Published in final edited form as:

Mol Reprod Dev. 2008 August ; 75(8): 1258–1268.

Mos 3' UTR Regulatory Differences Underlie Species-Specific Temporal Patterns of Mos mRNA Cytoplasmic Polyadenylation and Translational Recruitment During Oocyte Maturation

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Abstract

The Mos proto-oncogene is a critical regulator of vertebrate oocyte maturation. The maturation-dependent translation of Mos protein correlates with the cytoplasmic polyadenylation of the maternal Mos mRNA. However, the precise temporal requirements for Mos protein function differ between oocytes of model mammalian species and oocytes of the frog *Xenopus laevis*. Despite the advances in model organisms, it is not known if the translation of the human Mos mRNA is also regulated by cytoplasmic polyadenylation or what regulatory elements may be involved. We report that the human Mos 3' untranslated region (3' UTR) contains a functional cytoplasmic polyadenylation element (CPE) and demonstrate that the endogenous Mos mRNA undergoes maturation-dependent cytoplasmic polyadenylation in human oocytes. The human Mos 3' UTR interacts with the human CPE-binding protein and exerts translational control on a reporter mRNA in the heterologous *Xenopus* oocyte system. Unlike the *Xenopus* Mos mRNA, which is translationally activated by an early acting Musashi/polyadenylation response element (PRE)-directed control mechanism, the translational activation of the human Mos 3' UTR is dependent on a late acting CPE-dependent process. Taken together, our findings suggest a fundamental difference in the 3' UTR regulatory mechanisms controlling the temporal induction of maternal Mos mRNA polyadenylation and translational activation during *Xenopus* and mammalian oocyte maturation.

Keywords

Mos; oocyte; human; mRNA; translation; cytoplasmic polyadenylation; CPE

INTRODUCTION

Despite a critical role in the control of human fertility, the mechanisms regulating human oocyte maturation are not well characterized. In model organisms, accumulation of critical cell cycle

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regulatory proteins during oocyte meiotic maturation depends upon the regulated translation of maternally derived mRNAs (Wickens et al., 2000; Mendez and Richter, 2001). The maternal mRNA encoding the Mos proto-oncogene is subject to tight translational regulation in oocytes from a variety of vertebrate species. The Mos protein is a serine/threonine kinase which activates the MAP kinase cascade through direct phosphorylation of the MAP kinase activator MEK (Posada et al., 1993; Shibuya et al., 1996). In the mouse, Mos protein is absent from immature oocytes and maturation-dependent cytoplasmic polyadenylation correlates with the translational activation of the maternal Mos mRNA at or after germinal vesicle breakdown (GVBD; Gebauer et al., 1994). While mouse meiotic cell cycle progression does not depend on Mos mRNA translation, Mos protein function is necessary for arrest of the mature oocyte at meiotic metaphase II (Colledge et al., 1994; Hashimoto et al., 1994; Araki et al., 1996; Hashimoto, 1996). In the frog, *Xenopus laevis*, Mos mRNA cytoplasmic polyadenylation and translational activation is an early event occurring several hours prior to GVBD (Sheets et al., 1994; Ballantyne et al., 1997; de Moor and Richter, 1997; Charlesworth et al., 2004). In addition to a common requirement for meiotic metaphase II arrest shared with mammalian oocytes (Sagata et al., 1989; Daar et al., 1991), *Xenopus* Mos protein function is also required earlier during oocyte maturation to mediate the Meiosis I to Meiosis II transition (Gross et al., 2000; Dupre et al., 2002). It has not been determined if the differences in temporal activation of the Mos mRNA in *Xenopus* and mammals reflects distinct 3' UTR regulatory element composition or if *Xenopus* and mammalian oocytes exert differential temporal regulation of the same mRNA translational control pathway.

Meiotic cell cycle progression has been best characterized in *Xenopus*, where the cytoplasmic polyadenylation and translational activation of select maternal mRNAs occur in a strict temporal order (Sagata et al., 1988; Sheets et al., 1994, 1995; Ballantyne et al., 1997; de Moor and Richter, 1997; Ferby et al., 1999; Howard et al., 1999; Charlesworth et al., 2000; Nakajo et al., 2000; Hochegger et al., 2001; Dupre et al., 2002). The ability to regulate addition of a poly[A] tail extension in the oocyte cytoplasm requires 3' UTR regulatory sequences, including cytoplasmic polyadenylation elements (CPE) (reviewed in Mendez and Richter, 2001) and Musashi/polyadenylation response elements (PRE; Charlesworth et al., 2002, 2004, 2006). CPE sequences have been shown to repress mRNA translation in immature oocytes and to direct late class mRNA cytoplasmic polyadenylation and translational activation in maturing oocytes. Both aspects of CPE function appear to require the CPE-binding protein (CPEB1) (Fox et al., 1989; McGrew et al., 1989; McGrew and Richter, 1990; Paris and Richter, 1990; Sallès et al., 1992; Standart and Dale, 1993; Gebauer et al., 1994; Stebbins-Boaz et al., 1996; Stutz et al., 1998; Minshall et al., 1999; de Moor and Richter, 1999; Barkoff et al., 2000; Charlesworth et al., 2000; Tay et al., 2000). However, the translational activation of early class *Xenopus* maternal mRNAs is directed in a CPE- and CPEB1-independent manner (Charlesworth et al., 2002, 2004). In the case of the *Xenopus* Mos mRNA, early translational activation is directed by a PRE-dependent pathway (Charlesworth et al., 2002). The PRE-dependent translational activation of the *Xenopus* Mos mRNA has been recently shown to be regulated by Musashi, a Mos PRE-specific RNA binding protein (Charlesworth et al., 2006). Curiously, the ability of Musashi to direct mRNA translational activation during *Xenopus* oocyte maturation contrasts to the role of Musashi in neural stem cells where it exerts target mRNA translational repression (Okano et al., 2005). While consensus CPE sequences are present in the 3' UTR of the Mos mRNA from a variety of vertebrate species, the possible contribution of Musashi and PRE sequences to mammalian Mos mRNA translational control have not been addressed.

