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# Analysis of polysomal mRNA populations of mouse oocytes and zygotes: Dynamic changes in maternal mRNA utilization and function<sup>☆</sup>

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## Abstract

Transcriptional activation in mammalian embryos occurs in a stepwise manner. In mice, it begins at the late one-cell stage, followed by a minor wave of activation at the early two-cell stage, and then the major genome activation event (MGA) at the late two-cell stage. Cellular homeostasis, metabolism, cell cycle, and developmental events are orchestrated before MGA by time-dependent changes in the array of maternal transcripts being translated. Many elegant studies have documented the importance of maternal mRNA (MmRNA) and its correct recruitment for development. Many other studies have illuminated some of the molecular mechanisms regulating MmRNA utilization. However, neither the complete array of recruited mRNAs nor the regulatory mechanisms responsible for temporally different patterns of recruitment have been well characterized. We present a comprehensive analysis of changes in the maternal component of the zygotic polysomal mRNA population during the transition from oocyte to late one-cell stage embryo. We observe global transitions in the functional classes of translated MmRNAs and apparent changes in the underlying *cis*-regulatory mechanisms.

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**Keywords:** Embryo; Oocyte; Translation; Polysome; Microarray; Maternal mRNA; CPE

## Introduction

At the start of each life, the oocyte must accomplish a complex series of remarkable tasks, including completion of meiosis, resumption of a mitotic cell cycle, suppression of programmed cell death, remodeling of parental chromatin, transcriptional activation, and initiation of the embryonic developmental program, while simultaneously preparing itself for the metabolic demands of DNA replication and cell division (Latham and Schultz, 2001, 2003; Jurisicova et al., 1998). These events are mediated by a rich array of MmRNAs and

proteins deposited in the oocyte during oogenesis. MmRNAs are stored in an inactive, masked form, and recruited for translation in a stage-specific manner during oocyte maturation or early embryogenesis (Davidson, 1986). Their recruitment is accompanied by elongation of the poly(A) tail, followed by complex changes in protein synthesis patterns (Bachvarova et al., 1985, 1989; Paynton et al., 1988; Vassalli et al., 1989; Latham, 1999; Huarte et al., 1992; Salles et al., 1992, 1994; Gebauer et al., 1994; Sheets et al., 1995; Hwang et al., 1997; Oh et al., 1997, 1998; Knowles et al., 2003; Evsikov et al., 2004).

The unmasking and recruitment of MmRNAs are controlled by a combination of *cis*-acting regulatory sequence elements, proteins that bind to them or that mediate modifications of these mRNA binding proteins and other proteins involved in translation. Perhaps the most widely appreciated *cis*-regulatory element is the cytoplasmic polyadenylation element (CPE) in the 3' untranslated region of MmRNAs (Simon et al., 1992; Oh et al., 1997, 2000; McGrew and Richter, 1990). Another element, the polyadenylation response element (PRE), may also contribute to MmRNA translational control (Charlesworth et

**Abbreviations:** MmRNA, maternal mRNA; MGA, major genome activation; CPE, cytoplasmic polyadenylation element; PRE, polyadenylation response element.

<sup>☆</sup> Microarray tabular data are deposited at <http://www.ncbi.nlm.nih.gov/projects/geo/>, data set accession number: GSE3962.

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al., 2004). A third element, the hexanucleotide polyadenylation signal, is also needed for recruitment (Gebauer et al., 1994; Simon et al., 1992).

The CPE has been extensively studied, mainly within the context of oocyte maturation. During maturation, MmRNAs with CPEs can be recruited for translation whereas MmRNAs without CPEs become deadenylated (Fox and Wickens, 1990; Varnum and Wormington, 1990). Deadenylated MmRNAs either become degraded or persist in the ooplasm to become readenylated after fertilization (Bachvarova et al., 1985, 1989; Paynton et al., 1988). The CPE functions by binding the CPE binding protein, CPEB1 (Cao and Richter, 2002; Mendez et al., 2000). Dominant-negative, non-phosphorylatable forms of CPEB1 interfere with oocyte meiotic progression and a deficiency of CPEB1 arrests oogenesis at pachytene I, indicating that the correct translational regulation of MmRNAs is essential for completing meiosis (Hodgman et al., 2001; Tay et al., 2003). The CPE-mediated control of mRNA recruitment appears well conserved between *Xenopus* and mouse (Richter, 1999).

High-resolution two-dimensional protein gel electrophoresis studies reveal a complex pattern of temporal changes in protein synthesis during the first cell cycle in mouse embryos (Latham et al., 1991). Studies in a range of non-mammalian species reveal a variety of MmRNAs that direct morphogenesis (e.g., Melton, 1987; Macdonald and Struhl, 1988; Kim-Ha et al., 1993; Evans et al., 1994; Wilhelm and Smibert, 2005). In mammals, inhibition of protein synthesis with cycloheximide (Wang and Latham, 1997; Wang et al., 2001) or inhibition of MmRNA polyadenylation (Aoki et al., 2003; Hara et al., 2005) prevents embryonic gene transcription, indicating important roles for MmRNAs in creating and activating the embryonic genome (Wang and Latham, 2000; Wang et al., 2001; Latham and Schultz, 2001). Collectively, these observations indicate that the MmRNA population is heterogeneous and serves numerous functions.

A thorough analysis of the identities and functions of translationally regulated MmRNAs is essential in order to understand fully the mechanisms that regulate MmRNAs throughout early development. Numerous studies have examined the translational control of individual MmRNAs in oocytes and embryos of both amphibian and mammalian species, and these MmRNAs have proven to be valuable models for studying translation regulatory mechanisms. Other studies have employed quantitative or semi-quantitative reverse transcription and polymerase chain reaction assays (RT-PCR; Rambhatla et al., 1995; Alizadeh et al., 2005) or analyses of total mRNA populations of oocytes and early embryos (Wang et al., 2004; Hamatani et al., 2004; Zeng et al., 2004) using oligo(dT) priming for reverse transcription to examine changes in the total MmRNA population. Because the efficiency of oligo(dT) priming can be affected by the length of the poly(A) tails on mRNAs, leading to apparent changes in mRNA abundance related to polyadenylation (Rambhatla et al., 1995), these studies also provided suggestive evidence for translational recruitment of other MmRNAs. Although suggestive and indeed interesting, a complete picture of MmRNA recruitment has not yet emerged. A complete, direct examination of MmRNAs that are recruited for stage-specific translation is

needed. Subsequent analyses of the sequences of these mRNAs, their temporal order of recruitment, their potential regulatory elements, and the functions of their encoded proteins should provide new insights into the mechanisms controlling MmRNA utilization and a more complete understanding of what specific biological processes are controlled at this level.

To address these objectives, we completed a detailed analysis of polysomal MmRNAs of ovulated oocytes and late one-cell stage embryos. This analysis of the polysomal MmRNA population allowed for a direct examination of mRNAs that are undergoing translation, and more importantly mRNAs that are differentially translated at different stages. We focused our attention on the changes in the polysomal MmRNA population during the one-cell stage because this is the critical stage during which fertilization must initiate processes to convert the mature oocyte into a developing embryo, and during which development is almost entirely driven by the translational recruitment of maternal mRNAs and the proteins they encode. Our analysis revealed well over 2000 MmRNAs that are preferentially translated in mature oocytes or preferentially recruited for translation during the first cell cycle. We observe fundamentally different functional classes of translated MmRNAs at the two stages and find that this shift in the polysomal MmRNA population is accompanied by a shift in usage of *cis*-regulatory elements. These data can now be used to examine in greater detail those mechanisms that define distinct temporal patterns of MmRNA recruitment.

