Identification of RNA-binding proteins specific to *Xenopus* Eg maternal mRNAs: association with the portion of Eg2 mRNA that promotes deadenylation in embryos

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Summary

Maternal *Xenopus* Eg mRNAs have been previously identified as transcripts that are specifically deadenylated after fertilization and degraded after the mid blastula transition. Destabilizing *cis* sequences were previously localised in the 3′ untranslated region of Eg2 mRNA. In order to characterize possible *trans*-acting factors which are involved in the post-transcriptional regulation of Eg mRNAs, gel-shift and u.v. cross-linking experiments were performed, which allowed the identification of a p53-p55 RNA-binding protein doublet specific for the 3′ untranslated regions of Eg mRNAs. These p53-p55 proteins do not bind to the 3′ untranslated regions of either ornithine decarboxylase or phosphatase 2Ac mRNAs, which remain polyadenylated in embryos. These novel RNA-binding proteins are distinct from the cytoplasmic polyadenylation element-binding protein that controls the polyadenylation of maternal mRNAs in maturing *Xenopus* oocytes, and from previously identified thermoresistant RNA-binding proteins present in oocyte mRNP storage particles. The p53-p55 bind a portion of the Eg2 mRNA 3′ untranslated region, distinct from the previously identified destabilizing region, that is able to confer the postfertilization deadenylation of CAT-coding chimeric mRNAs. This suggests that the p53-p55 RNA-binding proteins are good candidates for *trans*-acting factors involved in the deadenylation of Eg mRNAs in *Xenopus* embryos.

Key words: *Xenopus laevis*, maternal mRNAs, post-transcriptional control, RNA binding proteins.

Introduction

In many species, early steps of development occur in the absence of transcription, gene expression being controlled exclusively at a post-transcriptional level from maternal mRNAs that have been stored during oogenesis (Davidson, 1986). In several cases, the *cis*-acting sequence motifs involved in this control have been localised in the 3 untranslated regions of these mRNAs. For instance, cytoplasmic changes in the length of the poly(A) tract of maternal mRNAs, which play a major role in regulating the translation of these mRNAs during oocyte maturation in the frog (Dworkin and Dworkin-Rastl, 1985; Dworkin et al., 1985; Hyman and Wormington, 1988; Shuttleworth et al., 1990) and mouse (Huarte et al., 1987; Vassalli et al., 1989), are directed by the cytoplasmic polyadenylation element (CPE) localised near the AAUAAA polyadenylation signal (Fox et al., 1989; McGrew et al., 1989; McGrew and Richter, 1990). This element specifically binds *trans*-acting factors, CPE-binding proteins (McGrew and Richter, 1990; Paris et al., 1991b). In *Drosophila* the nanos (nos) gene product is a *trans*-acting factor that can suppress the translation of hunchback and bicoid mRNA and the nos response element(s) are situated in the 3 untranslated regions of both mRNAs (Wharton and Struhl, 1991). In the case of bicoid mRNA, at least, the nos-regulated supression of translation is associated with a shortening of the poly(A) tail.

Although *trans*-acting factors implicated in the storage of maternal mRNA in an untranslated state in *Xenopus* oocytes (Darnbrough and Ford, 1981; Richter and Smith, 1984; Dearsly et al., 1985; Crawford and Richter, 1987; Murray et al., 1991) and in the control of cytoplasmic adenylation during oocyte maturation (McGrew and Richter, 1990; Paris et al., 1991b) have been identified, those implicated in the control of maternal mRNA polyadenylation in early *Xenopus* embryos are less extensively understood.

We have previously identified maternal *Xenopus* mRNAs whose poly(A) tail length is modified after fertilization: the family of Eg mRNAs are deadenylated whereas the poly(A) tract of those denoted as Cl mRNAs is lengthened (Paris and Philippe, 1990). The poly(A) tail of other maternal mRNAs, for instance those coding for ornithine decarboxylase (ODC), is not significantly changed after fertilization (Osborne et al., 1991). Deadenylated Eg mRNAs are released from the polysomal fraction whereas mRNAs

which, like ODC, remain polyadenylated also remain translatable and stable throughout development. Despite the fact that the consequences of cytoplasmic adenylation of an mRNA on its translation are similar during oocyte maturation and in early embryos, the mechanisms involved in targeting an mRNA for a lengthening or a shortening of its poly(A) tail are probably different. For instance, B4 (Dworkin and Dworkin-Rastl, 1985), cdk2-Eg1 (Paris et al., 1991a) and Eg5 (LeGuellec et al., 1991) mRNAs are adenylated during oocyte maturation, however, B4 mRNA remains adenylated in the embryo whereas cdk2-Eg1 and Eg5 mRNAs are deadenylated in the embryo.