Previous studies employing inhibition of protein synthesis in general, or targeted ablation of the endogenous Mos mRNA, have suggested a role for regulated Mos mRNA translational control during human oocyte maturation (Pal et al., 1994; Hashiba et al., 2001). However, while a human CPEB1 protein has been characterized and shown to be expressed in human oocytes

(Welk et al., 2001), it has not been determined if regulated cytoplasmic polyadenylation of the maternal Mos mRNA occurs during human oocyte maturation. In this study we report that the human Mos 3' UTR contains a functional CPE sequence, interacts with the human CPEB1 protein and directs maturation-dependent cytoplasmic polyadenylation of the endogenous Mos mRNA. Unlike the *Xenopus* Mos mRNA, we find no evidence for Musashi/PRE-directed regulation of the human Mos mRNA. We propose that species-specific differences in 3' UTR regulatory element composition contribute to the differential temporal activation of Mos mRNA translation during *Xenopus* and mammalian oocyte maturation.

MATERIALS AND METHODS

Human Mos 3' UTR Constructs and CPE Mutants

Construction of pGEM GST β -globin 3' UTR and pGEM GST *XeMos* 3' UTR has been described elsewhere (Charlesworth et al., 2000, 2002). pGEM GST hMos 3' UTR was constructed by amplifying the human Mos 3' UTR (119 bp) by PCR from HeLa cells and cloned into *Bam*HI and *Xba*I sites of pGEM GST (Charlesworth et al., 2000). The primers were designed to encode a BamHI site upstream of the endogenous stop codon (bold): 5'(+)
CGCGGATCCT**TAGCTG**AAAACCTGGT-CAAGATAAG and a 3' *Xba*I site: 5'(-)
CGGTCTAGA-TAAAGGAGTTTT**AGTAACTTTATTT**. Subsequent PCR mutagenesis of the hMos 3' UTR was performed using a QuikChange Site-Directed Mutagenesis kit as per manufacturer's instructions (Stratagene, Cedar Creek, TX). All hMos 3' UTR mutations were verified by DNA sequence analysis.

Electrophoretic Mobility Shift Assay (EMSA) Probe Constructs

The GST fragment in the wild-type and mutant pGEM GST hMos 3' UTR plasmids was deleted by digesting with Nco I and Xho I, klenow treated and blunt end ligated. The resulting plasmids, were used for the in vitro transcription of radiolabeled hMos 3' UTR EMSA probes as previously described (Charlesworth et al., 2000; Welk et al., 2001). Similarly, Nco I/Xho I deletion of the GST fragment in pGEM GST β -globin 3' UTR generated a template for in vitro transcription of the β -globin 3' UTR EMSA probe. All EMSA reactions, unlabeled probe competition and antibody supershifts were as previously described (Welk et al., 2001).

Human Oocyte Collection and RNA Extraction

Human immature (GV) and mature (MII) oocytes were obtained with informed consent from patients undergoing in vitro fertilization (IVF) and/or Intra Cytoplasmic Sperm Injection (ICSI) after standard ovarian stimulation (Mahadevan et al., 1998). Oocytes were denuded with Hyaluronidase (Sigma, St. Louis, MO) and mechanical pipetting. Oocytes were examined for nuclear maturity and classified as either mature (MII) or immature (GV). The oocytes were lysed in 500 μ l of RNA Stat-60 and frozen immediately in liquid nitrogen. Total RNA from these oocytes was extracted as follows: 100 μ l of chloroform was added, mixed vigorously, incubated at room temperature for 3 min and the upper aqueous phase was collected after centrifugation at 12,000g for 3 min. The RNA was precipitated by adding isopropanol, incubating at 4°C for 30 min and subsequent centrifugation at 12,000g for 15 min at 4°C. Following a 70% ethanol wash, total RNA was resuspended in RNase-free water (2.5 μ l/oocyte).

Xenopus Oocytes, mRNA Injections and Sample Preparation

Xenopus oocyte isolation and culture has been described (Machaca and Haun, 2002). To analyze progesterone-inducible translation, GST reporter RNA levels were analyzed on agarose gels and normalized within each experimental set prior to injection into oocytes (typically 0.1 ng of reporter RNA per oocyte) as previously described (Charlesworth et al.,

2000). Were indicated, oocytes were injected with in vitro transcribed RNA encoding a GST open reading framed fused to either the last 48 nucleotides of the *Xenopus* Mos 3' UTR (Charlesworth et al., 2002) or the entire 119 nucleotide human Mos 3' UTR generated in this study. Oocytes were stimulated with 2 µg/ml progester-one (Sigma) and the rate of germinal vesicle breakdown (GVBD) monitored morphologically by the appearance of a white spot on the animal hemisphere. Pools of 5–10 oocytes were harvested and immature control samples were prepared at the same time as the progesterone-stimulated oocyte samples. For analysis of both protein and RNA from the same oocyte samples, pools of oocytes were rapidly lysed in Nonidet P-40 buffer as above and then a portion was removed and immediately mixed with RNA STAT-60 as previously described (Charlesworth et al., 2002). Results shown are representative experiments that were repeated three times with similar results.

