

Cytoplasmic CstF-77 protein belongs to a Masking Complex with CPEB in *Xenopus* Oocytes

Christel Rouget, Catherine Papin and Elisabeth Mandart

From the Centre de Recherches de Biochimie Macromoléculaire, CNRS, 1919 route de Mende, 34293 Montpellier cedex 05, France

Running title: Cytoplasmic CstF-77 in masking mRNA

Address correspondence to Elisabeth Mandart: CRBM, CNRS, 1919 route de Mende, 34293 Montpellier cedex 05, France, Tel (33) 4 67 61 33 39; FAX (33) 4 67 52 15 59; E-mail:

elisabeth.mandart@crbm.cnrs.fr

Regulated mRNA translation is a hallmark of oocytes and early embryos, of which cytoplasmic polyadenylation is a major mechanism. This process involves multiple protein components, including the CPSF (Cleavage and Polyadenylation Specificity Factor), which is also required for nuclear polyadenylation. The CstF (Cleavage stimulatory Factor), with CPSF, is required for the pre-mRNA cleavage before nuclear polyadenylation. However, some evidence suggests that the CstF-77 subunit might have a function independent of nuclear polyadenylation, which could be related to the cell cycle. As such, we addressed the question whether CstF-77 might have a role in cytoplasmic polyadenylation. We investigated the function of the CstF-77 protein in *Xenopus* oocytes, and show that CstF-77 has indeed a role in the cytoplasm. The *Xenopus* CstF-77 protein (X77K) localizes mainly to the nucleus, but also in punctuate cytoplasmic foci. We show that X77K resides in a cytoplasmic complex with eIF4E, CPEB (Cytoplasmic Polyadenylation Element Binding protein), CPSF-100 and XGLD2, but is not required for cytoplasmic polyadenylation *per se*. Impairment of X77K function *in ovo* leads to an acceleration of the G2/M transition, with a premature synthesis of Mos and AuroraA proteins. However, the kinetic of Mos mRNA polyadenylation is not modified. Furthermore, X77K represses mRNA translation *in vitro*. These results suggest that X77K could be involved in masking of mRNA prior to polyadenylation.

Regulation of gene expression plays a central role in many cellular functions. In

eukaryotes, formation of the mature 3' end of a messenger RNA is a two-step reaction that involves cleavage of the nascent transcript and subsequent polyadenylation in the nucleus (reviewed in 1-3). In mammals, the cleavage reaction requires two sequences, the highly conserved hexanucleotide AAUAAA located 30-20 nucleotides upstream of the cleavage site, and the G/U rich sequence that lies downstream of the 3' cleavage site (DSE for Downstream Element). These sequences are bound by different multisubunit factors. CPSF binds to the AAUAAA sequence via the CPSF-160 subunit, and CstF recognizes the DSE element via the CstF-64 protein (4,5). CPSF and CstF are known to interact cooperatively at 3' end signals (6), and allow pre-mRNA cleavage with the cleavage factors I and II (CFI and CFII), the carboxy-terminal domain of RNA polymerase II and the poly(A) polymerase (PAP). CPSF consists of the 160K, 100K, 73K, 30K and hFip proteins (7-9), and CstF is an heterotrimeric structure composed of the 77K, 64K and 50K proteins (10,11). CstF-77 is the subunit required for integrity of the CstF complex. CstF-77 binds to CstF-64, CstF-50 (12) and CPSF-160 (5) and therefore helps to hold together the much larger complete polyadenylation complex.

CPSF is also involved in cytoplasmic polyadenylation (13,14), a mechanism of controlling translation which is often used when transcription is quiescent, such as in oocytes or early embryos. In *Xenopus* oocytes, several dormant mRNAs are stored with short poly(A) tails, which are elongated when oocytes are stimulated by progesterone, provoking their translation.

Cytoplasmic polyadenylation in frog oocytes also requires two sequences on the 3'UTR

of target mRNAs, recognized by multiple protein components (reviewed in 15). CPEB directly binds to the CPE (Cytoplasmic Polyadenylation Element) located upstream of the AAUAAA sequence, which is recognized by CPSF, as in nuclear polyadenylation (14). CPEB interacts with CPSF, which may help to stabilize its association with the AAUAAA element and a cytoplasmic poly(A) polymerase. GLD2, a poly(A) polymerase which is structurally distinguishable from nuclear PAP, was first identified in *Caenorhabditis elegans* (16). The *Xenopus* related protein (XGLD2) was shown to bind to CPEB and CPSF and to participate in cytoplasmic polyadenylation (17,18). Sequences other than the CPE, the PRE sequences (Polyadenylation Response Element), regulate early cytoplasmic polyadenylation of specific mRNAs including Mos (19,20). The CPEB protein has been shown to repress mRNA translation in immature oocytes and to direct cytoplasmic polyadenylation and translational activation in maturing oocytes. Indeed, mRNA translation repression is controlled by a complex composed of CPEB, Maskin and eIF4E. Maskin interacts with CPEB and eIF4E and prevents eIF4G binding to eIF4E. After stimulation, CPEB is phosphorylated and cytoplasmic polyadenylation ensues, allowing disruption of Maskin-eIF4E binding and recruitment of eIF4G (21,22).

The yeast *Saccharomyces cerevisiae* and *Drosophila melanogaster* homologues of CstF-77 are, respectively, the Rna14 and Su(f) proteins. Several genetic and biochemical studies have shown a role for Rna14 in 3' end processing (23-25), although other studies for Rna14 have suggested a function independent of nuclear polyadenylation. Indeed, rna14 mutants can be separated into two classes: the poly(A)-negative class, which contains mutants deficient in mRNA 3' processing, and the poly(A)-positive class, which includes mutants that are not impaired in nuclear polyadenylation (26,27). Rna14 and Su(f) localize both to the nucleus and the cytoplasm. In yeast, this cytoplasmic localization is mainly in mitochondria (26,28). However, no mitochondrial function for Rna14 has been described so far. In *D.melanogaster*, the Su(f) protein has been implicated in cell cycle progression (29). In *Xenopus* and human cells, studies have shown that CstF-77, CstF-64 and CPSF-100 are concentrated

in nuclear domains in Cajal or coiled Bodies (30-33) as well as various components required for transcription and processing of the three classes of nuclear transcript (reviewed in 34). However, nothing was reported concerning a possible cytoplasmic localization of the CstF subunits.

The aim of our work was to study the cytoplasmic function of CstF-77, bearing in mind possible roles independent of nuclear polyadenylation that could be related to the cell cycle. We therefore addressed the question of whether CstF-77 could have a role in cytoplasmic polyadenylation. The *Xenopus* oocyte is an appropriate model to study other functions of CstF-77, as there is neither transcription nor nuclear polyadenylation during oocyte maturation (15). In this report, we identify the *Xenopus* CstF-77 protein (X77K) and show that it is partially localized in the cytoplasm. We demonstrate that X77K belongs to a cytoplasmic complex with eIF4E, CPEB, CPSF-100 and XGLD2. However, the protein is not required for cytoplasmic polyadenylation *per se*. Inhibition of X77K function in oocytes accelerates meiotic maturation and precociously induces Mos and AuroraA protein synthesis, without modifying the kinetic of Mos mRNA polyadenylation. Moreover, X77K inhibits *in vitro* mRNA translation in a dose dependent manner. These results suggest that X77K could have a function in mRNA masking prior to cytoplasmic polyadenylation.

EXPERIMENTAL PROCEDURES

X77K cloning and protein alignment - A *Xenopus leavis* oocyte cDNA library in λ gt11 from Clontech (ZL5000b) was screened with a 1.030 base pair (bp) probe obtained by RT-PCR from *Xenopus* RNA with the following degenerated oligonucleotides (Eurogentec): 5'-ATGGCYMARGCWTAYGAYTTYGCACT-3' and 5'-ACKCKSGTRTTRTTRTCYTCRTTBAG-3' where Y=C+T, M=A+C, R=A+G, W=A+T, K=T+G, S=C+G and B=T+C+G. Several clones containing the cDNA of the *Xenopus* CstF-77 homologue (X77K) were isolated and sequenced. The largest cDNA (about 2.300 bp) was subcloned at the EcoR1 site of the pBluescript II KS. It contains an open reading frame encoding a protein of 719 amino acids. This X77K amino acid sequence was aligned with those of *Homo sapiens*

CstF-77 (NP001317), *Mus musculus* CstF-77 (AAH03241), *Drosophila melanogaster* Su(f) (P25991), *Caenorhabditis elegans* CstF-77 (AAA62311) and *Saccharomyces cerevisiae* RNA14 (NP013777) with the ClustalW program. The X77K sequence was submitted to Genbank with the following accession number: AM071387.

Xenopus oocytes and embryos - Stage VI oocytes were selected after surgical removal of ovaries from mature female *Xenopus laevis* and treatment with collagenase (Sigma) at 1mg/ml in OR-2 (5mM Hepes pH 7.2, 82.5mM NaCl, 2mM KCl, 1mM MgCl₂). All further manipulations of oocytes were performed in modified Ringer's Solution (MMR: 5mM Hepes pH 7.8, 100mM NaCl, 2mM KCl, 1mM MgSO₄, 0.1mM EDTA, 2mM CaCl₂). Progesterone was used at a final concentration of 1mM. For protein oocyte extracts, oocytes were homogenized at 5 µl per oocyte in lysis buffer (20mM Tris pH7.5, 50mM NaCl, 50mM NaF, 10mM β-glycerophosphate, 5mM Na₄P₂O₇, 1mM Na₃VO₄, 1mM EDTA, 1mM EGTA, 0.1mM PMSF, plus 10 µg/ml each of leupeptin, chymostatin, pepstatin and aprotinin) at 4°C, centrifuged at 13000 rpm for 3 minutes at 4°C and the supernatant was used for further analysis. For manually enucleated oocytes, nucleus were collected and boiled in 5X Laemmli buffer and cytoplasm was homogenized at 5 µl per cytoplasm in lysis buffer. *In vitro* fertilization and embryo cultivation was carried out as described (35). The quantification of proteins in oocyte and embryo extracts was done by Biorad protein assay, with quantified BSA as standard. For microinjections, the usual injected volume for antibodies, purified protein or RNA was 20 to 40 nl per oocyte and the number of injected oocytes is 35 for each condition.