## Materials and methods

### *Isolation and culture of ovulated oocytes and embryos*

Ovulated MII stage oocytes were isolated from the oviducts of CF1 females (Charles River), which had been superovulated as described (Wang and Latham, 2000). Cumulus cells were removed by treatment with hyaluronidase (120 IU/ml, Sigma-Aldrich, St. Louis, MO) in M2 medium. Fertilized one-cell embryos were obtained by superovulation and mating of CF1 females to (B6D2)F<sub>1</sub> males. Fertilized embryos were cultured in KSOM w/o amino acids medium containing 24 µg/ml  $\alpha$ -amanitin from 19 h post-hCG. MII stage oocytes and one-cell embryos were collected for lysis at 15 h and 28 h post-hCG, respectively. Previous studies revealed gene transcription during the latter portion of the one-cell stage (for a review, see Latham, 1999; Latham and Schultz, 2001). The  $\alpha$ -amanitin treatment was employed to ensure that mRNAs isolated from polysomes were maternal in origin. It can be noted that in our previous analysis of mRNA translation, we observed no significant effect of  $\alpha$ -amanitin treatment on the polysomal distributions of four MmRNAs or two embryonically transcribed mRNAs examined (Wang and Latham, 2000). For quantitative RT-PCR, embryos were also collected at the two-cell stage (45 h post-hCG).

### *Polysomal mRNA preparation*

Groups of 160–500 MII stage oocytes, fertilized one-cell stage embryos, or two-cell stage embryos were lysed in 300 µl of polysome lysis buffer (PLB) [10× TAM (200 mM Tris pH 7.5, 1 M NH<sub>4</sub>Cl, 50 mM Magnesium acetate), 5 mM DTT, 50 µl of NP40, 25 mg of deoxycholate in a volume of 5 ml] with 300 U of prime RNase inhibitor (Eppendorf), 98.4 U of RNAGuard (Amersham Biosciences), and 5 mM DTT. The zonae pellucidae were removed using acidic Tyrode's buffer prior to lysis and the cells washed in order to avoid any contamination of samples with somatic cells and to permit complete solubilization and release of polyribosomes into PLB. Cell lysates were applied in a volume of 150 µl onto 50 µl sucrose cushions in 200 µl polycarbonate tubes

and centrifuged at  $5 \times 10^4$  rpm ( $1 \times 10^5 \times g$ ) for 40 min using a Beckman TLA-100 rotor as described (De Sousa et al., 1993). This method pellets the polyribosomes while leaving the mono and non-polysomal mRNA fractions in the supernatant. The specificity of separation of polyribosomal mRNAs was previously confirmed by sucrose density gradient analysis of the pellet and supernatant fractions (De Sousa et al., 1993). The method pellets  $>80$  S polyribosomal mRNAs. It should be noted that the major core mRNA ribonucleoprotein (mRNP), YB-1, yields mRNPs that sediment at about 28 S when complexed with non-translating mRNAs at a high molar ratio (Skabkin et al., 2004), and mRNPs have been reported as 20–22 S in other systems (Assairi et al., 1976; Goldenberg and Scherrer, 1981). Non-translating mRNP complexes should thus be easily separated from polysomes using this method. The polyribosomal pellets were solubilized with 20  $\mu$ l of guanidine thiocyanate buffer pH 7.0 (5 M guanidine thiocyanate, 25 mM Tri-sodium citrate dihydrate, 0.5% Na-lauroyl sarcosinate, 200 mM DTT), ethanol precipitated with glycogen as carrier, solubilized in TE buffer (10 mM Tris-Cl, pH 8; 1 mM EDTA, pH 8), and ethanol precipitated once again to remove any remaining guanidine salts. The final pellet was solubilized in RNase-free water and employed either for RT-PCR or for RNA amplification for microarray analysis as described below. A total of four independent samples were obtained for each stage for microarray analysis, and another 3–4 samples for each stage for quantitative RT-PCR analysis.

#### RNA amplification and hybridization

Polysomal RNA from oocytes or embryos was used in each sample. The total RNA was used for linear, two round amplification by in vitro transcription (Affymetrix Small Sample Preparation Technical Bulletin, [www.affymetrix.com](http://www.affymetrix.com)). Quality control steps taken to ensure inter-array reproducibility were as follows: processing of the samples at the same time, by the same person, with same lot of reagents, and use of same lot of Gene Chips. Moreover, a cocktail of prokaryote mRNAs (Poly-A control kit, Affymetrix) was added to all samples in equal amount at the beginning of RNA preparation as a control for RNA recovery and processing loss. Biotinylated cRNA yields after the second round of amplification was 20–87  $\mu$ g. OD reading of the samples was between 1.87 and 2.20, and denaturing gel showed the expected range of dimension of in vitro transcribed, biotinylated cRNA. Fifteen micrograms of cRNA were fragmented and hybridized to Affymetrix GeneChips MOE430 v.2. After hybridization, arrays were washed and stained in fluidic stations and scanned at 3  $\mu$ m resolution according to manufacturer's specifications (GeneChip Analysis Technical Manual, [www.affymetrix.com](http://www.affymetrix.com)).

#### Microarray data

GeneChip tabular data are available at the GEO Repository (GSE3962), [www.ncbi.nlm.nih.gov/geo](http://www.ncbi.nlm.nih.gov/geo). Microarray Analysis Suite 5.0 (MAS, Affymetrix) was used to quantify microarray signals with default analysis parameters and global scaling to target mean=150 in order to allow comparisons between arrays. The MAS metrics output was loaded into TIGR-MEV (The Institute for Genomic Research Multi Experiment Viewer v.3.0.3). A filtered list of all genes detected was created based on presence (MAS “P” call) in at least three out of four samples in any of the stages.

The statistical algorithm SAM (Significance Analysis of Microarray; Tusher et al., 2001) identified transcripts with significant differences (false discovery rate=0.0001) in the two stages. Lists of genes were imported into EASE (version 2.0, [www.david.niaid.nih.gov/david/ease](http://www.david.niaid.nih.gov/david/ease)) to test for over-representation of annotated genes (Hosack et al., 2003). EASE tested each list against the population of genes detected on the GeneChips, and an EASE score was calculated, expressing the likelihood of over-representation in the Gene Ontology Consortium annotation categories. Over-representation analysis was also performed using Ingenuity Pathway Analysis 3.0 ([www.ingenuity.com](http://www.ingenuity.com)). Finally, using the literature, the genes obtained after filtering and microarray analyses were listed under the representative GO group.

