Positive and negative cis-regulatory elements directing postfertilization maternal mRNA translational control in mouse embryos

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Potireddy S, Midic U, Liang CG, Obradovic Z, Latham KE. Positive and negative cis-regulatory elements directing postfertilization maternal mRNA translational control in mouse embryos. Am J Physiol Cell Physiol 299: C818–C827, 2010. First published June 23, 2010; doi:10.1152/ajpcell.00166.2010.—Mechanisms providing for temporally complex patterns of maternal mRNA translation after fertilization are poorly understood. We employed bioinformatics analysis to compare populations of mRNAs enriched specifically on polysomes at the metaphase II (MII) stage oocyte and late one-cell stages and a detailed deletion/truncation series to identify elements that regulate translation. We used the Bag4 3’ untranslated region (UTR) as a model. Bioinformatics analysis revealed one conserved motif, subsequently confirmed by functional studies to be a key translation repressor element. The deletion/truncation studies revealed additional regulatory motifs, most notably a strong translation activator element of <30 nt. Analysis of mRNA secondary structure suggests that secondary structure plays a key role in translation repression. Additional bioinformatics analysis of the regulated mRNA population revealed a diverse collection of regulatory motifs found in small numbers of mRNAs, highlighting a high degree of sequence diversity and combinatorial complexity in the overall control of the maternal mRNA population. We conclude that translational control after fertilization is driven primarily by negative regulatory mechanisms opposing strong translational activators, with stage-specific release of the inhibitory influences to permit recruitment. The combination of bioinformatics analysis and deletion/truncation studies provides the necessary approach for dissecting postfertilization translation regulatory mechanisms.

expression array; oocyte; polyribosome; messenger RNA motif; zygote

EARLY METAZOAN EMBRYOS are endowed with a rich maternal legacy via the oocyte or seed that sustains early metabolic activities and directs early developmental decisions. This applies to both plants and animals (9, 10, 15, 39). Maternal mRNAs (MmRNAs) are stored in an inactive, masked form (14, 42, 43, 46) and recruited for translation in a stage-specific manner during either oocyte maturation or early embryogenesis. This results in a complex pattern of protein synthesis over the period preceding transcriptional activation of the embryonic genome (19, 22, 26). This regulated translation of MmRNAs stands as the key mechanism directing early transitions in expressed proteins in the mature oocyte and early embryo.

While mRNA stability, RNA binding proteins, and mRNA sequestration are all factors that contribute to translational control, the distinction between which MmRNAs are regulated in a stage-specific manner ultimately must be dictated by cis-regulatory elements within each MmRNA. The most thoroughly studied paradigm of MmRNA recruitment involves the cytoplasmic polyadenylation element (CPE), a cis-regulatory element typically located in the 3’ untranslated region (UTR) of MmRNAs (12, 29). The CPE binding protein (CPEB) binds to the CPE and recruits the poly(A) polymerase (PAP), which elongates the poly(A) tail. Poly(A) binding protein (PABP) binding to the elongated poly(A) tail recruits eukaryotic translation initiation factor 4G (eIF4G), which displaces a translation inhibitory protein called maskin with which eIF4G must compete for binding to eIF4E (3). Maskin binding to eIF4E is regulated by phosphorylation. eIF4G binding to eIF4E is rate-limiting in the translation initiation process, and as a result of release of eIF4E from maskin the MmRNA can be recruited. Studies as far back as 1987 revealed the eIF4 complex as a key regulatory component of maternal mRNA regulation (18, 21).

Other cis-regulatory elements are required for MmRNA recruitment for translation. One is the hexanucleotide nuclear polyadenylation signal (1, 35, 40, 50). Studies in the frog revealed that the spacing between the CPE and the hexanucleotide can affect the timing of MmRNA recruitment during oocyte maturation (41). The pumilio binding element (31) also controls CPE function (33, 38). Other studies in the frog revealed that some MmRNAs are recruited for translation at different times during oocyte maturation and employ a “CPE-independent” mechanism of recruitment. Subsequent studies identified the polyadenylation response element (PRE) as a CPE-independent regulator (4). These studies provide valuable indications that the cis-regulatory elements controlling the fine temporal patterns of MmRNA recruitment are not limited just to the CPE element. Other proteins may regulate the timing of MmRNA recruitment, either through specific binding to cis-regulatory elements (33) or through more global mechanisms involving messenger ribonucleoprotein (mRNP) complex sequestration in the cytoplasm. Among the latter possibilities is the protein MSY2, which may sequester MmRNAs into mRNP complexes (52, 53).