Western Blot Analyses

The preparation of protein lysates and ECL Western blot analyses were performed as previously described (Howard et al., 1999; Charlesworth et al., 2000; Welk et al., 2001). For all gels, equivalent total protein was loaded for each sample. Rabbit polyclonal antibody against Glutathione S-Transferase (GST) (Z-5) was obtained from Santa Cruz Biotechnology, Santa Cruz, CA. and mouse monoclonal antibody against tubulin was obtained from Sigma. GST protein accumulation was quantitated using a ChemiImager 5500 and AlphaEaseFC software (AlphaInnotech Corp., San Leandro, CA).

RNA Ligation-Coupled PCR Polyadenylation Assays

The polyadenylation status of GST reporter or endogenous *Xenopus* Mos and cyclin A1 mRNAs were assessed by RNA ligation-coupled PCR (Rassa et al., 2000) essentially as described previously (Charlesworth et al., 2002, 2004, 2006). Briefly, total RNA from *Xenopus* or human oocytes was isolated by RNA-STAT60 and a kinased primer, P1 (5' pGGT CAC CTT GAT CTG AAG C), was ligated to the RNA 3' termini. The P1 primer contained a 3' amino modification to prevent concatemerization. Subsequently, reverse transcription was driven using a primer complementary to the ligated P1 anchor sequence (P1', 5' GCT TCA GAT CAA GGT GAC CTT TTT). PCR amplification was then performed using 1 µl of cDNA (0.1 oocyte equivalents) and an appropriate forward primer specific to the GST (5' ACC ATC CTC CAA AAT CGG ATC TGC) or human Mos (5' CGG TTG CTC TGA GAA GTT GGA AGA) coding regions or the *Xenopus* Mos (5' GTT GCA TTG CTG TTT AAG TGG TAA) or cyclin A1 (5' CAT TGA ACT GCT TCA TTT TCC CAG) 3' UTRs and the P1' reverse primer complementary to all ligated mRNAs in the sample. For the analysis of human Mos polyadenylation, 5 µl of cDNA (0.4 oocyte equivalents) was used with the human Mos forward primer and reverse primer P1'. The PCR amplification conditions for all reactions were: 94°C for 2 min, and then 40 cycles of [94°C for 30 sec, 56°C 1 min, 72°C for 1 min and 30 sec] and PCR products were analyzed on a 1.5% agarose gel. An increase in PCR product size is indicative of poly[A] tail extension and where noted polyadenylation was verified by direct sequencing of the excised PCR products.

RESULTS

The Endogenous Mos mRNA is Polyadenylated in a Maturation-Dependent Manner in Human Oocytes

The previously reported human Mos cDNA sequence only included 22 nucleotides of the 3' UTR and lacked a consensus polyadenylation hexanucleotide (Watson et al., 1982). Consequently, an assessment of the possible role of CPE-directed polyadenylation in the control of human Mos mRNA translational activation has not hitherto been addressed. We amplified the 3' UTR of the human Mos mRNA from both HeLa cells and human oocyte total RNA by ligation-coupled PCR (Rassa et al., 2000). The DNA sequence of the Mos 3' UTR

was identical from both sources (Fig. 1). The entire 119 nucleotide human Mos 3' UTR was then cloned from Hela cells using UTR-specific PCR primers. Analysis of the human Mos 3' UTR sequence revealed a U₅A CPE consensus sequence 5' of the polyadenylation hexanucleotide (Fig. 1A). A similar CPE sequence is present at the same position in the rodent Mos 3' UTRs (Fig. 1B) and has been shown to be a functional CPE in the murine Mos 3' UTR (Gebauer et al., 1994). While the murine and rat Mos 3' UTRs contain a second U₅A CPE sequence closer to the polyadenylation hexanucleotide (Fig. 1B), the human and monkey Mos 3' UTRs lack the 5'-most U and only retain a U₄A sequence (Fig. 1A dashed oval). It was not clear if the U₄A sequence would be sufficient to function as a CPE in the human Mos 3' UTR.

Given the prevalence of Mos mRNA translational regulation in model organisms and the presence of a consensus CPE sequence in the human Mos 3' UTR, we wished to determine if the endogenous human Mos mRNA was subject to maturation-dependent poly-adenylation as an indicator of mRNA translational activation. To address this issue we employed RNA ligation coupled RT-PCR to assess the polyadenylation status of the Mos mRNA in vivo. We have recently employed this very sensitive technique to assess endogenous maternal mRNA polyadenylation during *Xenopus* oocyte maturation (Charlesworth et al., 2002, 2004). Using this assay, an increase in the size of the PCR product is indicative of an increase in the length of the poly[A] tail. Using a forward primer specific to the human Mos mRNA coding region and a primer which is complementary to the DNA oligonucleotide ligated to the end of all the RNAs in the population (P1', see Experimental Procedures) we observed a 520 bp PCR product from immature, germinal vesicle positive (GV) human oocytes and a heterogeneous population of PCR products ranging from 580 to 620 bp in mature Metaphase II (MII) human oocytes (Fig. 1C, arrowhead and bracket, respectively). The amplified Mos PCR products both from GV and mature MII oocytes were directly excised and subjected to DNA sequencing. The DNA sequence confirmed that only the human Mos 3' UTR was amplified and that the increased size of PCR product observed in MII oocytes was specifically due to increased length of poly [A] tail. This experiment has been repeated three separate times using oocytes obtained from three independent patients (in each case the GV and MII oocytes were derived from the same donor). The poly[A] tail of the Mos mRNA in immature oocytes was small (around 20 adenylate residues) whereas in the mature, MII oocytes poly[A] tail length is around 60–100 adenylate residues. This increase in Mos mRNA poly[A] tail length is similar to the increase Mos mRNA poly[A] tail length observed during mouse, rat and *Xenopus* oocyte maturation (Goldman et al., 1988; Sheets et al., 1994; Lazar et al., 2002). Our results indicate that the endogenous Mos mRNA undergoes maturation-dependent polyadenylation in human oocytes.

