5 ml min$^{-1}$ prior to loading the protein sample. Approximately 50 μl of sample (17 μM) was loaded on to the column and eluted with 1.5 column volumes of filtration buffer. Retention time was determined in relation to standards (Bio-Rad).

RNA labeling protocol: All of the RNA sequences used in this work were prepared by chemical synthesis and deprotected/lyophilized as the manufacturer directed (Dharmacon or Integrated DNA technologies). Lyophilized samples were resuspended in 300 μl of TE buffer, pH 8.0, and the concentration was measured by determining the absorbance at 260 nm using a calculated extinction coefficient based on the nucleotide content.

Fluorescein 5-thiosemicarbazide (FTSC, Invitrogen) was used to 3’-end label each RNA via the method of Reines and Cantor (29). A typical 50 μl reaction consisted of 0.5 nanomoles RNA, 100 mM NaOAc, pH 5.1, and 5 nmoles NaIO$_4$. After a ninety minute incubation at room temperature, the sample was ethanol precipitated with 1 μl RNase free glyccogen (Invitrogen 20 μg/μl), 5 M NaCl (1/20 the volume), and 2 volumes of 100% ice-cold ethanol. The resulting pellet was resuspended in 50 μl of 100 mM NaOAc, pH 5.1 containing 1 mM FTSC. This reaction was incubated overnight at 4°C and unreacted label was removed using a Roche G-25 spin column. The labeling efficiency was determined by calculating the ratio of fluorescein absorbance at 490 nm to RNA-fluoroscen absorbance at 260 nm. Typical efficiencies were 60–80%.

Electrophoretic mobility shift assays: Electrophoretic mobility shift assays were used to measure the binding activity of recombinant MEX-5 and MEX-6 to fluorescein-labeled RNA oligonucleotides. Typical reactions consisted of 2–4 nM labeled RNA equilibrated with varying concentrations of protein in equilibration buffer for 3 hours. Equilibration buffer is 0.01% IGEPAL CA630 (a mild detergent used to prevent adhesion or protein and RNA to tubes, microplates, and gel wells), 0.01 mg/ml tRNA (a polyanionic non-specific binding inhibitor), 10 mM Tris, pH 8.0, 100 μM Zn(OAc)$_2$, and 100 mM NaCl. The RNA was heated to 60 deg. C and allowed to cool to room temperature before use. Immediately prior to loading, one-fifth volume of 30% v/v glycerol, 0.01% w/v bromocresol green was added to each reaction as a dye marker. A 40 μl sample of each reaction (100 μl total) was loaded onto a 1% agarose gel (EMD Biosciences, some lot to lot variability was observed) in 1X TB buffer. The gels were run for 40 minutes at 120 volts then immediately scanned using a fluor-imager (Fujifilm FLA-5000) with a blue laser at 473 nm. The fluorescence intensity of unbound RNA was determined as a function of protein concentration using ImageGuage software. The data were fit to a sigmoidal dose response function (equation 1) in order to determine the half maximal saturation point ($K_{d,\text{app}}$):

$$\phi = b + (m - b) \frac{1}{1 + \left(\frac{K_{d,\text{app}}}{P}\right)^n}$$

where $\phi$ is the fluorescence intensity, m is the maximal signal, b is the minimal signal, P is the protein concentration, and n is the apparent Hill coefficient. It is important to note that $K_{d,\text{app}}$ is not equivalent to the thermodynamic equilibrium dissociation constant for RNA sequences that contain multiple overlapping binding sites. In all cases, the reported value is the average of at least three experiments and the reported error is the standard deviation.

Competition assays were performed as above except a constant concentration of sub-
saturating recombinant MEX-5 was used in the equilibration while varying concentrations of unlabeled competitor RNA were added to the reaction. The apparent dissociation constant of the competitor RNA was determined by a fit of the data to a quadratic solution of the Lin and Riggs equation (30,31) as described (32).

Fluorescence Polarization assays: Equilibration reactions (100 μL volume) were set up using the same conditions as the electrophoretic mobility shift experiments above in 96-well black plates (Greiner). The apparent fluorescence polarization was determined using a Victor 3 plate reader (Perkin Elmer) equipped with fluorescein sensitive filters and polarizers. A total of five reads were measured for each experiment and the average and standard deviation of the millipolarization value (mP) were calculated for each protein concentration. The data were fit to equation 1 (where ϕ represents polarization rather than intensity) in order to extract the apparent dissociation constant. The reported value is the average of at least three experiments and the error is the standard deviation.