The role of CPEs in promoting MmRNA recruitment has also been revealed for the mouse. During oocyte maturation and after fertilization, a variety of MmRNAs that are recruited have been studied in detail. CPE-like elements have been characterized based on the sequence analysis of the 3’UTRs of these MmRNAs (32). Some of these 3’UTRs can direct reporter mRNA translation at different times, and are clearly associated with poly(A) tail elongation at specific stages (32).

The studies of MmRNA regulation to date have focused primarily on understanding the translational recruitment of MmRNAs during oocyte maturation, with a much lesser emphasis on postfertilization control, but MmRNAs continue to...
be recruited at specific times after fertilization. During murine oocyte maturation, the total amount of mRNA bearing long poly(A) tails diminishes greatly, as actively translating mRNAs become either degraded or deadenylated (6). After fertilization, the amount of poly(A) mRNA in the cell increases dramatically, detectable by a rapid incorporation of radioactive ATP into poly(A) mRNA as stored MmRNAs undergo elongation. Our systematic approach combining bioinformatics analyses and translation in the mouse zygote. Our goal was to develop a broader approach to investigate the mechanisms that control MmRNA translation and direct recruitment of MmRNAs with fine temporal control. The discovery of novel cis-regulatory elements will lay the foundation for discovery of proteins that interact with them, thereby uncovering broader aspects of this regulatory mechanism.

A study of expressed sequence tags (ESTs) in mouse embryos revealed that only about one-third of the MmRNA ESTs analyzed contained CPE-like elements, indicating that elements other than the CPE are involved in MmRNA regulation after fertilization (11, 20). Further evidence of this came from our expression array studies of polysomal mRNA populations in eggs and one-cell embryos (34). The arrays of MmRNAs being translated at the two stages differed greatly, with >4,000 MmRNAs (29% of the total detected) displaying statistically significant and twofold or greater differences and >2,000 differing at the level of threefold or more. About three-quarters of these differences corresponded to MmRNAs entering the polysomes during the one-cell stage, attesting to the vast scope of MmRNA recruitment occurring after fertilization. The biological functions supported by the MmRNAs preferentially translated at the two stages differed considerably, indicating the biological significance of correct MmRNA regulation. Most (86%) MmRNAs preferentially translated in the egg contain known CPEs, but nearly half the MmRNAs (47.3%) recruited at the late one-cell stage lack a known CPE. All the MmRNAs enriched in polysomes at either stage contained a putative PRE element, discounting this element as a candidate for controlling the timing of MmRNA recruitment at the late one-cell stage. These data indicate that the cis-regulatory elements dictating translation repression of specific MmRNAs in the oocyte and stage-specific recruitment during the one-cell stage are unknown, and thus the molecular mechanisms controlling this vital process remain uncharacterized. Identifying the novel cis-regulatory elements is an essential step toward defining the translational regulatory mechanisms that dictate diverse postfertilization MmRNA translation patterns.

We report the results of a series of studies to identify novel elements that direct postfertilization MmRNA recruitment for translation in the mouse zygote. Our goal was to develop a systematic approach combining bioinformatics analyses and direct functional tests in order to identify novel cis-regulatory elements, determine their prevalence among the population of regulated MmRNAs, and evaluate the contribution of mRNA secondary structure to the regulation. To establish and validate this approach, we employed the 3’UTR portion of one MmRNA (Bag4) that is highly expressed and enriched on the polysomes of one-cell embryos compared with metaphase II (MII)-stage oocytes. The 3’UTR sequence employed lacks a known CPE or PBE (Pumilio Binding Element) but contains all the information required to dictate enhanced translation after fertilization when coupled to an enhanced green fluorescent protein (EGFP) reporter. Bioinformatics analysis revealed motifs within this 3’UTR that are significantly enriched in abundance among MmRNAs enriched in polysomes after fertilization. Analysis of truncation and deletion mutants confirmed the importance of the corresponding regions and revealed a complex set of multiple translation activating elements (TAEs) and translation repressing elements (TREs) controlling translation in the oocyte and during the early one-cell stage via a novel mode of translation control mediated primarily by negative effects of the TREs. Bioinformatics analyses revealed that, while enriched among MmRNAs, the overall conservation of these newly identified elements is limited, but mRNA secondary structure may be more highly conserved. The approach developed here can be expanded to identify novel cis-regulatory elements controlling MmRNA translation and directing recruitment of MmRNAs with fine temporal control. The discovery of novel cis-regulatory elements will lay the foundation for discovery of proteins that interact with them, thereby uncovering broader aspects of this regulatory mechanism.

MATERIALS AND METHODS

Informatics analysis. Sequences of MmRNAs differentially translated in MII oocytes compared with late one-cell-stage embryos identified by microarray (34) were retrieved from the NCBI nucleotide database. For bioinformatics analysis, 3’UTR sequences and up to 60 nt of flanking coding sequence (CDS) were used for identification of novel motifs of length 1–11 nt enriched in mRNAs differentially recruited onto the polysomes in MII oocytes and late one-cell embryos.