Quality control parameters for the samples were in the following ranges: scale factor 1.8–17.9 and background 31.8–44.2; percent genes detected, 20.0–35.4; actin 3'/5' signal ratio 4.0–11.6 for seven samples (one sample yielded a ratio of 66 for actin but was as expected for GAPDH); GAPDH 3'/5' signal ratio 3.5–9.4 for the eight samples. It should be noted that many housekeeping mRNAs are likely not well suited to this evaluation because these mRNAs

undergo extensive deadenylation and degradation during oocyte maturation, which may thereby reduce the quantitative resolution and accuracy of their measurement, and make them non-representative of the results obtained with the overall polysomal mRNA population (Bachvarova et al., 1989; Paynton et al., 1988; Rambhatla et al., 1995). The prokaryote control mRNA signals were all within expected ranges (mRNA added as exogenous standards prior to extraction). Initial evaluations of the microarray data revealed these data to be of excellent quality and reproducibility. Hierarchical cluster (HCL) analysis of samples was performed using TIGR MEV version 3.03.

#### Quantitative RT-PCR analysis

To compare the quantitative representation of specific mRNAs in the polysomal populations at different stages, polysomal mRNA from MII stage oocytes and  $\alpha$ -amanitin-treated one-cell and two-cell stage embryos was isolated as described above, and then subjected to quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis using the Quantitative Amplification and Dot Blotting (QADB) method (Rambhatla et al., 1995; Latham et al., 2000). This method involves reverse transcription followed by the quantitative amplification of the 3' terminal portions of the entire mRNA population, preserving quantitative representation of individual sequences in the amplified cDNA population (Brady and Iscove, 1993). The PCR reaction is performed using a special 61 nt primer denoted “XT”, which terminates with 23 dT residues. The first 38 nt serve to anchor the PCR reaction. The amplified cDNA libraries representing different stages are then applied to dot blots, and these dot blots are then hybridized with radiolabeled gene-specific probes. Phosphorimaging and quantitative analysis of hybridization signals were then employed to obtain quantitative measurements of the abundances of specific mRNAs within the mRNA population. The quantitative sensitivity, reproducibility, and reliability of the QADB method have been extensively documented in many previous studies (reviewed in Zheng et al., 2004). The primers employed to obtain cDNA probes for hybridization are mentioned in Supplementary Table 1.

#### Sequence analysis

Full-length sequences of the mRNAs with poly(A) tails were obtained from NCBI. The presence of previously described CPEs (Simon et al., 1992; Oh et al., 2000) in these mRNAs was evaluated by manual sequence examination. Only CPEs present in 3' UTRs were considered. The presence of putative PREs (Charlesworth et al., 2004) was evaluated using Clustal W.

## Results

#### Overview of microarray results

We obtained microarray data from four independent preparations of the ovulated oocytes and late one-cell stage embryos. A total of 13,969 Affymetrix IDs were detected. External (PolyA control kit, Affymetrix and Genechip Eukaryotic Hybridization control kit) and internal (GAPDH) control parameters were within the expected ranges. Hierarchical clustering revealed excellent reproducibility among the samples of either oocyte or one-cell polysomes (Supplementary Fig. 1). Moreover, there was no intermixing of individual samples between the two different clusters, thus revealing significant differences between the polysomal populations of the two kinds of cells.

#### Global measurement of change in polysomal MmRNA population

One of the major questions concerning MmRNA populations is to what degree the polysomal MmRNA population changes



during the transition from ovulated oocyte to embryo, as the embryo approaches MGA. Previous studies revealed that the total rate of L-[<sup>35</sup>S]methionine incorporation increases slightly (~30–40%) after fertilization (Schultz et al., 1979; KEL, unpublished). Thus, MII stage oocytes and fertilized embryo are both translationally active and must possess sizeable polysomal mRNA populations. Dynamic changes in the array of proteins synthesized do occur, however, with about 60% of the proteins detected on high-resolution two-dimensional protein gels changing by two-fold or more in rates of synthesis, and 27% changing by four-fold or more (Latham et al., 1991). Collectively, these results indicate that the array of MmRNAs being translated changes a great deal over the course of the one-cell stage. Our microarray analysis was therefore designed to determine both the magnitude of the overall change in the polysomal MmRNA population, and to identify specific MmRNAs that undergo changes in relative abundances among the polysomal MmRNA population.

We used the Significance Analysis of Microarray (SAM) test to identify populations of MmRNAs differentially translated between the oocyte and late one-cell stage. In previous studies from other laboratories, the range of cutoff values used to identify differences in mRNA populations was 1.4–2.0 (Zeng et al., 2004; Pan et al., 2005). Using the more stringent 2.0-fold change cutoff, nearly one third (29%) of the detected Affymetrix IDs demonstrated differential translation. This indicates an astounding degree of difference between the polysomal MmRNA populations of oocytes and late one-cell embryos. To focus on the major differences between the two polysomal MmRNA populations, we used an even more stringent 3.0-fold cutoff (false discovery rate=0.0001) (Table 1), yielding 569 Affymetrix IDs with greater signals in the oocyte polysomal MmRNA population, and 2123 with greater signals in the one-cell polysomal MmRNA population. These Affymetrix IDs corresponded to 495 and 1816 individual mRNAs, respectively. Thus, during the transition from ovulated oocyte to late one-cell embryo, there was a predominant trend towards the recruitment of MmRNAs, with a lesser degree of loss of MmRNAs from the polysomal population.

Functional relevance of changes in the polysomal MmRNA population

With such a vast amount of change in the polysomal MmRNA population, the question arises whether the changes in the polysomal MmRNA populations reflect protein synthesis devoted to addressing specific needs, or instead reflect changes in protein synthesis that are less specific and not related to specific

Table 1  
Number of probe sets and transcripts enhanced in the MII and one-cell stage polysomal populations

Stage	≥ 3-fold enrichment	
	Probe sets (n)	Transcripts (n)
II	569	495
Late one-cell	2123	1816

Table 2  
Over-representation of the gene categories shown by EASE analysis of MmRNAs enriched in MII oocyte polysomes

Gene category <sup>a</sup>	EASE score
Integral to membrane	5.23E-21
Extracellular space	8.29E-05
Metal ion transport	7.48E-04
Transmembrane receptor protein serine/threonine kinase activity	9.33E-04
Transforming growth factor beta receptor activity	9.33E-04
Porter activity	1.07E-03
Electrochemical potential transporter activity	1.28E-03
Transmembrane receptor protein kinase activity	1.55E-03
Cation transport	2.08E-03
Receptor activity	2.46E-03
Di- or trivalent cation transporter activity	2.53E-03
Transporter activity	2.54E-03
Carrier activity	5.06E-03
Transferring glycosyl groups	6.52E-03
Signal transducer activity	9.28E-03
Endomembrane system	9.80E-03
Di- or trivalent inorganic cation transport	1.01E-02
Ion transport	1.07E-02
Transmembrane receptor activity	1.14E-02
Cation transporter activity	1.89E-02
Morphogenesis of an epithelial sheet	2.22E-02
Manganese ion binding	4.46E-02

<sup>a</sup> The gene lists from SAM were imported into EASE (version 2.0) to reveal the overrepresentation of annotated genes. EASE analysis was performed with Bonferroni multiplicity correction. An EASE score was calculated, expressing the likelihood of overrepresentation in the Gene Ontology Consortium annotation categories—GO biological process, GO cell component, and GO molecular function.

processes. To distinguish between these possibilities, we undertook three independent analyses: (i) Expression Analysis Systematic Explorer v. 2.0 (EASE) analysis; (ii) Ingenuity Pathway Analysis (IPA), which uses a proprietary database to explore affected networks and processes; and (iii) manual assignment of affected MmRNAs to specific functional categories.