Stoichiometric binding experiments were performed as above except the reactions were supplemented with unlabeled RNA to a final concentration of 1.5 μM. The elevated concentration of RNA enables determination of the apparent stoichiometry by measuring the equivalence saturation point. This value was estimated by plotting polarization as a function of molar equivalents of protein to RNA and performing linear fits to pre- and post-saturation data. The equivalence point was determined by the intersection point of the two lines, and separately by a fit of the data to the quadratic equation as described (33).

UTR sequence analysis: C. elegans 3′-UTRs were retrieved from Wormbase release WS165. To determine the frequency of each possible octamer sequence in 3′-UTR-space, a Ruby script was written to enumerate each possible octamer and send it to the pattern searching tool PATSCAN (34). The PATSCAN output files were analyzed using standard UNIX text processing tools. The total number of occurrences of each class of A, C, G, or U-containing octamers was determined by summing up the number of occurrences of each octamer in that class. The theoretical distribution of each class was determined by a binomial distribution weighted by the fractional proportion of each base in all C. elegans 3′-UTRs.

RNAi: C. elegans strains expressing GFP-MEX-5 (JH1448) or GFP-PIE-1 (JH1327) were obtained from the Caenorhabditis Genetics Center and cultured by propagating animals with the roller phenotype. Embryos were harvested from young adult hermaphrodites grown on OP50 food by bleach treatment and then deposited on NGM plates seeded with OP50 or NGM-IPTG plates seeded with mex-3 RNAi food (generously provided by Dr. Craig Mello) and cultured as described (35). Embryos were collected from gravid adult hermaphrodites by dissecting the worms in M9 on a 4% agarose pad with a fine gauge needle. DIC and GFP images were collected with live specimens using a Zeiss Axioskop microscope with 40X or 100X objectives.

RESULTS

MEX-5 and MEX-6 bind to ARE repeat elements: To clarify the role of MEX-5 and MEX-6 in development, we set out to test whether these proteins, like TTP, bind with high affinity and specificity to RNA. A recombinant fragment of each protein comprising the TZF domain was purified from bacteria and tested for the ability to interact with an established TTP binding sequence – the AU-rich element (ARE) of TNF-α mRNA (26). Two approaches were employed to measure affinity to this RNA: electrophoretic mobility shift (EMSA) and fluorescence polarization (FP) assays. Both methods reveal that MEX-5 and MEX-6 bind to TNF-ARE RNA. The EMSA experiments show that multiple binding sites are present in this sequence. The apparent dissociation constant (Kₐᵥapp) of MEX-5 for TNF-ARE RNA is 17 ± 1 nM by EMSA and 14 ± 4 nM by FP (Fig. 2A, B, Table 1). Similarly, MEX-6 binds to this RNA with an affinity of 4 ± 3 nM by EMSA and 12 ± 3 nM by FP (Fig. 2A). Both proteins are capable of binding to RNA with high affinity. Furthermore, because the EMSA and FP results are nearly equivalent, it is clear that both
assays can effectively monitor RNA binding by these proteins.

TNF-ARE RNA contains several UUAUUUAUU repeat sequences and therefore can bind multiple molecules of TTP (26). To determine the affinity of MEX-5 for a shorter RNA variant containing only one TTP site, we repeated the binding analyses with ARE13 RNA (AUUUAAUUAAUUUA). The apparent dissociation constant for this sequence is 55 ± 15 nM by EMSA and 97 ± 4 nM by FP (Fig. 2). In contrast, TTP binds to ARE13 RNA with 5-10 fold tighter affinity (26). Together, the results show that MEX-5 binds to UUAUUUAUU repeat sequences, but with an overall affinity that is weaker than TTP.

**MEX-5 binds to regulatory elements in glp-1 and nos-2 3′-UTRs:** Prior work demonstrates that several factors are aberrantly expressed in mex-5 mutants (14). Ectopic expression of five proteins (SKN-1, PIE-1, MEX-1, POS-1, and PAL-1) and reduced levels of two others (GLP-1 and MEX-3) result from mex-5 mutation. Furthermore, MEX-5 is required to activate zif-1, which in turn targets TZF proteins for degradation (16). Lastly, recent studies reveal that MEX-5 is required for anterior degradation of mex-1, nos-2, and pos-1 transcripts (21,36). Although the results are consistent with MEX-5 regulating a network of maternal genes, this regulation has not been shown to be a direct result of MEX-5 binding to target mRNAs.

