Identification of a new deadenylation negative feedback loop that regulates meiotic progression

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ABBREVIATIONS

APC/C: Anaphase promoting complex/cyclosome

ARE: AU-rich element

AREBP: ARE binding protein

C3H-4: Cx8Cx5Cx3H fourth member

cdc: cell division cycle

cdk: cyclin dependent kinase/cell division kinase

cds: coding sequence

Chx: Cycloheximide

CPE: cytoplasmic polyadenylation element

CPEB: cytoplasmic polyadenylation element binding protein

CPSF: Cleavage and polyadenylation specificity factor

CSF: Cytostatic Factor

CstF: Cleavage stimulation factor

EDEN: embryonic deadenylation element

eIF: eukaryotic initiation factor

eEF: eukaryotic elongation factor

Emi: early mitotic inhibitor

eRF: eukaryotic release factor

GVBD: Germinal vesicle breakdown

Hex: hexanucleotide

IRES: Internal ribosome entry site

m7Gppp: 7-methil guanosine

MI: metaphase I

MII: metaphase II

MBT: Mid blastula transition

MPF: Maturation (or M-phase) promoting factor

mRNA: messenger RNA

mRNP: messenger ribonucleoprotein; mRNA-protein complex

ORF: open reading frame

PABP: poly(A) binding protein

PARN: poly(A)-specific ribonuclease

PBE: pumilio binding element

poly(A): polyadenosine

RRM: RNA recognition motif

TNF-α: tumor necrosis factor alpha

UTR: untranslated region

KEY WORDS

3' UTR
deadenylation
cyclin E
C3H-4
CPEB
development
Emi1
Emi2
feedback loop
maternal mRNAs
meiosis
metaphase
oocyte maturation
poly(A)
polyadenylation
translational control
Xenopus Laevis

ABSTRACT

Vertebrate immature oocytes are arrested at prophase of meiosis I (PI). During this growth period the oocytes synthesize and store large quantities of dormant mRNAs, which will drive the oocyte's re-entry into meiosis and progression through the two consecutive M-phases (MI and MII) to become arrested for a second time, as the result of the synthesis and activation of the Cytostatic Factor (CSF). Remarkably, these transitions occur in the absence of transcription and are fully dependent on the sequential translational activation of the maternal mRNAs accumulated during the PI arrest. The translational activation of these mRNAs is mainly induced by the cytoplasmic elongation of their poly(A) tails, which is mediated by the Cytopasmic Polyadenylation Element (CPE) pressent in their 3'UTRs. The time and extent of translational activation of the CPE-containing mRNAs is finely regulated resulting in differential rates of product accumulation that, combined with the control of protein degradation, establishes phase-specific peaks of expression of the factors that drive meiotic progression (Schmitt et al., 2002; de Moor and Richter, 1997; Ballantyne et al., 1997; Mendez et al, 2002; Charlesworth et al., 2002). Until now, no specific deadenylation was found to be required for meiotic progression. In this work we have identified, in a full-genome screening for cytoplasmically polyadenylated mRNAs, a new negative feed back loop required to exit from metaphase into interkinesis. Contrary to the previously identified negative feedback loops mediating this transition, this one takes place at the translational level by deadenylating mRNAs encoding for proteins that promote or stabilize Maturation-Promoting Factor (MPF) activation. This negative feed back loop is mediated by AU Rich Elements (AREs) and the ARE-binding protein C3H-4, which is synthesized during the PI-MI transition as a result of the early wave of cytoplasmic polyadenylation. In turn, C3H-4 recruits the deadenylase CCR4 to CPE and ARE containing mRNAs such as those encoding for Emi1 and Emi2, respectively, mediating deadenylation or preventing polyadenylation at MI. Disruption of this negative feedback loop prevents MI-interkinesis transition and causes pseudometaphase arrest.

INTRODUCTION

1. mRNA biogenesis.

Eukaryotic gene expression is a complex stepwise process that begins with transcription. Protein-coding genes are transcribed by RNA polymerase II (RNAP II) (Bentley, 1999), during transcription, the nascent pre-mRNA is capped at the 5' end, introns are removed by splicing, and the 3' end is cleaved and polyadenylated. The mature mRNA is then released from the site of transcription and exported to the cytoplasm for translation. Distinct machineries carry out each of the steps in the gene expression pathway, but it is now evident that the individual events are physically and functionally coupled (Maniatis and Reed, 2002); this is partially achieved by the C-terminal domain (CTD) of the largest subunit of the RNA polymerase II (RNAPII), which acts as a loading platform for several transcription and mRNA processing factors (Meinhart and Cramer, 2004; Proudfoot, 2004) (Fig. 1).

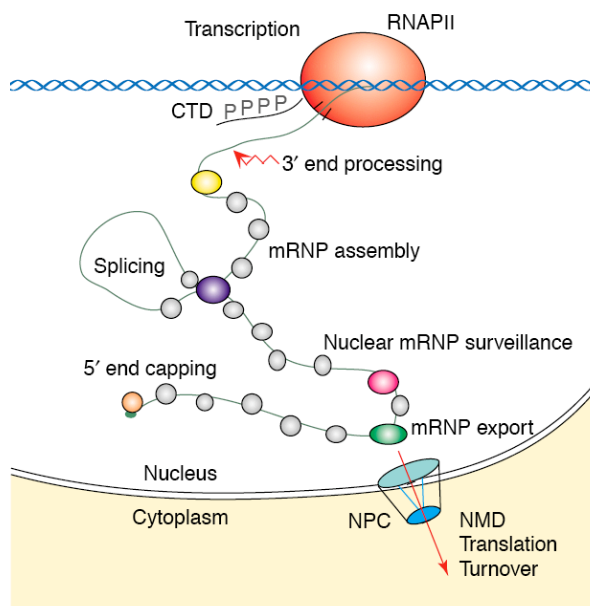


Figure 1. Simplified view of eukaryotic gene expression, co-transcriptional processing and mRNP assembly are shown. Taken from Aguilera, 2005.

During each of the processing steps protein complexes are deposited on the pre-mRNA, therefore mRNAs are never nude molecules, but rather are always coated by RNA binding proteins (RNABPs) constituting the messenger ribonucleoprotein particles (mRNPs) (Aguilera, 2005). The mRNP biogenesis is thus a co-transcriptional event influencing subsequent steps in mRNA processing. The assembly of the mRNP particle starts with the

association of the cap-binding protein complex, followed by the splicing-dependent assembly of the exon-junction complex (EJC) in intron-containing genes and by the binding of RNA-export adaptor proteins deposited by the cleavage and polyadenylation events (Fig. 1). Once in the cytoplasm cellular events can influence in the mRNP composition (Jensen et al., 2003). RNAs not assembled with the correct mRNP are not exported from the nucleus are turned over by components of an evolutionarily conserved multisubunit complex, the exosome (Schmid and Jensen, 2008).

2. The mRNA structure.

Mature RNAs are exported to the cytoplasm only after all nuclear processing events are completed successfully (for review, see (Maniatis and Reed, 2002; McKee and Silver, 2004; Stutz and Izaurralde, 2003)). The mRNAs consist of an RNA body flanked by two modifications, not coded in the DNA sequence, the 5' cap and the polyadenosine tail (poly(A) tail). The RNA body contains a translatable region, the open reading frame (ORF), preceeded and followed by two untranslated regions (UTRs) the five prime UTR (5' UTR) and the three prime UTR (3' UTR). These UTR regions are transcribed with the coding region and thus are exonic as they are present in the mature mRNA (Shatkin and Manley, 2000) .

2.1. The cap structure.

The 5' processing involves three enzymatic steps to add the 7-methylguanosine (m^7Gppp) cap to the 5' end of the transcript (Shatkin and Manley, 2000). Capping is critical to protect the nascent transcript from 5' to 3' exonucleolytic degradation, to promote splicing, to facilitate mRNA transport, and for recognition and proper attachment of mRNA to the ribosome, a requirement for efficient translation (Varani, 1997).

2.2. The poly(A) tail.

The 3' processing occurs in a coupled, two-step reaction that includes endonucleolytic cleavage of the nascent RNA, followed by the addition of a polyadenosine tract (Jensen et

al., 2003; Zhao et al., 1999) which is immediately coated with the nuclear poly(A) binding protein (PABP) (Baer and Kornberg, 1983). Nuclear polyadenylation is the last processing step before mRNA export to the cytoplasm, it requires the poly(A) polymerase as well as four protein complexes: cleavage polyadenylation specificity factor (CPSF), cleavage stimulation factor (CstF) and cleavage factors I and II. CPSF specifically binds the canonical AAUAAA signal upstream of the cleavage site, whereas CstF interacts with U-rich sequences downstream. Together, these factors recruit the cleavage factors and specify the site of cleavage. After the cleavage event, CPSF promotes poly(A) addition by poly(A) polymerase. The poly(A) tail protects the mRNA from degradation and enhances translation.

All mRNAs initially receive a tail of ~250 adenines, but upon export the length of the poly(A) tail can be modified by cytoplasmic proteins, therefore mRNAs are heterogeneus in poly(A) tail length, ranging from ~250 nucleotides (Brawerman, 1976) to ~30 nucleotides long (Sheiness and Darnell, 1973) (Ahlquist and Kaesberg, 1979). Histone mRNAs are an exception, their 3' end processing includes the cleavage but is not followed by the polyadenylation step, therefore the mature RNAs are not coated by PABP, otherwise, the stem-loop binding protein (SLBP) binding stimulates their export to the cytoplasm, where they reside without a poly(A) tail, regulates their translation and protects them from degardation (Marzluff, 2007).

2.3. The ORF.

The ORF constitutes the translated region of the mRNA, which starts with the AUG initiation codon and ends with one of the three stop codons (UAA, UAG or UGA).

2.4. The untranslated regions (UTRs).

Several roles in gene expression have been attributed to the untranslated regions, including mRNA stability, mRNA localization, and translational efficiency. The ability of a UTR to perform these functions depends on the sequence of the UTR which differs between mRNAs (Fig. 2).

The regulatory motifs in the 5'UTRs which influence translation are; secondary structure, by itself or as binding site for regulatory proteins, which repress cap-dependent translation by

inhibiting binding or scanning of the translational machinery. Upstream ORF (uORF) and upstream AUGs (uAUG) inhibit translation by restricting the access of ribosomes to the correct start codon, but when eIF2 is phosphorylated inhibiting general translation, the mRNAs containing uAUG are actively translated (Cigan et al., 1993). Internal ribosome entry sites (IRES) are also secondary structures that favor entry of the ribosome in a cap-independent manner. Finally, primary sequence binding sites for regulatory proteins can also influence translation (Wilkie et al., 2003)

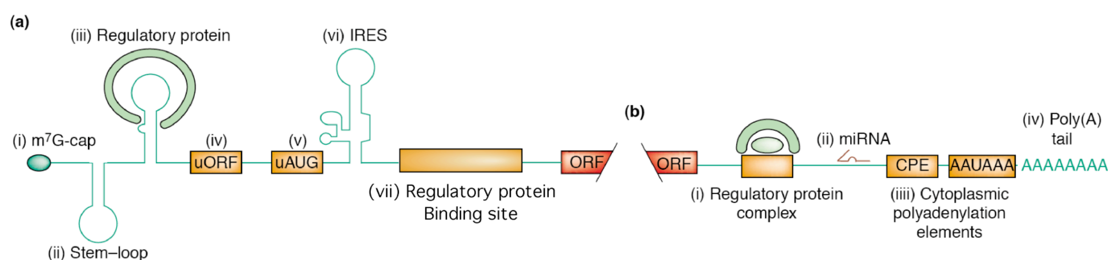


Figure 2. Regulatory elements in the 5'UTR **(a)** and 3'UTR **(b)**. Modified from Gray and Wickens, 1998.

The regulatory motifs in the 3'UTR also include primary sequence binding sites for regulatory proteins, that may affect the mRNA's stability or location in the cell, miRNA binding sites that affect the translation and stability of the mRNA, and finaly the cytoplamic polyadenylation elements which are involved in translation efficiency; the cytoplasmic polyadenylation element (CPE) and the hexanucleotide (Hex).

About half of mammalian genes use alternative cleavage and polyadenylation to generate multiple mRNA isoforms differing in their 3'UTRs (Beaudoing and Gautheret, 2001) (Zhang et al., 2005). Alternative polyadenylation occurs in both a splicing-independent form (multiple polyadenylation sites in a terminal exon) and in a splicing-dependent form (mutually exclusive terminal exons) (edwalds-gilbert et al. 1997). Very recently Sandberg et al. observed that when two versions of a 3'UTR were possible, the extended one showed reduced protein synthesis by affecting mRNA translation, also they observed that generally the usage of short UTRs correlated with proliferating cells while the extended ones correlated with arrested cells, suggesting that the mechanism or mechanisms dictating the alternative use of UTRs are regulated in a cell-cycle dependent manner.

3. The close loop model.

The closed-loop model refers to the bridging of the 5' end of mRNA with the 3' end of the same mRNA. The eIF4G interacts with the PABP, this interaction approximates both ends of the mRNA probably enhancing translational efficiency by eliminating the initiation step after the first round of translation takes place when the ribosome reaches the end of the mRNA. (Sachs and Varani, 2000; Mangus et al., 2003)

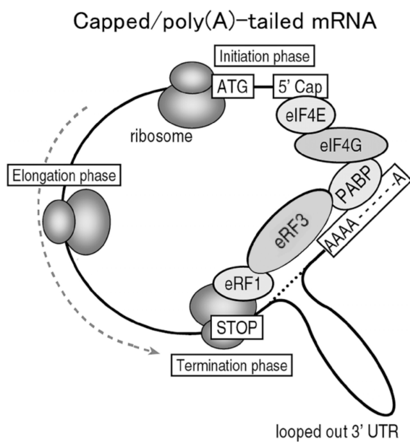


Figure 3. Re-initiation of translation. Circularization of the mRNA through a complex consisting of poly(A) tail-PABP-eIF4F-cap suggests the hypothetical model that a translation-terminating ribosome may be recruited to the next translation initiation. Modified from Uchida 2002.

4. Translation steps.

a. Initiation.

This step of translation is the most targeted for regulation. A working model for cap-dependent initiation, consistent with most of the current data, is depicted in figure 4. Although this model represents a general consensus, it is not unambiguously established (Hershey et al., 1996; Pain, 1996).

Following ribosomal subunit dissociation, assisted by a number of initiation factors (eIFs), including eIF1, eIF1A eIF-3 and eIF5, the small (40S) ribosomal subunit (carrying eIF-3) associates with a ternary complex to generate a 43S pre-initiation complex. The ternary complex contains the methionine-charged initiator tRNA, initiation factor eIF-2, and GTP. This 43S complex is then recruited, with the aid of the eIF-4F group of initiation factors already bound to the mRNA, to the 5' end of the mRNA. The eIF-4F group is composed of three

subunits; eIF-4E, which binds to the cap, eIF-4A, which has ATPase-dependent RNA helicase activity, and eIF-4G, which through its interaction with eIF-3 aids the binding of the 43S pre-initiation complex. The helicase activity of eIF-4F unwinds secondary structures from the 5' untranslated region (UTR), which would otherwise impede the initiation apparatus. This helicase activity is stimulated by eIF-4B, which binds simultaneously with, or very closely after, eIF-4F. The interaction of eIF-4B with eIF-3 may also aid in the binding of the 43S complex. Once bound, the scanning model proposes that the 43S complex migrates along the 5'UTR in an ATP-dependent process, until it encounters an initiator AUG codon, recognized by the tRNA basepairing, when a stable 48S is formed. Any AUG is not suitable for being an initiator codon, otherwise, there is an optimal context surrounding the AUG which determines it's use, the sequence context for AUG recognition is GCC(A/G)CC**AUG**G (Kozak, 2002) which depends on the eIF1 and eIF1A activities. Following AUG recognition, eIF-5 triggers hydrolysis of the GTP in the ternary complex, initiation factor release occurs, and the large (60S) ribosomal subunit enters. Then elongation begins.

b. elongation.

Three positions for tRNAs are available in the ribosome; an acceptor site (A site) where the amminoacyl-tRNA is placed, the peptidyl site (P site) which contains the tRNa with the growing polypeptide, and exit site (E site) where the empty tRNA exits the ribosome. Peptide chain elongation begins with a peptidyl tRNA in the ribosomal P site next to a vacant A site. An aminoacyl tRNA is loaded to the A site as part of a ternary complex with GTP and the elongation factor 1A (eEF1A). The selection of the correct eEF1A·GTP·aa-tRNA ternary complex is chosen by several steps involving codon-anticodon base pairing between the mRNA and the tRNA, conformational changes in the decoding center of the small ribosomal subunit, and GTP hydrolysis by eEF1A releases the aminoacyl tRNA into the A site in a form that can continue with peptide bond formation.

The ribosomal peptidyl transferase center then catalyzes the formation of a peptide bond between the incoming amino acid and the peptidyl tRNA (located in the P site). Elongation factor 2 (eEF2) with the hydrolysis of a GTP molecule, moves the mRNA three nucleotides, resulting in an empty tRNA in the E site, the elongated peptidyl-tRNA in the P site, and an

empty A site. This procedure is repeated until a stop codon is encountered and the process of termination is initiated.

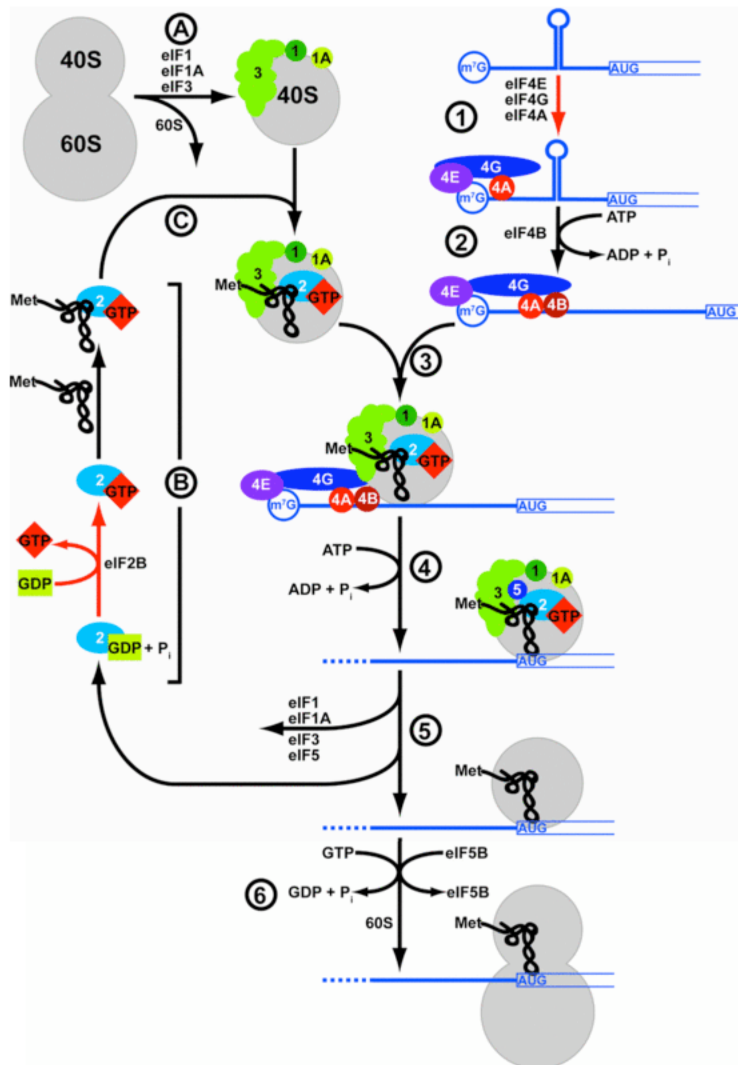


Figure 4. Translation steps. Taken from Smith et al., 2008.

c. termination and recycling of the ribosomes.

Termination is stimulated by the presence of a termination codon in the A site. The peptidyl transferase center of the ribosome is believed to catalyze the hydrolysis reaction to liberate the polypeptide chain from the P site tRNA, in response to the activity of the eRF1 and eRF3. Then GTP hydrolysis on eRF3 leads to the dissociation of the RF from the ribosome.

Ribososme reclycling in eukaryotes is largely misterious, some results have suggested a possible involvement of the ortholog of the bacterial IF3 in this process (Kapp and Lorsch, 2004). Indeed pisarev et al. have recently demonstrated that eIF3 is the only factor that could split postermination ribosomes on its own and th at this dissociating activity is strongly enhanced by eIF3j and less by eIF1 and 1A (Pisarev et al., 2007).

An arising model proposes that once termination takes place and the completed peptide is released, one or both eRFs remain bound to the post-termination complexs. Then eIFs 3, 1, 1A and 3j cooperatively dissociate such complexes into free 60S subunits and mRNA- and tRNA-bound 40S subunits. eIF1 then promotes dissociation of P site decetylated tRNA, after which eIF3j mediates the release of mRNA. eIF3 clearly initiates recycling, but the other in which other factors join the process is yet unknown. After dissotiation, eIF3 (and other eIFs, plays a key role remained unbound to recycled ribsomal smal subunits, protecting them from re-association.

Although, it is generally believed that the functionof eRF3 is solely to facilitate the release of completed peptides from the ribosome, it has been found that eRF3 also associates with eIF4G through PABP (Hoshino et al., 1999) (Uchida et al., 2002) suggesting a possible connection between the 5'end of an mRNA close to the termination site. The association of eRF1-eRF3- PABP-eIF4F might promote tracking of terminating ribosomes to the 5'end of the same mRNA (Uchida et al., 2002). This mechanism could explain a missing link for the participation of recycled 40S subunits in new round of initiation on the same mRNA strongly suggesting a movement of small ribosomal subunits over the poly(A) tail back to the 5'end of the mRNA- via the 5'-3' end associated factors, thus, allowing re-iniciation of translation rather than the first initiation event (Kapp and Lorsch, 2004; Uchida et al., 2004).