The EASE analysis reveals over-representation of specific GO categories of transcripts within a list of affected mRNAs, in this case MmRNAs showing enhanced translation at a specific stage. Such over-representation provides an indication that the corresponding biological process may have particular relevance to the underlying biology of the experimental system. The EASE analysis revealed that most of the MmRNAs enriched in the oocyte polysomal MmRNA population encoded proteins associated with homeostasis (Table 2). Top-ranking GO categories included integral to membrane, membrane, extracellular space, extracellular region, metal ion transport, growth factor receptors, transmembrane molecular transport, and signal transduction activities. The MmRNAs enriched in the one-cell stage polysomal MmRNA population (Table 3) revealed an over-representation of mRNAs encoding proteins related to biosynthesis. The four top-ranked categories were macromolecular biosynthesis, biosynthesis, protein biosynthesis, and cellular biosynthesis.

The IPA analysis seeks to identify biological pathways or networks of interacting gene products, or to identify specific affected processes in a manner similar to the EASE analysis. By associating lists of affected mRNAs with specific pathways or

Table 3  
Over-representation of the gene categories shown by EASE analysis of MmRNAs enriched in one-cell polysomes

Gene category	EASE score
Macromolecule biosynthesis	8.50E-05
Biosynthesis	3.4E-04
Protein biosynthesis	3.7E-04
Cellular biosynthesis	4.0E-04
Intracellular	4.7E-04
Cytosol	4.2E-03
Cytosolic ribosome (sensu Eukaryota)	5.2E-03
Structural constituent of ribosome	5.4E-03
Macromolecule metabolism	9.8E-03
Pyrophosphatase activity	1.1E-02
Cellular macromolecule metabolism	1.1E-02
Intracellular organelle	1.2E-02
Hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	1.3E-02
Nucleoside-triphosphatase activity	1.4E-02
Oxidation of organic compounds	1.5E-02
Ribosome	1.7E-02
Main pathways of carbohydrate metabolism	1.9E-02
Nucleosome	1.9E-02
Regulation of transport	2.4E-02
Transcription cofactor activity	2.6E-02
Primary metabolism	2.9E-02
Cytoplasm	3.0E-02
Cellular metabolism	3.1E-02
ATPase activity	3.2E-02
Gluconeogenesis	3.4E-02
Protein metabolism	3.5E-02
Metabolism	3.5E-02
Obsolete cellular component	3.7E-02
Protein domain-specific binding	3.8E-02
Cellular protein metabolism	3.8E-02
Mitochondrion	4.0E-02
Purine ribonucleotide metabolism	4.1E-02
Transcription factor binding	4.1E-02
Ribosome biogenesis and assembly	4.3E-02
Protein complex	4.4E-02
Purine nucleotide metabolism	4.5E-02
Non-membrane-bound organelle	4.7E-02
Intracellular non-membrane-bound organelle	4.7E-02
Double-stranded RNA binding	4.8E-02

The gene lists from SAM were imported into EASE (version 2.0) to reveal the overrepresentation of annotated genes. EASE analysis was performed with Bonferroni multiplicity correction. An EASE score was calculated, expressing the likelihood of overrepresentation in the Gene Ontology Consortium annotation categories—GO biological process, GO cell component, and GO molecular function.

processes, the IPA provides a second, independent means of identifying categories of affected mRNAs. The IPA yielded similar categories for the two classes of MmRNA as did the EASE analysis (i.e., enriched in oocyte or one-cell polysomes), which may reflect constraints within the annotation database (Fig. 1). However, the relative rankings and the significance values differed between the two stages. Protein synthesis was the top-ranked category at both stages but attained a greater level of significance in one-cell embryos. The categories of molecular transport, protein trafficking and DNA replication, recombination, and repair were also more highly ranked at the one-cell stage.

EASE and IPA analyses are limited by the degree and accuracy of annotations within GO categories. Moreover, EASE analysis relies solely on numerical relationships between gene lists and cannot account for differences in arrays of genes within categories. Consequently, whereas a positive result with EASE analysis can provide a strong indicator of a process that is likely to be affected, a negative EASE result does not exclude the possibility that other processes are also affected. We therefore undertook a third method of analysis involving the manual assignment of affected mRNAs to specific functional categories, followed by the evaluation of the relative size of each category. For this manual analysis, we devised 12 functional categories (Fig. 2; Supplementary Tables 2 and 3). Of the 2311 differentially translated MmRNAs, 870 have not yet been annotated in the available databases. We assigned 1430 (99%) of the remaining 1441 transcripts to one or more of these 12 categories. The largest category of mRNAs preferentially elevated in the oocyte polysomal MmRNA population was homeostasis (19%). Signaling, transcription, metabolism, and cell cycle control were also among the top-ranked categories. The largest category for the one-cell polysomal MmRNA population was metabolism. This category contained twice as high a percentage (21%) of mRNAs at the late one-cell stage as at the oocyte stage (9%). Overall, 19% (270 of 1430) of the differentially translated mRNAs were related to cellular metabolism. The relative contribution of genes related to homeostasis was reduced by nearly four-fold at the one-cell stage, accounting for only about 5% of the elevated transcripts. The categories of transcription, translation, and signal transduction were significant fractions of the one-cell polysomal MmRNA population, encompassing 39% (447 of 1430) of the differentially translated MmRNAs. Cell cycle control-related MmRNAs declined in relative abundance (7% versus 3%, respectively) among the differentially translated MmRNAs.

These results reveal that different functional categories are enriched in the polysomes at the two developmental stages. Moreover, dramatic changes occur between the two stages even within the same functional category.

#### Polysomal expression profiles of representative MmRNAs

We next examined the temporal profiles of expression of specific mRNAs within the polysomal populations during development. We collected polysomal mRNA from MII stage oocytes, late one-cell stage  $\alpha$ -amanitin-treated embryos, and two-cell stage  $\alpha$ -amanitin-treated embryos. These were analyzed using a quantitative RT-PCR method to assay 22 MmRNAs from different functional categories. Of these 22 MmRNAs, 11 were enhanced in oocyte polysomes and 11 were enhanced in one-cell polysomes. This analysis confirmed the microarray results for the vast majority of transcripts analyzed (20 of 22) (Fig. 3). Nine of the MmRNAs enhanced in oocyte polysomes displayed large decreases (range 1.5- to 82-fold) in translation in one-cell embryos and remained low at the two-cell stage. The apparent abundance for the *Cept1* mRNA was somewhat variable at the oocyte stage, declined at the one-cell stage, and then declined further at the two-cell stage. The *Abhd3*