Antibodies and Western blots- The anti-pTpY-MAPK was obtained from New England Biolabs (9106S). The anti-eIF4E was provided by S. Morley or was obtained from mouse antisera using the same peptide antigen. The anti-CPSF-100 and the anti-Pak5 were provided by E. Wahle and N. Morin's laboratories respectively. The anti-β-tubulin and anti-HA antibodies were obtained from E7 (Iowa hybridoma bank) and from the 12CA5 hybridomas respectively. The anti-RPA and anti-AuroraA were provided by J.M. Lemaître and C. Prigent respectively. The anti-

Mos, anti-ERK and IgG were obtained from Santa Cruz (sc-086, sc-94 and sc-2027, respectively). Other antibodies used against GST and *Xenopus* 77K, CPEB and GLD2 are rabbit polyclonal antisera and were affinity purified. The 77K Cter antibody is directed against the 17 amino-acids carboxyl terminal of the human 77K protein: NH₂-VPPVHDIYRARQQKRIR-COOH that was kindly provided by David Bentley, and the 573 antibody is directed against the following X77K peptide: NH₂-LKDDVDRKPEYKPD-COOH. The anti-CPEB antibody is directed against GST-CPEB fusion protein as described (36). The anti-XGLD2 antibody is directed against the GST-XGLD2 2/3 carboxy terminal fusion protein. For microinjections, dialyzed antibodies were used at 1 µg/µl. Western blots were probed with primary antibody at 50 ng/ml and the appropriate secondary antibody horseradish peroxidase (HRP) conjugate diluted according to manufacturer recommendations (Amersham) and revealed by ECL (Amersham).

RNA and recombinant proteins - The Mos 3'UTR construct was obtained by inserting the SpeI/XbaI fragment from the pT7-G UK XMos plasmid kindly provided by N. Sagata (37) into SpeI/XbaI-digested pCS2 (Dave Tumer, University of Michigan). This 320-nucleotide fragment contains Mos PRE, CPE and AAUAAA sequences. Mos 3'UTR RNA were prepared by linearizing the pCS2-Mos320 with XbaI and carrying out transcription reaction according to Papin and Smith (38). The pCSH vector corresponds to the pCS2 vector with two HA tags inserted in the multiple cloning site. Capped mRNA encoding HA-X77K, HA-XGLD2 and HA-CPEB were prepared by cutting the pCSH-X77K ORF, pCSH-XGLD2 ORF and pCSH-CPEB ORF respectively with NotI and carrying out transcription reaction.

Immunoprecipitations- Protein oocyte extracts corresponding to 20 oocytes were performed in extract buffer (100 mM KCl, 0.1 mM CaCl₂, 1mM MgCl₂, 10 mM Hepes pH7.6, 50 mM sucrose, plus 10 µg/ml each of leupeptin, chymostatin, pepstatin and aprotinin). For some experiments, the oocyte extracts were treated 10 min at room temperature with RNase A at 0,02 mg/ml. Oocyte extracts were incubated with 200 ng of antibodies over night at 4°C on wheel, supplemented with 20 µl of Protein A-Sepharose

beads and mixed for another 45 min at 4°C on wheel. The immunoprecipitates were then washed 3 times in XRB A (0.2% Triton X-100, 10 mM Tris pH8, 150 mM NaCl and 2 mM EDTA), 3 times in XRB B (0.2% Triton X-100, 10 mM Tris pH8, 500 mM NaCl and 2 mM EDTA) and 2 times in 10 mM Tris pH8, eluted by boiling in 2X Laemmli buffer, separated by SDS-PAGE and analyzed by Western blotting.

For immunoprecipitation of radio-labeled X77K, 1 µg of 77K antibody was incubated or not with 10 µg of its antigen (77K Cter peptide coupled with thyroglobulin) before being applied to protein-A Sepharose beads for 30 min at 4°C. The beads were then washed three times with PBS, supplemented with ³⁵S methionine X77K translated in rabbit reticulocyte lysates, and mixed 1 h on ice. The immunoprecipitates were then washed 3 times in XRB A, 3 times in XRB B, and 2 times in 10 mM Tris pH8, eluted by boiling in 2X Laemmli buffer, separated by SDS-PAGE, and analyzed by autoradiography.

Cap column and GST Pull-Down assay– The cap column assay was performed as described (22). Briefly, 30 oocytes were homogenized in homogenization buffer (HB), and treated or not 10 min at room temperature with RNase A (0,02 mg/ml). The oocyte supernatants were supplemented with 0.1 mM GTP or 5 mM ⁷mGTP and applied in batch to a ⁷mGTP-Sepharose (cap) column (Amersham) that had been equilibrated with HB plus bovine serum albumin (BSA, 0.1 mg/ml). Following mixing for 1h at 4°C in eppendorf, the cap column was extensively washed (100 bed volumes) with 0.1 M KCl in 50 mM Tris pH 7.5. The bound material was then eluted with 5 mM ⁷mGTP on column. The eluates and a fraction of oocyte lysates collected before and after mixing with the cap column were separated by SDS-PAGE and analyzed by Western blotting.

The GST pull-down assay was performed as described (39). Briefly, HA-tagged CPEB and HA-tagged XGLD2 were *in vitro* translated in rabbit reticulocyte lysates (Promega) in the presence of unlabeled amino acids. GST-X77K expressed in *Baculovirus* and GST expressed in *E.coli* were purified with glutathione sepharose (Amersham). 5 µg of purified GST-X77K or GST were bound to 20 µl of glutathione beads in

binding buffer. The beads were subsequently incubated 2h at room temperature in 400 µl of binding buffer plus 5 µg of the HA-CPEB or HA-XGLD2. The beads were then extensively washed with binding buffer and boiled in 2X Laemmli buffer. The eluates and inputs were separated by SDS-PAGE and analyzed by Western blotting.

Polyadenylation assays - Total RNA from oocytes was extracted using the Mini RNA Isolation II™ kit (Zymo Research) and the PAT (PolyAdenylation Test) assay was carried out according to Salles and Strickland (40) with minor modifications. Reverse transcription was done on total RNA with oligo(dT)-anchor (dT-PAT) as primer (5'-GCGAGCTCCGCGGCCGCGT₍₁₂₎-3'). Subsequent PCR was carried out using the dT-PAT primer and a specific upstream primer for Mos 3'UTR RNA (5'-GCACTGAAAATACAAGCAAGGATATG-3'), CyclinB1 ORF RNA (5'-GTGGAAATGGCCCGCCCACTC-3'), AuroraA 3'UTR RNA (5'-GGCTGTCACCGTACAACGCTACTTG-3') and actin type 5 3'UTR RNA (5'-CAAATGTTGCAGGTACACCTG-3'). The PCR products were resolved in a 2.5% agarose gel and visualized with ethidium bromide staining.

For *in vitro* polyadenylation assays, egg extracts were prepared according to Murray and Kirschner (41). Egg extracts were adjusted to 1 mM ATP, 1 mM MgCl₂, 7.5 mM creatine phosphate and 0.1 mM EGTA pH7.7. 30 ng of Mos 3'UTR RNA was added to the egg extract and the polyadenylation assay was carried out at 23°C for 1h, prior to RNA extraction as described in McGrew and Richter (42). Mos 3' UTR polyadenylation analysis was carried out as described above (PAT assay). For depletion experiments, 2 µg of antibodies were incubated 30 min at 4°C with 15 µl of Dynabead protein-A beads (Dyna). Beads were washed three times with PBS plus 100 µg/ml BSA and incubated with 15 µl of egg extract for 30 min at room temperature. After precipitation, depleted extract was collected and used for polyadenylation assay and Western blot analysis. Bead precipitates were eluted by boiling in 2X Laemmli buffer and analyzed by Western blotting.

In vitro translation in Rabbit Reticulocyte Lysate (RRL) - The luciferase translation assay

was performed according to manufacturer recommendations (Promega). 100 ng of Luciferase mRNA (provided in RRL kit) were added to 10 μ l of RRL in presence of [³⁵S]methionine (2 μ l, 1000Ci/mmol, methionine/ cysteine mix, Amersham) with or without the indicated quantities of GST-77K or GST proteins, for 1.5h at 30°C. The equivalent of 20% of the reaction was used for autoradiography and western blot analysis. The amount of proteins added to the reaction was analyzed by Coomassie staining. The luciferase RNA was analysed by RT-PCR after the reaction. Reverse transcription was done with random primers and the amplification with the following oligonucleotides: 5'-AAGCCACCATGGAAGA-3' and 5'-CTCTAGAATTACACGGCGATC-3'.

Cell culture and immunofluorescence - Mouse NIH 3T3 cells were cultured in DMEM supplemented with 10% FBS in a 37°C incubator with 5% CO₂. *Xenopus* XTC cells were cultured in 70% L15 Leibovitch's Media supplemented with 10% FBS in hermetic tissue culture flask at 25°C. Cultured cells were fixed using 4% paraformaldehyde (in PBS) for 15 min at room temperature. The cells were then permeabilized in PBS containing 1% Triton-X100 for 10 min at room temperature and blocked in PBS containing 0,1% Tween and 3% BSA (blocking reagent). Antibody incubations were done 1h at room temperature in blocking reagent followed by secondary Alexa-546-labeled goat anti-rabbit IgG (Molecular probe) used in identical conditions. DNA was visualized by DAPI staining.

RESULTS

Identification of Xenopus CstF-77 homologue - A *Xenopus laevis* oocyte library was screened with a probe made by RT-PCR from *Xenopus* oocyte RNA and degenerate oligonucleotides. A cDNA encoding the *Xenopus* homologue of CstF-77 was isolated. This cDNA contains an open reading frame (ORF) encoding a protein of 719 amino-acids, with a predicted molecular weight of 83kDa that we named X77K. We identified a domain organization similar to the known CstF-77 homologues, with 11 HAT (half a TPR) repeats, a bipartite nuclear localization signal (NLS) and a proline-rich domain at the C terminus (figure 1). Apart from the NLS, these

domains are known to mediate protein-protein interactions, and the HAT repeats have been described to be specific to a family of proteins involved in RNA processing (11,43). For the human CstF-77 protein, the proline-rich domain has been shown to be necessary both for binding other CstF subunits and also for self association (12). It is noteworthy that the CstF-77 proteins are highly conserved, since the amino acid sequence of X77K shares 94% identity overall with the human and mouse CstF-77 proteins, 57% with *D.melanogaster* protein and 27% with *S.cerevisiae* protein (figure S1).

CstF-77 is localized both in the nucleus and cytoplasm - To study the expression of X77K protein, we raised a polyclonal antibody against the human C terminal peptide (77K antibody). By Western blotting, this antibody recognizes the HA-tagged X77K expressed in oocytes, and the purified *Baculovirus* GST-X77K protein (figure 2A lanes 2 and 5). It also detects endogenous *Xenopus* CstF-77 protein from oocytes or cell lysates (figure 2A lanes 1 and 3 respectively) and endogenous mouse (lane 4) and human protein (not shown). Moreover, the *in vitro* translated X77K protein is immunoprecipitated by the 77K antibody but not by the 77K antibody pre-incubated with the peptide antigen (figure 2B). These results show the specificity of the 77K antibody for the 77K protein.