GSPT associates with eIF4G through PABP and that the GSPT-PABP interaction is involved in the multiple rounds of translation (Fig. 3). The synergistic enhancement of translation by cap and poly(A) is explained by the circularization of mRNA, which is mediated through a complex consisting of poly(A)-PABP-eIF4F-cap (21-23). This fact suggests the hypothetical model that a translation-terminating ribosome may be recruited to the next translation initiation. However, this idea is unsatisfactory since translation is terminated at stop codons that are not always close to the poly(A) tail of mRNA. Therefore, some factors are likely to

mediate the physical coupling between the terminating ribosome on the stop codon and the poly(A) tail. The fact that GSPT interacts with eRF1 and PABP at the same time (Fig. 3) suggests that GSPT may be the bridging protein to connect the stop codon with the poly(A) tail. In this hypothesis, a 3'-untranslated region, which locates between a stop codon and a poly(A) tail, could be looped out, and the terminating ribosome could be passed to the 5' cap structure through the novel protein bridge consisting of eRF1, GSPT, PABP, and eIF4F (Fig. 3). In addition to the role of PABP in translation, several lines of evidence suggest that PABP might affect translation in a poly(A)-independent manner. Furthermore, this function appears to be independent of its binding to eIF4G. The results presented here suggest that the GSPT-PABP interaction may also be involved in poly(A)-independent translation, though the exact mechanism is still unclear.

It is well established that PABP has another function; it prevents mRNA degradation by protecting the poly(A) tail. In general, mRNA degradation, an important aspect of gene expression, is a strictly regulated process that is often linked to translation, and translation-dependent deadenylation is an important step of this mechanism in which PABP is probably involved. Moreover, several reports show that GSPT is involved in nonsense-mediated decay, a mechanism by which mRNAs containing a premature termination codon are rapidly degraded. These mechanisms are not well understood, but it is conceivable that they are linked to the translation termination. Further studies on GSPT/eRF3 should be important for the understandings of not only translation machinery but also mRNA-decay mechanism.

5. Control of translation

Initiation is the rate-limiting step in translation and is the most common target of translational control. Changes of protein expression at a translational level can be achieved in a very fast manner, localized and when transcription is shut off, e.g. during S-phase and M-phase, contrary to what would happen if the changes would be at a transcriptional level. Translation inhibition of mRNAs can be targeted at two different levels, one includes all the mRNAs in the cell, and another is directed to a subset of mRNAs.

5.1. Global control of translation.

Global control is achieved by changes in the availavility of basal components of the translational machinery, which are required for all translation events in the cell. Examples of such regulation have been shown for eIF4G, eIF4E-binding proteins (4E-BPs) and eIF2 (Dever, 2002).

Some viruses use the internal initiation of translation as an infection strategy; they inactivate cap-dependent translation in the host cell by cleaving the eIF4G, therefore only the viral mRNAs can be translated. The proteolytic cleavage of eIF4G disrupts cap-dependent translation since it ultimately disrupts the bridging between the cap and the 40S, required for translation initiation (Fig. 4) (Lamphear et al., 1995).

The 4E-binding proteins (4E-BP) family down-regulates general cap-dependent translation in response to a variety of signals by binding to eIF4E and preventing its interaction with eIF4G, thus reducing the levels of eIF4F ternary complex available for initiation (Richter and Sonenberg, 2005). Upon certain stimulus the interaction between 4E-BP and eIF4E is lost and therefore translation is upregulated (Haghighat et al., 1995; Pause et al., 1994). The initiator factor eIF2 can be phosphorylated in its a-subunit by a small number of very specfic kinases in response to a number of signals. The phosphorylation interferes with the recycling of the eIF2·GDP complex to eIF2·GTP catalyzed by the guanine-nucleotide-exchange factor eIF2B. and leads to. The high affinity of phosphorylated eIF2·GDP for eIF2B causes accumulation of blocked eIF2·GDP–eIF2B complexes and therefore depletion of ternary complex (mohr and mathews 2008, ron and harding 2008). There are four different eIF2 kinases in mammals activated by different stresses, including PKR, PERK, HRI, and GCN2 (Sonenberg and Hinnebusch, 2007).

5.2. Specific control of translation.

Translational regulation can also only be targeted to a subset of mRNAs. For this purpose, characteristically the regulatory elements are only present in a number of mRNAs, they reside in the 5' and 3'UTRs which differ from one mRNA to another, although certain primary

sequences or secondary stuctures, with the ability to interact with RNA binding proteins, can be shared within a subset of mRNAs.

5.2.1. Translation regulation by poly(A) tail length: polyadenylation

The poly(A) tail has been shown to act synergistically with the cap to stimulate translation so that mRNAs that are both capped and polyadenylated are translated to a much greater extent than mRNAs that have been modified at just one end (Gallie, 1991; Preiss and Hentze, 1998). It has been established that translationally repressed mRNAs have a poly(A) tail not shorter than 40 nucleotides but the assembled mRNP obstaculizes the ribosome entry, not repressed mRNAs also have a poly(A) tail of 40 nucleotides , but in this case the cap is accesible for translation initiation although it will be inefficient since the poly(A) tail is not enough for efficient translation. Finally translationally active mRNAs contain a poly(A) tail of 70 nucleotides or more that is enough for enhanced translation efficiency (Richter, 2007).

Poly(A) tail length shortening and elongation are therefore mechanisms that regulate translation efficiency.

Cytoplasmic polyadenylation

Cytoplasmic polyadenylation is a highly regulated and conserved mechanism, first observed in clam oocytes (Rosenthal et al., 1983), that increases translation during meiotic maturation and after fertilization (Mendez and Richter, 2001; Richter, 1999, 2007). This phenomenon has subsequently been seen in a variety of other organisms including worms, starfish, flies, frogs, mice and somatic tissues (Fox et al., 1989; Groisman et al., 2002; Paris et al., 1988; Paris and Philippe, 1990; Rosenthal and Wilt, 1986; Salles et al., 1992; Salles et al., 1994; Standart et al., 1987; Vassalli et al., 1989).

Mos, cyclin B1, and several other dormant mRNAs in *Xenopus* oocytes contain short poly(A) tails (~20-40 nucleotides), and it is only when these tails are elongated (to ~100-250 nucleotides) that translation takes place (Mendez and Richter, 2001; Richter, 2007). The regulatory sequences that govern their translational activation and polyadenylation are primarily found in the 3’UTR of these maternal mRNAs. The best characterized of these sequences is the cytoplasmic polyadenylation element (CPE), which is a U-rich sequence. In

Xenopus, the consensus CPE is $U_{4-5}A_{1-2}U$, although some variation may be tolerated in the context of specific mRNAs (Mendez and Richter, 2001; Pique et al., 2008; Richter, 2007; Stebbins-Boaz et al., 1996). Cytoplasmic polyadenylation requires two elements in the 3'UTRs of responding mRNAs, the Hex AAUAAA or AUUAAA, which is bound by a variant of the cleavage and polyadenylation specificity factor (CPSF) and the nearby CPE, which recruits the CPE-binding protein (CPEB) (Colegrove-Otero et al., 2005; de Moor et al., 2005; Fox et al., 1989; Hake and Richter, 1994; McGrew et al., 1989; Mendez and Richter, 2001; Richter, 2007).

a. CPEB: the key regulator.

CPEB is the critical regulator for gene expression in early development. It was first cloned and characterized in *Xenopus* oocytes as a 62-kDa protein that bound specifically to the CPEs mediating cytoplasmic polyadenylation (Hake and Richter, 1994; Stebbins-Boaz et al., 1996). Later on, *Xenopus* CPEB has become the founding member of a large RNA-binding proteins family from *C. elegans* to humans (Bally-Cuif et al., 1998; Christerson and McKearin, 1994; Gebauer and Richter, 1996; Kurihara et al., 2003; Lantz et al., 1992; Liu and Schwartz, 2003; Luitjens et al., 2000; Theis et al., 2003; Walker et al., 1999).

Xenopus CPEB protein is composed of three regions (Fig. 5): the amino-terminal regulatory portion, two RNA recognition motifs (RRMs), and a cysteine-histidine repeat similar to a metal-coordinating region or zinc-finger (Hake and Richter, 1994). The N-terminal half contains consensus Aurora A kinase phosphorylation sites (LDS/TR), which are phosphorylated early during meiotic maturation (Mendez et al., 2000a; Mendez et al., 2000b). This region also contains a PEST sequence, a sequence enriched in proline (P), glutamic acid (E), serine (S) and threonine (T) that target proteins for rapid destruction (Rechsteiner and Rogers, 1996), which mediates CPEB degradation by ubiquitination in response to cell division cycle 2 (Cdc2) and *Xenopus* Polo-like Kinase 1 (Plx1) phosphorylation during later stages of meiotic maturation (Mendez et al., 2002; Reverte et al., 2001; Setoyama et al., 2007; Thom et al., 2003). The C-terminal half contains two RRM domains and two unusual zinc-finger regions ($C_4C_2H_2$) required for binding to CPE-elements (Hake et al., 1998).

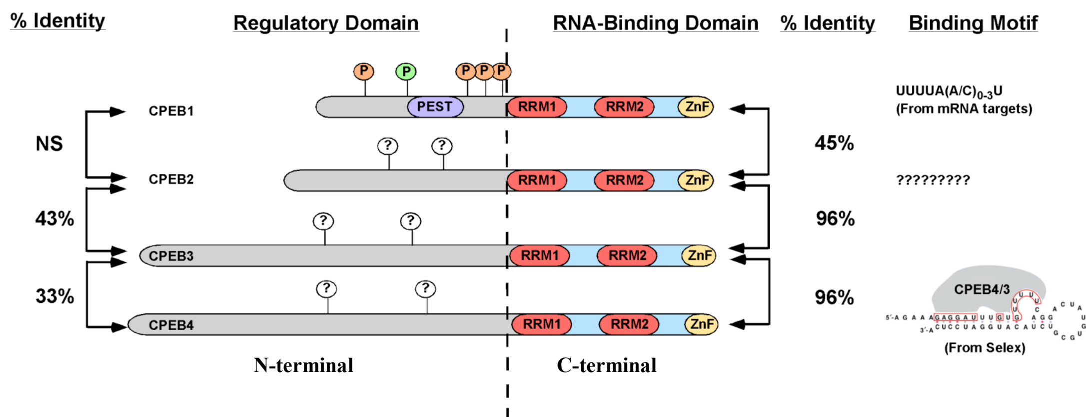


Figure 5. Comparison of CPEB family members. Schematic representation of the CPEB family of proteins. CPEB proteins contain an N-terminal regulatory domain and a C-terminal RNA-binding domain that consist of two RNA recognition motifs (RRM1, RRM2, red) and a Zinc-finger domain (ZnF, yellow). PEST box (blue), Aurora A-phosphorylation site (P, green) and Cdc2-phosphorylation sites (P, orange) are only present in CPEB1. Putative phosphorylation sites in the CPEB2-4 are shown (circled question marks). The percentage (%) of identity among the CPEB proteins as well the binding motifs in the targets mRNAs is also indicated. Whereas CPEB1 recognizes a primary sequence ($U_{4\text{-}5}A(A/C)_{0\text{-}3}U$), CPEB3-4 seems to recognize a secondary structure (U-rich loop within stem-loop structure) (Huang et al., 2006). CPEB2 binding motif is still unknown.

CPEB belongs to a family of proteins with four members (CPEB1, CPEB2, CPEB3 and CPEB4) (Fig. 5). The founding member of the CPEB proteins is sometimes referred as 'CPEB1' (in this thesis, it is referred to 'CPEB'). By sequence comparison within and across phyla, it has been shown that CPEB and CPEB2-4 constitute different branches of the CPEB family of proteins (Mendez and Richter, 2001). Mouse CPEB2 (mCPEB2), mCPEB3 and mCPEB4 isoforms are the most similar between them and show less homology to mCPEB1. The four CPEBs are expressed in different tissues (Theis et al., 2003) and interact with distinct RNA motifs (Huang et al., 2006). The transcript of mCPEB1 is abundantly expressed in brain and more weakly expressed in kidney, lung, heart and oocytes (Gebauer and Richter, 1996; Tay et al., 2000; Theis et al., 2003; Wu et al., 1998). mCPEB2 is abundantly expressed in testis and brain (Kurihara et al., 2003; Theis et al., 2003). mCPEB3 mRNA is strongly expressed in heart and brain, and finally, mCPEB4 mRNA in embryos and adult brain as well as kidney, lung and heart (Theis et al., 2003). Unlike mCPEB1, the other CPEB isoproteins lack PEST sequence and Aurora A kinase phosphorylation sites. However, alternative splice isoforms of CPEB2-4 posses putative phosphorylation sites for cyclic AMP-dependent protein

kinase (PKA), calcium-calmodulin-dependent protein kinase-II (CaMKII) and p^{70}S6 kinase (Theis et al., 2003). Human homologues of all four mouse proteins have been also identified (Kurihara et al., 2003; Welk et al., 2001).

In mammals not only meiosis and development, but also cellular senescence, axon guidance, synaptic plasticity and long-term memory consolidation are also regulated by CPE/CPEB translational control, reviewed in (Richter, 2007).

b. CPEB-interacting proteins.

Xenopus CPEB performs a dual role – it represses cap-dependent translation in arrested oocytes and activates translation, via cytoplasmic polyadenylation, in meiotically maturing oocytes, eggs and early embryos (de Moor and Richter, 1999; Mendez and Richter, 2001; Richter, 2007). However, there is some controversy about the CPEB-interacting proteins. Here, we summarize the CPEB-interacting proteins known to date.

Maskin

Maskin is member of the transforming acidic coiled-coil containing (TACC) family, which has important roles in cell division and cellular organization in both embryonic and somatic systems (reviewed in (Gergely, 2002)). Maskin not only associates with CPEB but also binds to the cap-binding initiation factor eIF4E. This configuration of factors precludes the interaction eIF4G-eIF4E and thereby inhibits translation by precluding 40S ribosomal subunit recruitment at the 5' end of the mRNA (Cao et al., 2006; Cao and Richter, 2002; Mendez and Richter, 2001; Richter, 2007; Stebbins-Boaz et al., 1999).

Pumilio

Xenopus Pumilio (Pum) is a RBP member of the Pumilio/Fem3-binding protein (PUF) family (Spassov and Jurecic, 2003; Wickens et al., 2002) that specifically associates to maternal mRNAs (Nakahata et al., 2001) as well as CPEB via its PUF domain, in a RNA-independent manner (Zamore et al., 1997; Nakahata et al., 2001; Nakahata et al., 2003). Pum possibly cooperates with CPEB repressing maternal mRNAs such as the one encoding for cyclin B1 (Nakahata et al., 2003).

RCK/Xp54

RCK/Xp54 is a DEAD-box RNA helicase (Minshall and Standart, 2004; Minshall et al., 2001), involved in splicing, RNA transport, degradation and translation (Weston and Sommerville, 2006). Xp54 is present at constant levels throughout oogenesis and is implicated in the nuclear assembly of stored mRNA particles in early oocytes, where it shuttles between nucleus and cytoplasm (Ladomery et al., 1997; Smillie and Sommerville, 2002; Thom et al., 2003). Xp54 associates with CPEB (Minshall and Standart, 2004) and seems to interact also with eIF4E (Minshall and Standart, 2004) suggesting a potential role in translation repression (Coller and Wickens, 2002; Minshall and Standart, 2004; Minshall et al., 2001).

4E-T

CPEB recruits the vertebrate Cup homolog 4E-T (4E-transporter), which in turn binds to an oocyte specific eIF4E isoform (4E1b) (Minshall et al., 2007) suggesting a CPEB-dependent repression complex. In this complex is also found the RNA helicase RCK/Xp54, and the P-body components P100 (Pat1) and Rap55 (Minshall et al., 2007).

PARN

The poly(A)-specific ribonuclease (PARN) is a cap-interacting deadenylase (Balatsos et al., 2006; Copeland and Wormington, 2001; Gao et al., 2000) that also interacts to CPEB (Kim and Richter, 2006). The 64-kDa isoform is thought to be responsible for shortening the poly(A) tail of CPEB-repressed mRNAs in oocytes (Kim and Richter, 2006).

GLD-2

Xenopus germ-line-development factor 2 (GLD-2) is a divergent poly(A) polymerase belonging to the same large family of DNA polymerase β nucleotidyl transferases, but with a limited additional homology to the classical poly(A) polymerases and lacking the RNA-binding domain. Although GLD-2 directly binds to CPEB in both immature and mature oocytes, it is only required for cytoplasmic polyadenylation-induced translation (Barnard et al., 2004; Kim and Richter, 2007; Rouget et al., 2006; Rouhana et al., 2005; Rouhana and Wickens, 2007).

CPSF

The cleavage and polyadenylation specificity factor (CPSF) is a four subunits complex (30, 73, 100 and 160-kDa) that not only mediates RNA cleavage but also subsequent nuclear and cytoplasmic polyadenylation (Barnard et al., 2004; Bilger et al., 1994; Dickson et al., 1999; Kim and Richter, 2006; Mandel et al., 2006; Mendez et al., 2000b; Proudfoot et al., 2002; Rouhana et al., 2005; Wahle and Ruegsegger, 1999). CPSF directly interacts to CPEB through the 160-kDa subunit both in immature and mature oocytes (Barnard et al., 2004; Kim and Richter, 2006; Mendez et al., 2000b; Rouget et al., 2006; Rouhana et al., 2005).

Symplekin

Symplekin is a nuclear protein that is present in complexes containing processing factors involved in 3' end RNA processing. It is a scaffold protein upon which multicomponent complexes are assembled in the nucleus (Hofmann et al., 2002; Takagaki and Manley, 2000; Xing et al., 2004). In *Xenopus* oocytes, however, Symplekin is also found in cytoplasmic complexes with the cytoplasmic polyadenylation machinery such as CPEB, GLD-2 and CPSF (Barnard et al., 2004; Kim and Richter, 2007).

CstF77

CPEB associates with the subunit of the cleavage stimulatory factor CstF77 (Rouget et al., 2006), which is the subunit required for integrity of the CstF complex (77, 64 and 50-kDa proteins) (Takagaki and Manley, 1994; Takagaki et al., 1990) involved, together with CPSF, in pre-mRNA cleavage before nuclear polyadenylation. In addition to its nuclear function, CstF77 may have a role in repression in *Xenopus* oocytes (Rouget et al., 2006). CstF77 also interacts with GLD-2 and CPSF (Rouget et al., 2006).

Aurora A

Aurora A/Eg2 is member of the Aurora family of serine/threonine protein kinases, which has important roles in cell cycle progression, bipolar spindle formation and chromosome segregation (reviewed in (Crane et al., 2004; Ducat and Zheng, 2004; Marumoto et al.,

2005)). Aurora A interacts with CPEB, and also phosphorylates CPEB on Ser174 during early stages of oocyte maturation (Mendez et al., 2000a) increasing the affinity of CPEB for the CPSF (Mendez et al., 2000b). This phosphorylation is the crucial event in polyadenylation-dependent translation of specific maternal mRNAs (Barnard et al., 2005; Charlesworth et al., 2004; Mendez et al., 2000a; Mendez et al., 2000b; Sarkissian et al., 2004).

XGef

The Guanine Nucleotide Exchange Factor, xGef, is a member of the Rho family of GTPase proteins and it is a CPEB-interacting protein (Martinez et al., 2005; Reverte et al., 2003). xGef has been also proposed to stimulate early CPEB phosphorylation. Due to xGef immunoprecipitates seem to contain Mitogen-Activated Protein Kinase (MAPK), it may be required to bring CPEB to the signaling complexes involved in its phosphorylation (Keady et al., 2007).

APLP

The mouse CPEB1 is found to bind the small intracellular domain of the transmembrane Amyloid precursor-like protein 1 (APLP1) and its relatives (Cao et al., 2005). Maskin, CPSF, GLD-2 and several other factors involved in polyadenylation are all detected by immuno-electron microscopy on membranes in the same fractions as APLP1, CPEB and CPE-containing mRNAs (Cao et al., 2005). While the association with amyeloid precursor proteins may have great significance for the role of CPEB in neurons, it is yet unclear whether APLP1 is required for polyadenylation in oocytes and even whether it mediates the membrane association of the polyadenylation machinery.

In *Xenopus* oocytes, CPEB might reside in several ribonucleoprotein-complexes and accomplishes its dual role in translation regulation depending on its association with the above mentioned interacting factors. In arrested immature oocytes, these CPEB-interacting proteins may function in redundant repression mechanisms and, thus, at present is difficult to choose among the multitude of models proposed for translational repression mediated by CPEB. In addition, a given mRNA can exist in more than one complex depending on the combination of factors that are recruited in time and space. Therefore, CPEB can assemble

two functionally opposing complexes: one mediating translational repression by association with Maskin, Pumilio, RCK/XP54 and PARN; and other driving polyadenylation and translational activation in response to progesterone with CPSF, Symplekin and GLD-2 (Barkoff et al., 2000; Barnard et al., 2004; Cao and Richter, 2002; de Moor and Richter, 1999; Kim and Richter, 2006; Minshall and Standart, 2004; Minshall et al., 2001; Nakahata et al., 2001; Nakahata et al., 2003; Rouhana et al., 2005; Stebbins-Boaz et al., 1999) (Fig. 6). The switch of the repression complex to the polyadenylation complex in response to progesterone is regulated by phosphorylation of their components. In addition to the above-described Aurora A phosphorylation of CPEB, all three CPEB, Maskin and Pum are regulated by other phosphorylation events (Barnard et al., 2005; Keady et al., 2007; Mendez et al., 2002; Nakahata et al., 2003; Sarkissian et al., 2004; Stebbins-Boaz et al., 1999).

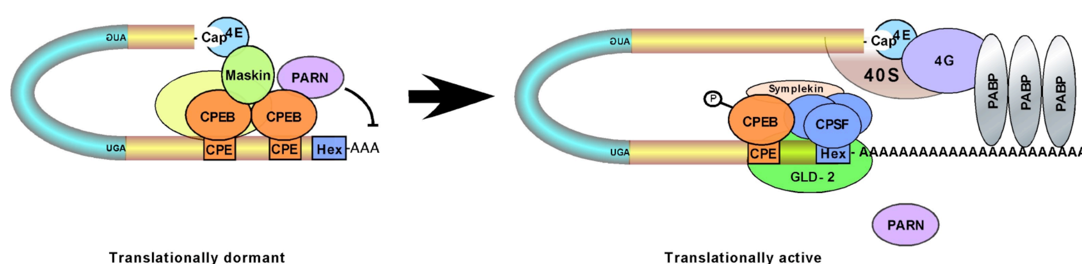


Figure 6. CPEB-mediated translational control. In immature oocytes, mRNAs containing cytoplasmic polyadenylation elements (CPEs) are translationally dormant (masked) and reside in a repression complex containing the CPEB, Maskin, eIF4E and PARN. (Note that other factors could be present (indicated in yellow)). Once maturation begins, newly phosphorylated CPEB (by Aurora A kinase) increases its affinity for the CPSF and expulses PARN from the complex resulting in GLD-2-catalized polyadenylation. The elongated poly(A) is then bound by multiple copies of PABP, which subsequently interacts with and helps eIF4G to displace Maskin from eIF4E allowing translation initiation.

c. Translational repression.