The present study was performed to identify RNA-binding proteins that specifically associate with the 3′ untranslated regions of maternal mRNAs that are deadenylated after ferilization (Eg mRNAs). We have identified a p53-p55 protein doublet that specifically binds the 3′ untranslated region of cdk2-Eg1, Eg2 and Eg5 mRNAs. These proteins bind to a 410 nucleotide distal portion of Eg2 mRNA 3′ untranslated region that specifically promotes the deadenylation of chimeric mRNAs. Conversely, the 497 proximal nucleotides of the Eg2 3′ untranslated region, which was previously shown to confer post-MBT instability

(Bouvet et al., 1991), neither promote the deadenylation of chimeric RNAs nor bind the p53-p55.

Materials and methods

Plasmid constructions and RNA synthesis

Plasmids used in this report are described in Fig. 1. The cDNAs corresponding to part of the cdk2-Eg1 (Paris et al., 1991a), Eg2, Eg5 (LeGuellec et al., 1991), ODC (Bassez et al., 1990) and *Xeno-pus* phosphatase 2A catalytic subunit (PP2Ac) (Cormier et al., 1991) mRNAs were cloned into the *Eco*RI site of Bluescript KS phagemid. For cdk2-Eg1, ODC and PP2Ac, these cDNAs contain all of the coding and 3′ untranslated regions. For Eg2 and Eg5, the cDNAs contains the last 80 and 47 codons, respectively, and the entire 3′ untranslated regions. Chloramphenicol acetyl transferase (CAT) constructions were based on the pSP64-CAT plasmid provided by Dr G. Huez where the CAT coding region is cloned between the *Hin*dIII and *Bam*HI sites of pSP64.

CATpA (Fig. 1A) was obtained by inserting the *Ssp*I-*Pvu*II fragment of cdk2-Eg1 cDNA clone into the *Sma*I site of pSP64-CAT. This fragment contains 65 adenyl residues preceded by the last nine nucleotides of cdk2-Eg1 cDNA, and followed by the *Eco*RI-*Pvu*II fragment of pBluescript KS containing the T3 promoter which was used for verifying the construction by sequencing.
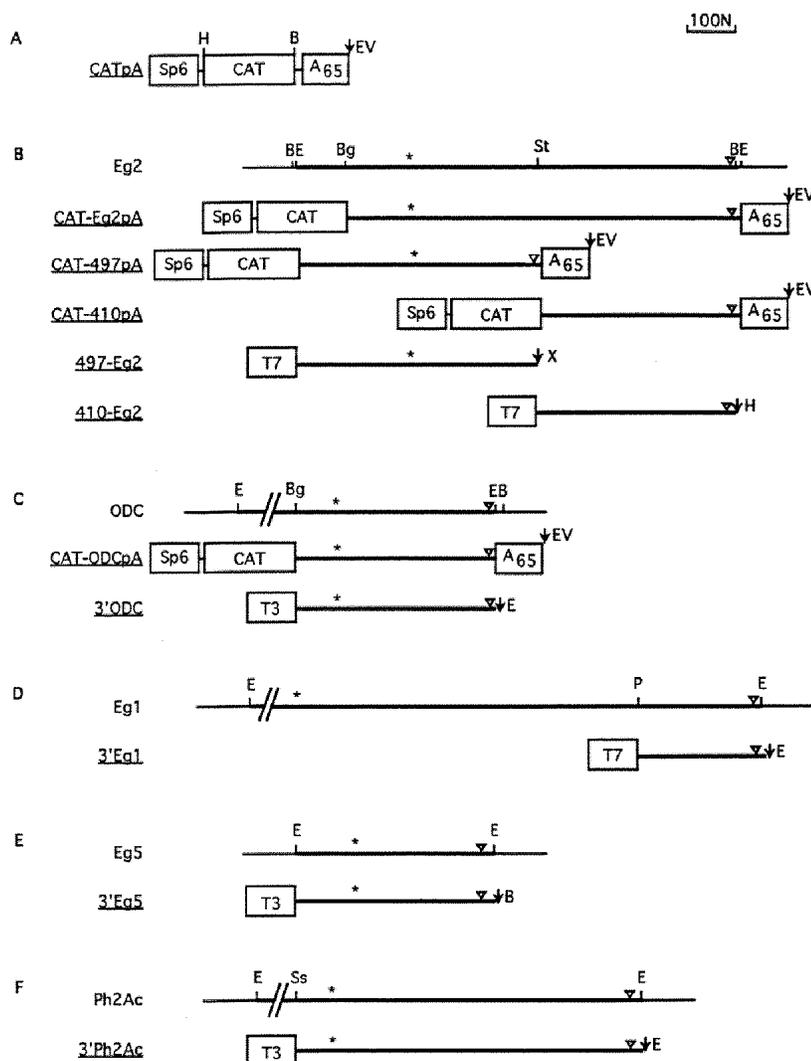