The number of occurrences and number of possible positions for each potential motif m of length L in the 3’UTR were counted. The number of possible positions for m in sequence s of length Ls is the number of sub-sequences (of length L) in s that we can compare to m, which is $L_s - L + 1$. Then for a set of 3’UTRs the total number of occurrences of m as well as the total number of possible positions was calculated. By dividing the total number of occurrences by the total number of possible positions for m, the frequency of motif m specific to the set of mRNAs was obtained. We also considered calculating the motif frequency as the number of distinct 3’UTRs in a set that contain m divided by the number of 3’UTRs in the set.

The log ratio was calculated from the frequency of occurrence of a motif at MII and one-cell stages. The log ratio of frequency for a particular motif is a measure of enrichment of the motif in mRNAs differentially translated at either MII or one-cell stage. Based on the total number of occurrences and total number of possible occurrences for a motif in MII- and one-cell-stage polysomal mRNA, the goodness-of-fit statistical test was performed to obtain a P value. The Benjamin-Hochberg method was applied to control the false discovery rate (FDR). A P value threshold of 0.01 was used, which indicates 1 of 100 discoveries as false positives. The above analysis was extended to identify motifs of length 6–11 nt with partial match by relaxing the criteria and allowing one nucleotide mismatch.

CONTRAfold (http://contra.stanford.edu/contrafold/) was used for prediction of RNA secondary structure. CONTRAfold predicted structures of histone-fold domain and iron-responsive element RNAs that were similar to the structures determined by nuclease mapping experiments and NMR spectroscopy (36). Also, footprinting experi-
mRNA was purified with the MEGA CLEAR kit (Ambion) or by LiCl transcription was performed with the mMESSAGE mMACHINE kit vector digested with EGFP ORF and 3′ = 3′ UTR of 647 nt (BC058518). A late to contain a comparatively short 3′ UTR was digested with C820 POSTFERTILIZATION TRANSLATIONAL CONTROL

mRNA was initially reported Bag4 mRNA expression analysis. Bag4 mRNA expression was analyzed by measuring GFP fluorescence. Late one-cell embryos first displayed measurable GFP fluorescence at 6 h after mRNA injection, indicating that 6 h is required to detect reliable mRNA translation. Images of oocytes and embryos were taken at ~3-h intervals starting from 6 h after mRNA injection. The camera settings for all groups and experiments for obtaining the images were held constant. Fluorescence intensity was measured with the Image J program from NCBI. Background subtraction was performed by subtracting the Image J value of un.injected embryos from the injected embryos in each experiment. The cumulative average was then calculated from the measurements of all oocytes or embryos for a specific time point obtained from all the experiments with a particular mRNA injection. The time point when the fluorescence first appeared was considered as the time of appearance of fluorescence (hours after mRNA injection). All injections were repeated three times and with at least two mRNA preparations.

Quantitative mRNA expression analysis. Bag4 mRNA expression was evaluated by two independent methods, quantitative real-time RT-PCR and quantitative RT-PCR amplification and dot blotting (QADB). For the real-time RT-PCR method, RNA was isolated from eggs and α-amanitin-treated one-cell embryos with the Pico Pure RNA Isolation Kit (Arcturus). cDNA for RT-PCR was synthesized from total RNA with random hexamers. PCR was performed with the Taqman PCR Master Mix and an ABI 7000 thermal cycler (Applied Biosystems) at typical amplification parameters (50°C for 2 min and 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min) with Bag4 primers from Applied Biosystems (Foster City, CA). The expression level of Bag4 mRNA was normalized to internal gene control histone H2A mRNA. Statistical analysis was performed by Student’s t-test. For QADB, polysomal RNA isolated from MII oocytes and α-amanitin-treated one-cell- and two-cell-stage embryos was used as described previously (25, 37). This technique preserves the quantitative representation of the individual mRNAs with respect to the entire population of mRNAs while amplifying the 3′ ends of the mRNA (2). The 3′-terminal portions of mRNA were amplified with a 61-nt primer with an oligo and 23 dT residues and blotted on a Nytran membrane to measure the expression of any specific gene with radiolabeled probes. cDNA probes complementary to the 3′-terminal portion of Bag4 mRNA were used for hybridization onto the blot. Normalized data were used to obtain the expression pattern of Bag4 mRNA.