In dormant oocytes, mRNAs irrespective of whether they contain or not a CPE, might acquire a long poly(A) tail (~200-250 nucleotides) in the nucleus as a typical RNA maturation process (Huarte et al., 1992; Kim and Richter, 2006; Sachs and Wahle, 1993). Following nuclear export, only CPE-containing RNAs are able to interact with CPEB and its binding partners that, in turn, remove most of the poly(A) tail to ~20-40 nucleotides. Both PARN and GLD-2 are present and active, in immature oocytes, but due to PARN is more active in the complex,

the poly(A) tail is removed as soon as it is added by GLD-2 (Kim and Richter, 2006). However, a short poly(A) tail by itself is not sufficient to fully repress translation. For this to occur, another factor, Maskin, is involved. Thus, CPEB through its bridging partner Maskin, repress translation precluding the association of eIF4G to eIF4E and therefore the 40S ribosomal subunit recruitment required for translation initiation at the 5' end of the mRNA (de Moor and Richter, 1999; Stebbins-Boaz et al., 1999) (Fig. 6).

Although CPEB can recruit PARN to the mRNA, deadenylation by PARN requires a 5' cap structure (Balatsos et al., 2006; Copeland and Wormington, 2001; Gao et al., 2000; Kim and Richter, 2006) leading to a conflict surrounding the presence of Maskin and PARN in the same complex.

d. Translational activation.

Progesterone-induced meiotic-resumption causes the translational activation of Ringo mRNA (Ferby et al., 1999; Gutierrez et al., 2006; Lenormand et al., 1999; Padmanabhan and Richter, 2006) and Glycogen Synthase Kinase (GSK-3β) inactivation leading to an activation of Aurora A kinase (Sarkissian et al., 2004). Thus, CPEB is activated by phosphorylation on Ser174 by Aurora A (Mendez et al., 2000a; Sarkissian et al., 2004). However, there is some discussion about the kinase mediating this early phosphorylation and MAPK has been recently shown implicated in prime CPEB for Ser174 phosphorylation or even in activate the possible Ser174 kinase (Keady et al., 2007).

This CPEB phosphorylation on Ser174 as a result of progesterone stimulation increases its affinity for CPSF (Mendez et al., 2000b), which, in turn, recruits the cytoplasmic poly(A) polymerase GLD-2 (Barnard et al., 2004; Rouhana et al., 2005), and also induces the ejection of PARN from the complex (Kim and Richter, 2006). Concomitantly, Maskin is phosphorylated by Cdc2 (Barnard et al., 2005) or Aurora A kinase (Pascreau et al., 2005) and thus is dissociated from eIF4E (but not from CPEB) (Cao and Richter, 2002). In addition, embryonic poly(A)-binding protein (ePABP), is recruited to the 3' end of the mRNA by a transient association with the polyadenylation complex, promoting the recruitment of and help eIF4G to displace Maskin from eIF4E enabling initiation of translation (Cao and Richter, 2002; Kim and Richter, 2007; Wakiyama et al., 2000) (Fig. 6).

e. Temporal control of translation, meiotic progression in *Xenopus* oocytes.

In most vertebrates, full-grown but immature oocytes are arrested at prophase of meiosis-I (prophase-I: PI; diplotene). During the long period of growth named oogenesis, these oocytes synthesize and store in theirs cytoplasm a complex population of mRNAs, which will drive oocyte re-entry in the meiotic cell cycle and later on early embryonic divisions (Mendez and Richter, 2001; Schmitt and Nebreda, 2002). Meiotic resumption, which includes germinal vesicle breakdown (GVBD), chromosome condensation and spindle formation marks the onset of oocyte or meiotic maturation and in *Xenopus* is stimulated by the hormone progesterone (Figure 6). Meiotic or oocyte maturation is comprised of two consecutive M-phases, meiosis-I and meiosis-II (MI and MII) without an intervening S-phase (Iwabuchi et al., 2000). At MII the oocytes become arrested for a second time, as the result of the synthesis and activation of the Cytostatic Factor (CSF), and await for fertilization (Sagata, 1996). Remarkably, in *Xenopus* oocytes these transitions occur in the absence of transcription, which does not resume until mid-blastula transition, and are fully dependent on the sequential translational activation of the maternal mRNAs accumulated during oogenesis (Mendez and Richter, 2001). Synthesis of the proto-oncogene c-mos, a serine/threonine kinase encoded by a cytoplasmically polyadenylated mRNA, leads to the activation of the MAPK, one of whose targets is $p90^{Rsk}$, reviewed in (Schmitt and Nebreda, 2002). Once fertilization takes place, meiosis finally ends and embryonic cell divisions start. Mitosis in the embryo is unlike any other and consists on a rapid succession of M- and S-phases without intermediate G1 or G2 phases.

5.2.2. Translation regulation by poly(A) tail length: deadenylation.

Deadenylation: AREs, EDEN and default

In mammals there are two main mRNA turnover pathways, 5’ to 3’ degradation, and 3’ to 5’ degradation. Which target aberrant and deadenylated mRNAs (Fig. 7). Regardless of the pathway initiating mRNA turnover and the target mRNAs they all converge to the use of the same machinery.

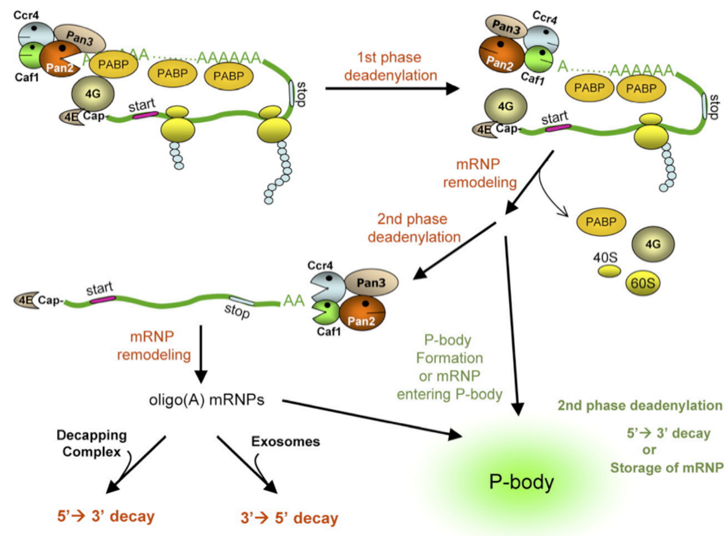


Figure 7. Pathways of eukaryiotic mRNA turnover. Taken from (Zheng et al., 2008).

a. Trans-acting factors.

Degradation of non-aberrant mRNAs begins with the shortening of the poly(A) tail at the 3' end of the mRNA by a variety of mRNA deadenylases. In mammalian cells two distinct phases trigger deadenylation; the first is characterized by a synchronus and stepwise shortening of the poly(A) tail, while in the second mRNAs become heterogeneous in their poly(A) tails lengths. This biphasic deadenylation is the consequence of sequnetial action of the Pan2 and CCR4 multisubunit complex activities (Yamashita et al., 2005). Following deadenylation, a decapping enzyme consisting of two subunits, Dcp1p and Dcp2p, removes the 5' cap structure, exposing the transcript to digestion by a 5' to 3' exonuclease, Xrn1p. Alternatively, after deadenylation, mRNAs can be degraded in a 3' to 5' direction by the cytoplasmic exosome, a complex of diverse 3' to 5' exonucleases. In this case, the resulting oligonucleotide cap structure is hydrolyzed by the DcpS scavenger decapping enzyme. (Parker and Song, 2004; Yamashita et al., 2005).

Four major deadenylase complexes have been described in vertebrates, variations of several components are found between species but have the same core components. Both in yeast and in mammals, the CCR4 complex was shown to bear the main catalytic activity responsible for cytoplasmic deadenylation (Daugeron et al., 2001; Tucker et al., 2001; Yamashita et al., 2005), whereas the second known cytoplasmic deadenylase, the Pan2–Pan3 complex, affects more particularly the initial phase of poly(A) shortening (Brown and

Sachs, 1998; Yamashita et al., 2005). Very recently these two complexes, CCR4-Not and the Pan2/Pan3, have been shown to interact via the CAF1 and Pan2 subunits respectively, and also to interact with PABP (Zheng et al., 2008).

The CCR4 complex, in different cell types or conditions is constituted by different proteins, therefore different sizes of the multicomplex subunit have been observed with molecular weights ranging from 0.65 to 2 mega daltons (Chen J et al., 2001).

The two minor deadenylation complexes are Nocturnin and PARN. Nocturnin was first described in xenopus retina were it is implicated in circadian regulation of mRNAs by deadenylation (Baggs and Green, 2003). Finally, PARN is unique since it interacts with the 5'cap structure during deadenylation (Dehlin et al., 2000; Monecke et al., 2008; Nagata et al., 2008), enhancing it's processivity. It is a nuclear and cytoplasmic deadenylase that may be involved in poly(A) tail shortening after mRNA processing (Kim and Richter, 2006). Pan2/3 is also nuclear in yeast and is proposed to be responsible for determining the poly(A) tail length of new transcripts in yeast.

Pan2/Pan3 complex is recruited and to the mRNA and stimulated by the PABP (Korner et al., 1998; Siddiqui et al., 2007; Zheng et al., 2008) and it also interacts with the translation factor eRF3 (Funakoshi et al., 2007). PARN interacts with the cap structure.

b. Cis-acting elements promoting deadenylation.

All transcripts are affected at some time by default deadenylation since it is the first step of the mRNA turnover pathway, still, specific transcripts can be targeted for deadenylation by several sequences in their 3'UTRs, such as EDEN (embryo deadenylation element), or AREs (A-U rich elements).

-EDEN: the consensus sequence is five repetitions of the UGU trinucleotide in a window of 35 nucleotides (graindorge et al. 2006). This sequences are bound by the *Xenopus* protein EDEN-BP a 53/55 kilodaltons doublet protein, but can also bind to AREs (Paillard et al.,

2002). CUG-BP is the EDEN-BP human homolog (Paillard and Osborne, 2003), which interacts with PARN to mediate deadenylation (Moraes et al., 2006).

-ARE: ARE motifs are the most studied cis-acting elements responsible for rapid turnover of unstable mRNAs in mammalian cells. They are located in the 3'UTRs of responding mRNAs. AREs, have been classified in three different categories (table 1) (Espel, 2005), although no real consensus sequence has yet been precisely defined for any class of ARE and, furthermore, these ARE classifications are based neither on the associated proteins nor on biological functions (Barreau et al., 2005), alternatively the classification is based in the deadenylation kinetics: class I and III AREs show synchronus poly(A) shortening (distributive kinetics), while class II AREs show asynchronus deadenylation (processive kinetics) (Xu et al., 1997). Most of the identified mRNAs containing AREs are cytokines or mRNAs implicated in inflamatory responses, but also there are cell-cycle related mRNAs which play important roles in development (p53, p21, cyclinA).

ARE Class	Consensus sequence	Example mRNA
I	AUUUA	c-fos, c-myc
II	UUAUUUAUU	TNFα
III	PolyU	c-jun

Table 1. ARE classification according to the cis-sequence. (Espel, 2005)

AREs are bound by ARE-binding proteins (ARE-BPs), to date, many have been described some promote destabilization by binding to an mRNA, but others prevent destabilization, therefore it seems that in some cases it is the relative abundance of the ARE-BPs in cells that determines the stability of ARE-containing mRNAs. Despite this, there is not a clear view of which proteins bind to which AREs, and what's more important the mechanism recruiting deadenylase complexes has not been elucidated for most of them (Barreau et al., 2005).
One of the most studied ARE-BPs is TTP, that negatively regulates the stability of TNFα, GM-CSF, IL2, IL3, c-fos and COX-2 mRNAs. Interaction with PARN (Lai et al., 2003), CCR4

(Rowlett et al., 2008), Xrn1 (Hau et al., 2007) and PABP (Rowlett et al., 2008) have been described, but there is contradictory data in this respect.

c. Deadenylation in Development.

During development the total amount of RNA remains unchanged (Golden et al., 1980), but it was observed that the total amount of polyadenylated (pA^+) RNA was reduced ~40% upon maturation (Darnbrough and Ford, 1976; Sagata et al., 1980). It was later shown (Colot and Rosbash, 1982) that this reduction in the pA^+ fraction was due to selective deadenylation of a subset of mRNAs such as cytoskeletal actins, ribosomal protein mRNAs and translation initiation factor 1α. The first explanation was that the subset of deadenylated mRNAs contained sequences in their 3'UTR that promoted the selective deadenylation (Hyman and Wormington, 1988), since introduction of CPEs in these 3'UTRs inhibited the deadenylation, it was concluded that mRNAs lacking CPEs were all deadenylated by a default process that does not require specific 3'UTR sequences (Varnum and Wormington, 1990). Later on, ARE-mediated deadenylation was described in oocytes by the microinjection of chimaeric RNAs (Voeltz and Steitz, 1998). Immature oocytes showed 20 fold less activity than mature oocytes or fertilized eggs, but yet no endogenous mRNA was identified. Voeltz and Steitz also observed that the increasing number of ARE elements would result in a stronger deadenylation and that until the midblastula transition (MBT), at the onset of transcription, deadenylation was uncoupled from mRNA degradation, unlike other studied conditions such as mammalian cells. Finally, default deadenylation is stimulated in maturing oocytes (Fox and Wickens, 1990; Varnum and Wormington, 1990), and this default deadenylation persisits after fertilization (Legagneux et al., 1995; Stebbins-Boaz and Richter, 1994). Default deadenylation in mature oocytes is attributed to PARN (Dehlin et al 2000).

During embryo development and until MBT, only EDEN-mediated deadenylationhas been shown (Legagneux et al., 1992), and it is regulated by phosphorylation of EDEN-BP upon fertilization (Detivaud et al., 2003). Some of the identified targets of EDEN-mediated deadenylation are the mRNAs of: Aurora A(Eg2), Aurora B, c-Mos, Cdk1, Cdk2(Eg1), casein Kinase 2 beta, Bub 3, Wee1, MELK(Eg3), Eg5/KLP and NEK2B, CPEB (Graindorge et al., 2008) .

6. Meiosis.

Meiosis consists of two consecutive M phases without an intervening S-phase. Most of the knowledge accumulated to understand meiosis, has been achieved from the study of the *Xenopus laevis* oocyte maturation. Vertebrate oocytes undergo a round of DNA replication at the beginning of the meiotic cell cycle, then they enter in the first prophase were they are arrested in the diplotene stage, for an uncertain amount of time. During the first period, named oogenesis, oocytes grow in size and synthethize a stockpile of mRNAs that remains stable until the mid blastula transition (MBT) where transcription starts again (Golden et al., 1980). Hormonal stimuli, provided by folicular cells surrounding the oocytes, release fully grown oocytes (Dumont Stage VI; sVI) from the arrest allowing re-entry to the cell cycle until new arrest at metaphase II, where the egg awaits for fertilization. As soon as maturation starts transcription is actively repressed, and translational mechanisms to regulate the expression of the stored mRNAs are activated (Mendez and Richter, 2001). From this moment and until MBT protein expression will only be regulated at the level of translation and protein degradation.

Cytoplasmic polyadenylation has been described as the key mechanism regulating translation during early development. This mechanism controls the translation of key molecules for meiotic progression such as mos, cyclins B, wee1, cdk2, cyclin A1, TPX2, Xkid and also histone B4, G-10, lamin B1 and FGFreceptor (Pique et al., 2008; Sheets et al., 1994) (Charlesworth et al., 2000; Culp and Musci, 1998; Eliscovich et al., 2008; McGrew et al., 1989; Paris and Philippe, 1990; Ralle et al., 1999; Stebbins-Boaz and Richter, 1994). But polyadenylation of other mRNAs than these are probably required for meiotic progression (Barkoff et al., 1998). There is a first stage of polyadenylation independent of protein synthesis and cdc2 activity, which takes place before GVBD. At this stage, mos cyclin B2 and B5 are polyadenylated. The second stage of polydenylation takes place at MI, this polyadenylation is dependent on protein synthesis and cdc2 activation (Pique et al., 2008).

Three key activities for meiotic progression are the maturation promoting factor (MPF), heterodimer of cdc2 and cyclinB, the anaphase promoting complex/cyclosome (APC/C), a large multisubunit complex (Buschhorn and Peters, 2006), and the cystostatic factor (CSF),

defined as the activity that maintains the MII arrest. MPF and APC/C activities are related and opposing; MPF activates APC/C and in turn APC/C inhibits MPF by triggering degradation of cyclins B. For MI entry high MPF activity is required, then APC/C activity is increased lowering the MPF activity and therefore allowing MI exit. During anaphase high APC/C activity is a hallmark but also some MPF activity is required to inhibit replication, which is achieved by the combination of degradation and high translation rate of cyclins B1 and B4 (Iwabuchi et al., 2000). At the end of the first meiotic division the polar body is extruded with half of the DNA content. For the second metaphase entry APC/C activity is decreased due to the activity of its inhibitors and then MPF rises again. The CSF activity, which begins before MII entry, will then establish and maintain the arrest at MII by stabilizing the MPF. Masui and Markert (1971) defined the CSF as an activity that appears during maturation, with the highest level at MII, that causes metaphase arrest when microinjected into embryonic blastomeres and that is inhibited by elevated intracellular calcium upon egg activation. The composition of the CSF has not been well defined yet, but what is known are some of the molecules that are required for the CSF establishment, and for CSF arrest mantainance. CSF establishment occurs before MII entry and the key molecules involved are mos/MAPK pathway, Emi2, cyclin E/cdk2. The mantainance of the CSF arrest is sustained by the activities of mos and Emi2. CSF release is triggered by the elevation of intracellular calcium, which activates CaMKII phosphorylation activity, to phosphorylate among other targets Emi2, in turn, this phosphorylation primes Emi2 for the recruitment and phosphorylation of Plx1, which triggers the degradation of Emi2 by the proteasome, allowing the destabilization of MPF and increase od APC/C activity, therefore resumption of meiosis (Rauh et al., 2005).

OBJECTIVES

1.Genome-wide identification of CPE-regulated mRNAs and their function in meiotic progresssion.

2. Identification and characterization of C3H-4 as an ARE-binding protein required for meiotic progression.

RESULTS

Xenopus laevis oocytes is the model system of choice for studying both meiosis and translational regulation by cytoplasmic polyadenylation. The features of this system allow the study of individual cells since they are big enough for manipulation (~1.3 mm), but also allow the use of a larger amount of oocytes because they can be obtained in large quantities. Oocytes are arrested at prophase I of meiosis, upon progesterone treatment meiotic ressumption can be induced and easily followed morphologically and biochemically, which allows the study of the process in detail. For instance, metaphase I occurs between 2 and 6 hours after progesterone treatment, it can be scored by the appearance of a white spot in the pigmented animal pole of the oocyte, this is the result of the displacement of the cortical pigment granules upon nuclear or germinal vesicle breakdown (GVBD). A biochemical marker for meiotic progression is the cdc2 activity, it has been very well characterized that this activity peaks upon metaphase I entry, then the activity falls to low levels during the anaphase I and peaks again for metaphase II entry, this activity can be measured by the phosphorylation of recombinant Histone H1 which is a substrate of cdc2 kinase. This system also offers the possibility of microinjection of molecules into the cytoplasm or into the nucleus. For instance microinjection of DNA oligonucleotides combined with the endogenous RNAseH activity of the oocytes, also microinjection of *in vitro* transcribed mRNAs can be used to express any protein.

Finally it is a very good system to study translational control because transcription is shut off and therefore the changes in protein expression are only due to translational regulation combined with protein degradation. Furthermore, mRNA degradation does not occur to protect the maternal mRNAs until transcription starts again, and this is probably due to the absence of Xrn1 a decapping enzyme, again, this feature provides a system where mRNA stability does not influence in protein expression.

Hence, in this thesis work we have used the *Xenopus laevis* oocyte system to study translational control during meiosis.

1. Identification of mRNAs polyadenylated during meiosis.

Maturation requires protein synthesis in the absence of transcription, only a few mRNAs have been reported to be regulated by cytoplasmic polyadenylation during meiosis although it is the major mechanism regulating translation during this period. The known CPE-regulated mRNAs are not enough to support meiosis, therefore our main goal was to identify all the polyadenylated mRNAs during meiosis. For this aim we designed a functional screening to identify them.

1.1. Poly(U) chromatography selection for RNAs that contain a poly(A) tails of 70 nucleotides or more.

From previous studies in our lab, it was determined that in *Xenopus* oocytes the minimal poly(A) tail length for efficient translational stimulation was 70 nucleotides (unpublished data). For this reason we set up a poly(U) chromatography, based on a previous report (Jacobson, 1987), that would retain mRNAs with a poly(A) tail of 70 nucleotides and longer, to then elute them, in a fraction we called elution. This method allowed us to selectively isolate mRNAs with a poly(A) tail of 70 nucleotides or longer. The specificity of this approach was validated by microinjection of reporter RNAs, which either contained or not CPE elements (+CPE, -CPE) combined with presence or absence of poly(A) tail of 73 nucleotides (+pA, -pA). The reporter RNAs were individually microinjected into stage VI oocytes. A pool of oocytes where also treated with progesterone to allow CPE-dependent polyadenylation of the reporters. Then the retention of the reporters in the poly(U) chromatography was evaluated by specific RT-PCRs for each construct; the flow through and the eluted fraction were analyzed. In the absence of progesterone, only the control-RNAs containing a poly(A) tail before injection (+CPE+pA, -CPE+pA) were recovered after selection by poly(U) chromatography (Fig. 8, lanes 1 and 3), whereas in the presence of progesterone the RNA containing a functional CPE but microinjected without a poly(A) tail (+CPE-pA) was also present in the pA+70 fraction (Fig. 8 lane 6). The reporter with neither CPE nor poly(A) (-CPE-pA) was not recovered after poly(U) chromatography in either case (Fig. 8, lanes 4 and 8). As a control of injection the flow through fractions were also analyzed, and similar amounts of the reporter

RNAs were found in all conditions (Fig. 8, lanes 9-12).

We concluded that indeed the poly(U) chromatography allowed us to selectively isolate mRNAs with a poly(A) tail length of at least 70 nucleotides.