Fig. 1. Schematic diagram of the different plasmids used. Only the transcribed regions are represented. Boxes indicate the positions of the prokaryotic promoters, the CAT coding region, and the track of 65 adenyl residuesas denoted. Thick lines represent the relative lengths of sequences derived from *Xenopus* mRNAs. Thin lines are plasmid sequences. Stars and triangles denote, respectively, the stop codons and the polyadenylation signals of *Xenopus* mRNAs. Vertical arrows denote the restriction sites used for run-off. (A) The CATpA plasmid. (B) Plasmids derived from Eg2 mRNA. (C) Plasmids derived from ODC mRNA. (D) The 3′ Eg1 plasmid. (E) The 3′ Eg5 plasmid. (F) The 3′ PP2Ac plasmid. In each case, plasmid names are underlined. In B-F, the 3′ terminal portion of the relevent *Xenopus* mRNA is figured at the top, with the restriction sites used for the different constructions: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; EV, *Eco*RV; H, *Hin*dIII; P, *Pst*I; Ss, *Ssp*I; St, *Stu*I; X, *Xho*I.

Before constructing the Eg2-derived plasmids (Fig. 1B), the filled-in *Hin*dIII site containing the last two A residues of the Eg2 nuclear polyadenylation signal (AAUAAA) was ligated to a *Bam*HI linker. For CAT-Eg2pA, the *Bgl*II-*Bam*HI(linker) fragment of Eg2 cDNA was inserted at the *Bam*HI site of pSP64-CAT. This 820 nucleotide fragment contains the 43 last codons and the entire 3′ untranslated region of Eg2 mRNA. The poly(A) tail was added by inserting the *Ssp*I-*Pvu*II fragment of cdk2-Eg1 cDNA clone into the *Sma*I site of pSP64 as described for CATpA. CAT-497pA was obtained by isolating the fragment of Eg2 cDNA clone between the Bluescript polylinker *Bam*HI site and the Eg2 *Stu*I site. The Eg2 Nuclear polyadenylation signal was added at the 3′ end of this fragment by blunt-ending with Klenow enzyme and ligating with the *Dra*I-*Bam*HI (linker) fragment (19 nucleotides). This composite segment was cloned into the filled-in *Bam*H1 site of CATpA. CAT-410pA was obtained by isolating the fragment of Eg2 cDNA clone between the Eg2 *Stu*I site and the *Bam*HI (linker) site. This fragment was blunt-ended with Klenow enzyme and cloned into the filled-in *Bam*H1 site of CATpA. 497Eg2 was made by cloning the 497 nucleotide Klenow-treated *Eco*RI-*Stu*I fragment of Eg2 cDNA clone into the *Hin*cII site of Bluescript KS phagemid. 410Eg2 was made by cloning the 410 nucleotide Klenow-treated *Stu*I-*Eco*RI fragment of Eg2 cDNA clone into the *Eco*RV site of Bluescript KS phagemid.

CAT-ODCpA (Fig. 1C) was obtained by cloning the *Bgl*II-*Bam*HI fragment of *Xenopus* ODC cDNA into the *Bam*HI site of pSP64CAT. This 380 nucleotide fragment contains the last 30 codons and the entire 3′ untranslated region of *Xenopus* ODC mRNA followed by the *Eco*RI-*Bam*HI segment of Bluescript KS phagemid. The poly(A) tail was added by inserting the *Ssp*I-*Pvu*II fragment of cdk2-Eg1 cDNA clone into the *Sma*I site of pSP64-CAT-ODC as described for CATpA. 3′ODC clone was obtained by digesting the complete cDNA clone with *Bgl*II and *Cla*I (polylinker site) enzymes, treating with Klenow enzyme and religating.

3′Eg1 clone (Fig. 1D) was made by digesting the complete cdk2-Eg1 cDNA clone with *Pst*I enzyme and religating.

3′Eg5 (Fig. 1E) corresponds to the *Eco*RI-*Eco*RI 420 3′ terminal nucleotides of Eg5 cDNA.

3′PP2Ac clone (Fig. 1F) was made by cloning the 710 nucleotide *Ssp*I-*Eco*RI fragment of PP2Ac cDNA between *Hin*cII and *Eco*RI sites of Bluescript KS phagemid. The orientations of these constructions was confirmed by sequencing.

Wild-type GbB4 and M6 mutant GbB4 plasmids were provided by Dr J. Richter (Paris et al., 1991b).

In vitro transcripts were made by using the Promega kit, according to the suppliers instructions, except that 20 μCi of -[^{32}P]UTP (Amersham, 800 Ci/mmol) was added when labelled transcripts were to be obtained. For unlabelled transcripts, trace levels of [^{3}H]UTP (Amersham) were added to facilitate the quantification. When made for injection experiments, transcripts were capped by adding a 10-fold excess of m^{7}G(5′)ppp(5′)G to the reaction. Promoters and restriction sites that were used for transcription and matrix linearization are indicated in Fig. 1. For u.v. cross-linking experiments, transcripts were purified by electrophoresis on a 4% polyacrylamide-urea gel.