RESULTS

Regulation of Bag4 mRNA translation. We previously reported (34) the results of an array analysis of MmRNAs that were differentially present on polysomes of MII-stage oocytes versus fertilized late one-cell-stage embryos. A large fraction of the MmRNAs differed between the two populations (495 and 1,816 at a threshold of 3-fold on the array). Strikingly, while the CPE was present in a vast majority of mRNAs enriched on MII oocyte polysomes, only about half of those enriched on polysomes of the late one-cell stage contained previously published CPE sequences. Our goal was to identify cis-regulatory motifs that dictate late one-cell-stage recruitment of MmRNAs by undertaking a detailed molecular dissection of the 3′UTR of an mRNA that was readily detectable, displayed a strong enrichment on the late one-cell-stage polysomes, and lacked a known CPE. The Bag4 mRNA satisfied these criteria, with a raw intensity value of 1,248, ~12-fold enrichment compared with the MII polysomes, and no known CPE in the 647 nt 3′UTR reported (GenBank accession no. BC058518).
Total Bag4 mRNA expression displayed a similar level of expression in MII-stage oocytes and one-cell-stage embryos (Fig. 1A), whereas the Bag4 mRNA polysomal content dramatically increased during progression to the late one-cell stage (Fig. 1B). Other mRNAs satisfied these criteria, but the comparatively limited length of the Bag4 mRNA 3′ UTR made it an ideal target for analysis, and it was selected as a starting point for developing our approach to identify novel cis-regulatory elements.

Bag4 mRNA 3′ UTR recapitulates endogenous mRNA regulation. To determine whether the Bag4 mRNA 3′ UTR contained the necessary elements to confer the same pattern of translation as the endogenous mRNA, we devised a construct coupling the Bag4 mRNA 3′ UTR to the EGFP reporter sequence and microinjected that construct (Fig. 2A, Bag4) into MII-stage oocytes. The injected oocytes were either retained without activation or parthenogenetically activated. Alternatively, oocytes were parthenogenetically activated before microinjection. Examination of EGFP expression at a fixed time after microinjection allowed translational recruitment in MII-stage oocytes, early one-cell-stage activated parthenotes, or late one-cell-stage activated parthenotes to be measured (Supplemental Fig. S1).1 Our analyses confirmed that different regions of the Bag4 3′ UTR stimulated or repressed translation, as evidenced by differences in EGFP reporter fluorescence intensities for different constructs. Quantitative measures of results are given in Fig. 2. To facilitate summary of results, values were classified as low, intermediate, or high. The Bag4 construct was translated exclusively in late one-cell-stage parthenotes, mirroring the behavior of the endogenous Bag4 mRNA displayed in the previous microarray analysis of fertilized embryos (Fig. 2A, Bag4). To ensure further that parthenogenetically activated embryos faithfully reflect what occurs in fertilized embryos, we evaluated expression in embryos produced with ICSI before or after microinjection (Fig. 2B, Bag4).

Again, we observed stage-specific recruitment and EGFP production at the late one-cell stage. Thus the 647-nt 3′ UTR of the Bag4 mRNA contains all the cis-regulatory information necessary to direct stage-specific recruitment at the late one-cell stage, and parthenogenetically activated embryos and ICSI embryos display the same manner of regulation for this test mRNA.

Conservation of motifs within Bag4 mRNA among other mRNAs enriched in late one-cell-stage polysomes. If specific 3′ UTR sequence motifs are involved in stage-specific regulation of a group of mRNAs, there should be specific motifs within the Bag4 mRNA that are overrepresented among mRNAs that show stage-specific translation. The mRNA populations displaying differential abundances in polysomes at the MII and one-cell stages (34) were examined to determine whether potential regulatory motifs could be identified within the Bag4 3′ UTR as explained in the informatics analysis. This revealed one motif with a log ratio >2 in the Bag4 3′ UTR that is enriched in mRNAs translated preferentially at the one-cell stage. The CUUACCUG is present in 5 mRNAs enriched in MII-stage polysomes compared with 60 mRNAs at the one-cell stage (P value ~0.0005). Functional studies (below) examined the role of this motif in translational control. The bioinformatics analysis also revealed two motifs with a >2 log ratio within the Bag4 3′ UTR and 65 terminal nucleotides of the ORF (marked with “x” in Fig. 3A) that were overrepresented in mRNAs that were preferentially translated in MII-stage oocytes. Because our emphasis was on understanding enhanced recruitment at the one-cell stage, these two motifs were not examined further.

The degree of conservation of the CUUACCUG motif and a second less highly conserved motif GGACCUC (see below, Fig. 3B) in Bag4 of different species was studied. Up to two nucleotide mismatches were allowed to identify the motifs, and the data were used to create sequence logos (Supplemental Fig. S2). These motifs were well conserved in the Bag4 3′ UTR across species.