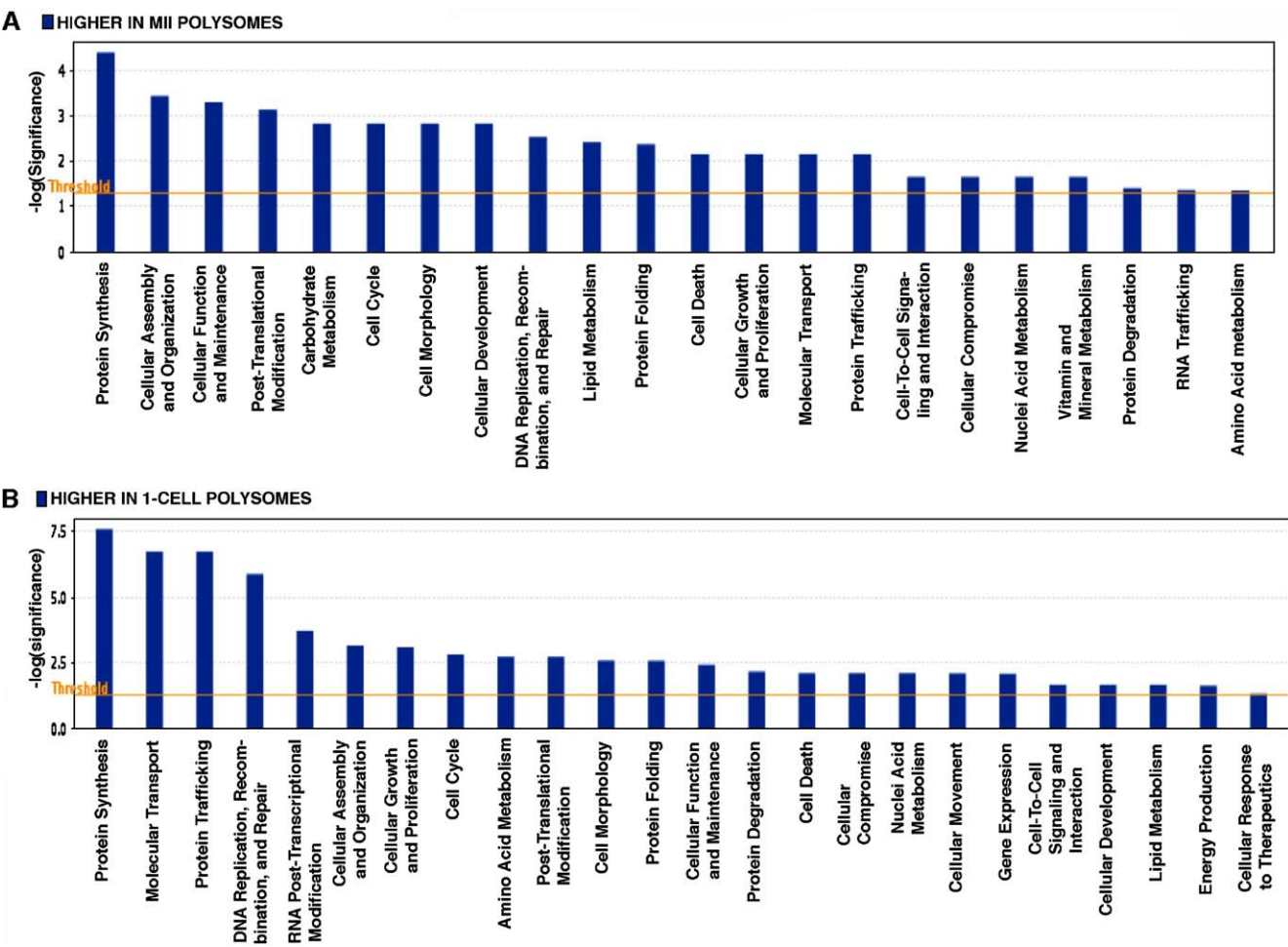


Fig. 1. Over-representation of functional categories in (A) MII stage oocyte and (B) one-cell embryos based on Ingenuity Pathway Analysis. Threshold of significance was set at  $p=0.05$ .

mRNA failed to display a decrease in polysome abundance. Ten of the mRNAs that were enhanced in one-cell polysomes displayed 1.6- to 231-fold increases in polysomal abundance. Most continued to display elevated polysomal abundances at

the two-cell stage relative to the oocyte, although several declined somewhat between the one-cell and two-cell stages. It should be noted that *Gtf3c2* is unusual among the genes analyzed in that it is represented by five probe sets on the array, only one of which yielded a differential hybridization signal. As different probe sets may target different splice variants, such a difference in translation of a single variant would not be expected to be revealed by the QADB method.

Several MmRNAs identified in our microarray analysis had been identified previously in a subtraction hybridization screen at the one-cell stage (KEL, unpublished). These include *Spry4*, *Abcf3*, and *Kpna2* (not shown). Additionally, it is worth noting that published results support the preferential translation of *Gdf9*, *Hlfoo*, and *Mos* in the oocyte and/or their degradation after fertilization (Rajkovic and Matzuk, 2002; Alizadeh et al., 2005) and preferential translation of the *Slc6a9* at late one-cell stage (Steeves et al., 2003), as shown here. These observations collectively provide further confirmation of our microarray results.

Analysis of polysomal mRNAs translated at both stages

Although the above analysis reveals stage-dependent changes in the polysomal MmRNA population and biological

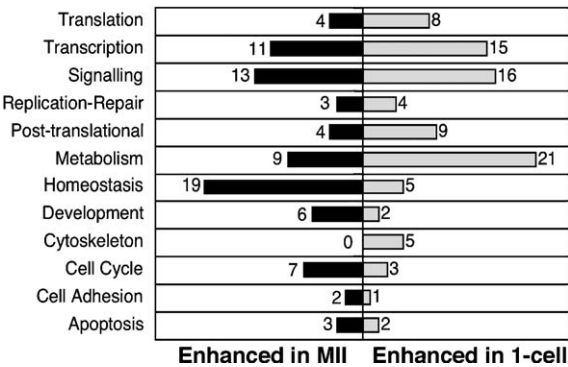


Fig. 2. Manual assignment to functional (GO) categories for MmRNAs preferentially translated in MII stage oocyte (left) or one-cell embryos (right). Data are expressed as percentage of total annotated transcripts. Non-annotated transcripts and transcripts with unknown function were not considered for the analysis. Categories with <1% representation in both groups were not included. Numbers next to the bars represent the percentage of that category at that stage.