Embryo injections and extracts

Eggs were obtained and fertilized by standard procedures and incubated in F1 solution as previously described (Paris et al., 1988). 2-cell-stage embryos were injected with 20 nl of in vitro transcripts (500 pg) and incubated for the indicated times at 22°C. Batches of five embryos at the appropiate stages were collected, rapidly frozen and stored at −70°C.

Oocyte, egg and embryo extracts were prepared essentially as described by Murray and Kirschner (1989) using Versilube F-50

oil (General Electric) to remove the buffer solution before crushing the biological material by centrifugation. The cytosol layer was clarified by a further centrifugation at 30 000 *g* for 15 minutes and stored at −70°C. For band-shift and cross-linking experiments, extracts were diluted 10-fold in extraction buffer (*N*-2-hydroxyethylpiperazine-*N*′-2ethanesulfonic acid 10 mM pH 7.7; KCl 100 mM; MgCl$_2$ 1 mM; CaCl$_2$ 0.1 mM; sucrose 50 mM) before use.

RNA analysis, band-shift experiments and u.v. crosslinking.

RNA was extracted from injected embryos using the proteinase K-phenol method (Harland and Mishner, 1988) and analysed on formaldehyde-agarose gels as previously described (Bouvet et al., 1991). In vitro deadenylation was achieved by oligo(dT)-directed RNAase H digestion (Osborne et al., 1991).

For RNA-protein complex analysis, in vitro ^{32}P-labelled transcripts (1 μl, 50 fmole) were incubated for 15 minutes at 25°C in 10 μl of diluted extract. For competition experiments, ^{3}H-labelled transcripts were mixed with the labelled transcripts before adding to the extracts. For u.v. cross-linking experiments, the samples (12 μl), when incubated, were placed 4 cm from a u.v.-light source and irradiated for 10 minutes at 4°C with 254 nm light. Samples were digested with RNAase A at a final concentration of 2 mg/ml for 30 minutes at 37°C and denatured at 100°C in the presence of SDS. The samples were analysed by denaturing polyacrylamide gel electrophoresis and autoradiography. For band-shift assays, glycerol (1 μl at 80%) and RNAase T1 (1 μl, 72 units) were added to the samples at the end of the incubation which was then continued for 30 minutes at 30°C. Samples were then loaded onto a non-denaturing polyacrylamide gel (0.6%, bisacrylamide/acrylamide ratio=1/60) and electrophoresed 2 hours at 200 V in 0.5× Tris-Borate EDTA buffer.