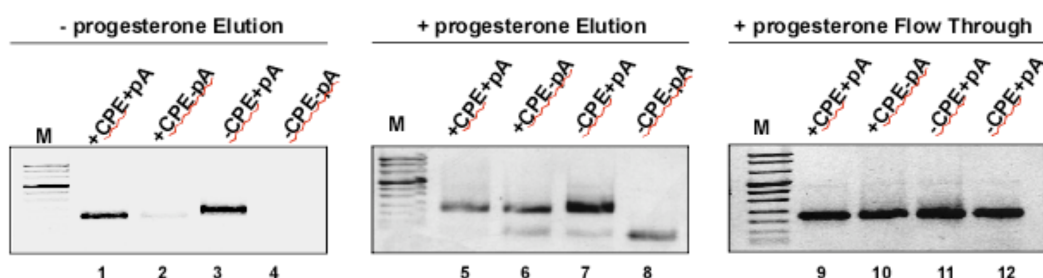


Figure 8. Setting-up of poly(U) chromatography for the selection of mRNAs with a poly(A) tail of at least 70 nucleotides. PCR analysis of reporter RNAs from the elution fractions in progesterone untreated input (lanes 1-4), progesterone treated input (lanes 5-8), and flow through from progesterone treated input (lanes 9-12).

1.2. Discrimination between early and late polyadenylated mRNAs.

As previously described, Cycloheximide (Chx) treatment or mos antisense injection prevents late polyadenylation (Ballantyne et al., 1997). Therefore we tested whether we could selectively isolate “early” versus “late“ polyadenylated mRNAs, using the poly(U) chromatography in combination with the cycloheximide treatment. For this purpose we carried out the poly(U) chromatography using two different input RNAs, one where the input RNA was total RNA from Chx and progesterone treated oocytes and another where the input RNA was total RNA from progesterone treated oocytes. The eluted fractions, containing the poly(A) + 70 mRNAs were analyzed by PCR for the presence of endogenous mos, cyclin B1, GAPDH and tubulin cDNAs. mos is a very well known “early” polyadenylated mRNA, while cyclin B1 is a very well described “late” polyadenylated mRNA (Ballantyne et al., 1997). GAPDH and Tubulin were used as negative controls since they are not translationally regulated by cytoplasmic polyadenylation. The results for this validation show that mos mRNA, but not cyclin B1 mRNA, was present both in the MI and MII libraries (Fig. 9b), while cyclin B1 mRNA was only retained in the poly(U) column in the MII library as expected. GAPDH and tubulin mRNAs were not purified in either case (Fig. 9b).

Therefore, we validated the prediction that the poly(U) chromatography of RNA from Chx and progesterone treated oocytes would selectively isolate early polyadenylated mRNAs, while poly(U) chromatography from RNA from progesterone treated oocytes would contain both early and late polyadenylated mRNAs.

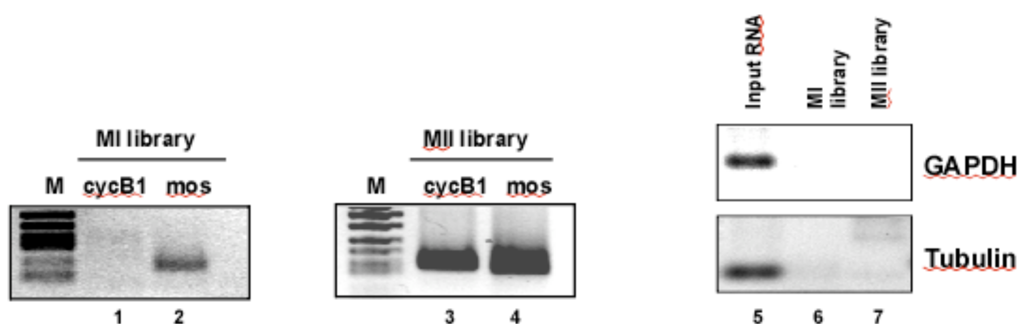


Figure 9. Discrimination of early vs. late polyadenylated mRNAs. Specific PCRs of cyclin B1 (cycB1), mos, GAPDH and tubulin were performed from pA+70 fraction of the poly(U) chromatography where in (a.) input RNA from Chx and progesterone treated oocytes was used, in (b.) input RNA is from progesterone treated oocytes, and in (c.) lane 6 is from Chx and progesterone treated oocytes and lane 7 from progesterone treated oocytes.

1.3. Screening to identify mRNAs polyadenylated during meiotic progression.

To describe which mRNAs other than the already known are polyadenylated during oocyte maturation, we designed a functional screening that would allow us to isolate RNAs that acquired a poly(A) tail during meiosis using the poly(U) chromatography procedure decribed in sections 1.1 and 1.2. (Fig. 10).

For this aim, a *Xenopus laevis* oligo dT primed cDNA library (Wellcome/CRC Institute), generated from total egg RNA, was *in vitro* transcribed, the resulting transcripts were deadenylated and microinjected into stage VI oocytes. Following stimulation with progesterone, total RNA was extracted and resolved by poly(U) chromatography (as described in section 1.1), selecting those RNAs that during the maturation process had acquired a poly(A) tail of 70 adenines or more. The screening was carried out both in the presence and absence of Cycloheximide (Chx), thus obtaining two different populations of polyadenylated mRNAs (Fig. 10). One corresponds to “early” polyadenylated mRNAs, obtained when including Chx treatment, referred from now on as MI library, and the other

population contained both “early” and “late” polyadenylated mRNAs, obtained in the absence of the Chx treatment, referred from now on as MII library.

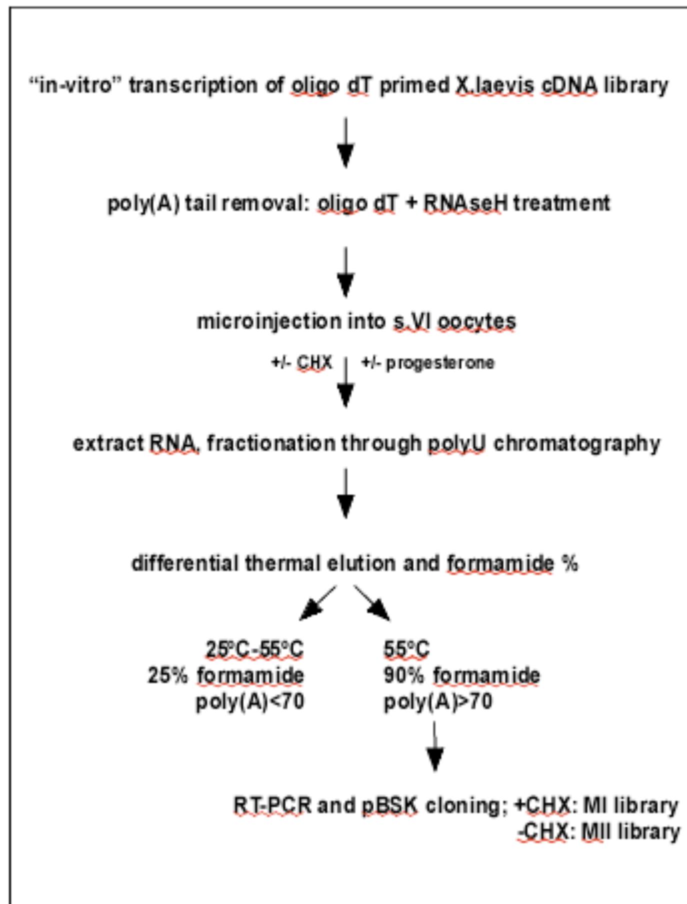


Figure 10. Scheme of the fuctional screening steps. By this method two libraries were generated; MI library containing only “early” polyadenylated mRNAs, and MII library containing both “early” and “late polyadenylated mRNAs.

1.4. Identification of the libray mRNAs and analysis of 3’UTR regulatory elements.

The RNAs recovered from the poly(U) eluted fractions were retrotranscribed and amplified for cloning (see methods for more details). The clones were sequenced and by blast search the corresponding mRNAs were identified. In the MI library, 55 different sequences were found (Appendix 1), within these by blast search we identified 21 mRNAs, 18 hypothetical proteins and 15 corresponded to sequences from which no ESTs were found.

In the MII library we found 54 different sequences (Appendix 2), from which only 15 sequences correspond to known mRNAs. Since we sequenced 5% of the clones and obtained 54 new sequences, we estimate that there will be arround 2000 mRNAs in this library and therefore polyadenylated during maturation.

We searched for putative translational regulatory elements in the 3'UTRs of the mRNAs recovered in the libraries, consistently all UTRs contain the sequences required for cytoplasmic polyadenylation, the Hex and at least one CPE. In the MI library some mRNAs contain consensus CPEs and others non consensus CPEs, also there are mRNAs with one CPE or multiple CPEs, and all correspond to "early" CPE arrangements. Moreover 55% of the 3'UTRs also contain an additional sequence that is involved in regulating the length of the poly(A) tail, AU-rich elements (AREs), that can either promote or inhibit deadenylation of the mRNA. The fate of these mRNAs is determined by the ARE-binding protein (AREBP) bound to the mRNA.

The 3'UTR sequences from the MII library contain "early" CPE arrangements 92% but also "late" CPE arrangements (6%). Also more than half of the sequences (59%) contain AREs.

a.

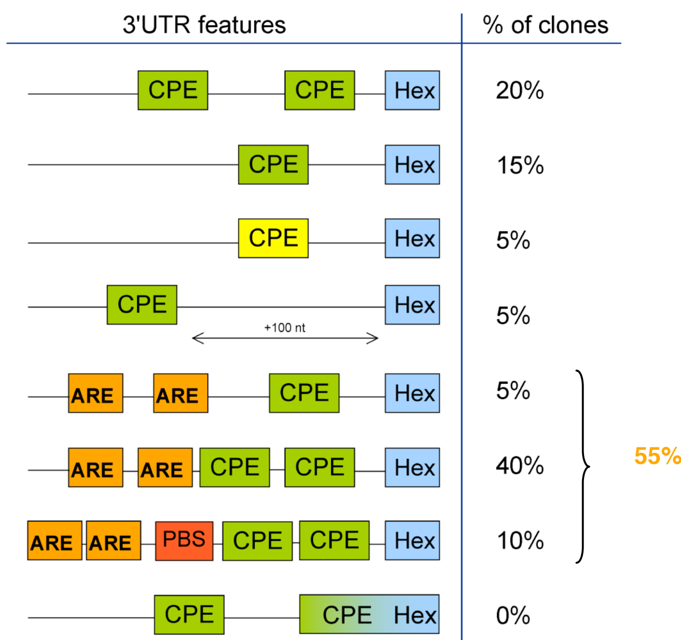
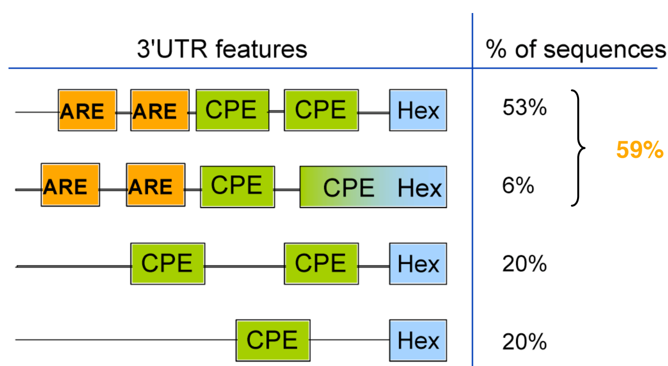


Figure 11. Scheme of the 3'UTR elements present in the full length UTRs of the mRNAs identified in the MI library **a.** and MII library **b.** mRNAs were classified according to features of the 3'UTR elements. The number of mRNAs in each group is represented by the percentage of clones.

b.



1.5. Cytoplasmic polyadenylation of “library mRNAs”

To functionally validate the screening for polyadenylated mRNAs, some of the library mRNAs were challenged to a polyadenylation assay. *In vitro* transcribed [^{32}P] UTP labelled RNAs, corresponding to the 3’UTRs, were microinjected into immature oocytes. The oocytes were then incubated in the presence or absence of progesterone, total RNA was extracted and resolved in 6% Acril-Urea gel, to finally visualize the RNA probes by autoradiography. This assay shows that all of the tested RNAs undergo polyadenylation to some extent upon progesterone treatment (Fig. 12). These results indicate that the libraries we have generated contain mRNAs which are polyadenylated during meiosis.

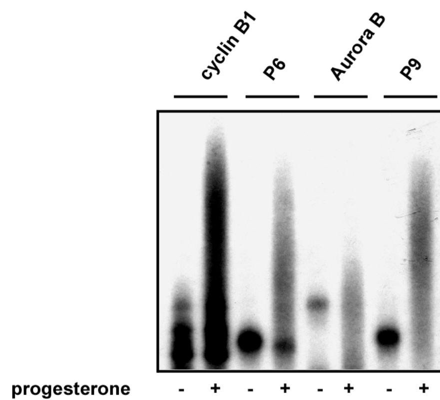


Figure 12. *In vitro* polyadenylation assay of “library mRNAs”. Oocytes microinjected with cyclin B1 radiolabelled 3’UTR as a control, and p6, aurora B, p9 radiolabelled library 3’UTRs, a pool was treated with progesterone (+) and others remained untreated. Total RNA was extracted from the different pools and analyzed in a denaturing 6% Acril-Urea Gel followed by autorradiography.

2. Identification of mRNAs required for meiotic progression.

We designed a second screening to identify whithin the newly-identified cytoplasmically polyadenylated mRNAs which of them were also required for meiotic progression. For this aim we designed antisense oligos that would target the 3’UTR of one mRNA to direct it’s RNAseH-mediated scission, consequently ablating specifically the translational control of the chosen target. The meiotic effects of the indicated microinjected antisense oligonucleotides, would be assessed by following chromosome dynamics, by Hoechst DNA-staining, at the

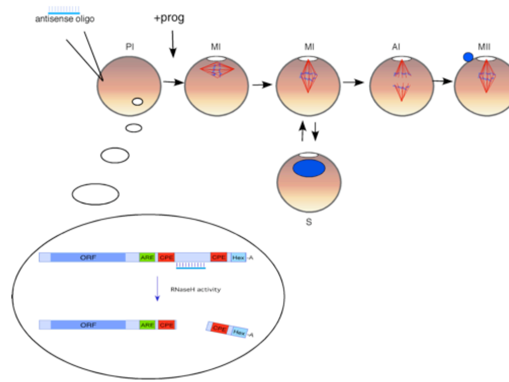
time when the control oocytes reached MII, since chromosomal events are very easily scored (Fig. 13 a). We tested five mRNAs chosen arbitrarily to be assesssed for meiotic progression.

2.1. Meiotic effects of individual translational inhibition of five mRNAs

To test the hypothesis that polyadenylation of other mRNAs than only mos are required for meiotic progression (Barkoff et al., 1998), we ablated the expression of five mRNAs, X71067, hsp90, tyrosine phosphatase 4a2, PP2C and C3H-4, within the newly identified mRNAs in section 1.4. Analysys of the meiotic effects upon the inhibition of the individual mRNAs, by staining the chromosomes and observing them under the microscope, indicated that while control oocytes reached MII arrest, defined by the presence of the second metaphase plate and the polar body (Fig. 13 a), X71067 and Hsp 90 mRNA-scissed oocytes were arrested in prophase I, which is shown by the presence of condensed chromosomes not organized in a metaphasic plate and the absence of polar body (Fig. 13 b). Also, Tyr phosphatase 4a2 and PP2C mRNA-scissed oocytes showed decondensed chromatin in the absence of polar body, consistent with the exit from meiosis into an S-like phase (Perez et al., 2002) (Fig. 13 b). Finally, C3H-4 depleted oocytes displayed a more unusual phenotype with partially decondensed chromosomes and no visible polar body (Fig. 13 b).

Therefore all the tested mRNAs’ translation is required for meiotic progression. They affect different phases in meiotic progression shown by the different phenotypes upon inhibition of their synthesis.

a.



b.

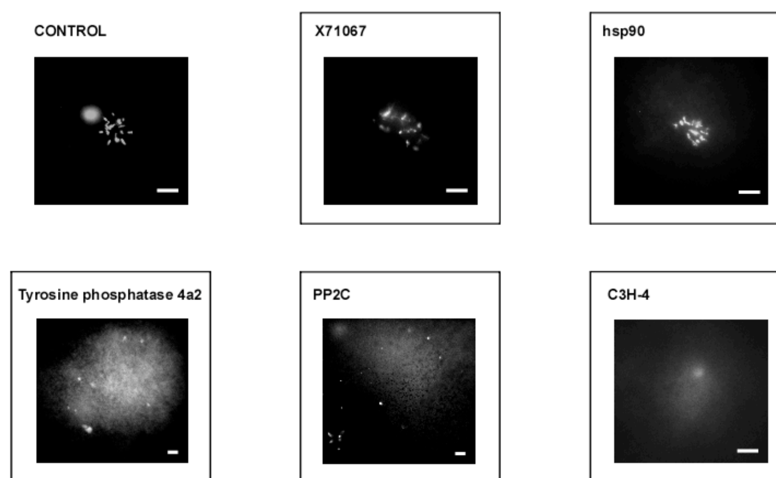


Figure 13. Meiosis requires translation of five newly identified mRNAs **a.** Schematic representation of a screening to identify mRNAs translation of which are required for meiotic progression. Chromosome dinamics of maturing oocytes and oocytes that exit to S-phase (S) during maturation are pictured. **b.** Meiotic effects of the indicated microinjected antisense oligonucleotides, assessed by Hoechst DNA-staining at the time when the control oocytes reached MII. Scale bars represent 20 µm.

3. The role of C3H-4 in meiosis.

Since more than half of the UTRs identified in our screening contained AREs, we focused in the role in meiosis of one of the newly identified mRNAs, C3H-4, which codes for an ARE-binding protein, with four Cx8Cx5Cx3H repeats, member of the Tristetraprolin (TTP) family of proteins (De et al., 1999; Lai et al., 2000). Considering the possibility that this protein could have a role in ARE-mediated deadenylation during meiosis.

3.1. Endogenous mRNA expression and polyadenylation profile.

Expression of C3H-4 has been described to be restricted to the ovaries, oocyte, egg and early embryonic stages until mid blastula transition (De et al., 1999). Also, in immature oocytes C3H-4 mRNA is localized to the animal pole of the oocyte (De et al., 1999). We have identified C3H-4 as a new mRNA that is regulated by cytoplasmic polyadenylation during meiosis. The 3'UTR of C3H-4 contains two potential CPEs, and an hexanucleotide (Fig. 14 a), which according to the combinatorial code of cis-acting elements (Pique et al., 2008) would commit the mRNA to be polyadenylated early during maturation. To analyze changes in the mRNA length during oocyte maturation, we first analyzed by northern blot the endogenous C3H-4 mRNA, and have seen that the mobitity of the mRNA is decreased in response to progesterone stimulation of the oocytes, but upon treatment of the total RNA with RNAseH and oligo dT, which would remove the poly(A) tail, the mobility is increased again indicating that the difference in mobility was due to the presence of a poly(A) tail (Fig. 14 b). To further determine at which time during maturation the mRNA is polyadenylated, we blocked polyadenylation of the late mRNAs by microinjection of mos antisense oligonucleotide. Then we assessed polyadenylation of the endogenous mRNA by RNA-ligation coupled to RT-PCR, we have seen that C3H-4 mRNA is also polyadenylated when late polyadenylation is blocked (Fig. 14 c), therefore it is an early polyadenylated mRNA in agreement with its CPE arrangement (Pique et al., 2008). The subtle difference in length of the poly(A) tail in mos microinjected oocytes compared to control oocytes, is due to the fact that blocking late polyadenylation also blocks a positive feedback loop for polyadenylation which is activated by Cdc2. Other early polyadenylated mRNAs also show this effect when late polyadenylation is blocked (Pique et al., 2008).

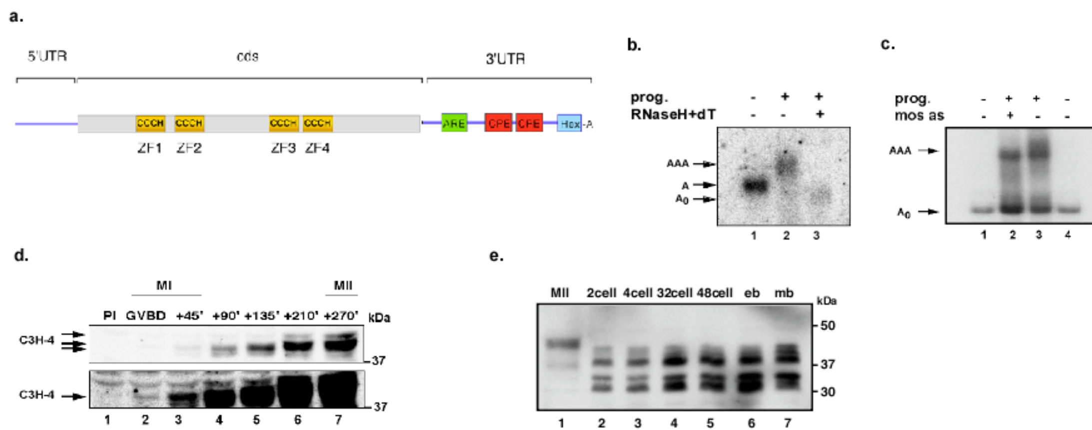


Figure 14. C3H-4 is polyadenylated and translated during oocyte maturation. **a.** Schematic representation of C3H-4 mRNA, the 5'UTR, the coding region (cds) which contains four zinc fingers (ZF1-ZF4), the 3'UTR which has ARE and CPE regulatory elements and and hexanucleotide (Hex). **b.** Northern Blot to show C3H-4 mRNA of progesterone treated oocytes (+) or not (-), and RNaseH and oligo dT treated (+) or not (-). A_0 shows deadenylated mRNA, A shows mRNA with short poly(A) tail, AAA shows mRNA with a long poly(A) tail. **c.** RNA-ligation coupled to RT-PCR, to show endogenous C3H-4 polyadenylation in mos antisense (mos as) injected oocytes (+) or not (+). **d.** Time course of maturation analyzed by Western Blot with anti-C3H-4 antibody, immature oocytes (PI), at GVBD and the indicated minutes after GVBD. The lower pannel corresponds to the same blot exposed for a longer period. **e.** Time course of embryonic development analyzed by Western Blot with anti-C3H-4 antibody. MII corresponds to a mature oocyte, then embryos at the 2 cells, 4 cells, 32 cells, 48 cells, early blastula (eb), and mid blastula (mb) stages were analyzed. Two embryos were loaded in each lane.

3.2. Endogenous protein expression.

To analyze the expression of C3H-4 during maturation, we raised antibodies against the protein. In accordance with the polyadenylation profile, we have shown, that the expression of C3H-4 starts early during the maturation process and increasing amounts of protein are accumulated towards the end of meiosis, MII (Fig. 14 d). The relative level of C3H-4 during early development was also examined by wertern blot using the anti-C3H-4 antibody. C3H-4 protein was present at high levels in MII arrested oocytes, the protein was also detected in embryos but had slower mobility relative to that shown in MII oocytes, this is most likely due to phosphorylation as the changes in mobility shown during maturation (Fig. 14 e). The significance of this observations remain to be determined.