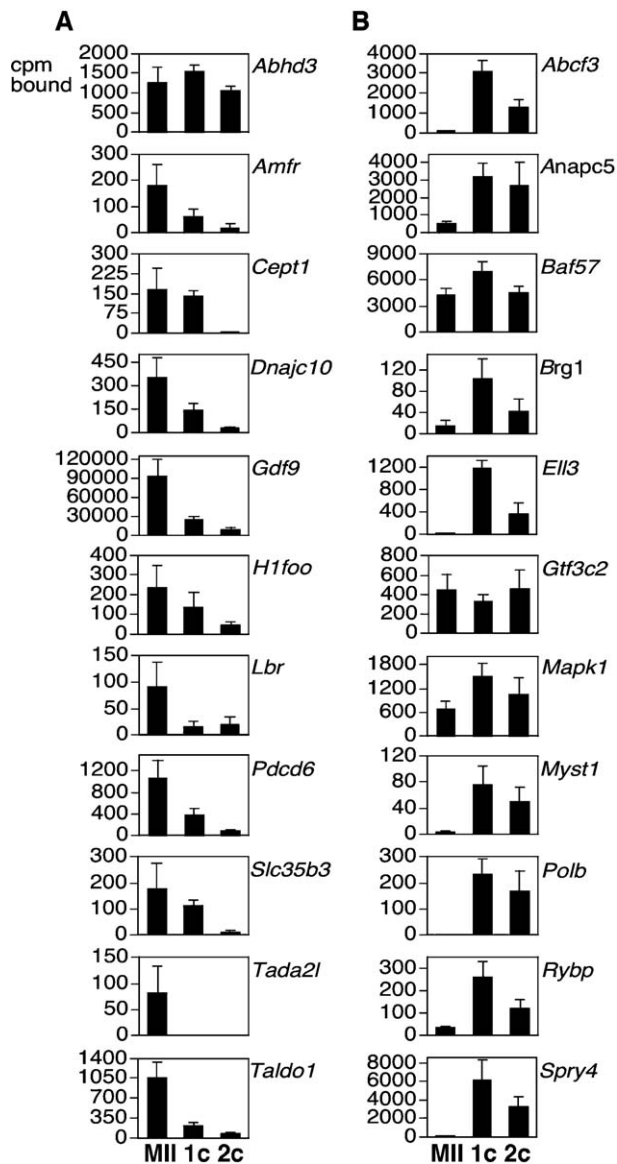


Fig. 3. Relative polysomal abundances of MmRNAs in oocytes and embryos. MmRNAs enhanced in the polysomal MmRNA population of (A) MII oocytes and (B) one-cell stage embryos. MII, MII-stage oocyte; 1C, one-cell stage embryo; 2C, two-cell stage embryo.

functions associated with these changes, our data can also provide insight into the functions encoded by MmRNAs that are translated in common (defined here as <three-fold differences) between MII stage oocytes and one-cell embryos. To explore this population of MmRNAs, we grouped these MmRNAs according to their gene ontology categorizations (Fig. 4). We observed categories encompassing mRNAs related to post-translational modifications, gene transcription, macromolecular and ion transport mechanisms, cell cycle control, DNA replication and repair, ubiquitination, mRNA splicing, and signal transduction. These are the same GO categories encompassed by those MmRNAs shared in common between the total MmRNA populations of both MII stage oocytes and one-cell embryos (Zeng et al., 2004). As a way of comparing the two populations further, we employed the IPA tools to produce a list of significantly over-represented functional

categories (Fig. 5). This comparison yielded several interesting results. First, there were fewer categories represented among the polysomal mRNAs than the total mRNA population. Second, the predominant categories in both populations were related to signaling functions. Other functions of interest included DNA repair and cell cycle control. Thus, IPA yielded several of the same categories revealed in Fig. 4. Third, there are some differences in the relative rankings of some of the categories, most notably nucleotide excision and repair. Thus, the polysomal MmRNAs that are translated throughout the transition from MII oocyte to one-cell embryo encode diverse functional categories, with some statistical differences between these MmRNAs and the total population of MmRNA expressed at both stages.

#### Translation regulatory elements in differentially translated MmRNAs

Specific *cis*-acting elements have been identified in MmRNAs from a variety of species. Chief among those

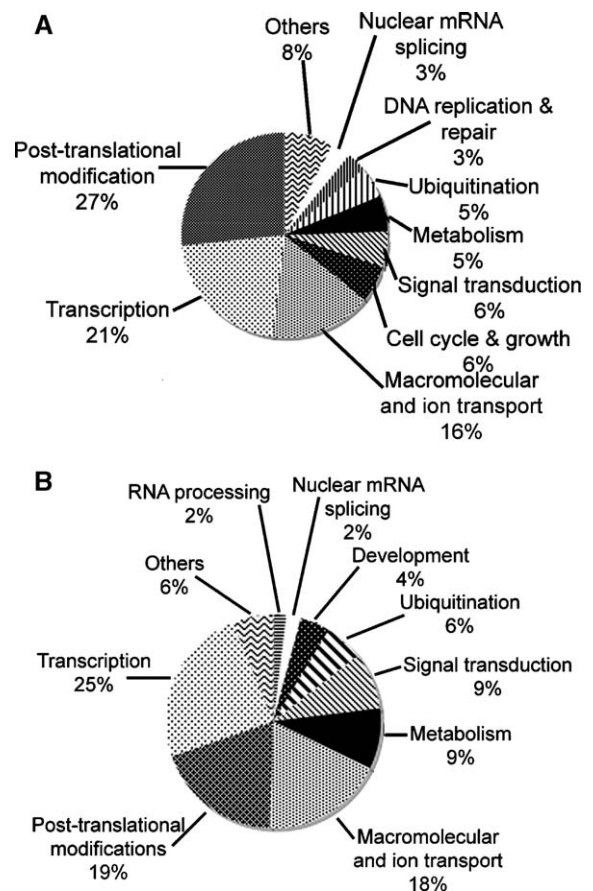


Fig. 4. Gene ontology categories for mRNAs (A) detected on polysomes with less than three-fold differences between MII stage oocytes and one-cell embryos and (B) detected as less than three-fold different between total mRNA populations of MII stage oocytes and one-cell embryos using microarray data produced by Zeng et al. (2004). For simplicity, functionally related GO categories have been combined. GO categories that could not be combined and that contained fewer than 50 entries for the polysomal data set were included in a single category entitled “others”. A total of 2996 annotated polysomal mRNAs and 2957 from the total mRNA populations (Zeng et al., 2004) were included in the analysis. Non-annotated mRNAs were excluded from the analysis.