Molecular dissection of Bag4 mRNA 3′ UTR. The motifs identified by bioinformatics analysis as enriched in one-cell-stage polysomal mRNAs may contribute to stage-specific regulation. To test this, we created a series of deletion/truncation mutants of the Bag4 3′ UTR that contained or lacked these motifs and evaluated the effects of these alterations on translation via EGFP reporter expression (Fig. 2A). We noted that all constructs tested were translationally recruited at the late one-cell stage. This indicates that the delay in maternal mRNA translation until the late one-cell stage must rest primarily with translational inhibitory mechanisms that operate in the MII oocyte and early one-cell-stage embryo. Further analysis of deletions and truncations provided insight into the position and identities of the inhibitory elements.

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The region 3′ to nt position 1752 appeared to contain translation suppressive activity, as constructs lacking this region (Bag4-1, Bag4-3, Bag4-13) were translated highly at all stages (Fig. 2A). The region spanning nt 1344–1423 (Bag4-13) supported high rates of translation at all stages. This region thus contains a potent TAE designated as TAE-1. As the unmodified Bag4 reporter lacked nt 1344–1395, the TAE-1 must be located within the 28-nt region 1396–1423. Two additional activators may exist within the Bag4 3′ UTR. The region from 1644 to 1748 nt (TAE-2, Bag4-7) supported a high level of translation at the MII stage, and the region from 1753...
to 2018 nt (TAE-3, Bag4-8,9) supported a high rate of translation at all stages. Truncating the latter region (Bag4-9) reduced translation at the early one-cell stage, indicating that the breakpoint may disrupt the activator.

The Bag4-8 construct containing the 1753–1890 nt region displayed reduced translation at MI and early one-cell stages, indicating the possible presence of a translation inhibitory element suppressing translation at these stages. The Bag4-19
Some mRNAs contained multiple occurrences of these motifs. 3.62% of those in the one-cell enriched polysomal population. mRNAs in the MII enriched polysomal mRNA population and some. The CUUACCUG motif was present in 1.15% of with 5.3% and 66% for mRNA enriched in MII-stage poly-

<table>
<thead>
<tr>
<th>Motif</th>
<th>Stage (total mRNAs</th>
<th>No. of mRNAs with Exact Motif</th>
<th>No. of mRNAs with Exact and Single Nucleotide Mismatch Motif</th>
</tr>
</thead>
<tbody>
<tr>
<td>GGACCUC</td>
<td>MII</td>
<td>23 (5.31%)</td>
<td>284 (65.58%)</td>
</tr>
<tr>
<td></td>
<td>One cell</td>
<td>128 (7.73%)</td>
<td>1,066 (64.41%)</td>
</tr>
<tr>
<td>C9UACCUG</td>
<td>MII</td>
<td>5 (1.15%)</td>
<td>204 (47.11%)</td>
</tr>
<tr>
<td></td>
<td>One cell</td>
<td>60 (3.62%)</td>
<td>677 (40.9%)</td>
</tr>
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UTR, untranslated region; MII, metaphase II.

The inhibitory effect of TRE-3 appears to be required to counter the strong activating effect of TAE-1. The regions immediately upstream and downstream of TRE-3 (Bag4-7, Bag4-9) cannot suppress translation at the MII stage, but translation at the early one-cell stage was reduced. These regions may contain additional TRE elements (TRE-2 and TRE-4) that suppress translation in early one-cell embryos. Comparing the Bag4-7 and Bag4-7,8 constructs, it appears likely that the inhibitor (TRE-2) within the Bag4-7 region (1644–1748 nt) cooperates with TRE-3 in translational repression and suppresses the weak activator (TAE-2). The informatics analysis revealed a motif (GAGGGAGAG) that appeared enriched in the TRE-2 region, but this motif did not pass statistical significance for being overrepresented in the overall population. The Bag4-7 region also appears to cooperate with an element (TRE-1) specific to the Bag4-10 region (1470–1748 nt) to suppress translation, particularly at the MII stage. Inclusion of the entire region from 1644 to 2018 nt (Bag4-7,8,9) yielded a high rate of translation at the MII stage, further indicating that TRE-3 may predominantly counter the effects of TAE-1. The lack of inhibition with the construct Bag4-8,9 indicates that TRE-3 may not be able to suppress the positive effects of TAE-3, further illustrating its principal role in countering effects of the other activators (TAE-1 and TAE-2).

Further studies of the Bag4-19 construct (lacking TRE-3) confirmed enhanced translation in ICSI early one-cell-stage embryos, contrasting with the strong level of inhibition seen with Bag4-24 in the ICSI embryos (Fig. 2B). Thus the strong inhibitor clearly functions in fertilized embryos as well as parthenotes.