A Western blot analysis on oocyte and embryo lysates shows that X77K is expressed at a constant level during *Xenopus* oogenesis and embryogenesis up to stage 40 (figure 3A and 3B). In *S. cerevisiae* and *D. melanogaster*, the CstF-77 homologues Rna14 and Su(f) respectively localize both to the nucleus and cytoplasm (28,44). In *Xenopus* oocytes, the nuclear localization of X77K has been well determined, where it is present in Cajal bodies and lampbrush chromosomes (32). However, nothing has been reported concerning a possible cytoplasmic localization. To investigate whether X77K could be cytoplasmic in *Xenopus laevis*, lysates from nucleus, cytoplasm or total oocytes were analyzed by Western blotting with 77K antibody. Figure 3C shows that X77K is mainly nuclear, but is also present in the cytoplasm. We verified that cytoplasmic extracts were not contaminated with nuclear extracts by probing the membrane for the nuclear protein RPA (Replication Protein A). This

double localization was confirmed by immunofluorescence experiments on *Xenopus* XTC and mouse NIH3T3 cell lines (figure 3D): CstF-77 protein is mainly in the nucleus but is also localized in punctuated bodies distributed in the whole cytoplasm. These results show that the CstF-77 protein is localized both in the nucleus and cytoplasm in vertebrates, as in *S. cerevisiae* and *Drosophila*.

As X77K has a functional NLS but partially localizes to the cytoplasm, we investigated whether the protein shuttles between the GV (Germinal Vesicle) and the cytoplasm, even if it does not present a NES (Nuclear Export Signal). To answer this question, oocytes were treated or not overnight with leptomycin B, a nuclear export inhibitor. They were then enucleated, to analyze whether the protein accumulated in the nucleus in presence of leptomycin B. In this condition, no change in the relative levels of X77K between the nucleus and the cytoplasm was observed, showing that X77K does not shuttle between the two compartments (figure 3E). Surprisingly, the CPEB protein was sensitive to leptomycin B and accumulated in the nucleus (figure 3E). This result validates the leptomycin B treatment and implies that CPEB shuttles between the cytoplasm and the nucleus. This observation was not expected since CPEB was not detected in the nucleus under normal conditions (35 and figure 3E) and suggests new functions for CPEB in the nucleus, or in nucleo-cytoplasmic transport.

X77K is part of a cytoplasmic complex with CPEB, eIF4E, CPSF-100 and XGLD2 - As we asked whether X77K could have a function in cytoplasmic polyadenylation, we checked for X77K partners in the cytoplasm. In *Xenopus* oocytes, CPEB plays a key role in translational repression in immature oocytes, and in directing cytoplasmic polyadenylation during oocyte maturation. We investigated whether X77K could belong to a complex which includes CPEB. We performed co-immunoprecipitations from total stage VI oocyte extracts, treated or not with RNase A, with antibodies directed against different proteins known to be in a cytoplasmic complex with CPEB. In this experiment, X77K, CPEB, eIF4E and CPSF-100 proteins were co-immunoprecipitated with 77K, eIF4E and CPEB antibodies but not with nonspecific IgG (figure 4A). As a negative control we used an antibody

directed against the Pak5 (P21 activated kinase) protein. This antibody did not immunoprecipitate any of the proteins tested. Similarly, Pak5 protein was not immunoprecipitated by the 77K, eIF4E or CPEB antibodies. These results show that X77K specifically interacts with eIF4E, CPEB and CPSF-100 independently of the mRNA.

To show that cytoplasmic X77K is involved in this complex, we enucleated oocytes and performed co-immunoprecipitation experiments from oocyte cytoplasm extracts treated with RNase A. The X77K, CPEB and CPSF-100 proteins were co-immunoprecipitated by X77K, eIF4E and CPEB antibodies (figure 4B). These results show that cytoplasmic X77K belongs to a complex with CPEB, eIF4E and CPSF.

It has been reported that XGLD2, a divergent poly(A) polymerase, directly binds to CPEB, even in stage VI oocytes (18). We asked whether XGLD2 was also in the complex with X77K in stage VI oocytes. As XGLD2 is expressed at a low level in oocytes (18), immunoprecipitations were performed from oocytes overexpressing XGLD2. HA-tagged XGLD2 mRNA was injected into oocytes and, after 14 hours of expression, the oocyte extracts were subjected to different antibody immunoprecipitations in presence or absence of RNase A. In these experiments, HA-XGLD2, X77K, eIF4E and CPEB proteins were co-immunoprecipitated with XGLD2, X77K, eIF4E and CPEB antibodies (figure 4C), showing that XGLD2 is also part of the complex. It is interesting to note that two forms of eIF4E were immunoprecipitated by the eIF4E antibody but that only the lower form is mainly co-immunoprecipitated (figure 4A and C).

To better characterize the complex and investigate whether the complex exists in metaphase II oocytes, we used another approach. Cap columns (^{7m}GTP chromatography), which allow eIF4E recruitment, were used to identify proteins present in the complex. Stage VI and metaphase II extracts, treated (figure 4D lanes 6 and 7) or not (lanes 2 and 3) with RNase, were supplemented with GTP to reduce non-specific adsorption. As a negative control, some extracts were supplemented with free cap (^{7m}GTP) (figure 4D lanes 4 and 5). The extracts were then applied on a cap column and the bound material was eluted with free cap and detected by Western blot. A fraction of both X77K and CPEB from stage VI and metaphase II

oocytes with or without RNase was retained on the column in presence of GTP but not when $^7\text{mGTP}$ was added in the extracts (figure 4D). This experiment was done using GTP at a lower concentration than $^7\text{mGTP}$ in the extracts. To confirm the specificity of the binding, a control experiment was done either, using the same concentration of GTP and $^7\text{mGTP}$ in the extracts, or, by eluting the bound proteins with GTP or $^7\text{mGTP}$ (figure S2). We observed that GTP did not compete for eIF4E, 77K and CPEB binding while $^7\text{mGTP}$ did (figure S2 compare lane 6 with lane 8) and that GTP only weakly eluted bound proteins compare to $^7\text{mGTP}$ (compare lane 4 with lane 2). A small amount of total X77K was retained on the resin (figure 4D and S2), which is coherent with the fact that only a small fraction of X77K is cytoplasmic and can bind to eIF4E. It is noteworthy that CPEB was also poorly retained on the resin (figure 4D and S2), even though it is abundant in the cytoplasm. However, this result could be explained by the fact that CPEB, known to be part of the masking complex, interacts only transiently with eIF4E (20). In metaphase II oocytes, the electrophoretic mobility of CPEB is shifted and the protein is partially degraded (45). However, the same proportion of total CPEB was retained on the column in stage VI and metaphase II extracts. As negative controls, we looked for the binding of ERK and β -tubulin proteins, and observed, as expected, that they were not retained on the column (figure 4D). We failed to detect endogenous XGLD2 on the resin, probably because of its low expression level.

To assess whether the interactions were direct, we performed GST-pull-down experiments with purified GST-X77K from *Baculovirus* and either HA-CPEB or HA-XGLD2 proteins translated *in vitro*. Figure 4E shows that GST-X77K bound to HA-CPEB and HA-XGLD2, whereas GST alone did not. In the same kind of experiment, a direct interaction between GST-X77K and HA-eIF4E was not conclusive, due to a high background in the GST control (data not shown).

Altogether, these results show that X77K belongs to a RNA-independent cytoplasmic complex with eIF4E, CPEB, CPSF-100 and XGLD2, and that X77K is capable of directly interacting with CPEB and XGLD2.

X77K is not required for cytoplasmic polyadenylation - We showed that X77K binds to

proteins required for cytoplasmic polyadenylation in oocytes. To address whether X77K is required for cytoplasmic polyadenylation, we performed *in vitro* polyadenylation tests in egg extracts that were subjected to 77K, or control IgG immunodepletions. As a control, we also did the experiment on egg extracts depleted for CPEB, a protein that is known to be required for cytoplasmic polyadenylation. Figure 5A shows that immunodepletions of X77K and CPEB completely removed the endogenous proteins (*left panel*), which were retained on the antibody beads (*right panel*). Moreover, even if CPEB was apparently not depleted by 77K antibody, and vice versa, we observed a co-immunoprecipitation between X77K and CPEB in egg extracts (figure 5A *right panel*) that confirmed our results obtained with oocyte lysates (figure 4).

To do the polyadenylation test, Mos 3'UTR RNA, bearing wild-type CPE and PRE elements, was incubated for one hour with egg extracts or X77K, CPEB or mock depleted extracts. The total RNA was isolated at the beginning of the reaction and one hour later. Then, Mos 3'UTR polyadenylation was checked by PAT (PolyAdenylation Test) assay, to measure the poly(A) tail length. Figure 5B shows that Mos 3'UTR RNA was polyadenylated in control and X77K depleted extracts, while it was not polyadenylated in CPEB depleted extracts (compare *lanes 2, 4, 6* with *lane 8*). These results show that depletion of X77K does not impair cytoplasmic polyadenylation *in vitro*.

To verify that this is also the case *in vivo*, we performed a polyadenylation test in oocytes. Oocytes were first injected with Mos 3'UTR RNA. Some of them were subsequently injected with 77K, CPEB or nonspecific IgG. One hour later, the oocytes were induced, or not, to mature with progesterone, and harvested when 30% of the control oocytes have undergone GVBD (Germinal Vesicle Break Down). The total RNA was isolated and scored for Mos mRNA polyadenylation. In control oocytes, Mos 3'UTR was polyadenylated only when the oocytes were submitted to progesterone (Figure 5C compare *lane 2* with *lane 3*). The change in electrophoretic mobility observed was due to the lengthening of the poly(A) tail, since the addition of oligo (dT) and RNaseH before the PAT assay suppressed the mobility shift (figure 5C *right panel*). As in egg

extracts, the impairment of X77K function did not modify the Mos 3'UTR polyadenylation in the oocytes (figure 5C compare *lane 6* with *lanes 3 and 8*). In CPEB antibody injected oocytes, Mos 3'UTR was polyadenylated to a lesser extent than in control oocytes (compare *lane 10* with *lanes 3 and 8*). This short poly(A) tail of Mos 3'UTR observed was probably due to a PRE-dependent polyadenylation. Indeed it has been shown that Mos 3'UTR could be precociously polyadenylated independently of CPE/CPEB, but the length of the poly(A) tail was shorter than in the presence of CPEB (20). The above experiment shows that X77K is not required for polyadenylation of exogenous Mos mRNA.