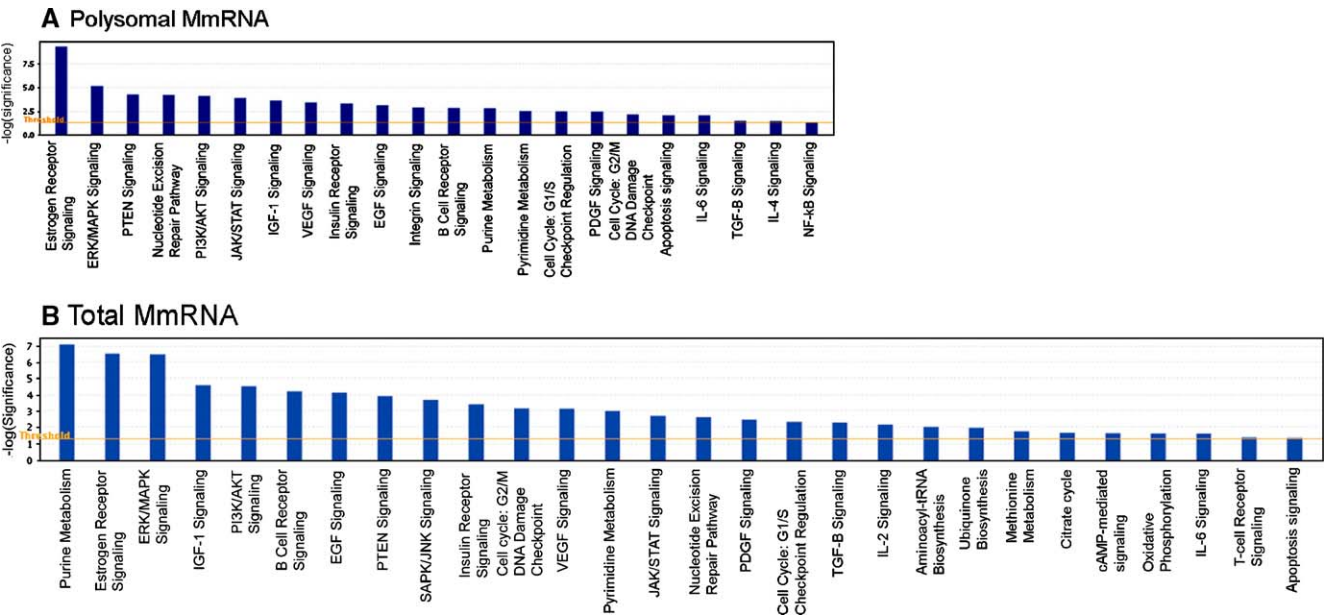


Fig. 5. Over-representation of functional categories in (A) MmRNAs detected on polysomes with less than three-fold differences between MII stage oocytes and one-cell embryos and (B) MmRNAs detected as less than three-fold different between total mRNA populations of MII stage oocytes and one-cell embryos using microarray data produced by Zeng et al. (2004). Threshold of significance was set at  $p=0.05$ .

believed to control MmRNA utilization are the CPE and PRE, and of course numerous other *cis*-regulatory elements have been identified as regulating mRNA stability or translation in somatic cells (e.g., Lopez de Silanes et al., 2005; Mazumder et al., 2005; Skabkina et al., 2005; Wu and Belasco, 2005; Padmanabhan and Richter, 2006; Weil and Beemon, 2006, to cite a few). The identification of over 2300 differentially translated MmRNAs provides a new database on which to base an analysis of putative translation regulatory elements within a population of MmRNAs that are preferentially translated at different stages. These putative regulatory elements can be subjected to functional evaluation on an individual basis.

We selected a total of 150 MmRNAs (50 translated more highly in oocytes, 100 translated more highly in one-cell embryos) for detailed sequence analysis to identify putative regulatory elements (Supplementary Tables 4 and 5). The MmRNAs were chosen without bias toward expression values. Both lists of transcripts were sorted by Affymetrix IDs. A population of transcripts was then selected randomly from these lists and used for the analysis, seeking only to ensure that the

selected mRNAs were distributed throughout each of the lists. Selections were made independently of such parameters as signal intensity, fold difference, or biological function. However, we used only transcripts for which the published full-length sequence included the poly(A) tail, ensuring that the crucial 3' UTR was present. Previously characterized CPEs were identified based on earlier studies of *Xenopus* and mouse MmRNAs (Simon et al., 1992; Oh et al., 2000). Genes with multiple transcript splice variants were scored positively if a known CPE was seen in any one variant. We also searched for putative PREs based on sequences published for *Xenopus* MmRNAs (Charlesworth et al., 2004). The proportion of transcripts containing putative CPEs identified based on those CPEs described previously differed noticeably between MmRNAs preferentially translated in the oocyte and those translated in the one-cell embryo (84% versus 41%, respectively) (Table 4). In contrast, 100% of the MmRNAs of both stages contained putative PREs.

Discussion

We have completed a detailed analysis of the polysomal MmRNA populations of mouse oocytes and late one-cell stage embryos. By focusing on the polysomal MmRNAs rather than total mRNA, and by focusing on the changes that occur during the first cell cycle, we were able to address four essential questions: To what degree does the polysomal MmRNA population change during the conversion from oocyte to embryo? What MmRNAs are recruited specifically during this transition? What biological processes are supported by the newly recruited MmRNAs? To what degree are different *cis*-regulatory mechanisms likely responsible for recruitment in oocytes versus fertilized embryos?

Table 4  
Prevalence of CPE and PRE elements in representative samples of MmRNAs enriched in MII and late one-cell stage polysomes

CPEs	Enriched at M-II (n=50) (%)	Enriched at one-cell stage (n=100) (%)
MmRNAs with known <sup>a</sup> CPEs	84	41
MmRNAs without known <sup>a</sup> CPEs	16	59
MmRNAs with PREs	100	100

n, number of polysomal MmRNAs analyzed.  
<sup>a</sup> Refers to CPEs previously identified in *Xenopus* or murine studies as indicated under Materials and methods.

The degree of polysomal MmRNA population change following fertilization has not been determined previously. Previous analyses of protein synthesis patterns (Latham et al., 1991) indicated that the array of synthesized proteins changes a great deal over the course of the one-cell stage. Those analyses, however, were limited to the most abundant proteins, and moreover the identities of the proteins were not determined. Other studies employing oligo(dT) primed RT-PCR assays, RT-PCR methods that selectively amplify polyadenylated mRNAs (Rambhatla et al., 1995; Alizadeh et al., 2005), or oligo(dT) primed microarray studies have also revealed some mRNAs that displayed transcription-independent increases in apparent abundance, potentially attributable to polyadenylation and recruitment during the one-cell stage (Hamatani et al., 2004; Wang et al., 2004; Zeng et al., 2004). These studies, however, can only reveal a fraction of recruited MmRNAs due to possible changes in translation initiation unrelated to polyadenylation (Hellen and Sarnow, 2001), translation-coupled degradation following recruitment (Wang and Latham, 2000), variability in poly(A) tail lengths before and after polyadenylation, and an overwhelming effect of the ongoing massive degradation of the MmRNA pool on relative abundances of more long-lived MmRNAs. Indeed, whereas our analysis revealed a total of 2692 differences between the polysomal MmRNA populations of oocytes and one-cell embryos, microarray studies examining total cell mRNA captured only a small fraction of these changes. For example, we evaluated a microarray data set of MII stage and  $\alpha$ -amanitin-treated one-cell embryos (Zeng et al., 2004) using the same analytical parameters as applied to our arrays and found only 1160 differences that reflected potential polyadenylation. Similarly, two other published studies (Wang et al., 2004; Hamatani et al., 2004) yielded many fewer changes in apparent MmRNA abundances over comparable developmental periods (93 and 232 affected mRNAs comparing these stages, respectively). Additionally, it is notable that a list of differentially expressed MmRNAs obtained by the analysis of total mRNA in previous studies is not equivalent to the list of preferentially translated MmRNAs. For example, of the 197 MmRNAs found by Zeng et al. (2004) to be enriched in MII oocytes, only 140 were included in our list of preferentially translated MmRNAs. Moreover, the much larger sizes of our lists of differentially translated MmRNAs versus the lists of differentially expressed MmRNAs observed by Zeng et al. (2004) points to the existence of a substantial number of MmRNAs that are expressed across both stages but undergo stage-specific translation. These comparisons illustrate the importance of examining polysomal mRNA populations directly, and the value of this study in advancing our understanding of the dynamic nature of the polysomal MmRNA population in the early embryo.

Our global analysis of the polysomal MmRNA population provides the most complete estimate to date of the fraction of MmRNAs that change significantly in the polysomal MmRNA population during the one-cell stage. A moderately stringent cutoff of two-fold difference revealed changes in nearly one third (29%), and a three-fold cutoff revealed changes in 15% of the mRNAs. By either measure, it is clear that the transition

from oocyte to one-cell embryo is accompanied by a dramatic change in the array of translating MmRNAs.