Extended UAUU sequence repeats similar to the TNF-ARE are not present in the 3′-UTR of any candidate MEX-5 regulatory target. However, several functional regulatory elements have been identified in the 3′-UTR of glp-1 and nos-2 mRNAs (Fig. 3A, B). Two translational control elements are present in the 3′-UTR of glp-1 mRNA, a spatial control region (SCR) and a temporal control region (TCR) (37). Furthermore, five elements (subA-E) found in the 3′-UTR of nos-2 mRNA coordinate translational silencing, mRNA localization, and 3′-end formation (21). To determine if MEX-5 binds these functional elements, we performed EMSA and FP experiments with overlapping thirty nucleotide fragments of the TCR and all five elements from the nos-2 3′-UTR (Fig. 3; Table 1). Surprisingly, MEX-5 binds with high affinity to all of the TCR fragments and three of the elements from nos-2 mRNA (TCR1-4, subA, subC, subE, $K_{d,app}$ ~ 25 – 100 nM). MEX-5 binds moderately to nos-2 subD ($K_{d,app}$ = 200 ± 20 nM) and poorly to nos-2 subB ($K_{d,app}$ 400 ± 50 nM by FP, > 1 μM by EMSA).

Because a single shifted species is observed with TCR2 RNA, we decided to investigate the stoichiometry of the complex by repeating the FP experiments with elevated TCR2 RNA concentration (Fig. 3C, D). The apparent stoichiometry is approximately one to one (equivalence point N is 0.9 ± 1 by a quadratic fit), demonstrating that there is only one binding site in this RNA and that the recombinant protein is nearly 100% active (Fig. 3D). Consistent with these results, analytical gel filtration chromatography reveals that the TZF domain is predominantly monomeric at concentrations well above the apparent dissociation constant for this RNA sequence (17 μM, $K_{d,app}$ = 31 ± 9 nM, Fig. 3D).

Inspection of the RNA fragments reveals that all of the interacting sequences contain a tract of six to eight uridines within an eight-nucleotide window. This feature is absent in nos-2 subB, suggesting that MEX-5 requires this element in order to bind. To test this model, a mutant version of the second TCR fragment where all of the uridines are replaced by adenosine was prepared. As expected, this sequence does not bind to MEX-5 (Table 1). The results show that MEX-5 does not require UAUU repeats in order to bind to RNA with high affinity, and suggest that MEX-5 can bind to sequences harboring an extended uridine tract.

**MEX-5 binds to polyuridine:** TTP displays an 80-fold preference for UAUU repeat sequences over polyuridine (26). In contrast, our data show that MEX-5 binds with high affinity to uridine-rich RNAs lacking canonical ARE motifs. To test whether uridine nucleotides are sufficient to promote MEX-5 binding, EMSA and FP experiments were performed with a thirty-nucleotide polyuridine sequence. MEX-5 binds to this RNA with an apparent $K_{d}$ of 29 ± 6 nM by EMSA and 23 ± 2 nM by FP (Fig. 4A). The data show that MEX-5 can bind to polyuridine with affinity similar to that of TNF-ARE. This demonstrates that MEX-5 binds with less specificity than TTP.
To further probe MEX-5 specificity, EMSA and FP experiments were performed with fifteen-nucleotide polyuridine, polyadenosine, polycytidine, and polyguanosine sequences. As before, MEX-5 binds to polyuridine with slightly weaker affinity than a similar length AUUUA repeat RNA (ARE13), but does not bind to polycytidine, polyadenosine, or polyguanosine (Fig. 4A, Table 1). Together, the results show that uridine tracts are both necessary and sufficient to promote MEX-5 binding.

In order to identify the shortest RNA fragment that can bind to MEX-5, competition EMSA and FP experiments were performed with even shorter polyuridine sequences (U6 and U9). Competition experiments were favored with shorter RNA sequences to prevent the possibility that steric hindrance by the 3′-fluorescein label would influence the apparent affinity. Disruption of the complex between MEX-5 and labeled ARE13 RNA was used to probe the affinity of unlabeled competitor sequences. The apparent dissociation constant of the competitor (Ke, app) was determined by a fit of the data to the Lin and Riggs equation (30,31). By self-competition, Ke, app for ARE13 is equivalent within error to the Kd, app determined by direct titration (Fig. 4B). In contrast, U6 and U9 are unable to compete for MEX-5 binding (Ke, app > 1 µM). Together, the results demonstrate that the minimal MEX-5 binding site is greater than nine nucleotides in length.

Uridine tracts are remarkably abundant in C. elegans 3′-UTR sequences: It is possible that MEX-5 specifically regulates only those maternal genes that contain uridine tracts in their 3′-UTRs. To identify potential MEX-5 targets, we searched every annotated 3′-UTR in Wormbase release WS165 for octamer sequences containing at least six uridines using the PATSCAN algorithm (34). Amazingly, 91% of 3′-UTRs harbor at least one potential MEX-5 binding site. This demonstrates that C. elegans 3′-UTRs are remarkably rich in uridine tracts, and implies that MEX-5 alone cannot specify mRNA targets for regulation.