To confirm this result, we performed a polyadenylation test on endogenous mRNAs from oocytes injected with IgG, 77K or CPEB antibodies, or non-injected (figure 5D). CyclinB1 and AuroraA mRNAs were polyadenylated in control and 77K antibody injected oocytes, which is in agreement with the result obtained with exogenous Mos mRNA. In CPEB antibody injected oocytes, there is no polyadenylation of cyclinB1 mRNA that has no PRE sequence, while we observed a short poly(A) tail on AuroraA mRNA which bears a PRE element (19). The polyadenylation observed with our PAT assay is a specific reaction, as endogenous actin mRNA, which is not subjected to polyadenylation during oocyte maturation, is not polyadenylated in our test (figure 5D). Furthermore, we observed a weak deadenylation of actin mRNA from 6 hours of progesterone addition, showing that the injected antibodies did not impair the deadenylation reaction during meiotic maturation. Altogether these results obtained *in vitro* and *in ovo* show that X77K is not required for cytoplasmic polyadenylation.

77K antibody injected into oocytes accelerates the G2/M transition - During the experiments of polyadenylation test in oocytes, we observed that X77K antibody injection accelerates oocyte maturation. The antibodies were injected into and maintained in the cytoplasm (figure 6A *insert*), arguing that 77K antibody affected the function of the cytoplasmic X77K protein. Moreover, as maturation is accompanied with a cessation of transcription (15), an effect on the nuclear polyadenylation could be ruled out. We repeated the experiment several times and the 77K

antibody injected oocytes always underwent 50% GVBD one hour or more before the control oocytes (figure 6A), depending on the batch of oocytes. This was also the case when we injected another antibody directed against X77K (the 573 antibody) (data not shown). In agreement with these results, this acceleration was accompanied by a premature synthesis of Mos protein and activation of MAP kinase (figure 6B). In 77K antibody injected oocytes, Mos protein is well detected 2 hours after progesterone induction, while in control oocytes (uninjected or IgG injected) it is clearly detected only 5 hours after progesterone addition. The MAPK activation, revealed by the bi-phosphorylation of ERK, is also earlier in 77K antibody injected oocytes (2 hours) than in control oocytes (4 hours). To investigate whether downstream events of maturation were accelerated, we checked for proteins where the synthesis is induced at GVBD. In 77K antibody injected oocytes, AuroraA synthesis increased 2 hours after progesterone induction while, in control oocytes (IgG injected and uninjected oocytes), this increase is well observed at 5 hours (figure 6B). This acceleration of protein synthesis also occurred for cyclinB1 (data not shown).

One supposition for this premature protein synthesis was that the impairment of X77K function in oocytes accelerates mRNA cytoplasmic polyadenylation. To test this hypothesis, we performed a time course of Mos 3'UTR polyadenylation in 77K antibody injected oocytes. The oocytes were first injected with Mos 3'UTR RNA and then with 77K antibody or IgG. Oocytes were harvested at different times after progesterone induction, total RNA was extracted and checked for Mos mRNA polyadenylation. In control oocytes and 77K antibody injected oocytes, Mos mRNA polyadenylation occurred at the same time, showing that injection of 77K antibody does not modify the kinetics of cytoplasmic polyadenylation (figure 6C). CPEB is known to belong to a masking complex with eIF4E and Maskin. It has been reported that mRNA cytoplasmic polyadenylation allows the disruption of Maskin/eIF4E interaction by eIF4G recruitment, and translation ensues (22). This suggests that the role of cytoplasmic polyadenylation is to allow the binding of eIF4G to eIF4E. In 77K antibody injected oocytes, Mos, AuroraA and CyclinB1 proteins were precociously

synthesized, while Mos mRNA polyadenylation was not accelerated. This phenotype, with the fact that X77K belongs to a complex with CPEB and eIF4E, suggests that X77K could have a role in mRNA masking before cytoplasmic polyadenylation.

X77K represses mRNA translation in vitro

- The above results document that X77K functions *in vivo* as part of a complex involved in mRNA masking, thereby preventing a premature translation of protein that must be synthesized at a precise time. To test a potential function of the X77K protein in mRNA translation inhibition, we performed an *in vitro* translation experiment. In this assay, we added increasing quantities of purified GST-77K or GST proteins in rabbit reticulocyte lysates programmed with a luciferase reporter, and checked for mRNA translation by measuring and quantifying ³⁵S methionine incorporation. Figure 7 shows that luciferase mRNA translation was inhibited by addition of recombinant X77K in a dose dependent manner, while no translation inhibition was observed with the GST protein. Importantly, this effect is not due to destabilization of Luciferase mRNA by the addition of the recombinant proteins. This result is in agreement with a role for X77K in mRNA masking.

DISCUSSION

In this study, we identified the *Xenopus* CstF-77 protein (X77K) and showed that the CstF-77 proteins are very well conserved in vertebrates (figure 1 and S1). X77K is expressed in oocytes and embryos at a constant level, and is localized mainly to the nucleus, but also in discrete cytoplasmic foci in vertebrate cells (figure 3). However, X77K does not shuttle between the two compartments (figure 3E). The cytoplasmic localization, together with other arguments (see introduction), suggested a cytoplasmic role for CstF-77 protein. We established that X77K is part of a cytoplasmic complex with eIF4E, CPEB, CPSF and XGLD2 (figure 4) but is not required for cytoplasmic polyadenylation *per se* (figure 5). Nevertheless, impairment of X77K function in oocytes, accelerates oocyte maturation and protein synthesis without modifying the kinetic of Mos mRNA polyadenylation (figure 6). Moreover, X77K represses mRNA translation *in vitro* (figure

7). Altogether, these results support a role for X77K in mRNA masking.

The known function of nuclear CstF-77 protein is to enhance cleavage by stabilizing the interaction between CPSF and AAUAAA in the pre-cleavage complexes (46). We envisage that cytoplasmic X77K could have the same function of complex stabilization on cytoplasmic mRNA. This could explain why cytoplasmic X77K is not required for cytoplasmic polyadenylation *per se*, since its nuclear counterpart is required for pre-mRNA cleavage but not for the subsequent poly(A) addition step of the reaction. In the nucleus, CstF-77 interacts with CPSF via its CPSF-160 subunit (5). In the cytoplasm, X77K also binds to CPSF and CPEB (figure 4B). Moreover, X77K directly interacts with XGLD2 and CPEB *in vitro*, showing that X77K, CPSF, CPEB and XGLD2 are part of the same cytoplasmic complex. CstF-77 has no RNA binding domain, thus, one or several binding partners must recruit X77K to increase the affinity of the polyadenylation complexes (nuclear or cytoplasmic) on the target RNA. In the nucleus, CstF-64 is the RNA binding protein that targets, with CPSF-160, CstF-77 on the pre-mRNA. In the cytoplasm, CPEB could replace CstF-64 for this function. Moreover, CstF-77 has a functional NLS, leading to its intranuclear targeting (31 and our unpublished data), and does not shuttle between the nucleus and the cytoplasm (figure 3E), which could explain the low level of its cytoplasmic counterpart. This suggests that CstF-77 can stay into the cytoplasm only in interaction with other proteins, and that a fraction of X77K newly synthesized can be recruited by CPEB, XGLD2 or CPSF-160 and probably other proteins (see below).

CstF-77 is not the only protein that belongs to both nuclear and cytoplasmic polyadenylation complexes: besides CPSF, shown to be involved in cytoplasmic polyadenylation (14), Symplekin was recently identified as part of the cytoplasmic polyadenylation machinery (18). Symplekin is a dual location protein that has been localized to the cytoplasmic plaques of tight junctions, and in the nucleus where it interacts with CPSF, CstF-77 and CstF-64 (12,47). However, Symplekin does not interact with the fully assembled CstF, suggesting that Symplekin and CstF-77 can compete for the same site in

CstF-64 (12). CPEB binds both X77K (this study) and Symplekin (18), strengthening the idea that CPEB could have the same function in the cytoplasm than CstF-64 in the nucleus.

A cytoplasmic localization for the other CstF subunits has not been described in vertebrates, and the *S.cerevisiae* homologue RNA15 is only nuclear (28). However, we cannot rule out a possible cytoplasmic function for the CstF-64 or CstF-50 subunits, as gametes express different protein isoforms from those present in somatic cells. Indeed, a second form of CstF-64 protein (tau CstF-64), expressed in meiotic and post meiotic male germ cells, has been described (48). Searches for specific CstF-64 or CstF-50 isoforms in oocytes, and their possible functions in the cytoplasm, have not been tested, due to a lack of purified antibodies recognizing the corresponding *Xenopus* proteins.

We observed an acceleration of the G2/M transition in 77K antibody injected oocytes submitted to progesterone. This phenotype is probably due to premature Mos protein synthesis, that activates the MAPK pathway and MPF (Maturation Promoting Factor). However, the Mos mRNA polyadenylation kinetic is not accelerated, suggesting that impairment of X77K function allows Mos mRNA recruitment on the ribosome independently of its polyadenylation state. It has been suggested that the function of cytoplasmic polyadenylation was to displace eIF4E from a masking complex (22). One possibility is that 77K antibody, by interacting with the X77K protein, disrupts the masking complex, allowing the binding of eIF4E to eIF4G, and the recruitment of the small ribosomal subunit by the mRNA. Thus, X77K could play a role in mRNA masking prior to cytoplasmic polyadenylation.

However, as X77K represses CPE-independent mRNA translation (figure 7), it probably has a more general function in mRNA translation inhibition. In that case, X77K would be targeted on the mRNA by one or several proteins other than CPEB. CPSF can recruit X77K on the mRNA by itself. Nevertheless, other RNA binding proteins, in cooperation with CPSF, could also address X77K on the messenger RNA. Xp54, a ubiquitous translational repressor that also interacts with CPEB (49), could be one of them. XPumilio (50,51) and XDAZL (52), other translational regulators independent of CPE

dependent cytoplasmic polyadenylation, are also potential candidates. Thus, CPSF and an X77K-containing complex could interact cooperatively to stabilize a RNA masking complex, until it is disrupted or modified by a signal allowing mRNA translation.

CstF-77 protein is localized in discrete cytoplasmic foci in NIH3T3 and XTC cells (Figure 3D). In oocytes, Xp54 and p47, the *Xenopus* and *Spisula* homologues of the yeast decapping activator Dhh1, are localized in stored mRNP and bind to CPEB (49,53,54). Moreover, it has been reported that human CPEB1 expression induces the assembly of stress granules, that in turn, recruit the processing bodies (PB) (55), and that eIF4E is localized in PB (56,57). Thus, proteins initially involved in mRNA translation can be localized in PB and, inversely, decapping activator can be stored in mRNP in oocytes. Recently, Brengues et al (58) have shown that mRNA molecules move from PB to polysomes and vice versa. These data show that stress granules, PB and stored mRNP are dynamically linked sites of mRNP remodeling.