The Human Mos 3' UTR Interacts With hCPEB1 In Vitro

We have previously reported that human CPEB1 (hCPEB1) is expressed in human oocytes where it may regulate the translational activation of maternal mRNAs during human oocyte maturation (Welk et al., 2001). To extend these observations we wanted to test if hCPEB1 could interact with the human Mos 3' UTR. To this end, GST and chimeric GST-hCPEB1 fusion proteins were prepared by coupled in vitro transcription and translation in rabbit reticulocyte lysates (Fig. 2A) as previously described (Welk et al., 2001) and incubated in vitro with either radiolabeled wild-type or mutant human Mos 3' UTR RNA probes (Fig. 2B) in RNA-EMSA studies.

Incubation of the human Mos 3' UTR probe with reticulocyte lysate expressing the GST-hCPEB1 fusion protein resulted in formation of a specific complex (Fig. 2C; lane 4, arrowhead). To confirm the specificity of hCPEB1 complex formation with the human Mos 3' UTR probe, the complex formation was challenged with 50-fold molar excess of unlabelled RNA probe competitors. Upon addition of unlabelled, wild-type human Mos 3' UTR probe (specific competitor) the formation of the specific complex was abolished (Fig. 2C, lane 5). Addition

of unlabelled human Mos 3' UTR probe with a disrupted U₅A sequence (mutant 2) had little affect on the formation of the specific complex (Fig. 2C, lane 6), but did eliminate the faster migrating non-specific complex. These findings confirm that the U₅A sequence is the hCPEB1 target site within the human Mos 3' UTR, since mutation of U₅A sequence abolishes the ability of the unlabeled Mos UTR probe to compete for specific complex formation. The non-specific complex was observed with either addition of un-programmed reticulocyte lysate or lysate expressing the GST moiety alone (Fig. 2C; lanes 2 and 3, open circle). The formation of non-specific complexes has been previously observed with rabbit reticulocyte lysates in EMSA assays when wild-type or CPE-disrupted *Xenopus* Mos and Wee1 3' UTRs were employed (Charlesworth et al., 2000; Welk et al., 2001). The specificity of the hCPEB1 complex was further verified by challenging with GST antibodies that resulted in a super-shift (enhanced retardation) of the GST-hCPEB1/RNA complex (Fig. 2C, lane 7). The GST antibodies did not affect the formation of the non-specific complex indicating that the GST fusion protein was not involved in this complex. As a further specificity control, antibodies to the unrelated B-Raf protein did not induce a super-shift of the GST-CPEB1 complex (Fig. 2C, lane 8). These results indicate that the hCPEB1 protein specifically interacts with the human Mos 3' UTR.

To verify that the U₅A sequence was the target of hCPEB1 within the human Mos 3' UTR, a series of EMSA reactions were performed using either wild-type or mutant human Mos 3' UTRs (Fig. 2D). Human Mos UTR mutant 1 encoded a disruption of the U₄A sequence adjacent to the polyadenylation hexanucleotide, Mos UTR mutant 2 encoded a disruption of the 5' U₅A CPE sequence and Mos UTR mutant 3 encoded disruptions of both the U₅A and U₄A sequences (see Fig. 2B). Specific complex formation with hCPEB1 was only observed when wild-type or Mos UTR mutant 1 probes were utilized (Fig. 2D, lanes 4 and 5). No specific complex formation was observed with Mos mutant 2 or 3 UTR probes (Fig. 2D, lanes 6 and 7). These results indicate that hCPEB1 can interact specifically with the U₅A CPE in the human Mos 3' UTR but that hCPEB1 does not interact with the U₄A sequence. As additional controls, no specific complex formation was observed with a β -globin 3' UTR probe (which lacks CPE sequences; Fig. 2D, lane 8) or when unprogrammed or GST moiety alone lysates were used (Fig. 2D, lanes 2 and 3 respectively).