To further explore this hypothesis, we repeated the PATSCAN search with every possible octamer sequence (4^8 sequences) in the C. elegans 3′-UTR database. There are 11,938 unique 3′-UTRs annotated in Wormbase. These UTRs are rich in uridine and adenosine but are relatively poor in guanosine and cytidine. To determine if tracts of uridine are overrepresented, we determined the theoretical distribution of octamer units weighted by the relative base content in 3′-UTRs using a binomial distribution. If a given base has a propensity to segregate into tracts of high and low base content, the frequency of octamers containing six to eight occurrences of the base will be greater than expected from the random distribution. Similarly, the frequency of octamers containing zero to three base occurrences will also be greater than the expected amount. Therefore, a plot of octamer frequency versus the number of base occurrences will appear more broad and shallow than the theoretical random distribution (Fig. 5A). In C. elegans 3′-UTRs, the observed distribution of uridine and to a lesser extent adenosine reveals a propensity to segregate into high and low base content tracts, while the observed distributions of guanosine and cytidine very closely match the random distribution (Fig. 5A). This is consistent with a selective pressure that favors runs of uridine and adenosine. When the distribution of observed octamer sequences is plotted as a function of relative frequency, we find that the most frequently observed octamer is U8, and 29 of the top 30 octamers contain at least six uridines (Fig. 5B). This highlights the preponderance of uridine tracts in the 3′-UTRs of C. elegans genes, and demonstrates that MEX-5 does not bind with sufficient specificity to select mRNAs for regulation on its own.

MEX-5 and PIE-1 localization do not depend on MEX-3: If MEX-5 regulates specific maternal transcripts, it must do so as part of a complex with a more specific RNA-binding protein. If so, a likely candidate is MEX-3. MEX-3 is a KH domain RNA-binding protein that is also required for anterior development (38). Mutation of mex-3 results in the same terminal phenotype as mex-5, and the protein displays a similar distribution throughout the 1-4 cell stages of development. Moreover, MEX-6, which is 70% identical to MEX-5, was identified in a yeast two-hybrid screen for MEX-3 interacting proteins (39). Finally, the localization pattern of MEX-3 protein is perturbed in mex-5 mutants (14).

Because the localization pattern of MEX-3 depends on MEX-5 (14), it is possible that the two
proteins localize in a complex. If so, then anterior MEX-5 accumulation might depend upon the presence of MEX-3. In addition, because MEX-5 activation of zif-1 drives posterior accumulation of PIE-1 (16), localization of this protein should be altered in the absence of MEX-3 if it serves as a zif-1 co-activator. To test these hypotheses, we knocked down MEX-3 levels using RNAi in worms expressing GFP-MEX-5 or GFP-PIE-1 by feeding them Escherichia coli expressing double stranded RNA targeted against mex-3 transcripts. The cellular distribution of GFP-MEX-5 and GFP-PIE-1 in embryos was determined by wide field fluorescence microscopy. No difference is observed in the expression pattern of GFP-MEX-5 or GFP-PIE-1 in mex-3 RNAi embryos (n = 135, n = 127, respectively; only embryos in the 1-8 cell stage were counted) compared to control embryos (n = 101, n = 111, Fig. 6). More than 90% of the embryos laid onto mex-3 RNAi plates failed to hatch (n > 500), indicating that RNAi was disrupting mex-3 function. We conclude that MEX-3 is not required for MEX-5 accumulation in the anterior or for activation of zif-1. The results argue against a functional role for a MEX-3/MEX-5 complex. However, we cannot rule out the possibility that they co-regulate other maternal transcripts.

A single residue in each finger defines MEX-5 RNA-binding specificity: The NMR structure of the mammalian TTP homolog ERF-2 reveals that each zinc finger recognizes adjacent UAUU repeats (27). In order to understand the molecular basis for differential MEX-5 RNA-binding specificity, we prepared a homology model of MEX-5 based on the ERF-2 structure using SWISS-MODEL (40) (Fig. 7A). The model reveals several amino acid differences in the RNA-binding interface that may contribute to the difference in specificity. Most notably, three adjacent residues (E, L, C) in each finger of ERF-2 combine to form an adenosine recognition pocket. In MEX-5, the glutamate residue is replaced with arginine (R274) in the first finger and lysine (K318) in the second (Fig. 1C). In the NMR structure, the glutamate side chain forms a hydrogen bond with the exocyclic amine of the adenosine base. In the homology model, the basic residues rotate away from the adenosine and form backbone contacts with adjacent nucleotides (Fig. 7B). The model predicts that loss of base-specific hydrogen bonds and formation of additional backbone contacts contributes to the relaxed specificity of MEX-5. If so, mutation of the basic residues to glutamate might confer TTP-like specificity to this protein.