The fact that: 1) X77K belongs to a complex with eIF4E, CPEB and XGLD2; 2) The impairment of 77K function leads to a premature protein synthesis in oocytes; 3) Recombinant X77K represses mRNA translation *in vitro* and 4) we observed CstF-77 in NIH3T3 and XTC cytoplasmic foci, suggest that cytoplasmic CstF-77 could have a ubiquitous function in mRNP remodeling events, for the transition of mRNA from inactive to active translation.

Acknowledgments- We thank the Thierry Lorca lab for reagents, advice and assistance, Cyril Bernis for egg extracts and Jamal Tazy for luciferase oligonucleotides. We are grateful to Simon Morley, Jean-Marc Lemaître, Nathalie Morin and Claude Prigent for the antibodies, Dr N.Sagata for the Mos vector, Dr D. Bentley for the CstF 77 carboxy terminal peptide and Elmar Wahle and Uwe Kuehn for the gift of unpublished anti CPSF-100 antibody. We also thank A. Bernet, J.M. Donnay, Y. Boublik and G. Herrada for technical assistance. We are grateful to D. Fisher for looking over the English. This work was supported by the Centre National de la Recherche Scientifique and the Association pour la Recherche sur le Cancer (contrat numbers 4469 and 3147 to E.M.). CR is supported by the Ministère de l'éducation nationale, de

REFERENCES

1. Zhao, J., Hyman, L., and Moore, C. (1999) *Microbiol Mol Biol Rev* **63**, 405-445
2. Edmonds, M. (2002) *Prog Nucleic Acid Res Mol Biol* **71**, 285-389
3. Proudfoot, N. (2004) *Curr Opin Cell Biol* **16**, 272-278
4. Wilusz, J., Shenk, T., Takagaki, Y., and Manley, J. L. (1990) *Mol Cell Biol* **10**, 1244-1248
5. Murthy, K. G. K., and Manley, J. L. (1995) *Genes Dev.* **9**, 2672-2683
6. Gilmartin, G. M., and Nevins, J. R. (1991) *Mol. Cell. Biol.* **11**, 2432-2438
7. Bienroth, S., Wahle, E., Suter-Crazzolara, C., and Keller, W. (1991) *J Biol Chem* **266**, 19768-19776
8. Murthy, K. G., and Manley, J. L. (1992) *J Biol Chem* **267**, 14804-14811
9. Kaufmann, I., Martin, G., Friedlein, A., Langen, H., and Keller, W. (2004) *Embo J* **23**, 616-626
10. Takagaki, Y., Manley, J. L., MacDonald, C. C., Wilusz, J., and Shenk, T. (1990) *Genes Dev* **4**, 2112-2120
11. Takagaki, Y., and Manley, J. L. (1994) *Nature* **372**, 471-474
12. Takagaki, Y., and Manley, J. L. (2000) *Mol Cell Biol* **20**, 1515-1525
13. Bilger, A., Fox, C. A., Whale, E., and Wickens, M. (1994) *Genes Dev.* **8**, 1106-1116
14. Dickson, K. S., Bilger, A., Ballantyne, S., and Wickens, M. P. (1999) *Mol Cell Biol* **19**, 5707-5717
15. Mendez, R., and Richter, J. D. (2001) *Nat Rev Mol Cell Biol* **2**, 521-529
16. Wang, L., Eckmann, C. R., Kadyk, L. C., Wickens, M., and Kimble, J. (2002) *Nature* **419**, 312-316.
17. Rouhana, L., Wang, L., Buter, N., Kwak, J. E., Schiltz, C. A., Gonzalez, T., Kelley, A. E., Landry, C. F., and Wickens, M. (2005) *Rna* **11**, 1117-1130
18. Barnard, D. C., Ryan, K., Manley, J. L., and Richter, J. D. (2004) *Cell* **119**, 641-651
19. Charlesworth, A., Cox, L. L., and MacNicol, A. M. (2004) *J Biol Chem* **279**, 17650-17659
20. Charlesworth, A., Ridge, J. A., King, L. A., MacNicol, M. C., and MacNicol, A. M. (2002) *Embo J* **21**, 2798-2806
21. Stebbins-Boaz, B., Cao, Q., de Moor, C. H., Mendez, R., and Richter, J. D. (1999) *Mol Cell* **4**, 1017-1027
22. Cao, Q., and Richter, J. D. (2002) *Embo J* **21**, 3852-3862.
23. Minvielle-Sebastia, L., Preker, P. J., and Keller, W. (1994) *Science* **266**, 1702-1705
24. Mandart, E., and Parker, R. (1995) *Mol Cell Biol* **15**, 6979-6986
25. Mandart, E. (1998) *Mol Gen Genet* **258**, 16-25
26. Rouillard, J. M., Brendolise, C., and Lacroute, F. (2000) *Mol Gen Genet* **262**, 1103-1112
27. Brendolise, C., Rouillard, J. M., Dufour, M. E., and Lacroute, F. (2002) *Mol Genet Genomics* **267**, 515-525
28. Bonneaud, N., Minvielle-Sebastia, L., Cullin, C., and Lacroute, F. (1994) *J. Cell. Science.* **107**, 913-921
29. Audibert, A., and Simonelig, M. (1999) *Mech Dev* **82**, 41-50
30. Schul, W., Groenhout, B., Koberna, K., Takagaki, Y., Jenny, A., Manders, E. M., Raska, I., van Driel, R., and de Jong, L. (1996) *Embo J* **15**, 2883-2892
31. Schul, W., van Der Kraan, I., Matera, A. G., van Driel, R., and de Jong, L. (1999) *Mol Biol Cell* **10**, 3815-3824
32. Gall, J. G., Bellini, M., Wu, Z., and Murphy, C. (1999) *Mol Biol Cell* **10**, 4385-4402

33. Bleoo, S., Sun, X., Hendzel, M. J., Rowe, J. M., Packer, M., and Godbout, R. (2001) *Mol Biol Cell* **12**, 3046-3059
34. Gall, J. G. (2003) *Nat Rev Mol Cell Biol* **4**, 975-980
35. Newport, J., and Kirschner, M. (1982) *Cell* **30**, 675-686.
36. Hake, L. E., and Richter, J. D. (1994) *Cell* **79**, 617-627
37. Sagata, N., Oskarsson, M., Copeland, T., Brumbaugh, J., and Vande Woude, G. F. (1988) *Nature* **335**, 519-525
38. Papin, C., and Smith, J. C. (2000) *Dev Biol* **217**, 166-172.
39. Nakamura, A., Sato, K., and Hanyu-Nakamura, K. (2004) *Dev Cell* **6**, 69-78
40. Salles, F. J., and Strickland, S. (1995) *PCR Methods Appl* **4**, 317-321
41. Murray, A. W., and Kirschner, M. W. (1989) *Nature* **339**, 275-280
42. McGrew, L. L., and Richter, J. D. (1990) *EMBO J.* **9**, 3743-3751
43. Preker, P. J., and Keller, W. (1998) *Trends Biochem Sci* **23**, 15-16
44. Audibert, A., Juge, F., and Simonelig, M. (1998) *Mech Dev* **72**, 53-63
45. Reverte, C. G., Ahearn, M. D., and Hake, L. E. (2001) *Dev Biol* **231**, 447-458
46. Weiss, E. A., Gilmartin, G. M., and Nevins, J. R. (1991) *EMBO J.* **10**, 215-219
47. Hofmann, I., Schnolzer, M., Kaufmann, I., and Franke, W. W. (2002) *Mol Biol Cell* **13**, 1665-1676
48. Wallace, A. M., Dass, B., Ravnik, S. E., Tonk, V., Jenkins, N. A., Gilbert, D. J., Copeland, N. G., and MacDonald, C. C. (1999) *Proc Natl Acad Sci U S A* **96**, 6763-6768
49. Minshall, N., and Standart, N. (2004) *Nucleic Acids Res* **32**, 1325-1334
50. Nakahata, S., Katsu, Y., Mita, K., Inoue, K., Nagahama, Y., and Yamashita, M. (2001) *J Biol Chem* **276**, 20945-20953
51. Padmanabhan, K., and Richter, J. D. (2006) *Genes Dev* **20**, 199-209
52. Collier, B., Gorgoni, B., Loveridge, C., Cooke, H. J., and Gray, N. K. (2005) *Embo J* **24**, 2656-2666
53. Lodomery, M., Wade, E., and Sommerville, J. (1997) *Nucleic Acids Res* **25**, 965-973
54. Minshall, N., Thom, G., and Standart, N. (2001) *Rna* **7**, 1728-1742
55. Wilczynska, A., Aigueperse, C., Kress, M., Dautry, F., and Weil, D. (2005) *J Cell Sci* **118**, 981-992
56. Andrei, M. A., Ingelfinger, D., Heintzmann, R., Achsel, T., Rivera-Pomar, R., and Luhrmann, R. (2005) *Rna* **11**, 717-727
57. Kedersha, N., Stoecklin, G., Ayodele, M., Yacono, P., Lykke-Andersen, J., Fitzler, M. J., Scheuner, D., Kaufman, R. J., Golan, D. E., and Anderson, P. (2005) *J Cell Biol* **169**, 871-884
58. Brengues, M., Teixeira, D., and Parker, R. (2005) *Science* **310**, 486-489

FIGURE LEGENDS

Fig. 1. *A*, Amino acid sequence of X77K. The HAT repeats and the Prolin-rich domain are underlined with solid and broken lines respectively. The bipartite nuclear localization signal (NLS) is in bold. Amino acids are numbered on the right. *B*, Schematic representation of X77K protein.

Fig. 2. 77K antibody characterization. *A*, Western blot analysis of stage VI oocyte extract (*lane 1*), HA-tagged X77K expressed in oocytes (*lane 2*), XTC and NIH (3T3) cell lines (*lanes 3 and 4*) and purified *Baculovirus* GST-tagged X77K (*lane 5*) probed with 77K antibody. *B*, Immunoprecipitates from ³⁵S methionine labeled X77K protein (1ng) with control IgG, or 77K antibody or 77K antibody pre-incubated with 77K antigen were analyzed by autoradiography.