Results

To determine whether factors that form specific complexes with the 3′ untranslated regions of Eg2 and ODC mRNAs could be detected, gel shift experiments were performed. First plasmids were constructed, which allowed the in vitro synthesis of short radiolabelled transcripts corresponding to the 3′ untranslated region of ODC mRNA (3′ODC) and two portions (497Eg2 and 410Eg2) of the 3′ untranslated region of Eg2 mRNA (see Fig. 1). The 497Eg2 portion corresponds to the last 80 codons and the 257 first nucleotides of the 3′ untranslated region of Eg2 mRNA terminating at the *Stu*I site. The 410Eg2 portion corresponds to the last 410 nucleotides of the 3′ untranslated region of Eg2 mRNA, starting at the *Stu*I site. These in vitro synthesized [^{32}P]UTP-labelled RNAs were incubated (15 minutes; 25°C) in extracts made from 4 hour (pre-MBT) embryos after which the samples were treated with RNAase T1 and loaded onto a native polyacrylamide gel (Fig. 2). Two major complexes, denoted A and B on Fig. 2, were observed with the 3′ODC RNA (lane 2) whereas no complex was detected with the 497Eg2 RNA (lane 10). It should be noted that the 497Eg2 portion was previously shown to contain *cis* element required to confer the degradation of chimeric RNAs in post-MBT embryos (Bouvet et al., 1991). Only one complex, denoted C on Fig. 2, was observed with the 410Eg2 transcript (lane 18), which migrates at the same apparent position as the complex B. The specificity of complexes A, B and C was tested by competition experiments in which
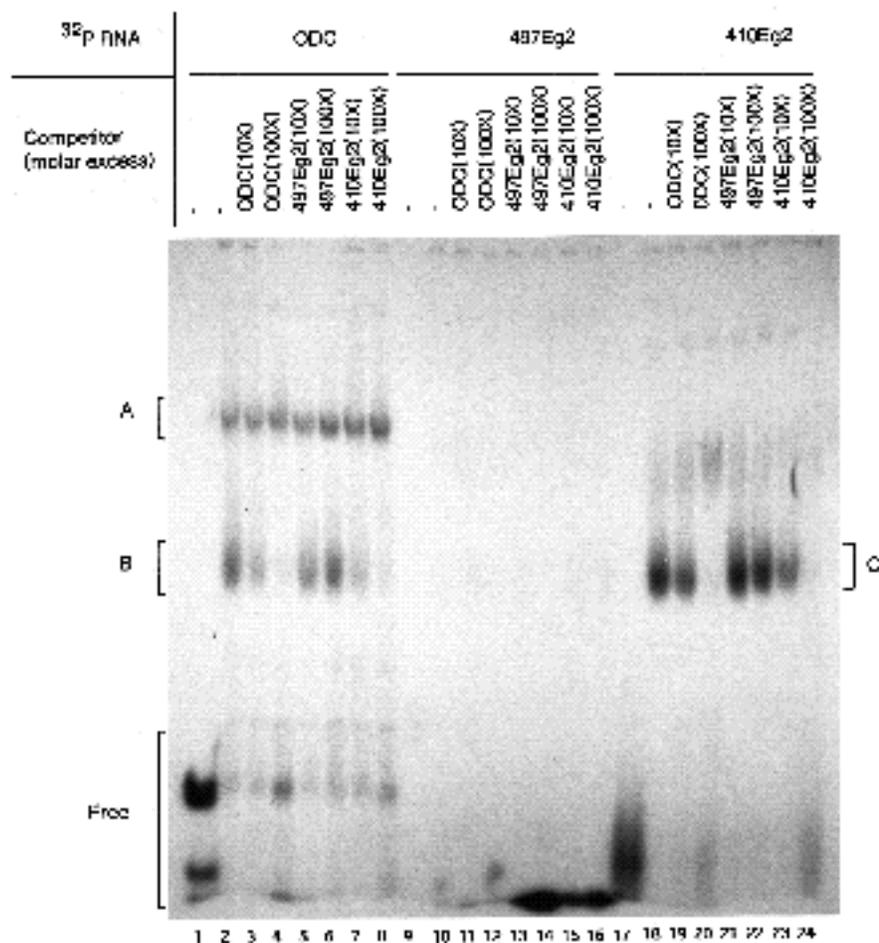
Fig. 2. Analysis of the RNA-protein complexes formed with the 3′ untranslated regions of ODC and Eg2 transcripts. ^{32}P-labelled transcripts corresponding to the 3′ untranslated region of ODC mRNA (lanes 1-8), the 497 nucleotide *Eco*RI-*Stu*I fragment of Eg2 mRNA (lanes 9-16) and the 410 nucleotide *Stu*I-*Eco*RI terminal fragment of Eg2 mRNA (lanes 17-24) were incubated in extracts made from 4 hour (pre-MBT) *Xenopus* embryos, treated with RNAase T1 and loaded onto a native 0.6% polyacrylamide gel. In lanes 1, 9 and 17, no extract was added. In lanes 3-8, 11-16 and 19-24, competitors unlabelled transcripts were added at a molar excess of 10- or 100-fold, as indicated. Brackets indicate the position of complexes A, B and C and that of free probe. In lanes 9-12, the free 497Eg2 probe partially electrophoresed out of the gel.

10- or 100-fold molar excess of homologous or heterologous unlabelled transcript was added. Complex A was not competed by any of the three unlabelled transcripts tested (lanes 3 to 8), indicating that this complex is probably not specific. Conversely, although complex B was not competed by the 497Eg2 RNA (lanes 5 and 6), it was efficiently competed by both the 3′ ODC (lanes 3 and 4) and the 410Eg2 transcript (lanes 7 and 8). A similar result was obtained with complex C (lanes 19 to 24), although the competition efficiency was slightly greater with the 410Eg2 transcript than with the 3′ ODC transcript. This suggests that at least some components of complexes B and C are common. However, one cannot exclude that complexes B and C also contain proteins that are specific for the ODC and Eg2 mRNAs, respectively.

In order to identify the different proteins that associate with the 3′ untranslated regions of ODC and Eg2 mRNAs, u.v. cross-linking experiments were performed. The in vitro synthesized [^{32}P]UTP-labelled 3′ ODC, 410Eg2 and 497Eg2 RNAs were incubated (15 minutes; 25°C) in extracts made from 4 hour (pre-MBT) embryos after which the samples were irradiated with 254 nm u.v. light, followed by RNAase A digestion. The ^{32}P-labelled proteins produced by this treatment were analyzed by SDS-PAGE electrophoresis (Fig. 3). Two major proteins of apparent relative molecular masses (M_r) of about 35×10^3 and 40×10^3 were found to cross-link with the 3′ ODC transcript, (lane 15). With the

497Eg2 transcript, only faint bands were observed (lane 1), which were detected with almost all the transcripts tested. Thus, these bands probably do not correspond to specific factors. Like the 3′ ODC transcript, the 410Eg2 transcript (lane 5) cross-linked with p35 and p40. Additional proteins were found to cross-link with the 410Eg2 transcript, having apparent relative molecular masses (M_r) of 53×10^3-55×10^3 and 60×10^3.