To test this hypothesis, we prepared mutant variants of MEX-5 where either or both of these basic residues were replaced with the glutamate residue present in TTP (R274E, K318E, and R274E/K318E). A substitution at an unrelated position in the RNA interface (M288Y) was prepared as a control. First, we examined the ability of the mutants to interact with the TNF-ARE sequence. The R274E and the K318E mutations do not affect the interaction, while the double mutation binds with four-fold reduced affinity to this sequence (Table 2). A small loss in affinity is expected due to increased electrostatic repulsion from the glutamate residues. The M288Y mutation has no effect on TNF-ARE affinity.

In contrast, the effect of glutamate mutations on binding to the thirty-nucleotide polyuridine RNA is significantly more drastic. The individual glutamate mutations reduce binding by two to four fold, while the double mutation significantly reduces binding (>15 fold by EMSA, 7.5 fold by FP, Fig. 7C, Table 2). As before, the M288Y mutation has no effect. Because all variants retain the ability to bind to TNF-ARE RNA, and because all are soluble and monomeric as determined by analytical size exclusion chromatography (Supplementary Fig. 1), we conclude that reduction in polyuridine binding results from a change in binding specificity rather than from protein misfolding effects. The data demonstrate that a single glutamate in each finger confers the ability to discriminate between UAUU repeat RNA and polyuridine, thus defining the molecular basis for the difference in RNA discrimination between MEX-5 and TTP/ERF-2.

DISCUSSION

Accurate post-transcriptional regulation of gene expression requires the ability to distinguish specific transcripts from the total pool of cellular RNA. Regulatory factors target mRNA through sequence-specific or structure-specific interactions (41). In the case of microRNAs, the mechanistic
basis for mRNA recognition is easy to conceptualize; complimentary base pairing of a seven or eight nucleotide seed drives mRNA discrimination (42). In contrast, the sequence code recognized by eukaryotic RNA-binding proteins usually includes a greater degree of degeneracy.

In C. elegans, early embryogenesis requires a class of divergent CCCH-type tandem zinc finger proteins. They segregate to opposite poles of the 1-cell embryo in response to PAR protein activity (14-16,43). Because they are homologous to mammalian TTP, most are thought to regulate maternal gene expression at the RNA level. Our data show that two of the C. elegans TZF proteins, MEX-5 and MEX-6, can in fact bind to RNA with high affinity but with relaxed specificity compared to TTP. The results are consistent with the hypothesis that they directly regulate maternal transcripts, but also suggest that accessory factors are required for mRNA targeting.

**Implications for the role of MEX-5 in development**

Uridine is by far the most common nucleotide present in the 3'-UTRs of C. elegans genes. We show that tracts of six or more uridines are present in the 3'-UTR of 91% of annotated transcripts, which is more frequent than expected from a random distribution. Therefore, contrasting with previous hypotheses, MEX-5 does not bind to RNA with sufficient specificity to drive regulation of a subset of maternal transcripts.

The relaxed RNA-binding specificity of MEX-5 has several implications for its function in development. It is possible that it is a general mRNA repressor, silencing all transcripts in the oocyte and the anterior lineage until it is destroyed after the 8-cell stage. This is unlikely because translation of glp-1 mRNA occurs in the anterior and appears to require MEX-5 (14,37). Alternatively, MEX-5 may be an affinity adapter for a more specific RNA-binding protein, regulating expression from just a few maternal transcripts as part of a cooperative complex. If so, then MEX-5 could behave like Drosophila melanogaster Nanos protein, which works in complex with Pumilio to regulate maternal mRNA expression (44-46). Finally, MEX-5 RNA-binding activity may not contribute to maternal RNA regulation. Instead, RNA-binding activity may be required to target MEX-5 to the posterior centrosome and to P-granules (Fig. 1A), two subcellular organelles that contain RNA (14,15,47,48). If so, then MEX-5 RNA-binding activity may play a role in germline maintenance in addition to its role in germline formation.

**Implications for RNA recognition by other CCCH-type TZF proteins**

The difference in specificity between MEX-5 and TTP is governed by a single amino acid substitution within a highly conserved region of each finger. Our data indicate that the amino acid identity of this discriminating position defines a code that predicts the specificity of other TZF proteins. We predict that glutamate residues encode selectivity for UUAUUAAUU RNA, while basic residues lead to promiscuous binding to uridine-rich RNA sequences including, but not limited to, UAUU-repeat sequences.

To determine the variability of the discriminator residue in TZF proteins, we performed a BLAST search of the C. elegans genome using the TZF domain of MEX-5 as a query (Fig. 8). Sixteen hits result from this analysis. Variability is observed at the discriminator position, but the other two residues that comprise the adenosine recognition pocket are conserved. Of the sixteen, only MEX-5 and MEX-6 contain basic amino acids in both fingers at the discriminator position. One protein, F38B7.1, has a glutamate at both positions similar to TTP and ERF-2. We predict that this protein binds specifically to UAUU repeat RNAs.