Fig. 3. Endogenous expression of CstF-77 protein. *A* and *B*, Immunoblot analysis of *Xenopus* oocyte extracts (25 µg by lane) *A* and embryo extracts (12,5 µg by lane) *B* with 77K and β-tubulin antibodies. The β-tubulin, consistently expressed throughout oocyte maturation and embryogenesis, serves as a loading control. *C*, Oocytes were manually enucleated and pools of nucleus and cytoplasm were collected separately. Proteins extracts were analyzed by Western blotting with 77K and RPA antibodies. The equivalent of one oocyte (StVI), one nucleus (N) or one cytoplasm (Cyto) was loaded. The Replication Protein A (RPA) is exclusively expressed in nucleus and serves as a control of enucleation. *D*, Immunofluorescence of NIH3T3 and XTC cells. Cells were fixed, stained with 77K antibody and with DAPI and observed by fluorescence microscopy. A nuclear and cytoplasmic expression of endogenous 77K is observed in both cell lines. *E*, X77K localization is not sensitive to leptomycin B. Oocytes were treated or not over night with leptomycin B (100 nM final) and were manually enucleated. Pools of nucleus and cytoplasm were collected separately. Proteins extracts were analyzed by Western blotting with 77K, CPEB, RPA and β-tubulin antibodies. The equivalent of one oocyte (StVI), one cytoplasm (Cyt) or 0.5 nucleus (N) was loaded. The RPA and β-tubulin proteins are respectively expressed in nucleus and cytoplasm and serves as enucleation and loading controls.

Fig. 4. X77K is part of a complex with eIF4E, CPEB, CPSF and XGLD2. *A*, StVI oocyte extracts, treated or not with RNase A as indicated, were immunoprecipitated with 77K, eIF4E, CPEB, Pak5 antibodies or with control IgG. The immunoprecipitates were analyzed by Western blotting with 77K, CPEB, eIF4E, CPSF-100 and Pak5 antibodies. *B*, Oocytes were manually enucleated and cytoplasmic extracts were treated with RNase A and immunoprecipitated with 77K, eIF4E, CPEB or control IgG. The immunoprecipitates were analyzed by Western blotting with 77K, CPEB and CPSF-100 antibodies. *C*, Oocytes were injected with mRNA encoding HA-tagged XGLD2 and, after 14 hours of HA-XGLD2 expression, protein extracts were treated or not with RNase A and immunoprecipitated with different antibodies as indicated. The immunoprecipitates were analyzed by Western blotting with HA, 77K, eIF4E and CPEB antibodies. The equivalent of 1 oocyte (*A* and *C*) and one cytoplasm (*B*) was loaded as input. *D*, Cap column assay. Protein extracts from StVI oocytes or mature oocytes (MII) treated or not with RNase A were supplemented with GTP or ^{7m}GTP and applied to ^{7m}GTP-Sepharose beads (cap column). After beads washes, the bound proteins were eluted with ^{7m}GTP. All of the eluted proteins (B: Bound), the equivalent 1/10 of StVI oocytes or mature oocytes extracts (input StVI or input MII), 1/8 of the supernatant of protein extracts after cap column binding (U: Unbound) were analyzed by Western blotting with eIF4E, 77K, CPEB, ERK and β-tubulin antibodies. *E*, GST pull-down assay. 5 µg of purified GST-X77K or GST were bound to glutathione beads that were subsequently incubated with *in vitro* translated HA-tagged CPEB (*left panel*) or HA-tagged XGLD2 (*right panel*). The bound proteins were boiled in SDS sample buffer. The eluates and the equivalent of 1/10 of the *in vitro* translated proteins (input) were analyzed by Western blotting with HA and GST antibodies.

Fig. 5. X77K is not required for cytoplasmic polyadenylation. *A* and *B*, Polyadenylation assay in egg extracts. *A*, Egg extracts were immunodepleted with 77K or CPEB antibodies or with control IgG. 1/10 of the egg extracts, immunodepleted or not (*left panel*) and 1/10 of the immunoprecipitates (*right panel*) were analyzed by Western blotting with 77K or CPEB antibodies. *B*, Egg extracts, immunodepleted or not as indicated, were incubated with exogenous Mos 3'UTR RNA. Total RNA extracted at 0 and 1 hour after Mos 3'UTR RNA addition was submitted to Mos polyadenylation analysis (PAT assay) using specific primers. *C* and *D*, Polyadenylation assay in oocytes. *C*, Exogenous Mos mRNA polyadenylation. *Left panel*, Oocytes were first injected with Mos RNA and 30 min later were injected with 77K, CPEB antibodies or with nonspecific IgG. After 1 hour incubation, maturation was induced or not with progesterone. Total RNA was extracted from pools of 5 oocytes collected at the time of progesterone addition (time point 0h) or when 30% of control oocytes have undergone GVBD (time point 6h). Total RNA was submitted to Mos polyadenylation analysis. *Right panel*, total RNA, corresponding to *lane 1* and *3* of the left panel, were submitted or not to RNaseH treatment before Mos polyadenylation analysis

(PAT assay). *D*, Endogenous mRNA polyadenylation. Oocytes were injected with 77K, CPEB antibodies or with nonspecific IgG. After 1 hour incubation, maturation was induced or not with progesterone. Total RNA was extracted from pools of 5 oocytes collected at the time of progesterone addition (time point 0h), when 30% of control oocytes have undergone GVBD (time point 6h), and two hours later (time point 8h). Total RNA was submitted to endogenous CyclinB1, AuroraA and actin type5 polyadenylation analysis (PAT assay) using specific primers. *C* and *D*, Lane 4 corresponds to RNA from a mix of oocytes injected with the different antibodies at time point 0. Fragment size (M) is indicated on the left in base pair (bp).

Fig. 6. Injection of 77K antibody accelerates oocyte maturation. *A*, Oocytes were injected with 77K antibody or nonspecific IgG or left uninjected and maturation was induced by progesterone one hour later. The percentage of GVBD was scored at the indicated times and graphed. For each treatment, 35 oocytes were injected. This graph is representative of six experiments. *Insert*, 5 hours after 77K antibody injection, oocytes were manually enucleated and pools of cytoplasm and nucleus were collected separately. Proteins extracts were analyzed by Western blotting with 77K and RPA antibodies. The equivalent of one uninjected oocyte (StVI), one cytoplasm (cyto) and one nucleus (N) from injected oocytes was loaded. The Replication Protein A (RPA) serves as a control of enucleation. An exclusive cytoplasmic localization of 77K IgG is observed. *B*, Western blot analysis of Mos and AuroraA synthesis, activated MAPK (ppERK) and β -tubulin in oocytes collected during an experiment similar to the one depicted in *A*. The β -tubulin serves as a loading control. *C*, Oocytes were first injected with Mos 3'UTR RNA and 30 min later were injected with 77K antibody or nonspecific IgG. 1 hour later, maturation was induced with progesterone. Total RNA was extracted from pools of 5 oocytes collected at the indicated times and was submitted to Mos polyadenylation analysis. Fragment size is indicated on the left in base pair (bp).

Fig. 7. X77K protein inhibits mRNA *in vitro* translation in a dose dependent manner. *A*, Luciferase mRNA was translated in rabbit reticulocyte lysates (RRL) in presence of ^{35}S methionine alone (Control) or with increasing amount of purified GST-77K or GST as indicated (0.5 μg to 3 μg). 20% of each treated RRL were analyzed by SDS-PAGE. Translated luciferase was revealed by autoradiography and β -tubulin, as a loading control, was analyzed by Western blotting. The integrity of luciferase mRNA in presence of the recombinant proteins was verified by RT PCR. The increasing amount of GST-77K and GST were analyzed by Coomassie staining. Two bands are detected for GST-77K, the lower one corresponding to GST-77K lacking the carboxy terminal end. *B*, Quantification of luciferase translation by Phosphoimager where control is referred as 100%.