The specificities and relative affinities of cross-linked proteins were assayed by competition experiments in which a 3-, 10- or 30-fold molar excess of homologous or heterologous unlabelled transcript was added. Proteins that weakly cross-link with all the tested transcripts were competed by neither homologous nor heterologous transcripts, confirming that they are not specific. According to this criteria, no specific factors associated with the 497Eg2 transcripts could be observed (compare lanes 1 to 4). The p35 and p40 proteins which cross-link with 3′ ODC transcript were efficiently competed by both the homologous transcript (lanes 22 to 24) and the 410Eg2 transcript (lanes 19 to 21). This confirms that the same p35 and p40 proteins bind to the 3′ ODC and 410Eg2 RNAs. When the cross-linking experiments were performed with the labeled 410Eg2 transcript, competition assays led to the same conclusions (compare lanes 9-11 and 12-14). The p53-p55 and the p60 proteins which cross-link to the 410Eg2 transcript were competed by the homologous unlabelled transcript
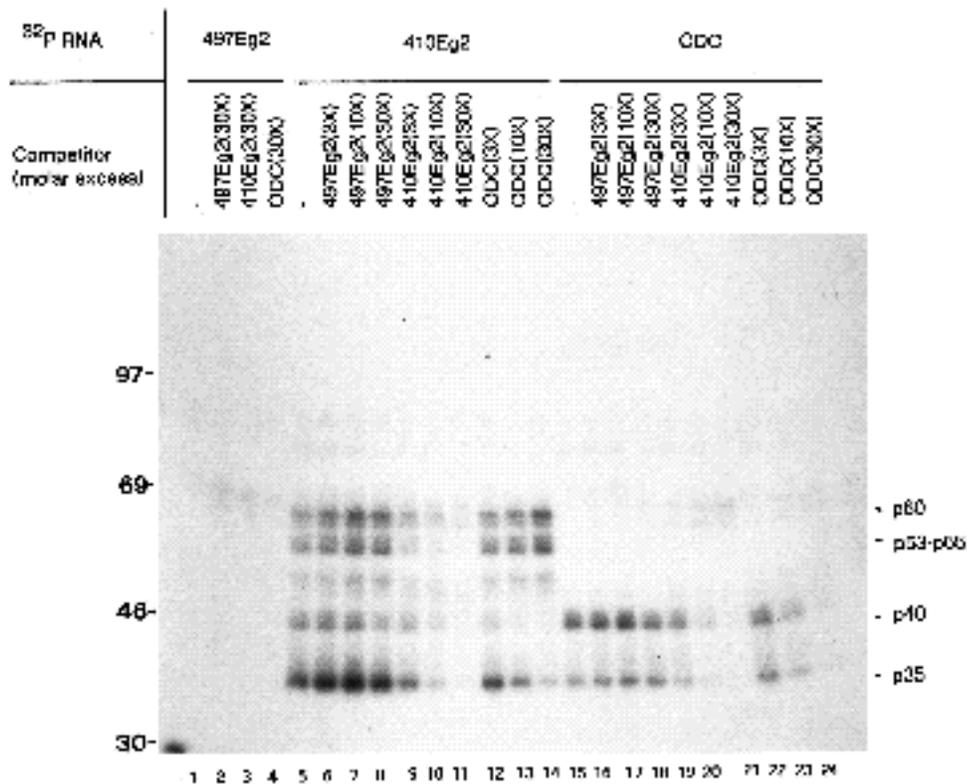
Fig. 3. Analysis of proteins cross-linked to the 3′ untranslated regions of ODC and Eg2 transcripts. ^{32}P-labelled transcripts corresponding to the 497 *Eco*RI-*Stu*I fragment (lanes 1-4), the 410 nucleotide *Stu*I-*Eco*RI terminal fragment (lanes 5-14) of the 3′ untranslated region of Eg2 mRNA, and the 3′ untranslated region of ODC mRNA (lanes 15-24), were incubated in extracts made from 4 hour (pre-MBT) *Xenopus* embryos, irradiated with u.v. light and digested with RNAaseA. The proteins rendered radioactive by cross-linking to the labelled transcripts were analysed by electrophoresis on a 10% polyacrylamide gels in the presence of SDS. Positions of the major cross-linked proteins are indicated on the right. The postion of the molecular weight markers are indicated on the left as $M_r \times 10^{-3}$. In lanes 2-4, 6-14 and 16-24, three, ten or thirty molar excess of unlabelled transcripts (Competitors) of the denoted sequences were mixed with the radiolabelled transcripts.