3.3. C3H-4 phosphorylation during maturation.

Because C3H-4 runs as a doublet in MII we tested whether the mobility shift was due to a phosphorylation. Phosphorylations may cause proteins shift to a higher apparent molecular weight on SDS-PAGE gels, in this case the presence of a phosphorylated residue can be detected by dephosphorylation of the protein and subsequent analysis of the size by western blot. Since C3H-4 is detected as a doublet in MII we first did an alcaline phosphatase treatment of MII extracts followed by detection of C3H-4 by western blot to test this posssibility. Indeed, alcaline phosphatase treatment yielded a faster migration of C3H-4 compared to untreated extracts (Fig. 15 a), suggesting that C3H-4 is phosphorylated in MII. Next, we analyzed the phosphorylation events during maturation, but since the amount of endogenous C3H-4 protein is almost undetectable in GVBD while very high in MII, such differences do not allow mobility comparison (Fig. 15b). Therefore to further study the phosprorylation we analyzed the SDS-PAGE mobility in C3H-4 overexpressed oocytes. Detection by western blot revealed that C3H-4 is first phosphorylated at GVBD and may be further phosphorylated at MII. Finally, we did in vitro kinase assays that would show all the phosphorylation events; both if they cause a shift in mobility or not.

Given the difficulty to express high levels of full-length C3H-4, the kinase assays were performed with different bacterially expressed C3H-4 fragments which did not contain the RRM motifs, that would probably be responsible for the difficulty of expressing high amounts in bacteria, but did contain putative phosphorylable residues; 178-277 residues and 178-221 residues. The purified protein fragments were incubated with oocyte extracts from different time-points during meiosis in the presence of [γ^{32}P] ATP, then the incorporation of radioactive phosphates was analyzed by electrophoresis and autoexposition. For the C3H-4 178-277 protein fragment we observed that it is phosphorylated at MI and at MII (data not shown). However the C3H-4 178-222 protein fragment phosphorylation was analyzed in the same way but during a maturaion time-course, this assay showed that in fact there is kinase activity which phoshorylates C3H-4 in PI arrested oocytes, then at MI there is a phosphorylation-induced SDS-PAGE mobility shift, next, during anaphase C3H-4 phosphorylation is reduced to very low levels, to finally, upon MII entry C3H-4 178-222 fragment is again phosphorylated, probably in at least two different residues; one that does not cause a decrease in mobility and

also in another that does, therefore at this time-point C3H-4 is shown as a doublet in an SDS-PAGE electrophoresis (Fig. 15 d). The histone H1 kinase assay done with the same extracts indicates the phase of the cycle of each time-point (Fig. 15 d). This results show that C3H-4 is phosphorylated during maturation, and probably in the serine-proline (SP) and tyrosine-proline (TP) rich region between residues 178 and 222.

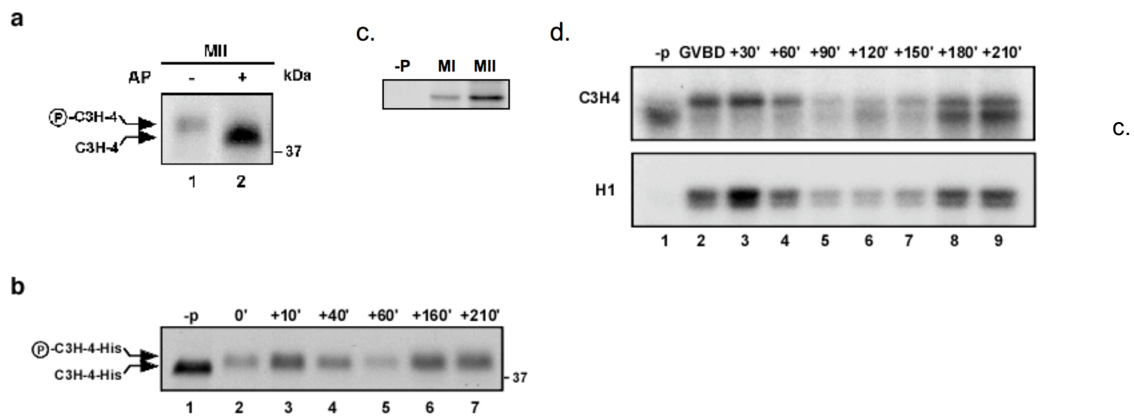


Figure 15. C3H-4 is phosphorylated during meiosis. **a.** Alcaline phosphatase (AP) treatment on MII oocyte extract analyzed by western blot with anti-C3H-4 antibody. **b.** Time course Western blot analysis with anti-C3H-4 antibody of overexpressed C3H-4 oocytes. **c.** C3H-4 178-277 fragment kinase assay in immature (-p) MI, and MII oocyte extracts. **d.** C3H-4 178-222 fragment time course kinase assay, upper panel. The same extracts as in the upper panel were used for Histone H1 time course kinase assay, lower panel.

3.4. C3H-4 translation is required for metaphase I exit.

To identify at which step of meiotic progression C3H-4 is required, we inhibited it’s expression and analyzed all the maturation steps. Meiotic ressumption can be assessed at different levels, the first one and most evident is the oocyte’s external morphology. Metaphase I or GVBD can be scored by the appearance of a white spot in the animal pole of the oocyte, metaphase II can also be scored by a rearrangement in the white spot pigments where the white spot is reduced in size and a second dark ring appears (unpublished observation). Also the changes that occur at the chromosomal level are the most direct way to follow meiotic progression and can be assessed by DNA staining and observation under a microscope (Castro et al., 2003). Finally meiotic progression can be followed by a biochemical marker, MPF activity; driven by the Cdc2 kinase activity that can be measured *in vitro* by the

phosphorylation of histone H1. Cdc2 activity increases abruptly upon MI entry, then drops to low levels during the MI-AI transition, required for replication inhibition (Iwabuchi et al., 2000), to later further increase until reaching a maximum stable level at MII held during the arrest (Huchon et al., 1993).

We had previously seen that C3H-4 synthesis is required for oocyte maturation (Fig. 13). By microinjection of an antisense oligonucleotide complementary to a portion of C3H-4 3'UTR, we ablated the expression of the protein by endogenous RNAseH cleavege of the mRNA basepairing to the microinjected oligonucleotide, by northern blot we followed the efficiency of the cleavege, a faster migrating band can be detected upon cleavege (Fig. 16 a), and by western blot we followed the expected absence of protein synthesis (Fig. 16 b). The effect of the antisense microinjection in oocytes was assessed morphologically, while the MI white spot appeared to be the same in control oocytes than in antisense microinjected oocytes, the MII white spot presented significant morphological differences for C3H-4 depleted oocytes; the pigment distribution was opposite than in control oocytes (Fig. 16c), indicative of possible cell cycle biochemical alterations. The effect of C3H-4 depletion in meiosis was further assessed by analyzing progression at a choromosomal level. Different antisesnse oligos directed against the 5'UTR (5'C3H-4-as), 3'UTR (3'C3H-4-as), and the coding sequence (3'C3H-4cds-as; data not shown) were microinjected into the oocytes, after O/N incubation at 18ºC the oocytes were treated with progesterone to trigger maturation, at the time that control oocytes reached MII, 4 hours after maturation was scored by the white spot, the oocytes were fixed and stained O/N with Hoechst. Then the choromosomes were analyzed under the microscope. Depletion of C3H-4, shown by western blot (Fig. 16 b), induced partial decondensation of the choromosomes in all of the designed antisense oligonucleotides (Fig. 16 d). Moreover, the same phenotype was shown when a mutant form of the protein that does not bind to RNA was microinjected (C3H-4DN). This phenotype was rescued by overexpressing C3H-4, achieved by microinjecting a synthetic RNA containing the cds and 3'UTR of C3H-4 (omiting the 5'UTR), therefore not targeted by the 5'UTR antisense oligonucleotide (Fig. 16 d, e). Next to further characterize the phenotype we analyzed the effects in meiosis of C3H-4 depletion at a biochemical level, for this we measured the Cdc2 activity. As expected in control injected oocytes Cdc2 activity sharply decreased after MI (Fig.

16), whereas C3H-4 depleted oocytes Cdc2 activity did not decrease to very low levels, but instead relative high levels were maintained (Fig. 16 f, g). Also oocyte maturation accompanied by Cdc2 activity took place one hour earlier in C3H-4 depleted oocytes than in control oocytes (data not shown).

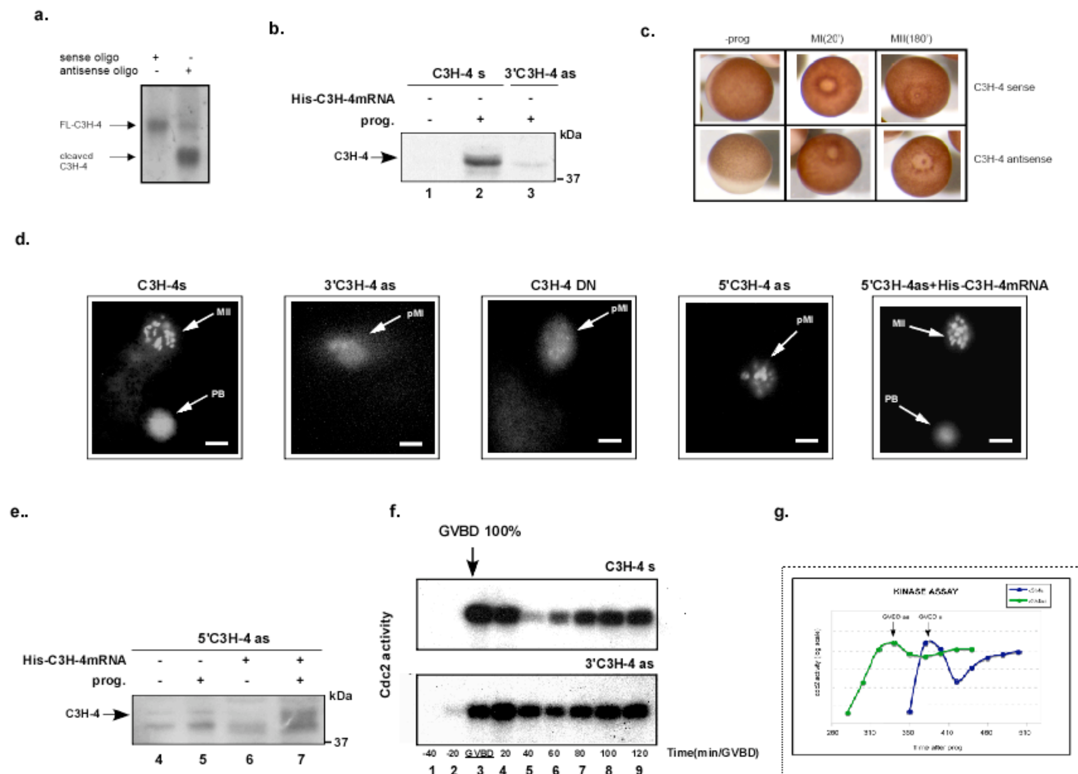


Figure 16. Polyadenylation-induced translation of C3H-4 is required for metaphase I exit. **a.** Northern blot showing C3H-4 mRNA in control sense oligonucleotide microinjected oocytes and antisense microinjected oocytes. **b.** C3H-4 protein levels in control (C3H-4 s) and in C3H-4 depleted oocytes (C3H-4 as). **c.** External morphology of the oocytes assessed in a. and b. **d.** DNA staining for visualization of metaphase plate (MII) and polar body (PB) or pseudometaphase plate (pMI) 6 hours after GVBD in control oocytes (C3H-4s) or microinjected with a 3'UTR antisesnse oligo (3'C3H-4as), a dominant negative form of C3H-4 (C3H-4DN), a 5'UTR antisense oligo (5'C3H-4as), and microinjected with an antisense oligo and a C3H-4 mRNA to rescue the expression (5'C3H-4as+HisC3H-4mRNA). **e.** C3H-4 protein levels in C3H-4 depleted oocytes (5'C3H-4 as), in the presence or absence of progesterone (prog) and in rescued (+ His-C3H-4mRNA) or not oocytes (- His-C3H-4mRNA). **f.** Histone H1 kinase assay to measure cdc2 activity in control (C3H-4s) and in C3H-4 depleted oocytes (C3H-4as) at the indicated time points in minutes. **g.** Cdc2 activity in f. depicted in a graph.

3.5. C3H-4 mediates deadenylation of ARE-containing mRNAs during meiotic progression.

Because C3H-4 is an ARE-binding protein, and it has been shown to bind to the ARE containing 3'UTR of TNFα (Blackshear, 2001), we next sought to determine whether C3H-4 is involved in regulating the deadenylation of ARE containing mRNAs in oocytes, for this

purpose we first analyzed the poly(A) status of a typical substrate for ARE-mediated deadenylation such as the TNFα, in control oocytes (Fig. 17 a) and in C3H-4 depleted oocytes (Fig. 17 b). A polyadenylated radiolabelled probe was microinjected into the oocytes, some were kept untreated and others were treated with progesterone. Total RNA was extracted from these oocytes and the length of the poly(A) tail was analyzed by denaturing gel electrophoresis. As shown in figure 17 b while in control oocytes progesterone stimulates total deadenylation of the probe, in C3H-4 depleted oocytes the TNFα probe is only partialy deadenylated, indicating that the deadenylation is inhibited under this condition.

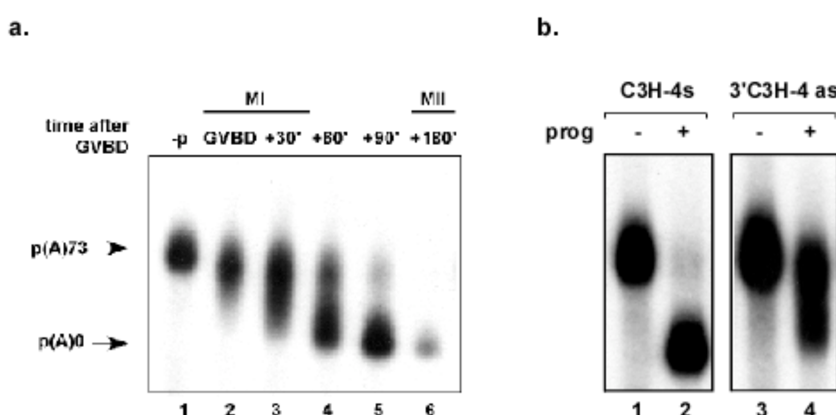


Figure 17. C3H-4 mediates TNFα deadenylation in oocytes. [32P]UTP labeled and polyadenylated TNFα probe microinjected into immature oocytes **a.** Oocytes were stimulated with progesterone and at the indicated times were collected. Polyadenylation of the probe was analyzed in a 6% Acril-Urea gel electrophoresis. Also unstimulated oocytes were analyzed (-p). b. Oocytes were first microinjected with either control (C3H-4s) or antisense oligo (C3H-4as), after O/N incubation TNFα radiolabeled probe was microinjected and analyzed the polyadenylation in unstimulated oocytes (-prog) or 3 hours after GVBD (+ prog).

3.6. C3H-4 recruits the CCR4/Not deadenylation complex to trigger deadenylation of target mRNAs.

To determine the molecular mechanism that underlies deadenylation of ARE containing mRNAs mediated by C3H-4, we tested whether a deadenylase was being recruited to the target mRNAs. There are four major deadenylases in vertebrates, PARN (also known as DAN), CCR4, PAN2 and nocturnin. PARN is found both in the nucleus and the cytoplasm (Copeland and Wormington, 2001), it interacts with the cap structure (Monecke et al., 2008)

and is involved in default deadenylation during meiotic maturation (Korner et al., 1998), also it is involved in deadenylation of CPE containing mRNAs in resting oocytes (Kim and Richter, 2006). Pan2 was first identified in yeast (Boeck et al., 1996), and is responsible for the shortening of the poly(A) tail of newly synthesized mRNAs in the nucleus (Brown and Sachs, 1998). CCR4 is involved in mediating deadenylation of cell-cycle related ARE-containing mRNAs in vertebrates (Collart, 2003; Morita et al., 2007). Lastly, Nocturnin is a deadenylase which in Xenopus is only expressed in retina in a circadian manner, which does not require RNA upstream sequence specificity (Baggs and Green, 2003). CCR4 therefore seemed a likely candidate to control ARE-dependent deadenylation in oocytes as well. To investigate the involvement of CCR4, we did C3H-4 and CCR4 CoIP in MII oocyte extracts. Figure 18 shows that indeed CCR4 coimmunoprecipitates with C3H-4 and viceversa. As previously shown (Fig. 14 d and Fig. 18 a) C3H-4 runs as doublet, in the C3H-4 immunoprecipitation the faster migrating band is detected (Fig. 18 c, lane 4) while in the CCR4 immunprecipitation the slower C3H-4 migrating band is detected (Fig. 18 c, lane 5). CCR4 is also detected in C3H-4 and in CCR4 immunoprecipitations (Fig. 18 b, lanes 1 and 2).

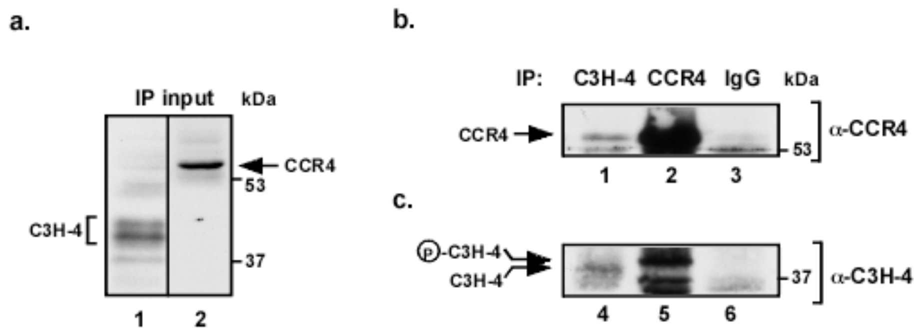


Figure 18. C3H-4 mediated ARE-dependent deadenylation in oocytes by recruiting the CCR4 deadenylase to the mRNAs. **a.** Cytoplasmic extracts used for immunoprecipitations in where C3H-4 (lane1) and CCR4 (lane2) levels are shown. **b.** CCR4 protein levels in C3H-4, CCR4 and immunoglobulin (Ig)G immunoprecipitates (IP). **c.** C3H-4 protein levels in the same IPs shown in b.

3.7. Identification of C3H-4 targets.

To explain the phenotype we observed upon C3H-4 depletion we sought to identify C3H-4 regulated mRNAs. We used a candidate approach searching for ARE and CPE-containing mRNAs encoding proteins that when overexpressed in MI would cause meiotic arrest, similar

to what we see upon C3H-4 depletion. We focused on the APC inhibitors Emi1 and Emi2. Emi1 is transiently expressed at low levels during the PI-MI transition and degraded between MI and MII (Ohsumi et al., 2004; Tung et al., 2005). Depletion of Emi1 delays or even prevents MI entry, whereas premature overexpression promotes MI entry (Marangos et al., 2007; Tung et al., 2005). After that, Cdc2 triggered destruction of Emi1 is required for progression beyond prometaphase (Margottin-Goguet et al., 2003; Reimann et al., 2001). Emi2 is synthesized during interkinesis (Liu et al., 2006; Ohe et al., 2007; Tung et al., 2007) and required for the establishment and maintenance of the CSF (Inoue et al., 2007; Liu et al., 2007; Nishiyama et al., 2007). Accordingly, premature expression of Emi2 at MI causes MI arrest (Ohe et al., 2007; Tung et al., 2007).

Emi1 3' UTR (Fig. 19 a) contains five potential CPEs and multiple potential AREs. The arrangement of CPEs suggests weak-activation by "early" cytoplasmic polyadenylation (Pique et al., 2008). Emi2 3' UTR (Fig. 19 a) contains four CPEs, in an arrangement indicative of a "late" and strong polyadenylation in MI (Pique et al., 2008), and multiple potential AREs. A recent report (Tung et al., 2007) shows that the 3' UTR of Emi2 contains CPEs, although the specific configuration of elements that determine the particular timing of polyadenylation of this mRNA was not determined.

To test if Emi1 and Emi2 mRNAs could be regulated by changes in their polyadenylation status during meiosis, we compared the polyadenylation kinetics of microinjected labeled probes for Emi1 3' UTR, containing weak-early CPEs and AREs, Emi2 3' UTR, containing strong-late CPEs and AREs, B1 3' UTR, which contains strong-late CPEs but no AREs and C3H-4 UTR, Which contains strong-early CPEs and AREs. Each probe was injected in 60 oocytes, after O/N incubation meiotic ressumption was stimulated with progesterone. Oocytes were pooled in groups of five when GVBD was scored (in maximum intervals of five minutes), then the oocytes were collected at the indicated time points after GVBD. A pool of five oocytes remained unstimulated with progesterone and collected at the end of the experiment. Total RNA was extracted from the pools of oocytes and resolved in a denaturing Acril-urea gel, autoexposition of the gel revealed the polyadenylation status of each probe. Emi1 was weakly polyadenylated during the PI-MI transition and sharply deadenylated between MI and MII (Fig. 19 b). Cyclin B1 was already maximally polyadenylated at MI whereas Emi 2, which

contains a similar arrangement of CPEs, was not maximally polyadenylated until interkinesis, with a pattern delayed from B1 (Fig. 19 b). C3H-4 was polyadenylated in MI, slightly deadenylated between MI and MII and fully readenylated in MII (Fig. 19 b). We concluded that the specific arrangement of CPEs combined with the presence or absence of AREs defines specific patterns of poly(A) tail length during meiosis. Thus, for Emi1 the weak "early" polyadenylation was reverted once C3H-4 is synthesized in MI whereas for Emi2 the strong "late" polyadenylation and C3H-4-mediated deadenylation were activated simultaneously at MI displacing polyadenylation to later stages of Meiosis.

Figure 19. Polyadenylation profile is defined by the CPE arrangement and the presence or AREs. **a.** Schematic diagram of the 3'UTRs of the indicated mRNAs showing the regulatory elements CPE (C), ARE (A), Hex (H). **b.** The indicated radiolabeled RNAs were microinjected into oocytes and then incubated in the presence or absence (-P) of progesterone. At the indicated times after maturation (min), the oocytes were collected and total RNA was extracted and analyzed in an 4-6% Acri-Urea gel electrophoresis followed by autoradiography. The percentage of polyadenylated RNA is indicated. Cdc2 kinase activity at each point was determined.