Fig 2

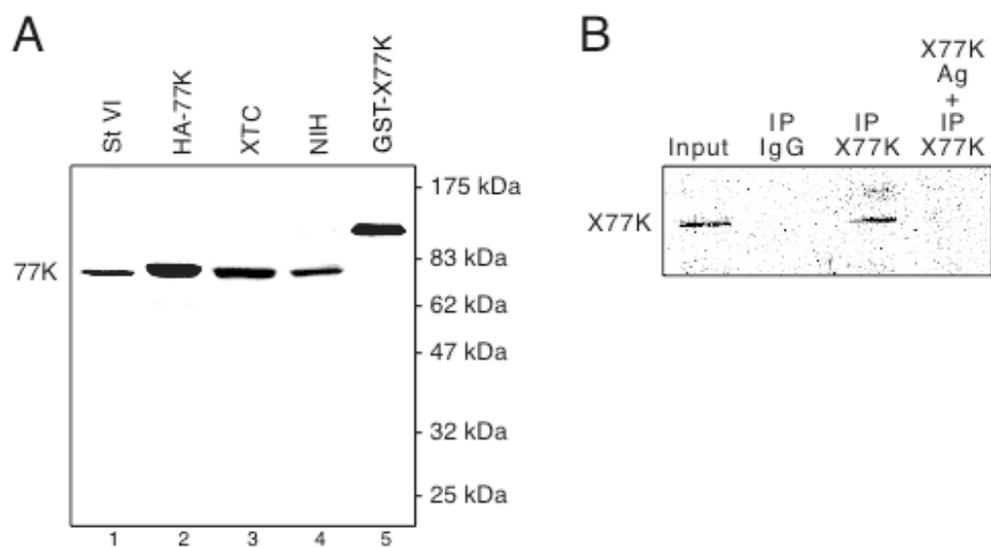


Fig 3

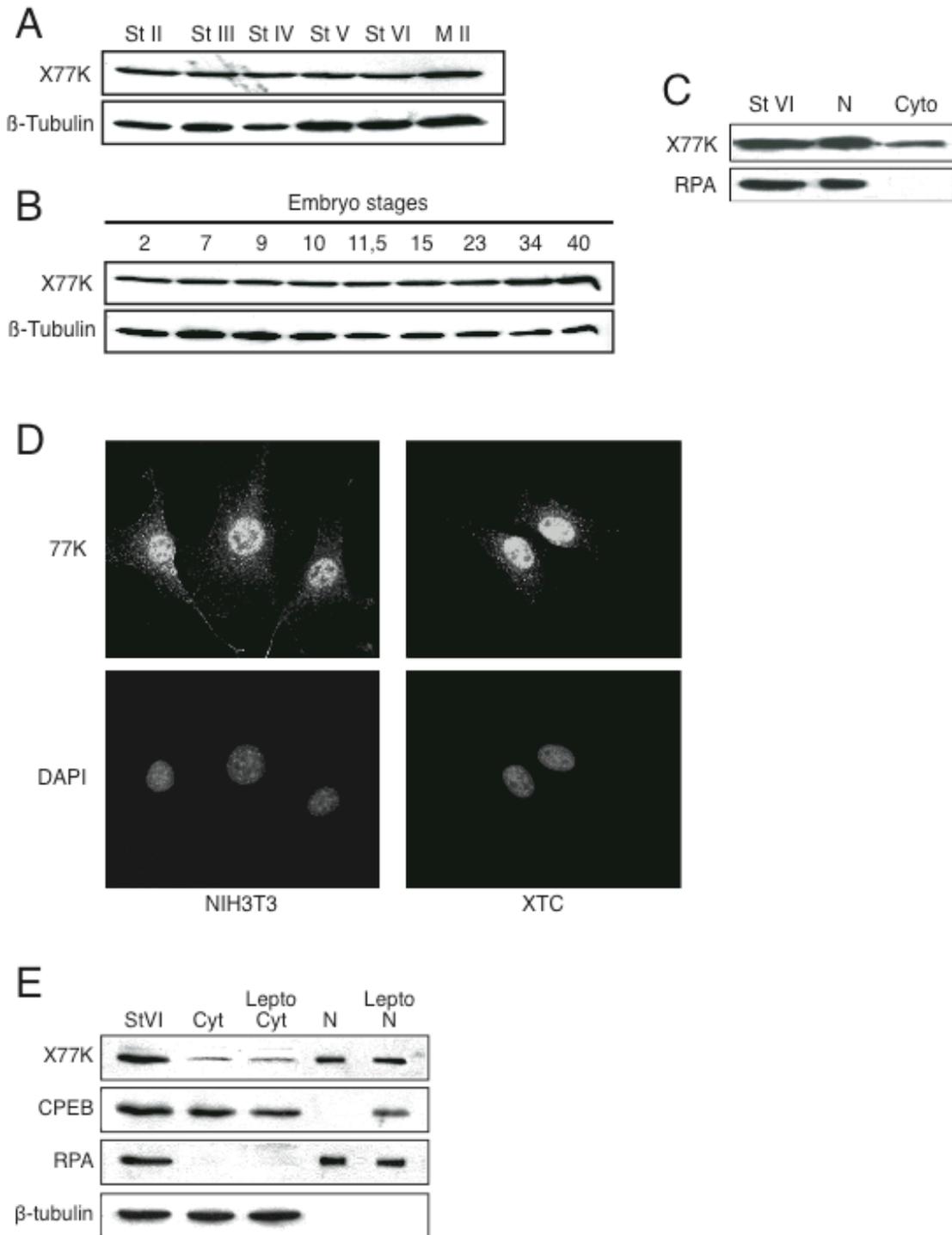


Fig 4

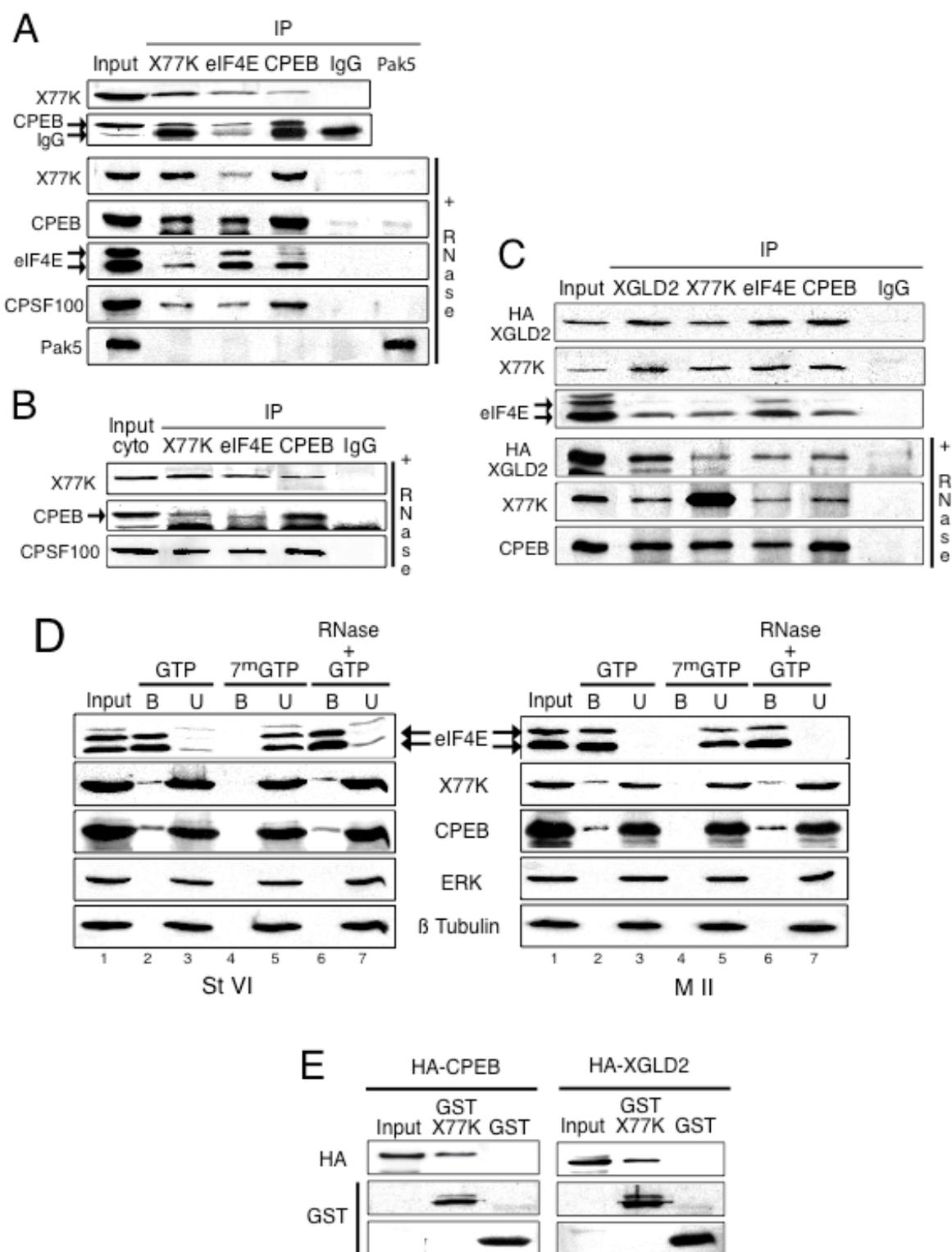


Fig 5

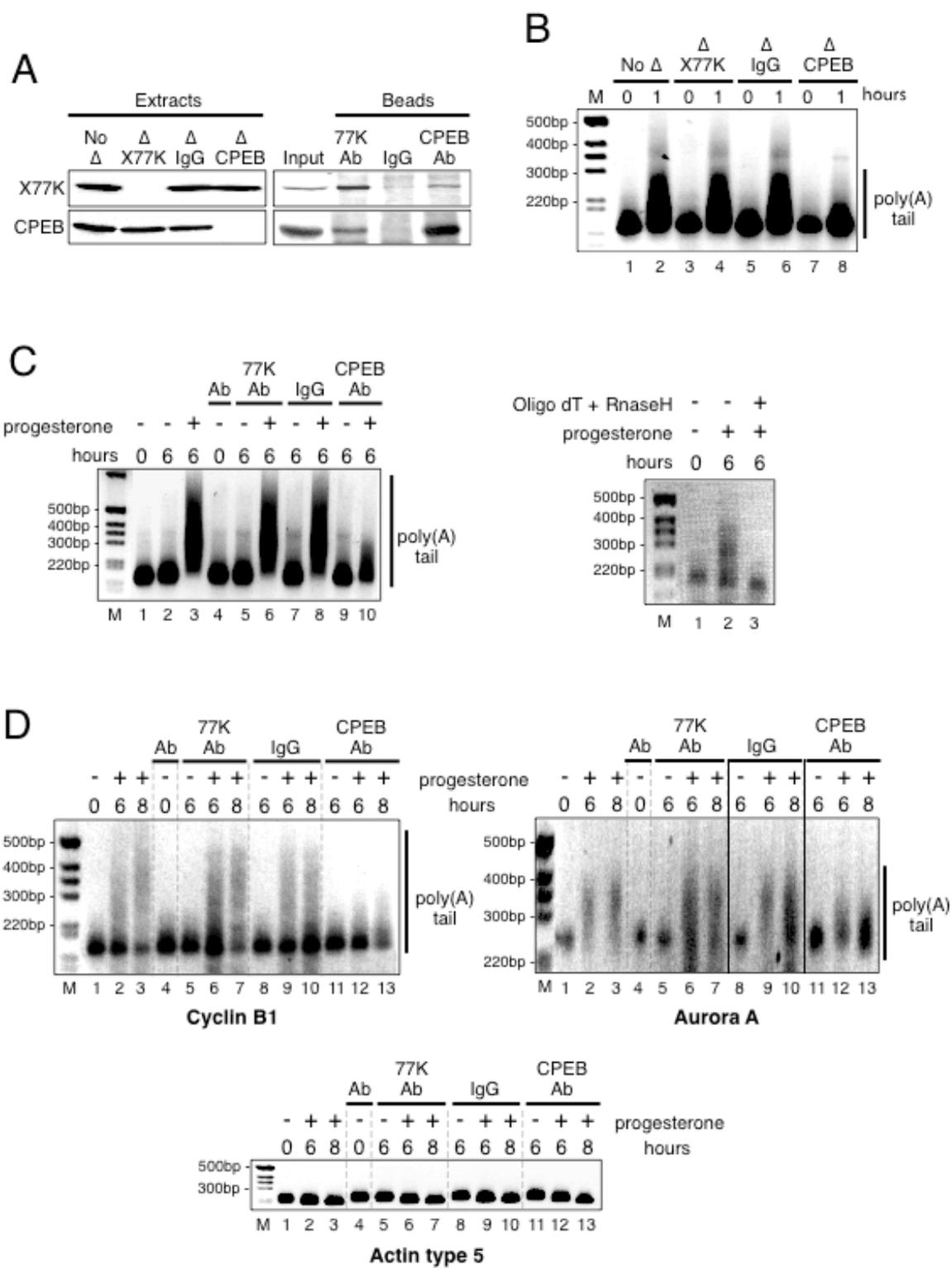


Fig 6

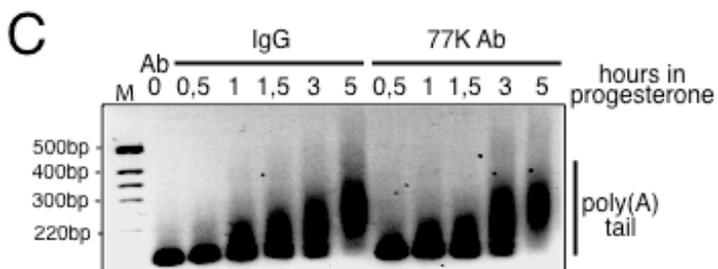
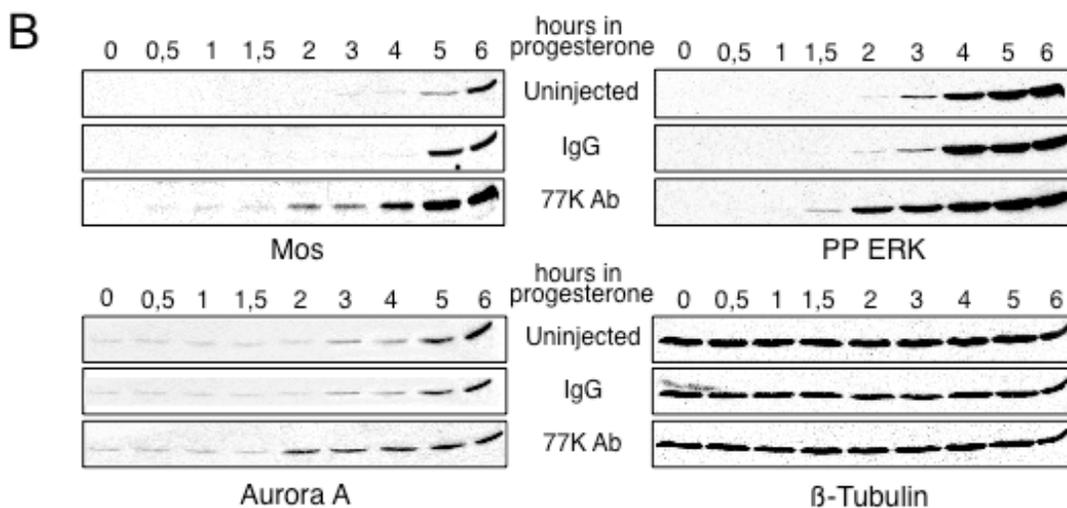
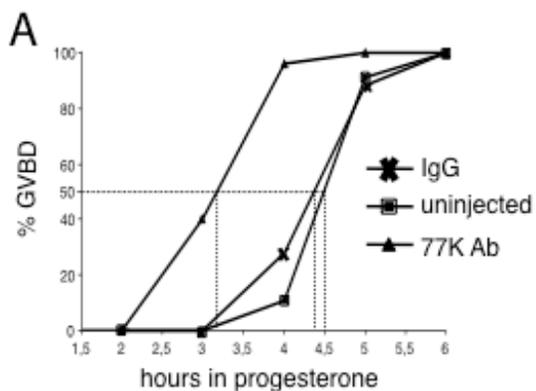


Fig 7

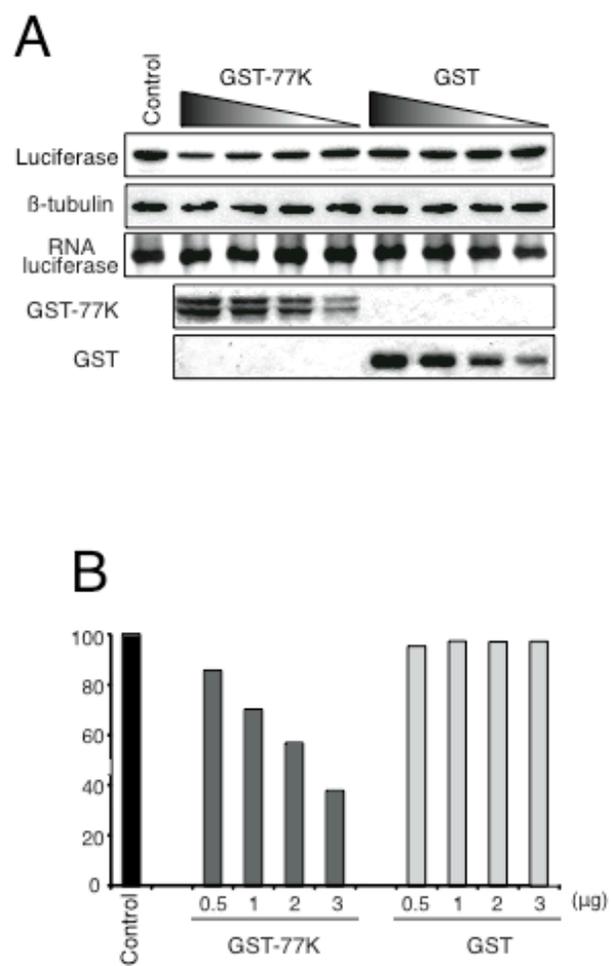


Fig S1

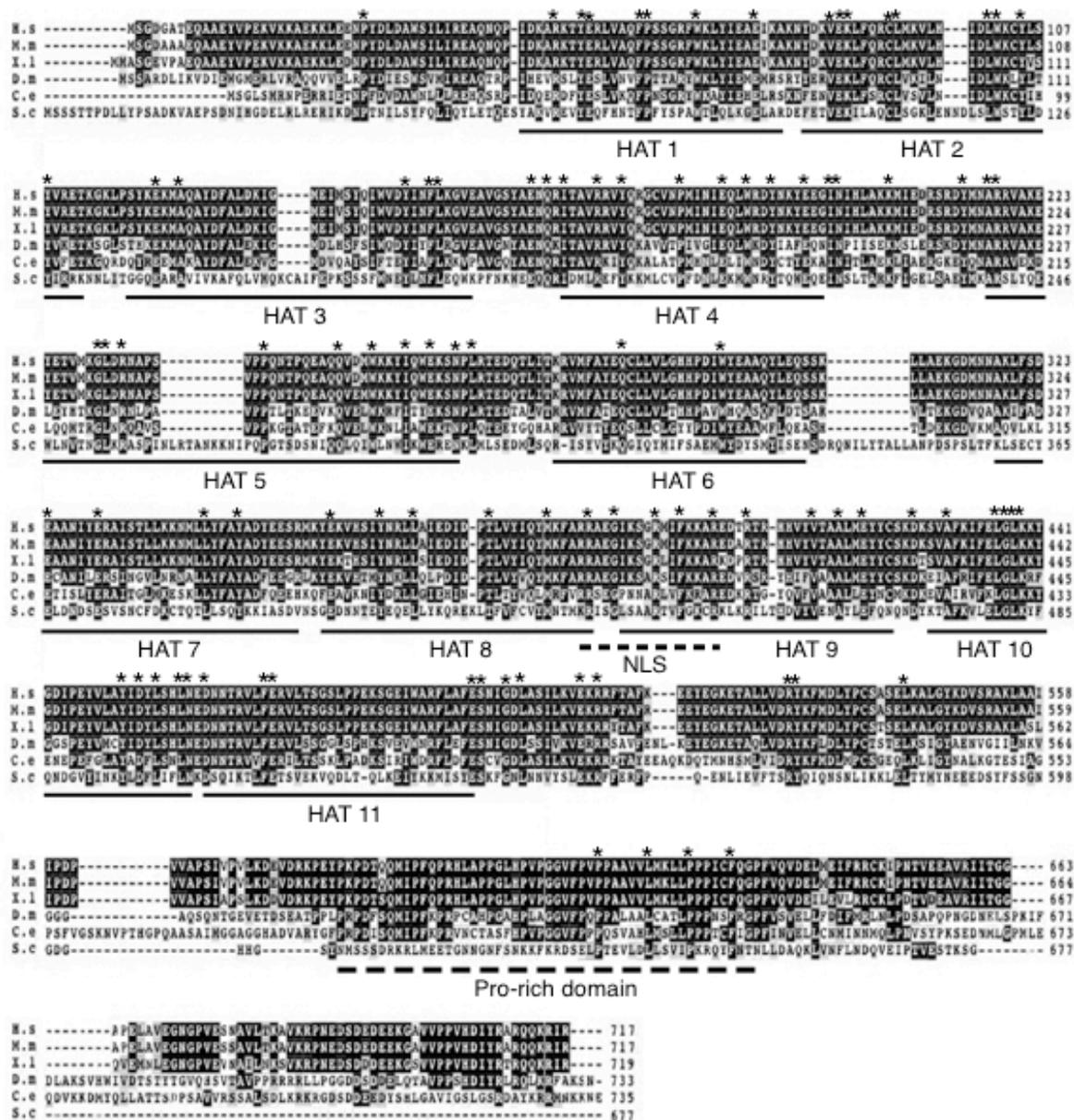


Figure S1. X77K alignment with other CstF 77 family members. Amino-acid sequence of the *Xenopus laevis* (X.l) 77K is optimally aligned with those of the human (H.s), mouse (M.m), *Drosophila Su(f)* (D.m), *Caenorhabditis elegans* (C.e) and *Saccharomyces cerevisiae* RNA14 (S.c) proteins. Identical and similar residues between the proteins are indicated in black and grey respectively. The asterisk (*) indicates amino acid conserved in