Interestingly, five proteins (POS-1, Y116A8C.19, Y116A8C.20, Y57G11C.25, and F38C2.5) have small hydrophobic residues in both discriminator positions, an alanine in finger one and a valine in finger two. Experiments with recombinant POS-1 protein indicate that it binds to RNA with specificity that is different from both MEX-5 and TTP (B.M.F. and S.P.R, unpublished results). Therefore, small hydrophobic residues at the discriminator position may define a third specificity class for TZF proteins.

The remaining TZF proteins have a combination of basic, small hydrophobic, or acidic/polar amino acids at the discriminator position. Three proteins required for oocyte maturation (OMA-1, OMA-2, and MOE-3) have a valine in the first finger and a lysine in the second.
We predict these proteins will display hybrid specificity between POS-1 and MEX-5. In contrast, PIE-1 contains an arginine in finger one and a glutamine in finger two. Like glutamate, glutamine can accept a hydrogen bond from adenosine, but it can also donate a hydrogen bond to the O6 carbonyl of a guanosine base as well. F38C2.7, C35D6.4, and MEX-1 have the combination of a small hydrophobic residue in one finger and a polar amino acid (serine or asparagine) that could theoretically accept and/or donate a base-specific hydrogen bond in the RNA complex. The variations of the discriminator residue imply differences in RNA-binding specificity by the divergent C. elegans TZF proteins. Further experiments will determine the possible RNA interactions allowed by each discriminator amino acid within this class of proteins.

Remarkably, the variations in the TZF protein discriminator residue found in C. elegans are absent in higher eukaryotes. Blast analysis reveals that every vertebrate CCCH-TZF protein in GenBank has a glutamate residue at both discriminator positions. In contrast, homologs with variable discriminators are present in Caenorhabditis briggsae and Caenorhabditis remanei, and in more divergent Nemata including parasitic species. Since the identity of the discriminator correlates with its RNA-binding specificity and thus its molecular function, the critical role of divergent TZF proteins during early development must be a special adaptation of this phylum.

The role of the PAR proteins in establishing cell polarity is conserved from worms to flies to mammals (49). Asymmetric expression of signaling proteins and transcription factors from maternal mRNAs is also highly conserved (2). Yet the cassette of RNA-binding proteins that connect these two layers is clearly highly divergent (50). Defining the basis of RNA-binding protein function in early development will provide a framework by which mechanistic differences in the regulation of maternal mRNAs contribute to variability of metazoan body plan.

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The authors thank Dr. Mary Munson and Dr. Ruth Zearfoss for critical comments concerning the manuscript and Dr. Craig Mello for providing mex-3 RNAi food. We are indebted to Dr. Nicholas Rhind for advice and assistance with fluorescence/DIC microscopy. The nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR). S.P.R. is a scholar of the Worcester Foundation for Biomedical Research.

The abbreviations used are: TZF, CCCH-type tandem zinc finger; TTP, tristetraprolin; UTR, untranslated region; ARE, AU-rich element, FTSC, Fluorescein-5-thiosemicarbazide.
Table 1: Analysis of MEX-5-TZF binding to RNA

| RNA ID  | Sequence                          | Gene/3'-UTR Position | EMSA $K_d$, app (nM) | FP $K_d$, app (nM) |
|---------|-----------------------------------|-----------------------|----------------------|--------------------|
| TNF-ARE | GUGAUUAAUUAAAUUUAAUUAAUUAAUUAAUUUAG | TNF-α 459–470         | 17 ± 4               | 14 ± 4             |
| ARE13   | AUUUAAUUAAUUAA                         | N/A                   | 55 ± 15              | 97 ± 4             |
| TCR1    | UUGUUUUAUUCUUUUUCUUUAUUAAACUGUUU      | glp-1 242–272         | 29 ± 3               | 25 ± 7             |
| TCR2    | UUUCCUUUAACUUGUUAACUUUUGAA            | glp-1 256–285         | 37 ± 18              | 31 ± 9             |
| TCR3    | UUAACUUUUGGAACUUUUCUUUUUGACAA        | glp-1 270–300         | 66 ± 22              | 52 ± 12            |
| TCR4    | UUCCCUUUUUUGACAGCUUUUAUACUGUAA       | glp-1 286–319         | 46 ± 15              | 62 ± 11            |
| subA    | CAUACUUUUUAUACUGGCUCCAACCGUUUA       | nos-2 15–47           | 67 ± 12              | 94 ± 2             |
| subB    | UACUUCGUUUCACAAAACAGAUAGUUUUU       | nos-2 53–80           | >1000                | 400 ± 50           |
| subC    | CCGUUCUAGCCUUUAUUAGAUUCCAAUUU      | nos-2 88–118          | 68 ± 11              | 78 ± 13            |
| subD    | CCAUCUCACACUUUUCUCGCUUUAA           | nos-2 119–143         | 400 ± 60             | 200 ± 20           |
| subE    | ACCUUUACUUUUUCUGCUUAUUACUUAAUA      | nos-2 144–180         | 62 ± 12              | 70 ± 12            |
| TCR2 U-A| AAACAAAAAAACAGGAACAAAAAAAGAAA       | N/A                   | >1000                | >1000              |
| U30     | UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUU     | N/A                   | 29 ± 6               | 23 ± 2             |
| U15     | UUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUUU | N/A                   | 150 ± 4              | 140 ± 4            |
| C15     | CCCCCCCCCCCCCCCC                     | N/A                   | >1000                | >1000              |
| G15     | AAAAAAAACAAAAAA                      | N/A                   | >1000                | >1000              |
| A15     | GGGGGGGGGG                          | N/A                   | >1000                | >1000              |
Table 2: Comparison of mutant MEX-5 RNA-binding specificity