To determine if these mRNA specific patterns of polyadenylation were indeed defined by C3H-4, we first tested whether U-labeled probes from Emi1 and Emi2 3' UTRs were able to crosslink C3H-4 and CPEB, using as controls a TNFα 3' UTR derived probe that contains well characterized AREs but no CPEs and a cyclin B1 3' UTR derived probe, which contains well

characterized CPEs but no AREs (Fig. 19 a). UV-crosslinking of these probes in cytoplasmic extracts from C3H-4 overexpressing oocytes, followed by C3H-4 immunoprecipitation or CPEB purification, showed that all three Emi1, Emi2 and TNFα, but not cyclin B1, probes bound C3H-4 (Fig. 20 a). On the other hand, CPEB was crosslinked to Emi1, Emi2 and cyclin B1, but not to TNFα probes (Fig. 20 b). We next sought to determine whether the endogenous mRNAs were also associated to C3H-4 and CPEB by immunoprecipitation with either C3H-4 or CPEB antibodies followed by RT-PCR for Emi1, Emi2 and cyclin B1 mRNAs. Indeed the endogenous Emi1, Emi2 and cyclin B1 mRNAs were associated with CPEB in MI oocytes (Fig. 20 c), whereas Emi1 and Emi2, but not cyclin B1, mRNAs were bound to C3H-4 (Fig. 20 c). Thus, we concluded that the predicted CPEs and AREs from Emi1 and Emi2 mRNAs are functional elements capable of recruiting, respectively, CPEB and C3H-4.

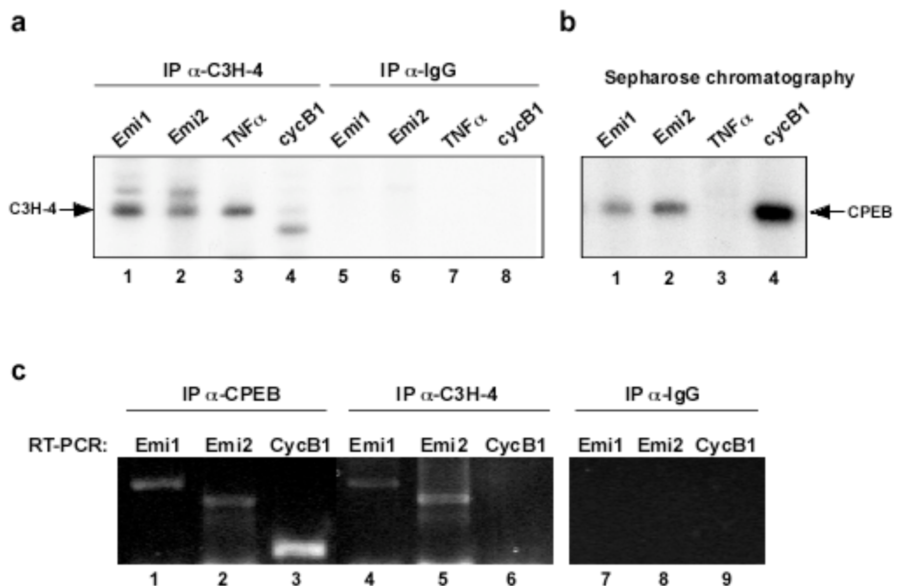


Figure 20. CPEB and C3H-4 proteins interact with Emi1 and Emi2 mRNAs. **a.** The indicated radiolabelled probes were incubated in oocyte extracts overexpressing C3H-4, were ultraviolet-crosslinked and immunoprecipitated with anti-C3H-4 antibody or control IgGs. The immunoprecipitates were analyzed by SDS-PAGE followed by autoradiography. **b.** The same RNA probes and ultraviolet-crosslinked extracts were analyzed by SDS-PAGE followed by autoradiography. **c.** Cytoplasmic extracts from MI oocytes were immunoprecipitated with anti-CPEB, anti-C3H-4 and control-IgG antibodies. The immunoprecipitates were analyzed for the Coimmunoprecipitation of the indicated mRNAs by RT-PCR.

Therefore, we next asked whether Emi1 and Emi2 mRNAs were subjected to specific regulation by cytoplasmic polyadenylation at particular phases of meiosis and whether C3H-4 controlled this specific pattern of polyadenylation. For this purpose we directly measured the poly(A) tail length of the endogenous mRNAs at PI, MI and MII, from either control oocytes or C3H-4 depleted oocytes, now also including cyclin E mRNA which is known to be translated at later stages of meiosis, also containing both CPEs and AREs in it's 3'UTR, (Fig. 21). In control oocytes Emi1 mRNA was deadenylated in PI, polyadenylated in MI and deadenylated again in MII whereas in C3H-4 depleted oocytes, Emi1 was not deadenylated in MII but rather further polyadenylated. In the same control oocytes, Emi2 mRNA was also deadenylated in PI but now the maximum polyadenylation was not detected until MII, whereas in the absence of C3H-4, this high polyadenylation was shifted from MII to MI to be then hyperpolyadenylated in MII. Finally, cyclin E displayed exactly the same polyadenylation profile than Emi2. Thus, we concluded that Emi1 is activated early during the PI-MI transition to be later deadenylated as result of the synthesis of C3H-4. Emi2 is polyadenylated during interkinesis as the result of the synthesis of C3H-4, which displaces Emi2 polyadenylation from MI to the MI-MII transition. And cyclin E is also polyadenylated during intekinesis because C3H-4 displaces the polyadenylation a later stage in meiosis near MII entry.

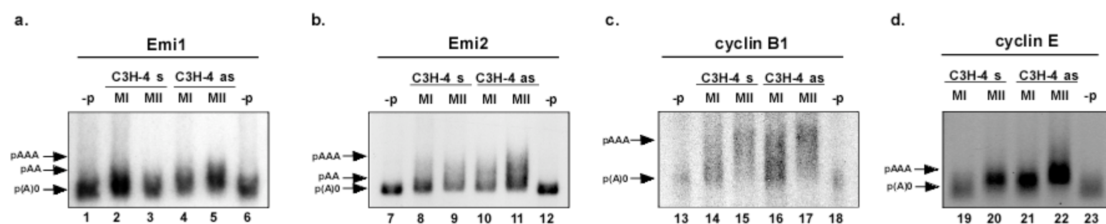


Figure 21. C3H-4 regulates the polyadenylation profile of Emi1, Emi2 and cyclin E during meiosis. Oocytes microinjected with 3'C3H-4 antisense oligonucleotide (C3H-4as) or C3H-4 sense oligonucleotide (C3H-4s) were treated with progesterone and collected at metaphase I (MI) or metaphase II (MII), or were left untreated (-p). Total RNA was extracted and the polyadenylation status of endogenous mRNAs was measured by RNA-ligation coupled to RT-PCR **a.** Emi1 mRNA. **b.** Emi2 mRNA. **c.** cyclin B1 mRNA. **d.** cyclin E mRNA.

Finally to further prove that Emi1 and Emi2 misstimed polyadenylation is responsible for the meiotic arrest shown in C3H-4 deplete oocytes and also to correlate the polyadenylation profile with protein translation we analyzed the protein expression of Emi2 in control oocytes and in C3H-4 depleted oocytes. It has been described that Emi2 is expressed arround 90 min after GVBD (MI to MII transition), in C3H-4 depleted oocytes we can see that the expression of Emi2 is early (Fig. 22), in accordance to the premature polyadenylation, therefore inhibiting APC prematurely. In rescued oocytes where C3H-4 expression is restablished, Emi2 expression is also restablished and it is only expressed 80 minutes after GVBD, as expected in control oocytes (Fig. 22).

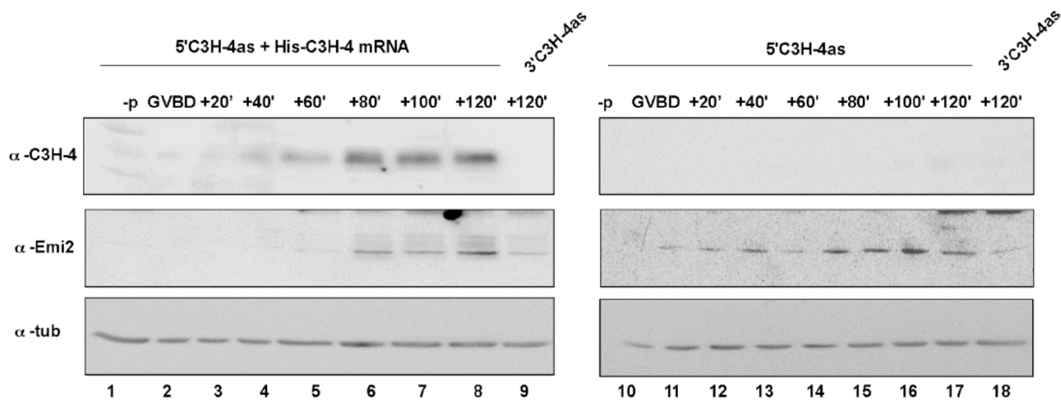


Figure 22. Emi2 protein levels during maturation are regulated by C3H-4. C3H-4 and Emi2 expression were analyzed in a maturation time curse in C3H-4 depleted oocytes (5'C3H-4as and 3' C3H-4as) and in rescued oocytes (5' C3H-4as +His-C3H-4 mRNA). Tubulin was used as a loading control.

DISCUSSION

Meiosis is a very tightly regulated process, which requires very specific patterns of protein synthesis. Translational activation of maternal mRNAs occurs at different phases of the cell cycle, which in combination with protein degradation, establish the different protein expression profiles driving meiotic progression. The best characterized mechanism known to regulate translational activation of maternal mRNAs is cytoplasmic polyadenylation, which controls the time and the extent of translational activation of maternal mRNAs. Cytoplasmic polyadenylation requires two elements in the 3'UTRs of responding mRNAs, the hexanucleotide AAUAAA (Hex) (Sheets et al., 1994), which is bound by the cleavage and polyadenylation specificity factor (CPSF) (Dickson et al., 1999) and the nearby cytoplasmic polyadenylation element (CPE), which recruits CPEB (Fox et al., 1989; Hake and Richter, 1994; McGrew et al., 1989). CPEB is phosphorylated and activated by the kinase Aurora A (Mendez et al., 2000a) as a result of the progesterone induced GSK-3 inactivation (Sarkissian et al., 2004) (although see (Keady et al., 2007)). This CPEB phosphorylation increases its affinity for CPSF (Mendez et al., 2000b), which, in turn, recruits the cytoplasmic poly(A) polymerase GLD-2 (Barnard et al., 2004; Rouhana et al., 2005), and decreases the affinity for PARN, a poly(A)-specific ribonuclease (Kim and Richter, 2006). In addition, embryonic poly(A)-binding protein, is recruited to the CPE-regulated mRNAs by a transient association with the polyadenylation complex, promoting the recruitment of eIF4G and displacing Maskin (Kim and Richter, 2007). Individual CPE-containing mRNAs display specific translational behavior during meiosis suggesting that individual features within their 3'UTRs determine their response to CPEB-mediated translational control. Thus, not every CPE-containing mRNA is masked (Barkoff et al., 2000; de Moor and Richter, 1999) and the activation of all CPE-containing mRNAs does not occur at the same time. Instead, the polyadenylation of specific mRNAs is temporally regulated (Ballantyne et al., 1997; de Moor and Richter, 1997; Mendez et al., 2002). The relative position of the CPE elements define the timing of translation, where overlapping CPE with the hexanucleotide commits the mRNA to “late” polyadenylation (required for MI-MII transition), while non-overlapping CPE commits the mRNA to “early” polyadenylation (required for the PI-MI transition). On the other hand, the extent of polyadenyation is defined by the distance of the CPE element to the hexanucleotide,

therefore mRNA polyadenylation can be also be classified as “weak” or “strong” (Pique et al., 2008).

Despite all the knowledge accumulated, this current model does not fully explain all the patterns of protein synthesis that have been observed, i.e translation of cyclin E and emi2 occurs towards the later stages of meiosis, for which polyadenylation of the mRNAs should occur later than the described “late” stage, in a putative third stage of polyadenylation, which takes place during interkinesis and that we have named “late-late”.

In this thesis we have performed a genome-wide identification of CPE regulated mRNAs and investigated the role of deadenylation in establishing the patterns of meiotic translational activation of maternal mRNAs.

Multiple mRNAs are polyadenylated during meiotic progression

A genome-wide screen allowed us to estimate that around 2000 mRNAs are polyadenylated throughout meiosis. Specifically, we have identified 55 mRNAs polyadenylated during the “early” stage of polyadenylation, which takes place before GVBD, and 104 mRNAs covering the whole maturation process, therefore, including mRNAs polyadenylated during the three stages of polyadenylation; “early” (before GVBD), “late” (at MI) and “late-late” (during the MI to MII transition). While only half dozen mRNAs regulated by cytoplasmic polyadenylation during maturation were previously reported, in accordance with our results, a recent bioinformatic screen has predicted that around 1700 mRNAs in the X.tropicalis genome contain CPE elements and therefore are potentially regulated by cytoplasmic polyadenylation (Pique et al., 2008). The 2000 mRNAs predicted in this thesis work will now probably account for the stage specific protein synthesis requirements of maturation. The massive amount of mRNAs identified was unexpected, but now we estimate that probably there are even more since most of the mRNAs were present only once in the libraries we generated, and none of the already known CPE-regulated mRNAs were detected by clone sequencing but rather detected by specific RT-PCR from the eluted poly(U) fractions.

When we analyzed the full length 3’UTR sequences in the libraries found as expected, that the MI library only displayed “early” CPE-arrangements while the MII library showed both “early” and “late” CPE-arrangements, which confirmed that the method for obtaining the early

library efficiently negatively discriminated the late polyadenylated mRNAs. Unexpectedly, more than half of the 3'UTRs of both libraries contain AREs, since this sequences have been described to be involved in regulating the stability of an mRNA by recruiting or preventing the recruitment of deadenylases to the target mRNA, we worked on the hypothesis that AREs could be involved in establishing the polyadenylation profiles during meiosis.

Antisense oligonucleotide microinjection targeting five early polyadenylated mRNAs from the libraries, tyrosine phosphatase 4a2, protein phosphatase 2c, hsp90, X71067 and C3H-4, were microinjected into oocytes to test whether they were required for meiotic progression. As a result, any of them successfully resumed meiosis, indicating that the translation of all tested mRNAs is required for meiotic progression. Typically, when an important factor for meiotic progression is disrupted, oocytes are either not released from the PI-arrest or re-enter the cell cycle unsuccessfully exiting it to S-phase in an attempt to resume meiosis. Both of these phenotypes were observed for hsp90, X71067, PP2C and TyrP4a2 depletions, while the C3H-4 depleted oocytes were arrested in a pseudometaphase-like stage.

For the effect of X71067 depletion, since it codes for a hypothetical protein, no expectations were prestablished, but yet it is required for meiotic progression in an unknown mechanism. Otherwise, for the mRNAs encoding the phosphatases, somehow the effect of their depletion in meiotic progression was expectable although not reported previously in a CPE dependent manner or in *Xenopus* meiotic progression. Cell cycle progression is driven by the cyclin/cdk heterodimer activity, reversible phosphorylation of cdks is necessary for their kinase activity and determine the activation state. Therefore, phosphorylation of cdks is a very tightly regulated process during cell cycle progression (Alonso et al., 2004). The phosphorylable residues can either trigger activation or inactivation of the kinase activity, likewise, dephosphorylated residues have the same dual function. Meiosis is driven by the cyclin/cdk heterodimer cyclinB/Cdc2; Protein Phosphatase 2c (PP2C; early weak + AREs) removes an activatory phophorylation in Thr 161 of Cdc2 in PI-arrested oocytes (De Smedt et al., 2002). The effect we have seen inhibiting posterior synthesis suggests that although PP2C protein is already present and has a role in maintaining the PI arrest in stage VI oocytes, new synthesis could be required at MI to inactivate Cdc2 in the MI-MII transition until MII entry. The "early

weak” CPE arrangement and the presence of AREs in the PP2C 3’UTR would commit this mRNA to be polyadenylated until MI. Consequently, if this phosphatase would be required for the inhibition of Cdc2 in the MI-MII transition, the absence of this phosphatase would result in a failure to inactivate Cdc2 in the MI-MII transition and therefore S-phase exit would be expected as a consequence of high Cdc2 activity during anaphase. Tyrosine Phosphatase 4a2 (TyrP4a2; early + AREs) belongs to the PRL family of small protein phosphatases, which in humans are upregulated in tumors, they are involved in tumor invasion and metastasis, but also in liver regeneration (Fiordalisi et al., 2006). PRL tyrosine phosphatases are in the signal transduction pathway that regulates cell growth control. The suggested mechanism for regulating this process is that PRLs dephosphorylate tyrosine residues in Cdc2, or in MAPK, which in turn regulate Cdc2 activity (Diamond et al., 1994), therefore TyrP4a2 may work in a similar way in oocyte maturation, which upon depletion abnormal Cdc2 activity would be expected.

The results for Hsp90 are also in agreement with the known Hsp90 function in meiosis, it has been reported that hsp90 is required for mos accumulation, it’s binding to mos allows Ser3 phosphorylation which stabilizes the protein (Fisher et al., 2000). But what was not known is that new synthesis of hsp90 is required for accomplishing this function.

A combinatorial code of cis-acting elements defines the time and extent of translational control

The results of this thesis together with the previous work by Piqué et al. allow us to postulate a set of rules that can be used to predict the translational behavior of CPE-containing mRNAs during meiosis (Fig. 23).

(1) Translational repression requires a cluster of at least two CPEs, irrespective of its position along the 3’UTR, where the distance between adjacent CPEs defines the extent of repression with an optimal distance of 10-12 nucleotides. This implies that the recruitment of Maskin must be mediated by a CPEB dimer and that the efficient repression mediated by multiple CPEs corresponds to the recruitment of this heterotrimer rather than multiple CPEB-Maskin heterodimers.

(2) Translational activation requires, at least, a single consensus CPE or a non-consensus CPE together with a Pumilio Binding Element (PBE). The CPE must be closer than 100 nucleotides from the Hex, but not overlapping.

(3) The distance CPE-Hex determines the extent of polyadenylation and translational activation (either "weak" or "strong"), with an optimal distance of 25 nucleotides, which would represent the more relaxed positioning of the CPEB-CPSF complex interacting respectively with the CPE and the Hex. Other less optimal distances would likely involve bending of the RNA, introducing tension that would destabilize the binding of the CPSF-CPEB complex. Additional PBEs or CPEs have a positive effect except for an overlapping CPE, which has a negative effect.

(4) "Early" or Cdc2-independent cytoplasmic polyadenylation requires CPE(s) non-overlapping with the Hex (such as in mos, cyclin B2 and B5, C3H-4 and emi1 mRNAs), whereas "Late" or Cdc2-dependent polyadenylation is driven by at least two CPEs, with one of them overlapping the Hex (such as in cyclin B1, B4, emi2 and cyclin E mRNAs). This effect is directly mediated by the fact that a CPE overlapping with the Hex has a dominant negative effect in polyadenylation and subsequent translational activation detected only in the presence of low CPEB levels. Thus, during the PI to MI transition, where the levels of CPEB are very high, multiple CPEs are occupied, including the one overlapping the Hex, preventing the recruitment of CPSF to the Hex. However, after Cdc2 is activated at MI most of the CPEB is degraded (Mendez et al., 2002) and stochastically only one CPE would be occupied. Because the non-overlapping CPE has a higher affinity for CPEB than the overlapping CPE-Hex that would imply that now the single CPEB would be preferentially recruited to CPE and free to recruit CPSF to the Hex and promote polyadenylation.

(5) The presence of AU-Rich Elements (AREs), a feature of mRNAs regulated by deadenylation (Voeltz and Steitz, 1998), further defines the effect on polyadenylation dictated by the different arrangements of CPEs. During meiosis, these AREs recruit a zinc-finger protein named C3H-4 that is encoded by a CPEB-regulated mRNA activated during the "early" wave of cytoplasmic polyadenylation. In turn, C3H-4 recruits the CCR4/Not deadenylase complex to the ARE-containing mRNAs opposing CPEB activity on mRNAs containing both CPEs and AREs. The effect of the C3H-4-mediated deadenylation on the

target mRNAs is defined by the arrangements of CPEs. Thus, for an mRNA, such as emi1 mRNA, that was polyadenylated by the “early” activation of a “weak” CPE, the deadenylation overrides the polyadenylation inactivating the mRNA after MI. For “early-strong” CPEs polyadenylation is displaced to MI, whereas for mRNAs containing a “late-strong” CPE arrangement, which would be polyadenylated in MI, C3H-4 is not able to completely neutralize the polyadenylation but causes a delay in the poly(A) tail elongation until later meiotic stages, generating a third wave of polyadenylation in interkinesis, such as for emi2 and cyclinE mRNAs.