all species. Amino acids of each protein are numbered on the right. The Prolin-rich domain (except for RNA14), the HAT repeats and a putative nuclear localisation signal (NLS) of the proteins are indicated.

Fig S2

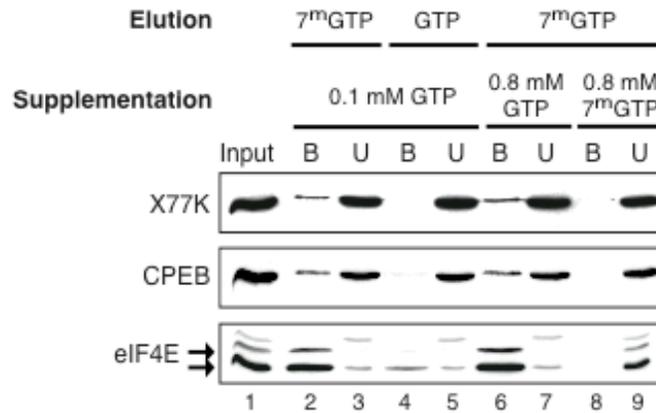


Figure S2. X77K, CPEB and eIF4E bind specifically to cap column. Protein extracts from StVI oocytes were supplemented with 0.1 mM GTP (lanes 2 to 5) or 0.8 mM GTP (lanes 6 and 7) or 0.8 mM 7^mGTP (lanes 8 and 9) and applied to 7^mGTP-Sepharose beads (cap column). After beads washes, the bound proteins were eluted with 1mM 7^mGTP (lanes 2,3, 6 to 9) or with 1 mM GTP (lanes 4 and 5). All of the eluted proteins (B: Bound), 1/20 of StVI oocytes extracts (input) or 1/20 of the protein extracts supernatant after cap column binding (U:Unbound) were analyzed by Western blotting with eIF4E, CPEB and 77K antibodies.