| Protein          | Polyuridine 30 RNA |  |  |  |  |  |  |  |  |  |  |  |
|------------------|-------------------|---|---|---|---|---|---|---|---|---|---|---|
|                  | K_d, app (nM)     | Relative Affinity | K_d, app (nM) | Relative Affinity | Fold Discrimination |
| MEX-5            | 29 ± 6            | 1             | 18 ± 1         | 1             | 1.6             |
| M288Y            | 28 ± 0.5          | 1             | 12 ± 7         | 0.7           | 2.3             |
| R274E            | 28 ± 4            | 1             | 16 ± 2         | 0.9           | 1.8             |
| K318E            | 110 ± 30          | 3.9           | 28 ± 1.5       | 1.6           | 4.0             |
| R274E/K318E      | >1000             | >35           | 70 ± 7         | 4             | >15             |
| MEX-5 (FP)       | 23 ± 2            | 1             | 14 ± 4         | 1             | 1.5             |
| R274E/K318E (FP) | 170 ± 7           | 7.5           | 23 ± 3         | 1.6           | 7.5             |
Figure Legends:

Figure 1: C. elegans tandem zinc finger proteins. A. Reciprocal gradients of the TZF proteins MEX-5, MEX-6, POS-1, PIE-1, and MEX-1 from the 1–4 cell stages of development. MEX-5 and MEX-6 are represented in red, POS-1, PIE-1, and MEX-1 are represented in blue. DIC and fluorescence images of GFP-MEX-5 in a live 4-cell embryo are shown. The pattern was observed in live embryos by Seydoux and co-workers with GFP reporters (15) and by Priess and colleagues using immunofluorescence in fixed embryos (14). The protein accumulates predominantly in the cytoplasm of the anterior blastomeres (a, ABa and ABp), but is also in P-granules (p) and on the centrosome of P2 (c). The cells are labeled in the DIC image. B. Domain structure of MEX-5. The location of the two CCCH zinc fingers (ZF1 and ZF2) is shown in blue. The numbers represent the primary amino acid sequence. Alignment of C. elegans zinc finger domains MEX-5, MEX-6, and POS-1 with mammalian TTP and ERF-2. The C. elegans proteins are boxed. Gray bars denote the three cysteines and one histidine that coordinate the zinc ion. The asterisks represent points of contact with RNA observed in the NMR structure of ERF-2 (27). The adenosine recognition pocket is boxed. The discriminator position is colored red for acidic side chains, blue for basic side chains, and green for hydrophobic side chains. Amino acids in italics were mutated in the present work.

Figure 2: MEX-5 is an RNA-binding protein. A. MEX-5 binds to the ARE element of TNF-α mRNA 3’-UTR and a shorter variant termed ARE13 by electrophoretic mobility shift. The interaction of MEX-6 with TNF-ARE RNA is shown for comparison. The unshifted RNA is denoted by an asterisk for each gel. The sequences of the RNA are shown. A fit of free RNA fluorescence intensity as a function of MEX-5 concentration is presented below each gel. The Kd, app and fit error is given for the specific gel above. B. Fluorescence polarization analysis of MEX-5 binding to TNF-α ARE and ARE13 RNA. Raw polarization values are presented. A fit of the data is shown, and the Kd, app and error are as in panel A.