The Human Mos 3' UTR Exerts Translational Regulation in *Xenopus* Oocytes

We next sought to determine if the U₅A CPE sequence in the Mos 3' UTR could direct translational regulation. Prior studies have shown that the heterologous *Xenopus* oocyte and embryo systems are extremely useful to examine evolutionarily conserved mRNA translational regulatory elements (Gebauer and Richter, 1996; Verrotti et al., 1996; Thompson et al., 2000; Knaut et al., 2002). We thus fused the entire 119 nucleotide human Mos 3' UTR downstream of a GST reporter RNA and injected the chimeric RNA into immature *Xenopus* oocytes. As controls for this experiment, immature *Xenopus* oocytes were separately injected with GST reporter RNAs fused to either the 153 nucleotide *Xenopus* β -globin 3' UTR or the terminal 48 nucleotides of the *Xenopus* Mos 3' UTR. The β -globin 3' UTR is not subject to regulated mRNA translational activation (Hyman and Wormington, 1988; Charlesworth et al., 2000). The last 48 nucleotides of the *Xenopus* Mos 3' UTR contains both PRE and CPE sequences and directs translational repression in immature oocytes and early (PRE-dependent) translational induction in maturing oocytes (Sheets et al., 1994, 1995; Stebbins-Boaz et al., 1996; Charlesworth et al., 2002). In this latter regard, the 48 nucleotide Mos 3' UTR recapitulates the translational activation of the endogenous Mos mRNA during progesterone-stimulated *Xenopus* oocyte maturation (Charlesworth et al., 2002). The injected oocytes were then split into two pools and either left untreated (immature) or stimulated with progesterone to induce oocyte maturation. Total RNA and protein lysates were prepared from the same pooled oocyte samples after 16 hr of culture and polyadenylation of the reporter RNA assessed by RNA ligation coupled PCR. Reporter RNA translation was assessed by Western blot for

GST protein accumulation. As expected, the GST reporter RNA fused to the β -globin 3' UTR was not polyadenylated in progesterone-stimulated oocytes (Fig. 3A). By contrast, the human Mos 3' UTR behaved similarly to the *Xenopus* Mos 3' UTR and directed polyadenylation (retarded mobility) in response to progesterone stimulation. DNA sequence analysis confirmed that the increased size of the PCR products in progesterone-stimulated oocytes was due to an increased size of the poly[A] tail.

Analysis of the protein lysates from the same pooled oocyte samples analyzed for polyadenylation in Figure 3A, revealed that GST protein accumulation under the control of the β -globin 3' UTR was similar in immature and progesterone-stimulated oocytes as expected, whereas the human Mos 3' UTR exerted translational regulation of GST protein accumulation (Fig. 3B). The human Mos 3' UTR, like the *Xenopus* Mos 3' UTR, directed translational repression in immature oocytes (where the level of GST accumulation was less than that observed with the β -globin 3' UTR) and this repression was relieved in progesterone-stimulated oocytes.

We next utilized the wild-type and mutant human Mos 3' UTRs shown in Figure 2A, to determine if the cytoplasmic polyadenylation and translational control exerted by the human Mos 3' UTR was regulated by the U₅A CPE. Chimeric GST reporter RNAs fused to the wild-type or mutant human Mos 3' UTRs were injected into immature *Xenopus* oocytes. As can be seen in Figure 3C, the length of the wild-type and mutant Mos 3' UTRs were indistinguishable in immature oocytes (Fig. 3C). Following progesterone stimulation, only the reporter mRNAs retaining the U₅A CPE (wild-type and mutant 1 human Mos UTRs) were able to direct maturation-dependent polyadenylation. Disruption of the U₅A CPE sequence (mutant 2) abolished progesterone-stimulated cytoplasmic polyadenylation of the GST reporter RNA, indicating that the U₅A sequence is a *bona fide* CPE. Curiously, the length of poly[A] tail was significantly longer in the absence of the polyadenylation hexanucleotide adjacent U₄A sequence (mutant 1).

Consistent with the polyadenylation data observed in Figure 3C, translational regulation of the GST reporter RNA also required the 5' U₅A CPE sequence. Both the U₅A CPE-containing reporter mRNAs (wild-type and mutant 1 Mos UTR) repressed GST accumulation in immature oocytes and this repression was relieved in progesterone-stimulated oocytes (Fig. 3D). By contrast, the mutant 2 UTR reporter chimera which lacked the U₅A CPE lost the ability to exert translational regulation as evidenced by similar levels of GST protein accumulation in immature and progesterone-stimulated oocytes. The mutant 1 Mos UTR consistently directed accumulation of GST to levels that exceed the levels controlled by the wild-type human Mos 3' UTR. Our results indicate that the U₅A CPE sequence is necessary for the translational regulation exerted by the wild-type human Mos 3' UTR. Moreover, CPE-directed polyadenylation correlates with the relief of repression and translational induction in mature oocytes.

Differential Temporal Polyadenylation of the Human and *Xenopus* Mos 3' UTRs in Maturing Oocytes

The *Xenopus* Mos mRNA is polyadenylated and translationally activated early during progesterone-stimulated oocyte maturation. This early polyadenylation significantly precedes GVBD and is regulated by a Musashi/PRE sequence, in a CPE-independent manner (Charlesworth et al., 2002, 2004, 2006). In contrast to Musashi/PRE-directed polyadenylation, CPE-directed polyadenylation was shown to occur later during maturation, around the time of GVBD (Charlesworth et al., 2002, 2004). The available evidence suggests that the late acting CPE sequence in the *Xenopus* Mos 3' UTR functions to maintain the extended poly[A] tail after oocyte GVBD. Our recent work has identified Musashi as the Mos PRE-specific binding protein and shown that Musashi function is necessary for early class mRNA translational