Overall, these results indicate the presence of multiple activators and multiple inhibitory elements that control translation in the MII-stage oocyte and early one-cell embryo. The strongest elements are the activator TAE-1 within the 5’ region (1396–1423 nt) and the strong inhibitor TRE-3 within position 1858–1869 nt, the latter encompassing the conserved motif (CUUACCUG) identified in the bioinformatics analysis (Figs. 2A and 3A).
Secondary structure analysis of Bag4 mRNA 3’ UTR. It was noted that the TREs identified above may interact with each other, given the increased translation repression seen with constructs Bag4-7,8 and Bag4-10 (comparing Bag4-7 and Bag4-8 to Bag4-7,8 and Bag4-7 to Bag4-10, respectively). We also noted a lack of translation suppression by TRE-3 in Bag4-8,9 and a lack of MII suppression in Bag4-7,8,9, which could reflect a disruption in secondary structure related to the deletions. To determine whether mRNA secondary structures might contribute to such effects, we examined the predicted secondary structures of the endogenous Bag4 mRNA and some of the deleted/truncated EGFP chimeric constructs, using the CONTRAfold software program (8) (Fig. 4). Comparison of the native Bag4 mRNA and the EGFP chimeric construct Bag4-7,8 and Bag4-24 mRNAs to the Bag4-7,8,9 EGFP construct revealed secondary structure interactions that correlated with inhibitory activities. Specifically, the region at 1827–1869 nt with the inhibitory TRE-3 element and the motifs that aid in regulation formed a distinctive hairpin structure in the native Bag4 mRNA, and in the Bag4-7,8 constructs, which show strong suppression in MII oocytes and early one-cell embryos. This same region formed a double-stranded structure in Bag4-24, which also showed strong inhibition at MII and early one-cell stages. However, this structure was predicted to be disrupted or less stable in Bag4-7,8,9, which displayed an enhanced level of translation in MII oocytes (Fig. 4). These data indicate that strong interactions of the 1827–1869 nt region with the surrounding nucleotides is associated with the inhibitory activity. Interestingly, the Bag4-8,9 and Bag4-8 constructs also displayed a prominent stem-loop region as seen in the other constructs, seemingly at odds with the lack of translational inhibition achieved with this construct. These results indicate that the secondary structure encompassing TRE-3 likely contributes to translational inhibition, although interactions with other elements (e.g., Bag4-7,8) and the presence of other elements (e.g., TAE-3, Bag4-8,9) affect the overall outcome on translational regulation.

To test further the presence of stem-loop structures associated with TRE-3 in translationally regulated MmRNAs, we examined the predicted secondary structures of other mRNAs enriched in polysomes at the one-cell stage and that contained the CUUACCUG (TRE-3) motif (Fig. 5). mRNAs for prediction were selected based on the presence of the inhibitor with no preference for the length of 3’ UTR or the location of the inhibitor. Secondary structures were predicted for 11 mRNAs enriched in one-cell-stage polysomes, one of which (NM_010266) contained two CUUACCUG motifs. We found that the different mRNAs displayed a range of potential interactions between the CUUACCUG motif and the surrounding sequences. We observed substantial stem-forming or base pairing interactions involving CUUACCUG (NM_025333, XM_488539, NM_024467, NM_020497, NM_023215, NM_010266, and NM_153794). This indicates that secondary structures involving CUUACCUG motifs may play important roles in translation control of multiple mRNAs within the regulated population.

Identification of conserved novel motifs from mRNAs enriched in one-cell polysomes. The bioinformatics analysis was successful in identifying novel motifs including the CUUACCUG
motif subsequently shown to be a key element (TRE-3) within the inhibitory region in Bag4 mRNA. This validated the informatics approach of searching for additional conserved elements that may regulate translation among the large population of MmRNAs preferentially translated at the different stages. By comparison of the two polynomial populations, the frequencies of motifs within individual translationally regulated mRNAs were determined. This informatics approach was expanded to a total of 44 other MmRNAs enriched on one-cell polysomes in order to search for other novel motifs that might play a role in regulation of translation. These 44 MmRNAs were chosen for analysis first based on their fold change comparing MII-stage and one-cell-stage polynomial mRNA populations and second sorted based on overall abundance (raw intensity values) and fold changes, except that only annotated mRNAs were included. The number of mRNAs with a particular motif, the number of occurrences in an mRNA, and the relative abundance among MII and one-cell-stage polynomial mRNA populations were determined. The 44 mRNAs analyzed contained 97 novel motifs that were enriched in the MII polysomal mRNA population, and 119 novel motifs were identified as enriched in one-cell polysomal mRNAs. Nearly half (19 of 44) of the mRNAs analyzed contained between 4 and 10 of these motifs, and seven had >10 motifs in their 3′UTRs (Table 2, Supplemental Table S1). Some of the novel motifs displayed multiple occurrences in the 3′UTRs of mRNAs enriched in one-cell stage, implicating these motifs as key features of these MmRNAs with potential roles in translational control. Two mRNAs contained 95 (Strx8) and 46 (Plxnad4) motifs in their 3′UTRs. The Strx8 mRNA displayed 4 novel motifs repeated 20 times each. Some of the motifs identified in the analysis of these 44 mRNAs are also shared between multiple mRNAs; 31 were shared between 2 of the 44 mRNAs and 13 between 3 of the 44 mRNAs.