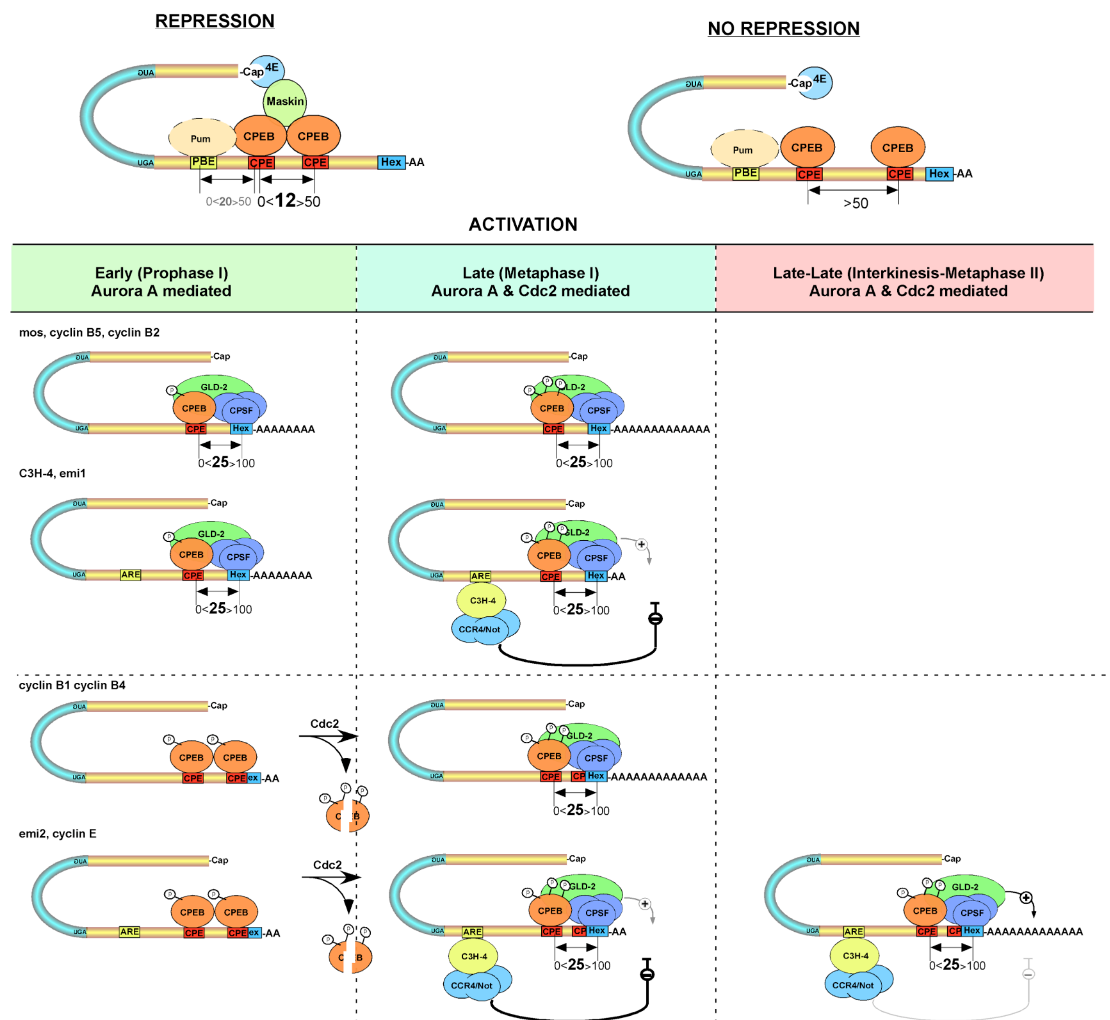


Figure 23. Model for CPE/ARE-mediated translational control. Schematic representation of the *cis*-elements and trans-acting factors recruited, with their covalent modifications. The distances required for translational repression and activation as well as the time of activation are indicated. Optional factors/elements are displayed with dotted lines. AA indicates short Poly(A), AAAAAAA indicates long poly(A) and P indicates phosphorylation.

Sequential polyadenylation and deadenylation drives meiotic progression

Meiotic progression is a switch-like irreversible process where the successive meiotic phases are discrete states sustained by multiple positive and negative feedback loops that require protein synthesis (Abaza et al., 2006; Ferrell, 2002; Matten et al., 1996; Xiong and Ferrell, 2003) and keep the oocyte from slipping rapidly back and forth between cell cycle phases (Brandman et al., 2005; Ferrell, 2002). The hierarchical translation of specific subpopulations of mRNAs at each meiotic phase is regulated through sequential waves of polyadenylation and deadenylation (Figure 25).

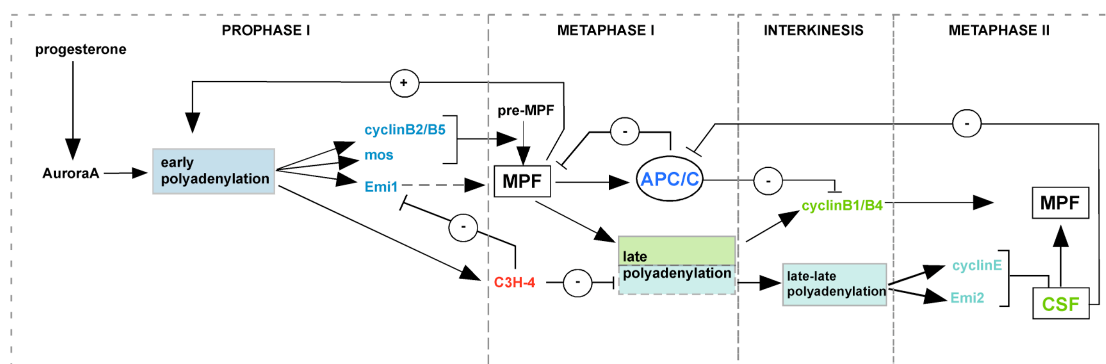


Figure 24. Schematic diagram showing the sequential waves of polyadenylation and deadenylation driving meiotic progression. Maturation-Promoting Factor (MPF), Anaphase-Promoting Complex (APC) and Cytostatic Factor (CSF) activities are indicated. The three waves of cytoplasmic polyadenylation (Early, Late and Late-late) are also depicted.

In PI arrested oocytes, the CPE-regulated mRNAs are either inactive with a short poly(A) tail or even actively repressed by a dimer of CPEBs. As the result of progesterone stimulation, CPEB is phosphorylated by Aurora A (Mendez et al., 2000a) inducing a first wave of “Early” or Cdc2-independent cytoplasmic polyadenylation of mRNAs such as the ones encoding the MPF components cyclins B2 and B5, the MPF activator Mos and the APC inhibitor Emi1 (Pique et al., 2008), which are required for the PI-MI transition. The switch-like activation of MPF is sustained by multiple positive feedback loops in the p42 MAPK/Cdc2 network (Ferrell, 2002; Matten et al., 1996), which require protein synthesis (Xiong and Ferrell, 2003) and that also target the re-activation of the “early” wave of polyadenylation through the synthesis and activation of Aurora A (Frank-Vaillant et al., 2000; Howard et al., 1999; Ma et al., 2003;

Matten et al., 1996). At the same time, a negative feedback loop, which opposes CPEB activity on mRNAs containing both “early-weak” CPEs and AREs, is switch on through the “early” polyadenylation activated translation of C3H-4 mRNA. C3H-4 generates a deadenylation wave that inactivates Emi1 translation in MI allowing for the activation of the APC and the transition to interkinesis. As the result of MPF activation in MI, CPEB is phosphorylated by Cdc2 and PLK1 triggering its partial destruction and generating the second wave of “Late” or Cdc2-dependent polyadenylation of mRNAs such as the ones encoding Cyclin B1 and cyclin B4. These cyclins are required to sustain an intermediate MPF activity during interkinesis, and for the reactivation of MPF in MII (Mendez et al., 2002; Mendez et al., 2000b; Pique et al., 2008; Setoyama et al., 2007). In addition, the partial destruction of CPEB together with the synthesis of C3H-4 generates the third wave of “late-late” cytopasmic polyadenylation. This wave targets mRNAs containing “late-strong” CPEs and AREs, such as the ones encoding the CSF components Emi2 and cyclin E, which are synthesized during interkinesis. CSF, in turn, inhibits the APC allowing the full reactivation of the MPF, now with cyclins B1 and B4, and maintaining the oocyte arrested in MII until fertilization takes place (Abaza et al., 2006).

Post-translational regulation of C3H-4 activity.

The results presented in figures 15 and 18 indicate that C3H-4 may be regulated by phosphorylation(s) taking place at two different meiotic stages. First, during the PI-arrest there is a kinase that phosphorylates C3H-4 (Fig. 15 c). We hypothesize that this would be an inhibitory phosphorylation, which may be important for synchronization of C3H-4 activation and synthesis. Otherwise, premature translation of C3H-4 would probably abrogate meiotic progression, inhibiting and activating phosphorylations would regulate the timing of activation. The fact that overexpression of C3H-4 in stage sVI oocytes does not have any effect, would support this hypothesis because the inhibitory phosphorylation in sVI would prevent premature C3H-4 activity. Later, upon MI entry, C3H-4 would be phosphorylated by a different kinase (Most likely one of the proline-directed kinases activated during MI, such as MAPK, Cdc2 or Plk1), which would cause a mobility shift and correlate with the recruitment of CCR4 by the slower migrating form of C3H-4 (Fig. 18), favoring the hypothesis that this

phosphorylation is required for activation. Finally, at MII C3H-4 display a mixed population of fast and slow migrating phosphorylated forms and low deadenylation activity. This inactivation can be caused by either a dephosphorylation event or by a third kinase activated at MII. Another possibility would be that CPE-mediated polyadenylation is much stronger towards the end of meiosis and therefore it overrides the ARE-mediated deadenylation without being necessary to inhibit deadenylation, but in that case it would not be in accordance with the deadenylation of an early mRNAs such as Emi1, which is deadenylated but not readenylated later. The data therefore indicate that inhibition of C3H-4-mediated deadenylation would also be required for MII arrest. The regulation of CCR-4 recruitment by the different phosphorylated forms of C3H-4 is still ongoing and we are currently mapping the C3H-4 residues phosphorylated at each meiotic phase.

CONCLUSIONS

1. We have identified 104 new mRNAs cytoplasmicaly polyadenylated during meiosis. In the course of a genome-wide screening we have estimated that over 2000 mRNAs are potential targets for CPEB-mediated translational control.

2. We have identified the mRNA encoding the zinc finger protein C3H-4 as a maternal mRNA that has to be activated by cytoplasmic polyadenylation to complete meiosis beyond metaphase I.

3. We have characterized C3H-4 as an ARE-binding protein that mediates deadenylation in response to progesterone.

4. We have defined a new negative feed back loop where C3H-4 opposes CPEB activity in MI inactivating early polyadenylated mRNAS and displacing polyadenylation of late-activated mRNAs to interkinesis.

5. We have identified that Emi1, Emi2 and cyclin E mRNAs are regulated by both CPEB and C3H-4. The sequential translational regulation of these mRNAs, together with previously identified CPEB-target mRNAs, define a molecular circuit driving meiotic progression.

MATERIALS AND METHODS

Oocyte manipulation and microinjection

Stage VI oocytes were obtained by surgical removal of ovaries from adult *Xenopus* females. To remove follicular cells and extracellular conective tissue ovary lobes were treated for 2 hours with 0.8 mg/ml collagenase and 0.48 mg/ml dispase II in Modified Bath's Saline media (1X MBS; 88 mM NaCl, 1 mM KCl, 1 mM $MgSO_4$, 2.5 mM $NaHCO_3$, adjust pH to 7.8, add fresh 0.7 mM $CaCl_2$.). Then the oocytes were thoroughly rinsed and then cultured in 1X MBS. Stage VI oocytes (~1,3 mm diameter) were sorted using a dissecting microscope. Microinjection of oocytes was performed using the Nanoject II Drummond microinjector. Maturation was induced by incubating the oocytes in 1X MBS containing progesterone (10 μM).

Oocyte DNA staining

Oocytes were fixed for 1 hour in Hepes buffer (100 mM KCl, 3mM MgCl2, 10 mM Hepes pH 7.8) containing 3.7% formaldehyde, 0.1% glutraldehyde and 0.1% triton X-100 (Castro et al. 2003), then 20μg/ml of Hoechst was added to the media and incubated O/N. Alternatively oocytes were fixed for O/N in 100% methanol containing 20μg/L of Hoechst dye. Stained oocytes were transferred to petri dish containing 1X MBS buffer free of Hoechst stain. Then individual oocytes were and mounted in a slide and coverslip and viewed from the animal pole with UV epifluorescence immersion objective (63X/0.9W U-V-I; Leica). Pictures were taken with Leica DFC300FX camera.

Overexpressed C3H-4 *Xenopus* oocytes extracts

500 stage VI oocytes were microinjected with 100ng of in-vitro transcribed RNA with the coding sequence of C3H-4 and a polyA tail of 73 nucleotides, after O/N incubation at 18ºC the oocytes were mixed with other 3000 non injected oocytes, then the oocytes were rinsed twice with Extract buffer (10 mM Hepes-KOH pH 7,5, 100 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 50 mM sucrose suplemented with Complete-EDTA free protease inhibitors from ROCHE), the oocytes were packed at 500 rpm for 5 minutes, the excess of buffer was removed and then the sample was centrifuged at 13,000 rpm with maximum acceleration and deceleration without brake in a Beckman Coulter OptiMax Ultracentrifuge. The cytoplasm was then recovered by inserting a 18-gauge needle through the wall of the centrifuge tube.

Obtention of embryos

Xenopus embryos of were obtained by first priming *Xenopus* females with injection of 50U of PMSG (pregnant mare serum gonadatropin) on day 1, on day 3 the frogs were injected again with 25U of PMSG. Finally, on day 8 ovulation was induced by the injection of hCG (human chorionic gonadatropin). The induced xenopus females were placed in 1X MMR media (5 mM Hepes, 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 0.1 mM EDTA, pH to 7.8) at 18ºC, aproximately 14 hours later they layed eggs. Testes were isolated from Xenopus males, that were first sacrificed by submerging in 0.05% benzocaine for 30 minutes to 1 hour. Then the testis were surgically removed and emmbebed in 1X MMR and stored at 4ºC. Prior to fertilization remove the buffer from the eggs, then with a pair of forceps tear a piece of testis and rub the tissue over the eggs, alternatively crush part of the testis tissue in 1XMMR and then with a pasteur pipette pour over the eggs. After contact with sperm the eggs were flooded with 0.1X MMR and collected at the different embryonic stages.

Xenopus cDNA library

NAME: Wellcome/CRC pSK egg

LIB_ID: 1545

ORGANISM*: Xenopus laevis*

ORGAN: egg

HOST: GeneHogs DH10B

VECTOR: pBluescript SK-

V_TYPE: phagemid

RE_5': EcoRI

RE_3': NotI

DESCR:

cDNAs were oligo-dT primed and directionally cloned. Library was constructed by N. Garrett, P. LeMaire, A.M. Zorn, and J.B. Gurdon (Wellcome/CRC Institute).

Purification of polyA(+70) RNA with poly(U)-sepharose

The *Xenopus* oligo cDNA library, “Wellcome/CRC pSK egg”, was in-vitro transcribed with the T3 mMessage mMachine kit (Ambion), then the RNAs were treated with RNAse H and oligo (dT) to remove their poly(A) tail. These RNAs were microinjected (46nL/oocyte) into stage VI oocytes, alternatively some oocytes were treated cycloheximide to a final concentration of 100μg/ml, that were treated with progesterone, and when mature, total RNA was extracted with Ultraspec (Biotecx). The polyA(+80)RNA fraction was purified by polyU chromatography: total RNA was incubated with sample buffer (0.01M Tris-HCl pH7.5, 1mM EDTA, 1% SDS) 5’ at 65ºC, then the sample was chilled on ice and binding buffer was added (0.05 M Tris-HCl pH7.5, 0.7 M NaCl, 10 mM EDTA, 25% (v/v) formamide), then the sample was loaded into the polyU-agarose (sigma) chromatography column and incubated 30’ at room temperature with agitation, the column had been previously washed with swellling buffer (0.05 M Tris-HCl pH 7.5, 1 M NaCl) and packed. Then the column containing the sample was washed 3 times at 25ºC and 3 times at 55ºC with washing buffer (0.05 M Tris-HCl pH7.5, 0.1 M NaCl, 10 mM EDTA, 25% formamide) and finally the remaining p(A)70+RNA was eluted with elution buffer (0.05 M Hepes pH 7, 10 mM EDTA, 90% (v/v) formamide). The eluted RNA was precipitated after Phenol-Chloroform extraction. Reverse transcription (RevertAid M-MuLV retrotranscriptase, Fermentas) of the purified RNA was performed with 3’Raceas oligo (TAATACGACTCACTATAGGGCGGATCCTTTTTTTTTTTTTTTTTTTTTTTTVN) and then PCR (Ecotaq polymerase, Ecogen) with T7as (GTAATACGACTCACTATAGGGC) and T3zorn (GGAAATTAACCCTCACTAAAGGGCAGAAT-AAACGCTCAACTTTGGC) primers was performed to amplify the library. To clone the products into a pBSK vector, the PCR products were digested with BamHI and EcoRI.

The constructs used to set up the method; +CPE-pA, +CPE+pA, -CPE-pA, -CPE+pA, are described in Piqué et al. 2008.

To validate the presence of early, late and control mRNAs in the MI and MII library PCRs were performed with the primer T7as (GTAATACGACTCACTATAGGGC) and the specific primers: mos s (ATGTGTTGCATTGCTGTTTAAGTGG), cycB1s (GGAGATCTTGTTGGCAC-

CATGTGCTTC), tub s (CTTGCGTTGTGCCCTTTTTC), gapdh s (GTCGCCCATCCTGCTA-GTC).

Antisense oligonucleotide experiments

To ablate the expression of a specific mRNA we designed the following 18-mer oligonucleotides: 3'C3H-4as (GGCTTCTCAACTGCTGGC), 5'C3H-4as (CCAGCTTTGACTC-CCCAT), ATGC3H-4as (TATCTCCATTGCCAGCTT), C3H-43'cds (GCAAGAGGTAGGAG-GAGG), C3H-45'cds (GCAAGCCATAGTTACAGG) and a complementary sequence as a control 3'C3H-4s (GCCAGCAGTTGAGAAGCC), 3'hsp90as (GGTGCAGAGAAAGGATAC), 3'PP2Cas (CCAATCCCCCCTCCAATG), 3'X71067as (AATTTTCGATAGTCCCTC), 3'tyrP4a as (GGTGGGGGCAATGCTTAG) the oligonucleotides were dissolved in water at 4,3μg/μl, and 23nl were injected per oocyte (98ng), after O/N incubation at 18ºC, progesterone was added and meiotic progression was assayed.

RNA-ligation coupled RT-PCR

4 μg of total oocyte RNA from pools of 6 oocytes, was ligated to 0.4 μg SP2 anchor primer (5' -P-GGTCACCTCTGATCTGGAAGCGAC-NH2- 3'), in a 10 μl reaction using T4 RNA ligase (New England Biolabs) according to manufacturers directions. The whole 10 μl RNA ligation reaction was used in a 50 μl reverse transcription, using the RevertAid M-MuLV retrotranscriptase (Fermentas), according to manufacturer's directions using 0.4 μg ASP2T (GTCGCTTCCAGAT-CAGAGGTGACCTTTTT) as the reverse primer. 1 μl of this cDNA preparation was used in each 25 μl PCR reaction using EcoTaq polymerase (Ecogen) and [^{32}P] dATP to label the product. The specific primers used were emi1s (GCAAACAGAATTTACGGAGGTTATAG), emi2s (GGGGCTTTGGGGGTGTGTATGATCC-G), 3'C3H-4s (GCCAGCAGTTGAGAAGCC), cycE (GTAGCTGGACTTCAGATG), cycB1MN (GTGGCATTCCAATTGTGTATTGTT). Then the PCR products were resolved in polyacrylamide gels and autoexposed.

Cloning

C3H-4 (AF061983) cDNA was cloned by RT-PCR (using the RevertAid M-MuLV Reverse Transcriptase from Fermentas for RT, and Taq plus precission polymerase from Stratagene for PCR) with C3H-4s (GGGAATTGCGAGATATCAAATGACAGTCTGG) and C3H-4as (GGGTCGAC-GTATAAAGTTAGCCGAGGACAG) primers, from total *Xenopus* oocyte RNA. The PCR product was cloned into a pGEX4T-3 expression vector, and into pBSK+pA73 vector for in-vitro transcription.

Emi1 (AF319594) and Emi2 (AF450296) 3'UTRs were cloned into pBSK by performing a PCR from the IMAGE clone 6641423, GeneBank accession number BU914851, and IMAGE clone 3405254, GeneBank accession number BG486873, respectively with specific primers containing the SmaI restriction site in the sense primer and the EcoRI restriction site in the antisense primer. The plasmid and the PCR products were digested with SmaI and EcoRI and ligated. The primers used for the amplifications were for Emi1 3'UTR; 3'Emi1s (TCCCC-CGGGGGACAGAATTTACGGAGGTTATAGTT) and 3'Emi1as (CGGAATTCCGGGCAAT-AATTTATTTAGCACAAAAAAA), Emi2 3'UTR; 3'Emi2s (TCCCCCGGGGGAGGAATTTGAA-GAGACTTTGAAGC), 3'Emi2as (CGGAATTCCGGTTTATTAAAAATGCATGCTAACCTAG).

The TNFα construct was generated from the IMAGE clone 718000 GenBank accession number AA261175 by PCR with the TNF-sense (GGAGATCTCAATGCACAGCTTTCCTCAC) and the TNF-antisense (CCGGATCCTTCCAACACTGGGTCTTCAG) oligonucleotides. The PCR product was cloned, by BamHI and Bgl II digestion, into the pLucassette plasmid (Pique et al. 2008) after the T3 promoter for transcription and before a poly(A) tail of 73 nucleotides to obtain a TNFα p(A)73 RNA.

Antibodies and Immunoprecipitations

C3H-4 antibodies were raised in rabbits against the XC3H-4 124_137 peptide (CSLPPRRYGGPYRER) by Invitrogen. C3H-4 Abs were affinity-purified from the antiserum using agarose beads (MicroLink Protein Coupling kit, PIERCE) coupled to the immunogenic peptide, as described by the manufacturer. The dCCR4 antibody was a gift from E. Wahle, Institut of Biochemistry and Biotechnology, University of Halle.

UV crosslinking followed by immunoprecipitations were done as described in Hake L 1998 with minor modifications, using 50 fmol of Emi1 and Emi2 RNAs and 100 fmol of TNFα and cyclinB1 RNAs.

Immunoprecipitation folowed by RT-PCR was performed as described in Aoki et al. 2003, with minor modifications. Immunoprecipitations were done with 8.5 μg of C3H-4 affinity purified antibody and 4μl of anti-CPEB antisera. MI oocyte fresh extracts were used for immunoprecipitations, specifically lysates of 50 oocytes at MI were used for each reaction, the oocytes were homogenized in lysis buffer (20 mM Hepes pH 7.5, 100 mM KCl, 2 mM $MgCl_2$, 0,5 mM PMSF, 1 mM DTT) and cleared by centrifugation at 13,000 rpm. The lysates were then incubated with C3H-4 or CPEB antibody bound to Protein A-sepharose (GE Healthcare) for 2 hours at 4 ºC. After extensive washing with wash buffer (20 mM Hepes pH 7,5, 175 mM KCl, 2 mM $MgCl_2$) the protein-RNA complexes were eluted with proteinase K digestion in the presence of tRNA for 30 minutes at 30 ºC, then total RNA was extracted with phenol-chloroform and half of the RNA was used for retrotranscription (mMLU-v from Fermentas) with the 3'race oligo (TAATACGACTCACTATAGGGCGGATCCTTTTTTTTTTT-TTTTTTTTTTTTTVN). One tenth of the reaction was used for specific PCRs using the T7as (GTAATACGACTCACTATAGGGC) primer and 3'emi1s (TCCCCCGGGGGACAGAATTTA-CGGAGGTTATAGTT), 3'emi2 (TCCCCCGGGGGAGGAATTTGAAGAGACTTTGAAGC), cycB1s (GGAGATCTTGTTGGCACCATGTGTTC).