Figure 3: MEX-5 interacts with regulatory elements in the 3’-UTR of glp-1 and nos-2 transcripts. A. Regulatory elements in glp-1 transcripts. Plots of normalized polarization as a function of MEX-5 concentration are shown. Uncorrected mP values ranged between 263 and 530. B. Regulatory elements in the 3’-UTR of nos-2 mRNA. Curves are as in panel A. The mP values ranged between 274 and 530 C. EMSA of MEX-5 binding to TCR2 RNA. The fit shown below is as per figure 2. The Kd, app and napp are given for the experiment shown. Errors represent the uncertainty of the fit. D. Stoichiometric binding of MEX-5 to TCR2 RNA. The total RNA concentration is shown. The lines represent linear fits to the pre- and post- saturation data. The stoichiometry is approximated from the intersection of the two lines, and from a fit of the data to the quadratic equation. The equivalence point N is given from the quadratic fit. The error represents the uncertainty of the fit. D. MEX-5 is predominantly monomeric at a concentration well above the Kd, app for TCR2 RNA (17 μM). The calculated molecular weight of MBP-MEX-5 (gray) and five standards (dashed line) are shown.

Figure 4: MEX-5 binds to polyuridine sequences. A. Interaction of MEX-5 with polyuridine 30, polyuridine 15, and polycytidine 15 sequences by fluorescence polarization. The curves are as in figure 3. B. Competition experiments of unlabeled ARE13 RNA and U6 RNA into the complex of MEX-5 with labeled ARE13 RNA.

Figure 5: Analysis of the distribution of base content in octamer windows of all annotated C. elegans 3’-UTRs. A. A plot of the relative frequency of octamer sequences with zero through eight occurrences of a given base (dark line) compared the expected frequency based on a random distribution weighted by the nucleotide distribution (dashed line). The fraction of each base present in C. elegans 3’-UTR space is shown above each chart. B. The identity of the top 30 most frequent octamer elements present in C. elegans 3’-UTRs and the frequency of occurrence in Wormbase release WS165 is shown.
Figure 6: MEX-5-GFP and PIE-1-GFP expression in mex-3 RNAi embryos. A. DIC images of a 4-cell and a comma stage embryo compared to the terminal mex-3 RNAi phenotype. The anterior and posterior pole are labeled in the 4-cell embryo. B. Comparison of the 4-cell stage localization pattern of GFP-MEX-5 or GFP-PIE-1 in untreated or mex-3 RNAi embryos.

Figure 7: A discriminator amino acid in each finger defines RNA-binding specificity. A. A homology model of MEX-5 bound to RNA based upon the NMR structure of ERF-2 (27). Amino acids that are conserved between the two are colored green. Amino acids that are different are in red. The RNA (5’-UUAUUAUU-3’) is denoted in blue. The box denotes the adenosine recognition pocket. B. Recognition of adenosine by MEX-5 (red) and ERF-2 (green). The numbering corresponds to the sequence in MEX-5. E denotes the position of the discriminator residue, which is an arginine in MEX-5 zinc finger 1 and a glutamate in ERF-2 zinc finger 1. C. Electrophoretic mobility shift of wild-type MEX-5 with polyuridine 30 RNA and the R274E/K318E mutant with polyuridine 30 and TNF-ARE RNA. Unshifted RNA is denoted by an asterisk. A fit of the normalized free fluorescence intensity as a function of R274E/K318E concentration is shown for TNF-ARE and polyU30 RNA. Fluorescence polarization data are shown for R274E/K318E mutant with TNF-ARE and polyU30 RNA.

Figure 8: Identity of the discriminator amino acid (bold) in the adenosine recognition pocket of each finger for all C. elegans TZF proteins (boxed) is presented. The acidic, basic, hydrophobic, and mixed TZF protein classes are labeled.
Figure #1
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Figure #2
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Figure #4
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Figure #5
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Figure #6
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Figure #7
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| Gene Identity | ZF1 A-pocket | ZF2 A-pocket |
|---------------|--------------|--------------|
| TTP           | ELC          | ELC          |
| ERF-2         | ELC          | ELC          |
| F38B7.1       | ELC          | EAC          |
| MEX-5         | RLC          | KLC          |
| MEX-6         | RLC          | KLC          |
| POS-1         | ALC          | VLC          |
| Y116A8C.19    | ALC          | VLC          |
| Y116A8C.20    | ALC          | VLC          |
| Y57G11C.25    | ALC          | VLC          |
| F38C2.5       | ALC          | VLC          |
| F38C2.7       | SLC          | VLC          |
| C35D6.4       | SLC          | VLC          |
| Y116A8C.17    | SLC          | VLC          |
| OMA-1         | VIC          | KLC          |
| OMA-2         | VIC          | KLC          |
| MOE-3         | VIC          | KLC          |
| MEX-1         | ALC          | QLC          |
| PIE-1         | RLC          | QIC          |

Figure #8
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J. Biol. Chem. published online January 30, 2007

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