activation (Charlesworth et al., 2006). Indeed, the Mos PRE contains a consensus Musashi binding site (A/G)U₁₋₃AGU (Imai et al., 2001) that is essential for Mos PRE function (Charlesworth et al., 2006). However, no (A/G)U₁₋₃AGU consensus Musashi binding sequence is present in the rat, mouse, monkey or human Mos 3' UTR sequences suggesting that the temporal control of Mos mRNA translational induction may be fundamentally different between *Xenopus* and these mammalian species. To directly assess possible temporal differences in the ability of the *Xenopus* and human Mos 3' UTRs to induce maturation-dependent cytoplasmic polyadenylation, we injected GST reporter RNAs into immature *Xenopus* oocytes and analyzed the time course of induced polyadenylation in response to progesterone stimulation (Fig. 4), rather than simply analyze the fully mature oocytes samples as shown in Figure 3A,C. Consistent with earlier studies, Musashi/PRE-dependent early polyadenylation of the *Xenopus* Mos 3' UTR begins prior to GVBD (Fig. 4, upper left panel, 1 hr after progesterone stimulation). The length of the *Xenopus* Mos 3' UTR continued to increase until the oocytes completed GVBD. In the same RNA sample preparations, the endogenous CPE-dependent *Xenopus* cyclin A1 mRNA was polyadenylated later in maturation at a time when the oocytes have completed GVBD (Fig. 4, lower left panel) consistent with prior studies (Sheets et al., 1994; Charlesworth et al., 2004). In contrast to the *Xenopus* Mos 3' UTR, polyadenylation regulated by the human Mos 3' UTR occurred significantly later in maturation, at a time when the oocytes had completed GVBD. The profile of polyadenylation directed by the hMos 3' UTR was similar to the endogenous *Xenopus* cyclin A1 mRNA analyzed from the same sample preparations (Fig. 4, right panels). Thus, when placed in a cellular context competent to mediate early mRNA translational activation, the human Mos 3' UTR directed only late, CPE-dependent control. These findings are consistent with the human Mos 3' UTR lacking a Musashi/PRE sequence. We conclude that 3' UTR regulatory differences between the *Xenopus* and human Mos 3' UTRs confer distinct temporal regulation of mRNA translational activation as assessed during *Xenopus* meiotic cell cycle progression.

DISCUSSION

In this study we report that the human Mos mRNA undergoes cytoplasmic polyadenylation in human oocytes in a maturation-dependent manner. This finding supports a model in which human oocyte maturation is regulated by polyadenylation and translational activation of select maternal mRNAs, including the Mos mRNA. Indeed, it has been shown that inhibition of protein synthesis with cyclohexamide or microinjection of Mos antisense oligonucleotides perturb human oocyte maturation (Pal et al., 1994; Hashiba et al., 2001). Furthermore, analyses of the 3' UTR database (Mignone et al., 2005) indicate that at least 11% of human 3' UTRs have a U₅A₁₋₂U CPE consensus sequence implying that in addition to regulating translation of the Mos mRNA, CPE-dependent translational control may regulate translation of multiple mRNAs during human oocyte maturation.

Using the heterologous *Xenopus* oocyte system we demonstrate that the human Mos 3' UTR has a single functional CPE of sequence U₅A. This CPE directs translational repression of a reporter RNA in immature oocytes and this repression is relieved as the oocytes re-enter the meiotic cell cycle and progress through maturation. The relief of repression correlates with the maturation-dependent cytoplasmic polyadenylation of the human Mos 3' UTR. It is interesting to note that unlike lower vertebrates, primate (human and monkey) Mos mRNA 3' UTRs appear to have one functional CPE sequence. The murine Mos 3' UTR has two functional CPE sequences, either of which can direct maturation-dependent cytoplasmic polyadenylation (Gebauer et al., 1994). Similarly, rat and pig Mos 3' UTR sequences have been reported (van der Hoorn and Firzlauff, 1984; Newman and Dai, 1996) and are predicted to contain two functional U₅A CPEs. While mutational disruption of the first CPE sequence (U₅A) completely ablated maturation-dependent polyadenylation of the human Mos mRNA (Fig. 3C), we show that the second U₅A CPE in rodent and porcine Mos 3' UTRs is a GU₄A sequence in primates

(Fig. 1) and fails to interact with the human CPEB1 protein (Fig. 2C). The GU₄A sequence does not direct maturation-dependent polyadenylation in *Xenopus* oocytes (Fig. 3C) or mediate translational repression in immature *Xenopus* oocytes (Fig. 3D). Nonetheless, the GU₄A sequence does appear to influence the translational regulation exerted by the human Mos 3' UTR. Mutational disruption of the GU₄A sequence (mutant 1 UTR) resulted in an increased length of poly[A] tail in response to progesterone-stimulation (Fig. 3C) and increased translation of GST above the levels seen with the wild-type human Mos 3' UTR (Fig. 3D). It is possible that the GU₄A sequence functions in some way to antagonize CPE-directed polyadenylation and temper Mos mRNA translation in primates when compared to other mammalian species. The GU₄A sequence may influence the secondary structure of the Mos 3' UTR and/or the affinity of CPEB1 for the upstream U₅A CPE, or perhaps serves as a specific target for a CPEB-independent regulatory protein. It is of note that the functionality of a CPE has been previously reported to be context dependent in model organisms, where the presence of other sequences in the 3' UTR can modulate the extent of CPE-directed polyadenylation and translational activation (McGrew and Richter, 1990; Simon et al., 1992; Gebauer et al., 1994; Stebbins-Boaz and Richter, 1994; Ballantyne et al., 1997; Charlesworth et al., 2000). Future studies will be required to determine the mechanism by which the GU₄A sequence influences the translational activation of the human Mos 3' UTR and whether this mechanism is more generally employed to modulate translation of other CPE-dependent mRNAs.