Histone H1 kinase assay

Protein kinase activity was measures on clarified extracts prepared from groups of five oocytes. Oocytes were homogenized in 10μl per oocyte of cold H1 linase buffer (80 mM sodium β-glycerolphosphate pH 7.4, 20 mM EGTA, 15mM $MgCl_2$, 0.5 mM Na_2VaO_4) containing protease inhibitors (complete EDTA-free protease inhibition cocktail, ROCHE: Chymotrypsin, 1.5 µg/ml, Thermolysin, 0.8 µg/ml, Papain,1 mg/ml, Pronase, 1.5 µg/ml, Pancreatic extract, 1.5 µg/ml, Trypsin, 0.002 µg/ml). Lysates were centrifuged at 12,000 g for 10 minutes at 4 ºC, and 30-40 μl of clarified extracts were recovered and transfered to a new tube. 10 μl of extract are incubated with 4 μg of Histone H1 (Sigma) and 2 μCi of [γ^{32}P] ATP,

during 15 minutes at room temperature. After the reaction is analyzed in a 12% SDS-PAGE electrophoresis and autoradiography,

Polyadenylation and Deadenylation assays

The RNA probes were in-vitro transcribed with a T3 RNA polymerase (Fermentas) in the presence of [α^{32}P]UTP. The RNAs were microinjected into oocytes which were incubated in the presence or absence of progesterone. Pools of 6 oocytes were collected at different time-points after maturation, for analysis of the length of the polyA tail. Total RNA was extracted with Ultraspec RNA Isolation System (Biotecx Laboratories, Inc.) reagent and precipitated with isopropanol, the equivalent amount of RNA in 1,5 oocytes was solved in a 6% or 4% Acril-Urea gel electrophoresis and autoexposed for visualization.

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APPENDIX 1. MI library

numbers of hits	sequences	mRNA; accession number
1	UUUUUUUUUGGAUUAAAUUUUAGUUUUUAAUGAACAUGUAAACG	BI312850
4	AAGCAAUGUGGCGUCAAAUAAUAAAAGUUGUUUUUCUUUCCGUAAAAAAAAAAAAAA	Taf16-P1; BC123259
1	CUUUCAUUUCAUACAGAAUAAACAAAAACCUAAAUAAAA	hypotetical protein MGC83807; BC070773
4	UCCAGGUUUUCUUAACUUGUGAUAGUGUUGUGUUUUUAAUAAAAUUGAUUUACUCCAAAAAAAAAAAAAAAA	cyclin A1; X53745
18	CUAAUUAAUUAAAAGUGUGAUUUUAUUAAAUCUGUUUUUAAGCAAA	c3h4; AF061983
2	CUUUAUUUAUUUUUUGUUGUAAAGUUAAAAAUAUUGCGCAAAAUAAAGAUCUUUUUACAGUAAA	hsp 90, beta; AY785160
1	UUUUUUUUGUGAGUGAAAGCUCAUUUAGUUAUAUUAAAAUACGCCAAUAAAA	unknown
1	UAAUUUGCUUUUAUUAAAAAAUAAAUUUUAUUAUGAAAA	unknown
1	UUUUUUUAAAUAAAGUAAACGUAAA	methyl-CpG binding protein MBD3; BC077740
1	AAUUUUUAAAUAUAAAGCAAUAUUAUUGCA	unknown
1	AUAAUGUGUAAGAUUUGUUAUUUAAUAAAAUGAGAGAGUUAAAAAAAAUUUUCAAAA	unknown
1	GCAAAUGUAUUUGCAACUAUGUGGUCAAUGCUUUGUCAUACAAAUGUACUUUUUCAAUGUAUACUUUGACUUUUAAAAUGCCUGCUUUGUGCUUUACAAUAAAUAAUAUCAAA	FUSE 1 binding protein; BC044277
1	CUUUUUUUAAACAAA	similar to testis-specific gene; BC046649
1	AAAAUAUUUUACAAAAUUAAA	tyrosine phosphatase 4a2; BC044707

1	CCUUUAAAUUAAUUUUUUAUCCCAUUANGAGAAAA	unknown
1	AAACAUCAAACUUUUUCAAAUAAAAUUAUUUUUUAAA	unknown
1	UUUUUUUAAUUUUUUCUCUAA	unknown
1	CUUUUAUAAUAAAGCAAUCGCUAUGUUUGGACAAAA	Protein phosphatase 2C gamma isoform (now 1G); BC041734
1	GGAUCAUCAGCUCCAUCUGUUUUAUUUGNUGUGUAUUAA UAAAGCGACUGUGCCCAAA	hypothetical protein; BC046704
1	AUUUUAAAAUUAAACGCAAA	unknown
1	AUUUUAUUGCUGUUUAAUAAACUUAUUGAGGUCCAAAGUA AA	unknown
1	ACUUAUUUUUACAUUAAAAAAAAUCAUACAUUUGAUUGAC UUCUAUAACAGACUGCUAUAAUACAAUAAAUAGCAACAGU GUCAGGUA	Ribonuclease P protein subunit p40; BC081021
1	ACAAUUUACACAUAAAUAAAAUAUUUAAAAAAA	CV118076
1	UGAACUCUUGUCUUUAUAUACAAUAAACGUGCCUUUUUCU UCUGGAAAAAAA	BQ397846
1	UUUUUUUAUUUGUAAAUAUUUUUACUACUAGUUAUGGUU GCACAUUUUUAUUUAUUUUAAUAAAAUUACAGCAAAAAAA	BX845423
1	UGUACACUUUUAUAAAUAAAAUUACUUGGCUUAUGAAAAA AAA	BX846364
1	UUUUACAAUAAACACAAAAUUCAGCAUCAAGUUUGUUUUU ACUGUGCAAUUUUAAUUGGAAAUGUAUCUGAACUUUUAUC UGAAUAAACGGAAUAAAUGUAAAAAAA	hypothetical protein ATPase PPloop superfamily; BC124921
1	AAAUCCUUAUGCAAAAGCAAAUAAAUUCAGUUUUUAUGAC UUGAUCCCAAAAUAAA	serum paraoxonase/aryleste rase; BC044294
1	CACGNGACUUUUUUUUUUUUUUCAUUUUUAAAUGGGAAU UGUACAUCGAA	unknown
1	UUUUGUUUUACAUCAAAAAUGUGAUUUGCAGUUUCUUGU UUUUAUUAUAGAAUGUAAUAAUAAAUAGUUUUUUGUCUUA AUCUCUUGAACAAUCAAGAUGGCUCCACA	CB197999

1	CGAANAUGUGUAUUUGUGAAAUAAACAUUUUAUUAUAAAA UUAAAAAGCAC	splicing factor 3b; Y08997(prot, mRNA O57683)
1	GUGUCCAACUUUAAUGUAUGUUUAGUGCUGUUUUUAAUG UGAAUAAAUAUUUUUUUAUCUAUGAAA	Histone H2b; BX854298
1	UUUUAAUUAUUUUGAGGGACUAUCGAAAAUUUUAAAUAUC AAA	X71067
1	ACAAUUUACACAUAAAUAAAAUAUUUGCAGAA	similar to growth arrest specific6 gas-6; BC060355
1	UUAGCAUUUAUUAAGCUAUUAUUUAUAAAA	BI448218
1	AAAAAAUAAAUGUUAUGCAGUUGUAUUUAAAG	unknown
14	ACGGGGAAUAAACUUAUUUUUAAUCAAA	srp9; BC077407
1	UACUGUAUAUUUGUAGAAUUAAAAAGAAUUUUUAAACAAA	BF614176
1	UAAACCCUGUAUUUUAAAUACUUUGAAGAUGAACUUUUUA CAAUAACCAUUAUAAUAAAAAUAUCAUCCUUGAAA	CR761341
1	AGAUUUUCCAGAGGUGCAUUCUGAGCGCACACAUAGAUU GGUCACAAGACCCACAUCUAACAUGUCUGCUGUUGAAGA UGAAGGAGUCUCCUUUUCAGUGACCAGCUGUAUAUAUUG AAAUAUUUUUCUACAAUAUUUUUGUCUGUAAACUCUGAAC	CV120363
1	AAAGGUCAUCCCAAAAAAUUAUUAUUUUAUGACUAAUAAA AGAAUACAUUAUGCUUUUUUCACA	unknown
1	UUUUAGAUACAAUGUGAUAUUUUAAUAGUACUGAAAUAUU AAAACCUUUCUGUUUGAUAAAAA	p32INGL; AY014017
5	GGAUCAUCAGCUCCACCUGUUUUAUUUGUUGUGUAUUAA UAAAGCGACUGUGCCCAAAAAA	BC046704
1	AAAAAAUAAAUAAAUAAAAAAUGAAAUUUUCCUCAAA	unknown
1	UUUUAAUUAUUUUAAAAAAACUUUGAUUUGCUUUUUCCAU UUUAGUGAUUCUUUAAAGGGAAAAUAUCACUGAGAAAUUA AAUUGAAUUUACUUUGCUAUCAAAAAAA	MGC82524 protein; BC072974
1	AGAUAAAGNUUAUGUGCACUUAAAUGUUUAAUAAAAUCUA CAUAUUUUAGAUGAAAAAA	TAK-1 binding protein; AW635150

1	CAUUUUUUUUUAAAAUAAAGUUGCUUAAAAUCAAA	hypothetical protein; BC130172
1	GGAAAAUCCCAGAUAUAAUAAAAAUAAAGUUCAUUAAA	CA971336
1	GACUGUAUUCCAUUGUAUUGUUUUUACUGUCUAAUAAAAAUUUUAAACUACAAAAAAAAAA	glutamine synthetase; D50062
2	AACUUCCGCAUAACAUCCCUAAAAACUUUUUCACAACAAUCUGAUGCCAAGAAUGUUUUUAUAAAUAAAUUUUUUAAGACAAUCAUUUGAAAAAA	p120 catenin; AF150743
1	AGAUCAAUUUUAUGAAAAGUGUUUGGGCCAAGGCAGCCAAA	unknown
1	UUUUUUUUUUUUAGAAGAAAAGAGUUUAAUUAUGUGGAAGCAGUUUCCUAAAUAAAGUUAAAUAUAGAAAAAA	unknown
1	AUUUACAGCUAGACUGUAUGAUGGAAUAAAAUGUUUUUUUAUUAA	msh2; BC089046
3	AAAAGGACCUUCAUUUGUUUUAUGAUCUGUCAAAGCUUCCCAUUGUGACUUUCAUAACUUAUGGUUUUUAUAUGGUUGCAUUCAUAAUGAAAUUAAAAAGUGAACUCA	arginine methyltransferase AY330768

Supplementary Table2: MII library

	sequences	clone; mRNA
	ACCAAAAGUGGUGUCAAUAAUAUUUCUUUUUAAUUGUUAGCUGAAUUUUUAAUAAAAAUAUGGACACAAAAAAAAAAAA	>Phosphatase 2A
	AUUUUUUUUUUUAUAUAUUUUUCCCAUUGAUAUGUAAAAUGUAUAGUACAAGCUGUGUAUUAGUGUUGUAACUUUUUUCCAAUAGUAAAAUUUCACUAAAAUGCGAAAA	>HNRNP G
	AACCUUAUUAUUUUGCGUAAAGAAAUAUAAUAAUUUUUUUCCUAAUAAAACUNUGGAACCCCAAAAAAAAAAAAA	>TFIID
	AAUCUGCAAAACUACCAUUAAAUAAAAUCUAAUCUCUGGAGUGGGGAGGACAUGUAAUACUUGGAUGUUUUCUUUGUAAAUAAAGUAUUGUAAUCUUCCAAAAAAAAAAA	>Aurora B
	AAUGUUACAUUUUAUUUUUGUUUAAAGUAAGUGGAAUUUAAAUCGAUAAAAUGUAACUUUAAAGGUUUUUAAAUAAAGUGUUCAAAUCAAAAAAAAAA	>unknown
	ACUUACUAAAAUUGAUUAUAAAAUAAACCUAAGACUUUUAUACAAAAAAAAAAAAAAAAA	>P6; unknown
	GAUGCUGCCCAAUAGCUUCAGAGAAGUUUGUGGAGCUAUUGGGCAGUGUUGAAAAAACAUUUUUUUUAUAAUUGCGCUACAGACUAGGUUGAUAUGUCUAGUAUUUGUAUAUUGUUUUAUUUAGAUGCAUUAAUAAAAGUUGUGAAAGAAAAAUAAAAAAA	>18-p2a Similar to splicing factor, arginine/serine-rich 6
	CUUUAUUUUAUUUUUUGUUGUAAAGUUAAAAAUGUUGCGCAAAAUAAAGAUCUUUUUACAAAAAAAAAAAAAAA	>P9; Hsp90
	CAAUGAUUUAAUAAAAUGGAUGUUUGUUUUAAAAAAAAAAAAAAAA	>Claudin4L2 tight junction protein
	AAAGCUCAUUUUUAAUCCUUUUUGUUCCUUUCUUUUAUAAUAAAGCAAUCGCUAUGUUUGGUCUUAAAAAAAAAAAAAAAA	>pyro5 phosphatase 1G: cdc2
	UACAUUUUGCAUCAACAUUUAUUUGAAAAAUAAAGGAUUCUUUCAUAUGUUUUAUCAAAAAAAAAAAAAAAAAA	>pyro6 hyp prot
	CCCCCAGGGUGAACUCUUGUUCUUUAUAUAACAAUAAACGUGUCCUUUUUCUUCUACAGAAAA	>bA3(Est)
	CCGAGCGACGUAUCGAUAAGCUUGAUAUCGCAAGUUCCCCGGGUCCUUUUUUUUAAAUAAA	>bA7
	CGUCAGUUAUUAUUAGUUUAGUUAGUUAAUUAAAGUUAAGUAGUCAGCAAGUCAAAAUAAGUAUUCUUUUUUAAUCCAAGGUUUAAAAAA	>bA9

	UAUAUCAGUCAGUCCAGGUUCCAGGUCAAUUAAAUUUAAAAGUUAAAGGUUAGUUAGUUUAAUUUUUAAAUUUCAAAUUUCGUUUUUAAGGUUUUUCAAGGUCAGU	>bB4
	UAGUUAGUUUAAUUUUUAAAUUUCAAAUUUCGUUUUUAAGGUUUUUCAAGGUCAGU	
	AUCGCAGUCCGGGAGUGUGUAUUGUAGAAAUAAUCAUUUAUUAAUAAAUUAAAAGCAA	>bB8
	AGGUAGUAGAUAUAUUAUAUCAGUCAGUCCAGUCAAGUAAUCAUUAUUUUUAAUUAAGAA	>bB12
	CAAAGUCAUUCAUUUCAUUCAAUUCCUUUUUGUUUCCUUUUCUUUUAUAAAUAAAGCAAUCGCUAUGUUUGGUCCCACAA	>bC3;cdc2
	AAGUCUGAUAUCGCAAGUCCCGGGAAGCAAUGUGGCAGUCAAAUAAUAAAGUUGUUUUUCUUUGC	>bC4(Est)
	AGUAAGUCAGUGUAUAUCAGUCAAGUUCCCAGGUUCCCGUUUCAUUUUCAAUUCAACAAGAAAUAAAACAAAAACCAAUCAUUUAAU	>bC5
	CCCAAGGUUUCCCCAGGGGUUUCCCAGGGUUUUCAUUUCAUCGUUGUGUAGUAGUUGUUGUUAGUUUUAAUUAAAUUAGUAUUAUUCAAGAA	>bC6
	CAGUCAGUCCAGUCAGUAAUUAAUUAAGUAGUGUAUUUAAUUAAUUCAUUGUUUUAAGUCAGCCAAAAUUUAAAAUCAUAAA	>bC8(Est)
	CGAGCGUAUCAGAUAGUCUUGAUAUCAGUCAAGUUCCCAGGAAAUAAAUCAUUUAUUUUUUAAAUUAAAA	>bC9(Est)
	CAGGUCAGGUCAGGUUCAGUCAGUAAGUUAAGUCUUAGUAAUUCAUCAGUCCAAGUUCCCAGGAAAUAAAUCUUAUUUUUAAUCAA	>bD10
	CCCCAGGGUCAAAGGUCCAAUUCCAAAAAUUAAUUAUUUUAUGACAUAAAUAAAAGAAUACAUUAUGCUUUUUUCACA	>bE9
	AAUAAUCAAUAGUAUUAAUUUUAAUUAAGUACUAGAAAUAAUUAAAAUCCAUUUCUUGUUUGUAUAAA	>bF4
	AUAUCAGUCAGUUCCCGGUCAUAAUUAAAUUAAAAGUGUGUAUUUUUAAUUUAAAAUUCUUGGUUUUUUUAAAGGUCCGGUUUUAAAAAAAUUAAAAAA	>bF5
	CAGGUCAGCAGGUCAGUCAGUCAGUAAGUCAGUGUAGUAUCAGUCAAGUCCCAAGGUCCAAGUAAAUCAAUUAUUUUUAAUUUUAAAU	>bF7
	AUAUCAUCAGUUCCAGGUCAGGGUUCAGGUCAAGUCCAGGUUCAAGUUCAAGUAAAAUUAAAAAGUCAAAUUUAAGUAAAUUAAGUUCAAGUUUUCCAAUUUCCCCAAGG	>bF9

	UUUCAUCCCGGCUAAUUAAUUAAAAGUAGUGUAUUUUAAUUUAAAUUCAUUGUUUUUUAAGGUUUAAAA	>bG2
	AGGUUAAAUUUCAAGUUCCAAGGUCCAAAGGUUUCCCCCCCAGGGGUCCAUUAAAAUUUUAAAAAUUUAAAAAAGGUUGUGUAUUUUUAAUUAAAUCUUGUUUUAAGUAAUUAAA	>bH2
	AGCGUAGUAGUAUAUUUAUAUCAGUCCAGUCCCGGUAUUUUUUAAAAUAAAGUUGCUUAAAAUCAA	>bH3
	AUAUAUCGCAGUCCGGAGCAUGUGGCGUCAAAUAAAUAAAAGUUAGUUUUUCUUUUCCAAAGUAAA	>bH4
	CAUUGUAUAUCAGUCAAGUUCCCCGGGCAUAAUUAAUUAAAGUGUGAUUUUAUUAAAUCUGUUUUUAAGCA	>bH8; c3h-4
	CAGUCCGGUCAGUUUCUACUUGUGAUAGUGUUGUGUUUUAAUAAAUUGAUUUACUCA	>bH9; CyclinA1
	CCAGGGGUUCAAGUUCCAGGUAAGUUAAAGUCAGUUUGUAAUUAAUUCAGUCAAAGUUCCCCCAGGGUCAAAUAAAAUCAUUAUUUUUUAAUUUGAUAAA	>aA7
	AGUCAGGUAGUCAGUAAUAAGUCUUGUAUAUCAGUCAAUUCCCCAGGAAUAAACUUAUUUUUAAUUAACA	>aB1;SRP9
	AUUAUCUUUGACUUUUAAAAUGCCUGCUUUGUGCUUUACAAUAAAUAAUA	>aB4; FUSE-BP1
	CAGGAGCAGCGUGUAGUAGUAAGUCAUGUAGUAUCAGUCAAGUUCCCAAGGUCCAAAGGUAAAAUUUUAAUUUCAAAAUUCAAAAAUUAA	>aC1
	AGCGUAGUCGUAUAAGUCAUGUAUAUCAGUCAAGUUCCCCAGGUCCAGUUUAAAUUUAAAUUUUUUAAUUUCCAAAUUUCAAAAAGUCAAAAA	>aC5;Xenopuslaevis programmed cell death6
	CAGUAAGUCAAGUUCGUUAGUAAUUCAGUCAAGUUCCCAGGUUCCCCGGUUUCCAUUUUCCAAUUUCAAUCAAGCAAAGUAAAAUCAAAAAACCAAAAUCCAAAUUAAAAAU	>aD4
	AUUAUAUCAGUCAGUCAGUUCCAGGUUAAUUAAGUUAAGUGUGUAUUUUAAUUAAUCUUGUUUUUUUAAGGCCAA	>aD7
	CAGUCAUCAGCUCCAUCUGUUUUAUUUGUUGUGUAUUAAUAAAGCGACUGUGCCCA	>aD8 eIF3 subunit 8
	CUCGAGGUCGAGCGGUAGUCAGAUAAAGUCAUUUGUAAUUAAUUCCAGGUCAAAAUUUUCCCCCAUUUCCGGUAAAAA	>aD10
	AGGGUCGACGGUAUCGAUAAGCUUGAUAUCGAAUUCCCCGGGAUUUUAAAAUUAAACGCA	>aD12

	CGAGCGGUAGUCAGAUAAGUCAUUGUAUAUCAGUCAAGUUCCCCAGGUCAAUAAAUCAUUAUUUUUUAAAUUUAAAA	>aE10
	CAGUAAGUCAAGUCAGUUAGUAAGUUCAGUUCAGUUCAAGUUUCCCAGGUUAAUUUUAGUUUUUAGUUAAUUAAAAAUCAUUUUAAAUAACAAACAAAA	>aE12
	CCGUCCAGGGUCCAGGGGUCAGGGUCAGGUCAGGUCAGGUUCAGUCAAGUAAGUCAGUGUAGUAUCAGUCAGUCCAGUCCGUUUAAUUUUAAAUAA	>aF5
	CAGUCAGUCCAGUUCCAGUUCAUUUAUUUUAGUUUUAGUUAAGUUAAAAGUUAAAAGUUCGGUUCAGCAAGUCAAAAUUAAAGUUAAUUCUUUUUUUAACCA	>aF10
	GUCGCAGUCGGUAGGUUCGGAAGUCAAGGUCAUUCGGUAAUUAAAGUCCGUUAAGUUCCCAGGUCAAAUUCAAAGCAAUUUUCCCCAGGUUUUUCAAAAU	>aG1
	CAGUUCAGUAAGUAAAGUCAGUUGUAUAUCAGUCAAGUUCCCCAGGGUCAGCAAUUUUAAUCACAAUAAAUAAAAAUAAUUUAAAU	>aG2
	aAUAAAUCAGUCGGCCUUUUUUCCCUUUUUUCCUUUCGGGGUAAAAAG	>aG3
	CAGUUCAGUCAAGUAAAGUCAGUUGUAUAAUUCAGUCAAGUUCCCCAGGGUCCGUAAUUAAAUUAAAAGUAGUGAUUUUAUUAA	>aH11