Our analysis also revealed changes in the functional classes of proteins encoded by recruited MmRNAs. Most striking is the switch from an emphasis on homeostatic processes in the oocyte to biosynthetic processes in the late one-cell embryo. The largest category of MmRNA preferentially translated in oocytes was related to homeostatic processes, but this category diminished greatly in one-cell embryos, indicating that the ovulated oocyte devotes a higher relative degree of activity to maintaining its cellular integrity. By contrast, we observed an increase, from 9% to 21%, in the proportion of preferentially translated MmRNAs involved in metabolism from oocyte to late one-cell stage, respectively. Combining the MmRNAs at the two stages revealed that about one fifth (19%) of the change in MmRNA utilization affects genes in this category. This indicates that metabolic processes likely change a great deal during this period. We also observed that 5% of the MmRNAs preferentially translated at the late one-cell stage were related to oxidoreductase activity, electron transporter activity, etc. The recruitment of these MmRNAs might help to meet the increasing metabolic demands of the one-cell embryo. Additionally, there were significant changes in the specific arrays of MmRNAs being translated within some functional classes (e.g., metabolism, transcription, translation, signal transduction), further attesting to dynamic changes in developmental requirements.

This switch from an emphasis on translating MmRNAs related to homeostasis to one encoding proteins related to metabolism is somewhat reminiscent of differences revealed by EASE analysis of microarray data obtained using total mRNA (Zeng et al., 2004). For example, major categories represented among the “maternal” and “maternal-zygotic” groups included those related to signal transduction, electron transport, and metabolism, whereas the “transient one-cell” group included mRNAs related to protein biosynthesis and modification, proliferation, transcription, and DNA metabolism. Our observations, combined with the observed decrease in abundances of the MmRNAs in the study by Zeng et al. (2004), indicate that many of the MmRNAs related to homeostasis are recruited during oocyte maturation and then progressively degraded. This decline in the ability of the cell to provide for basic cellular homeostatic processes must then be reversed after fertilization by the subsequent recruitment of MmRNAs encoding proteins to promote biogenesis.

One class of particular interest among the MmRNAs recruited during the one-cell stage encodes proteins related to gene transcription. Stage-dependent recruitment of MmRNAs encoding transcription factors may help initiate gene transcription (Wang and Latham, 1997; Wang et al., 2001). The late one-cell embryo indeed acquires the ability to undertake gene transcription (Latham, 1999; Latham et al., 1992), and specific transcription factors appear at the two-cell stage at the time of the MGA (Wang and Latham, 2000; Kaneko et al., 1997). We observed a number of transcripts for gene products involved in transcription (e.g., E2F transcription factor 1, RNA polymerase 1–3, MYST histone acetyltransferase 1, and SWI/SNF-related

family proteins) recruited at the late one-cell stage, providing further support for this model. Other transcription factor mRNAs may be recruited at the two-cell stage.

Twenty-five transcripts involved in apoptosis are translated more highly in one-cell embryos, compared to nine translated more highly in MII oocytes. Among these 25 MmRNAs, only three (Bcl2-like 1, MKL (megakaryoblastic leukemia)/myocardin-like 1, glutaminyl-tRNA synthetase) encode anti-apoptotic proteins. Earlier studies suggested that it is critical for the embryo to express its genome and direct the production of additional anti-apoptotic factors during the first two cell cycles (Jurisicova et al., 1998). This requirement may provide a selective mechanism to promote the elimination of unfit embryos (Jurisicova et al., 1998). Because our analysis was undertaken with  $\alpha$ -amanitin-treated one-cell embryos, our results focus specifically on MmRNAs and thus would not be expected to include newly transcribed mRNAs that suppress apoptosis. Our analysis, however, indicates that the population of recruited MmRNAs may be biased towards apoptosis, making transcription of anti-apoptotic genes an important early event.

Other genes recruited in one-cell embryos included cell cycle regulators, such as cyclins (M2, M4, B3, I, F) and anaphase promoting complex. The cyclins bind to and activate cyclin-dependent kinases (CDKs), controlling nuclear cell division cycles (Grana and Reddy, 1995). The anaphase-promoting complex prepares the cell for division by allowing it to progress through metaphase to anaphase and thus must be available for the first mitosis.

The stage-dependent increase in abundance of transcription factors and other proteins required for cellular function is likely facilitated by an increase of proteins required for translation. It was interesting to find that many transcripts encoding translation initiation, elongation, and termination factors were recruited by the late one-cell stage. A total of 18 transcripts were directly involved in these mechanisms in the late one-cell polysomal MmRNA population. A total of 41 ribosomal proteins (including 11 mitochondrial ribosomal proteins) out of a total of 89 expressed transcripts involved in translation are enriched in the late one-cell stage polysomal MmRNA population. The recruitment of these MmRNAs likely increases the translation capacity of the cell. This may help to increase further the recruitment of other MmRNAs, as well as promoting genome activation by the production of transcription factors (Worrad et al., 1994; Latham, 1999), and then recruitment of early embryonic transcripts. These events thus serve to guide the transition from an oocyte phenotype to an embryonic one, ensuring the initiation of the developmental program and driving it forward.

Our data provide an estimate of the overall proportion of translated MmRNAs regulated by previously described putative CPEs before and after fertilization. There is a dramatic change in the abundance of such putative CPEs among stage-specifically translated MmRNAs from the oocyte to the late one-cell stage. Most (84%) MmRNAs preferentially translated in oocytes contained such previously described CPEs, compared to only 41% of the MmRNAs recruited during deve-

lopment to the late one-cell stage. In contrast, putative PREs were found in all preferentially recruited transcripts at both stages. This indicates that the CPEs that have been described to date may be preferentially employed for MmRNA recruitment in oocytes. Additional CPEs may be needed to support the time-dependent recruitment of other MmRNAs at multiple different points during the first cell cycle in order to produce the observed complex pattern of protein synthesis (Latham et al., 1991). The existence of an array of previously uncharacterized CPEs, if confirmed, would be useful for exploring the molecular mechanisms that regulate translation in the early embryo. The PRE may also assume a larger role during the one-cell stage as well.

It is also of interest that the translation of the mRNAs encoding CPEB1 and CPEB4 is elevated in the oocyte. This may facilitate translational recruitment of CPE-containing mRNAs during oocyte maturation. A reduction in the rates of synthesis of these proteins in the one-cell embryo could be related to the shift in reliance upon different CPEs for translational recruitment in the zygote.

Overall, by applying the microarray technology to a specific functional class of mRNA rather than the total cell mRNA population, our analysis has revealed a dynamically changing polysomal MmRNA population of the mouse embryo and provided a novel resource for the design of new functional studies to understand the role of MmRNAs in development. Further, this analysis revealed specific biological processes that are likely critical for preparing the fertilized embryo for subsequent development. The analysis of polysomal MmRNA populations at additional stages in early embryo development should provide an even greater understanding of the mechanisms that regulate cell phenotype prior to genome activation. Solving this puzzle constitutes one of the major remaining goals in understanding early development in a variety of species. Finally, our analysis of the polysomal MmRNA population should increase our understanding of the molecular determinants of oocyte and embryo quality and should benefit both applied and clinical areas of reproductive biology.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.ydbio.2006.06.024](https://doi.org/10.1016/j.ydbio.2006.06.024).

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