Our analysis of the human Mos 3' UTR revealed a distinct difference from *Xenopus* Mos mRNA 3'UTR in control of timing of translational activation. Unlike the *Xenopus* Mos 3' UTR which contains a Musashi/PRE and directs early, Musashi/PRE-dependent cytoplasmic polyadenylation and translational activation prior to GVBD (see Fig. 4 and Charlesworth et al. (2002,2006)), the human Mos 3' UTR lacks a functional Musashi/PRE and directs late, CPE-dependent polyadenylation coincident with GVBD (Fig. 4). Thus, the temporal differences in *Xenopus* and mammalian Mos mRNA translational activation likely reflects distinct 3' UTR regulatory element composition. The differential temporal control exerted by the *Xenopus* and human Mos 3' UTRs is in contrast to the *Xenopus* and mammalian cyclin B1 3' UTRs, which exert late CPE-dependent translational activation at or after GVBD in both *Xenopus* and murine oocytes (de Moor and Richter, 1999; Barkoff et al., 2000; Tay et al., 2000).

These differences in timing of Mos mRNA translational activation likely reflect the differential temporal requirements for Mos protein during oocyte maturation in these species. Early *Xenopus* Mos mRNA translation is required to mediate the Meiosis I to Meiosis II transition, in addition to a later role in the maintenance of fully mature oocytes in meiotic II metaphase arrest (Gross et al., 2000; Dupre et al., 2002). By contrast, Mos function in mammalian oocytes appears dispensable for Meiosis I to Meiosis II transition but is required to maintain meiotic metaphase II arrest and prevention of parthenogenetic activation (Colledge et al., 1994; Hashimoto et al., 1994; Araki et al., 1996). Thus, while clearly important for *Xenopus* oocyte maturation, we find no evidence to support a role for Musashi/PRE-dependent cytoplasmic polyadenylation and translational activation of the Mos mRNA during mammalian oocyte maturation. However, these observations do not preclude a requirement for Musashi/PRE-dependent regulation of other mRNAs during mammalian oocyte maturation. Indeed, Musashi expression has been reported in the murine ovary (Sakakibara et al., 1996), although further studies will be necessary to resolve the role and requirement for Musashi function during mammalian oocyte maturation.

Current in vitro fertilization (IVF) regimes employ in vivo matured oocytes or in vitro maturation of aspirated GV positive oocytes, though both sources of oocytes present inherent limitations (Chian et al., 2004; Ali et al., 2006). The success of IVF protocols with in vivo matured oocytes has a significant dependency on the age of the oocyte donor where successful fertility outcomes decrease with age (Krey et al., 2001), while in vitro human oocyte maturation

is associated with a loss of developmental competence as indicated by the reduction or absence of specific proteins in oocytes cultured to metaphase II (Moor et al., 1998; Trounson et al., 2001). Oocyte GVBD is a useful morphological marker of meiotic progression but does not necessarily indicate oocyte maturity and acquisition of full developmental potential (Trounson et al., 2001). Analysis of endogenous Mos mRNA polyadenylation as an indicator of maternal mRNA translational activation may present a useful molecular marker of oocyte developmental competence. While the RNA ligation coupled PCR assay we employ herein is an invasive technique, it may prove useful in the optimization of developmental competence for improved rates of successful IVF outcomes.

CONCLUSIONS

We provide evidence that regulated cytoplasmic polyadenylation occurs during human oocyte meiotic maturation as it does in model organisms. Unlike the *Xenopus* Mos mRNA which is translationally activated early by Musashi-regulated PRE function, the human Mos 3' UTR directs late, CPE-regulated translational activation in the heterologous *Xenopus* system. Although our findings clearly indicate that the human Mos 3' UTR lacks an early acting Musashi/PRE sequence, we do not exclude the possibility that other regulatory elements may exist in the human Mos 3' UTR that do not function in the heterologous *Xenopus* system. Based on our findings reported here, we propose that species-specific differences in the composition of 3' UTR regulatory elements contributes to the differential temporal activation of Mos mRNA translation during mammalian and *Xenopus* oocyte maturation.

ACKNOWLEDGMENTS

We thank Dr. Michael Miller, Dr. Dean Moutos, and Dr. Francisco Batres for patient recruitment and oocyte aspiration. A.M.M. was supported by the American Cancer Society (RPG 101279), the National Institutes of Health (HD35688) and the Arkansas BioSciences Institute; M.C.M. was supported by NIH grant RR020146.

Grant sponsor: American Cancer Society; Grant number: RPG 101279; Grant sponsor: National Institutes of Health; Grant number: HD35688; Grant sponsor: NIH; Grant number: RR020146.

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Abbreviations

CPE, cytoplasmic polyadenylation element; CPEB1, cytoplasmic polyadenylation element binding protein; PRE, polyadenylation response element; GST, glutathione S-transferase; 3' UTR, 3' untranslated region; EMSA, electrophoretic mobility shift assay; MAP kinase, mitogen activated protein kinase; GVBD, germinal vesicle breakdown.