**DISCUSSION**

Translational control of MmRNAs is essential for development. Identifying the mRNA motifs that dictate different temporal patterns of translation is key to understanding this process. The results here illustrate a successful approach for identifying novel translation regulatory motifs that regulate MmRNAs in mammalian oocytes and early embryos, particularly after fertilization. Bioinformatics analyses identified a potential regulatory motif within the Bag4 3′UTR. The correspondence of this motif to a small region identified through functional studies to be important for regulation was established. Additional regulatory regions exist within the Bag4 3′UTR, identified through functional studies, and these play roles in translational control of the Bag4 mRNA. The novel motifs identified by the two approaches are distinct from the well-established CPE, which regulates translation during oocyte maturation in both mammals and amphibians.

### Table 2. Distribution of motifs in RNAs

<table>
<thead>
<tr>
<th>No. of Motifs in Each RNA</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4–10</th>
<th>&gt;10</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of RNAs</td>
<td>2</td>
<td>4</td>
<td>5</td>
<td>7</td>
<td>19</td>
<td>7</td>
</tr>
</tbody>
</table>

A total of 44 RNAs were analyzed (see Supplemental Table S1).

Our previous studies (34) revealed that the known CPEs that regulate MnRNA translation during oocyte maturation are not present in many MnRNAs that are translationally recruited after fertilization, and other studies of ESTs (11) likewise revealed a limited presence of CPEs in MnRNAs remaining after fertilization. After fertilization, MnRNAs are regulated in a strict temporal manner until genome activation. The mechanisms directing this complex pattern of MnRNA recruitment have remained unknown. However, a prerequisite for such regulation is that MnRNAs to be regulated in different patterns must contain sequence information that directs this. Hence, different regulatory motifs are needed to direct temporally complex patterns of translation, which in turn are essential for proper early embryo development.

Our identification of novel motifs constitutes a first step toward dissecting the mechanism responsible for postfertilization translational control, and it suggests several key features of the underlying mechanisms. First, we have demonstrated that translational control is mediated by a complex combination of both positive and negative regulatory elements, which we have designated as translation activating elements (TAEs) and translation repressing elements (TREs). The Bag4 3′UTR contains an estimated three TAEs and four TREs. The major TAE lies within a narrow 28-nt region at the 5′ end of the 3′UTR (AAUACCUUGGAUUGCCACGCUUG). The major TRE was localized to a narrow region of 12 nt (CUUACCUCAGG) that contains the conserved motif CUUACCUG, with one additional, less conserved nearby motif. The identification of these motifs provides an important basis for future studies to identify regulatory proteins that interact with them and in turn how the activities of those proteins are regulated.

Second, our data reveal interactions between different regions of the 3′UTR, wherein certain TREs appear to be required to offset effects of specific TAEs. Consistent with such interactions, our analysis of mRNA secondary structure indicates that this likely plays a role in successful repression of translation by TRE-3. Constructs in which base pairing at TRE-3 is less stable display reduced ability to suppress translation at early stages.

Third, our data reveal that the translational control of the total population of MnRNAs after fertilization cannot be explained on the basis of just a few motifs. At least seven regulatory motifs likely exist in the Bag4 3′UTR, but the informatics analysis revealed that only one of these is overrepresented among the total population of MnRNAs enriched on one-cell-stage polysomes. That one conserved motif corresponded to a small region subsequently shown to play a key inhibitory role. This illustrates the value and validity of the informatics approach, but it also highlights the diversity and lack of simple sequence conservation among regulated MmRNAs. Furthermore, the bioinformatics analysis indicated that many different motifs could be identified within a group of 44 MnRNAs analyzed, and confirmed that the individual motifs are present in relatively small numbers of mRNAs, albeit repeated multiple times within some of the mRNAs. Thus, in contrast to the widespread effect of known CPE elements and the additional effects of other common elements such as the
PRE and PBE, the translational control of the MmRNA population after fertilization likely involves a complex array of motifs. This complex array of motifs may be required to direct temporally diverse patterns of regulation. Additionally, the diversity of these motifs may be necessitated by the need to satisfy a requirement for mRNA secondary structure in translational control. Potential long-range effects of distant regions on the secondary structures can arise. As a result, as sequences vary within the 3’UTR or in the coding regions of individual mRNAs, this variation can affect portions of the secondary structure. Diverse motifs may thus be needed to support creation of a specific secondary structure that is recognized by translational regulators.