(lanes 9 to 11) but not at all by the 3′ ODC transcript (lanes 12 to 14) showing that these proteins are specific for the Eg2 transcript.

The RNA-binding proteins that are specific for the Eg2 transcript (i.e. the p53-p55 and the p60 proteins) do not bind to the 497Eg2 portion implicated in the post-MBT degradation process (Bouvet et al., 1991). This suggests that they may be involved in the postfertilization deadenylation of Eg mRNAs. From this hypothesis two predictions can be made: firstly, these factors should bind a portion of the Eg2 mRNA that confers postfertilization deadenylation; secondly, these proteins should specifically associate with other Eg mRNAs but not with maternal mRNAs like ODC that have a different behaviour.

The first prediction was tested by identifying *cis*-acting regions that control the poly(A) tail length of maternal *Xenopus* Eg mRNAs in embryos. Plasmids were constructed that allowed the in vitro synthesis of chimeric polyadenylated mRNAs containing the CAT reporter gene with or without different portions of the 3′ untranslated regions of *Xenopus* Eg2 or ODC mRNA (see Fig. 1). CATpA RNA contained the last nine transcribed nucleotides of cdk2-Eg1 3′ untranslated region and a 65 nucleotide poly(A) tract downstream of the CAT coding region. In CAT-ODCpA, the 30 last codons and the entire 3′ untranslated region of *Xenopus* ODC mRNA were placed between the CAT coding region and the poly(A) tract. In CAT-Eg2pA, the 43 last codons and the entire 3′ untranslated region of Eg2 mRNA were similarly situated. When injected into embryos, these chimeric mRNAs were trans-

lated producing an easily measurable CAT activity (data not shown).

The effects of these 3′ untranslated regions on cytoplasmic adenylation was tested by injecting the in vitro synthesized chimeric mRNAs into one blastomere of 2-cell-stage embryos. RNA was extracted either immediately (0 hour), one (1 hour) or three (3 hours) hours after injection and, in some experiments, the size of the transcript estimated by northern analysis was compared to that of the deadenylated transcripts produced by oligo(dT)-directed RNAase H digestion (Fig. 4A).

The CAT-Eg2 RNA was markedly shortened one and three hours after injection (lanes 1, 2 and 3). That this shortening was due to deadenylation was verified by RNAase H/oligo(dT) treatment. Although the CAT-Eg2pA RNA in the zero hour sample was clearly shortened when treated by RNAase H in the presence of oligo(dT) (compare lanes 4 and 5), the effect of such a treatment was diminished in the one hour sample (lanes 6 and 7) and completely abolished in the three hour sample (lanes 8 and 9). Therefore, the CAT-Eg2pA RNA is deadenylated when injected into embryos. In contrast, no change in the size of the CAT-ODCpA RNA was observed during this same period (lanes 10, 11 and 12). In the three hour sample, this RNA was shortened by RNAase H/oligo(dT) treatment (compare lanes 13 and 14) confirming that the CAT-ODCpA RNA remained adenylated three hours after injection. The data given in Fig. 4A (lanes 15-18) show that, like the CAT-ODCpA RNA, the adenylation status of CATpA RNA had not been modified three hours after injection. Therefore, the
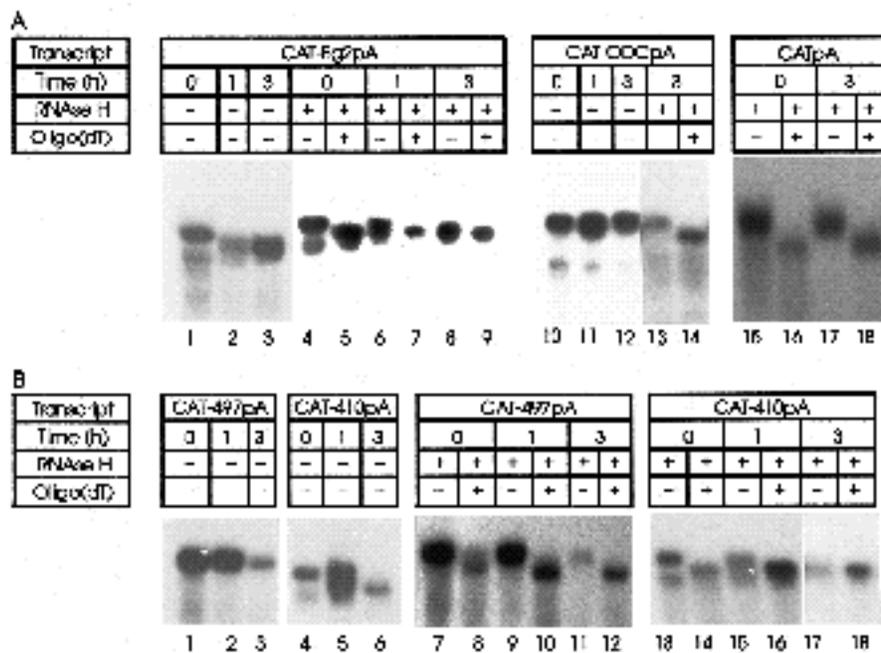
Fig. 4. Analysis of chimeric CAT RNAs injected into *Xenopus* embryos. 500 pg of capped chimeric RNAs were injected into 2-cell embryos. RNA was extracted from lots of five embryos just after injection (0 hour) or after one hour and three hours, as described in Materials and methods. Part of each of these samples was retained for direct analysis and the remainder was treated with RNAase H in the presence or absence of oligo(dT). One embyro equivalents of the RNA was subjected to northern analysis using a ^{32}P-labelled CAT cDNA probe. (A) Chimeric RNAs transcribed from the CAT-Eg2pA, CAT-ODCpA and CATpA plasmids. (B) Chimeric RNAs transcribed from the CAT-497pA and CAT-410pA plasmids.