Fig. 1.
The human Mos mRNA contains a cytoplasmic polyadenylation element (CPE). **A:** Schematic representation of the position of candidate CPE sequences relative to the polyadenylation hexanucleotide sequence (HEX, AAUAAA). **B:** Sequence alignment comparison of primate and rodent Mos 3' UTR sequences. Candidate CPE sequences are circled, the polyadenylation hexanucleotide sequence is boxed. **C:** Two immature germinal vesicle positive (GV) and two fully mature, metaphase II arrested (M II) human oocytes were obtained from the same patient undergoing IVF treatment (see Materials and Methods Section). Total RNA was prepared and the polyadenylation status of the Mos mRNA assessed by RNA ligation coupled PCR. The PCR products were excised and subjected to DNA sequence analysis. The size of the PCR products is indicated. This experiment was repeated with similar results using both GV and M II oocytes obtained from three separate patients.

Fig. 2.

The human cytoplasmic polyadenylation element binding protein (hCPEB1) specifically interacts with the CPE in the human Mos 3' UTR. **A:** GST Western blot to demonstrate the relative expression levels of the GST moiety or GST-hCPEB1 fusion protein in programmed rabbit reticulocyte lysates. The GST moiety alone was expressed to slightly higher levels than the GST-hCPEB1 fusion protein. UP, unprogrammed lysate. Arrowheads indicate position of the expressed proteins. **B:** Schematic representation of the mutant human Mos 3' UTRs generated in this study. **C:** RNA EMSA using a radiolabelled wild-type human Mos 3' UTR and either unprogrammed reticulocyte lysate (UP), or reticulocyte lysate programmed with mRNA encoding the GST moiety alone (GST) or an mRNA encoding a GST-hCPEB1 fusion protein. Where indicated, a 50-fold excess of unlabelled wild-type human Mos 3' UTR (specific competitor) or a 50-fold excess of human Mos mutant 2 UTR (non-specific competitor) RNA probe were also added to the binding reaction. The specific complex could be super shifted by addition of GST antiserum but not by addition of an irrelevant antiserum (B-Raf). **D:**

Radiolabeled β -globin 3' UTR, wild-type human Mos 3' UTR, or various mutant human Mos 3' UTR probes (see (B)) were analyzed for interaction with the GST-hCPEB1 fusion protein by RNA EMSA as described for (C). Specific complex formation was only observed with the wild-type and mutant 1 human Mos 3' UTR probes. For both (C) and (D), the position of the specific complex is indicated by an arrowhead, free probe by a filled circle and background non-specific complexes by an open circle. It should be noted that in the case of the mutant 1 UTR probe in (D, lane 5), an additional non-specific high-molecular weight complex near the top of the gel was observed regardless of whether unprogrammed, GST moiety-alone or GST-hCPEB1 lysates were used. Representative results are shown.

Fig. 3.

The human Mos 3' UTR directs cytoplasmic polyadenylation in *Xenopus* oocytes. *Xenopus* immature oocytes were injected with a GST reporter mRNA coupled to the indicated 3' UTRs. Oocytes were then either left untreated (I) or stimulated with progesterone (P) for 16 hr. Pooled oocyte samples were prepared from each condition and both total RNA and protein lysate prepared from each pooled sample. **A:** Samples were then analyzed for progesterone-dependent cytoplasmic polyadenylation by RNA-ligation coupled PCR. An increase in PCR product size is indicative of polyadenylation. In progesterone stimulated oocytes, the *Xenopus* Mos 3' UTR received an average of 40 adenylate residues and the human Mos 3' UTR received 32 adenylate residues. The migration of molecular size markers are indicated. **B:** A Western blot analysis of protein lysates prepared from the same oocyte sample pool analyzed in (A) to visualize GST protein accumulation controlled by the indicated 3' UTR. GST protein levels were quantitated and the relative levels are indicated (normalized to the levels controlled by the β-globin 3' UTR in immature oocytes). As a control for total protein loading, a tubulin Western blot of the same samples was performed (**lower panel**). The experiment was repeated three times with similar results. **C:** Immature *Xenopus* oocytes from the same frog were injected with GST reporter RNA coupled to the indicated 3' UTR. The injected oocytes were then split into two pools and either left untreated (I) or stimulated with progesterone (P). Pooled oocyte samples were analyzed for cytoplasmic polyadenylation by RNA ligation coupled PCR (C) or for GST protein accumulation and tubulin by Western blotting (**D**). In response to progesterone stimulation, an average of 35 adenylate residues were added to the *Xenopus* Mos 3' UTR, 30 adenylate residues to the wild-type human Mos 3' UTR and 77 adenylate residues to the human Mos mutant 1 UTR. GST protein levels were quantitated and the relative levels are indicated

(normalized to the levels controlled by the human Mos mutant 2 UTR). The experiment was repeated four times with similar results.

Fig. 4.

Differential temporal regulation of *Xenopus* and human Mos 3' UTR-directed polyadenylation in maturing oocytes. Immature *Xenopus* oocytes were injected with GST reporter RNA coupled to either the *Xenopus* (**Panel A**, XeMos) or human (**Panel B**, hMos) 3' UTRs. For each GST reporter RNA, the injected oocytes were then split into two pools and either left untreated (Imm) or stimulated with progesterone for the indicated times. Pooled oocyte samples were prepared as described in the legend to Figure 3 and analyzed for cytoplasmic polyadenylation by RNA ligation coupled PCR using a GST forward primer common to both injected RNA constructs. For each experimental time point, endogenous cyclin A1 mRNA polyadenylation was also assessed. At the 5 hr time point, 50% of the oocyte population had reached GVBD and the oocytes were pooled based on whether they had (+) or had not (–) completed GVBD. All the injected oocytes had matured by 7 hr. A retarded mobility of the PCR product above the dotted reference line is indicative of polyadenylation.