Not only does the array of motifs vary, but the number of such motifs also varies widely among MmRNAs. In the case of the Bag4 3’UTR, the number of motifs appears rather modest (3 TAEs and 4 TREs identified by functional studies and 3 conserved motifs identified by informatics analysis). For other MmRNAs, many more motifs can be detected with our bioinformatics analysis tools. Of 44 mRNAs, bioinformatics analysis revealed that 43% of the mRNAs had 4–10 motifs. Some MmRNAs can display a very complex 3’UTR with many potential regulatory elements, such as 95 and 46 motifs in Stx8 and Plxna4 mRNAs, respectively. It is interesting to note that the Stx8 mRNA 3’UTR contained 4 motifs at 20 copies each and an additional 14 motifs, while the Plxna4 mRNA contained many different motifs, mostly single occurrences, with the most abundant being repeated 4 times, and despite the large number of motifs contained in each of these two mRNAs only 3 motifs were shared between them. This illustrates the vast difference that exists between different MmRNAs, and the need for further study to decipher the regulatory logic controlling MmRNA translation. The informatics analysis applied here should be a great aid in identifying some of the putative regulatory motifs; however, functional studies of individual mRNAs remain essential.

Fourth, our data illustrate that the mechanisms mediating the complex pattern of MmRNA translational control after fertilization are likely eliminated by the late one-cell stage. All of the test mRNAs injected were efficiently translated at the late one-cell stage, regardless of their regulation at earlier stages. This indicates that the proteins that underlie MmRNA regulation are largely eliminated by the late one-cell stage. We propose that this likely enables a wave of global translational recruitment, leading ultimately to the translation and degradation of many MmRNAs. This could contribute to the dramatic elimination of MmRNA that occurs between the late one-cell stage and mid-two-cell stage and the overall transition from maternal control of development mediated by MmRNAs to embryonic control of development mediated by new embryonic transcripts. It is likely that the timing of this loss of translational control varies with species just as the timing of embryonic genome activation varies with species. The mechanisms that coordinate the two events (recruitment and elimination of MmRNAs and genome activation) are poorly understood, although coordination with cell cycle regulators is a possibility.

The U1 small nuclear RNA (snRNA) contains a conserved sequence UUACCUG required for recognition of the 5’ splice site (44). Communication between splicing and polyadenylation exists, and the U1 small nuclear ribonucleoprotein (snRNP) is involved in this coordination (48). The TRE-3 (CUUACCUG) identified by bioinformatics analysis has significant similarity to the 5’ splice site recognition sequence of U1 snRNA. The presence of the UUACCUG motif in the Bag4 3’UTR indicates that proteins interacting with the U1 snRNP UUACCUG motif might also interact with the Bag4 mRNA 3’UTR and possibly regulate translation.

Other studies revealed that the spacing between certain regulatory motifs can affect the temporal pattern of recruitment (33). The identification here of multiple positive and negative regulatory motifs suggests that additional mechanisms operate that rely on complex combinations of motifs, and possibly spacing between these different motifs as well. Other studies have also indicated that association of MmRNAs with other proteins and structures within oocytes and embryos may contribute to their regulation (7, 24, 45, 49). As with the CPE, however, these observations are largely limited to studies of recruitment during oocyte maturation. The combination of multiple motifs regulating MmRNAs after fertilization could provide an opportunity for the involvement of these other proteins and structures, and a means of determining the underlying role of specific sequences and secondary structures contributing to such interactions.

Our data illustrate the complexity of regulatory motifs and attendant mechanisms that control MmRNA regulation after fertilization. Our data indicate that translational control is driven primarily by mechanisms that suppress translation at early stages, so that temporal specificity most likely rests with controlling when inhibitory factors associated with the MmRNAs become dissociated to release those MmRNAs for translation. The negative regulatory motifs may specifically counter the activities of specific positive-acting motifs. Our functional studies modeling the Bag4 mRNA indeed revealed interactions between multiple inhibitory and stimulatory elements. On the basis of the bioinformatics analysis, it is clear that different MmRNAs contain diverse motifs in widely varying numbers and combinatorial sequence complexities. Additionally, our data indicate that secondary structure is key in translational regulation. Thus the complexity seen in sequence motifs likely reflects an underlying temporal complexity combined with a need to satisfy specific secondary structure requirements within the context of unique mRNA sequences. Our analysis also illustrates how a combination of bioinformatics and functional studies can be applied in order to identify components of the postfertilization translation regulatory mechanism, which remains poorly understood. The motifs that can be identified by this approach will provide the basis for further biochemical studies.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES

The Ecology of Regeneration in Plant Communities