3′ untranslated region of Eg2 mRNA but not that of ODC mRNA is able to promote the deadenylation of CAT chimeric RNA in embryos.

To circumscribe better the portion of the 3′ untranslated region of Eg2 mRNA that is involved in its deadenylation, the CAT-497pA and CAT-410pA plasmids were constructed that allowed the in vitro synthesis of chimeric RNAs containing, between the CAT coding region and the poly(A) tract, respectively the 497Eg2 or the 410Eg2 portions of the 3′ untranslated region of Eg2 mRNA previously used for the u.v. cross-linking experiments (see Fig. 1). Both contain a nuclear polyadenylation signal (AAUAAA) between the 3′ untranslated regions and the poly(A) tract.

When injected into one blastomere of 2-cell-stage embryos, the size of the CAT-497pA RNA did not significantly change during the three hour incubation period (Fig. 4B, lanes 1, 2 and 3) whereas that of CAT-410pA was measurably reduced (compare lanes 4, 5 and 6). That the differences in the behaviour of the CAT-410pA and CAT-497pA mRNAs is due to a deadenylation of the CAT-410pA mRNA was confirmed by treating the samples with RNAase H in the presence of oligo(dT). This treatment provoked a shortening of the CAT-497pA mRNA in the zero, one and three hour samples (compare lanes 7 and 8; 9 and 10; 11 and 12). In contrast the size shift caused by the RNAase H/oligo(dT) treatment of the CAT-410pA mRNA, although evident in the zero hour sample (compare lanes 13 and 14) was less clear in the one hour sample (lanes 15 and 16) and completely abolished in the three hour sample (lanes 17 and 18). Therefore, the CAT-410pA mRNA but not the CAT-497pA mRNA is deadenylated when injected in the 2-cell-stage embryos. Thus the distal 410 nucleotide portion (that binds the p53-p55 and p60 proteins) but not the 497 nucleotide portion of the 3′ untranslated region of Eg2 mRNA contains sequence element(s) that promote chimeric mRNA deadenylation. This suggests

that the p53-p55 and p60 proteins may play a role in the deadenylation process.

To be a possible candidate for a factor involved in the deadenylation process, the factors that specifically bind to the 3′ untranslated region of Eg2 mRNA should also associate with other maternal mRNAs that are deadenylated in postfertilization embryos, and not with that of mRNAs that remain polyadenylated. To test this second prediction, the factors that specifically associate with the 3′ untranslated regions of two other *Xenopus* Eg mRNAs, cdk2-Eg1 (Paris et al., 1991a) and Eg5, a kinesin-related protein (LeGuellec et al., 1991), were also analysed (Fig. 5). cdk2-Eg1 and Eg5 were chosen among the different Eg cDNAs that have been isolated because their structures have been fully analysed and particularly their 3′ untranslated regions have been unambigously identified. For cdk2-Eg1 and Eg5, the in vitro ^{32}P-labelled transcripts corresponded respectively to the last 187 and 401 nucleotides of the maternal mRNAs (see Material and methods). Transcripts corresponding to the 3′ untranslated region of ODC and the catalytic subunit of *Xenopus* phosphatase 2A (PP2Ac), which are not deadenylated in embryos, were also tested as controls. Fig. 5A shows that the p53-p55 factor complexes with the 3′ untranslated regions of all the Eg mRNAs that were tested (lanes 6, 11 and 16). Amongst the different factors that specifically associated with the 3′ untranslated region of Eg2 RNA, the p53-p55 factor is the only one that is clearly detected with the cdk2-Eg1-derived transcript (compare lanes 6 and 11). Thus the p53-p55 factor appears to be specific for maternal RNAs that are deadenylated after fertilization. It should be noted that the portion of cdk2-Eg1 mRNA 3′ untranslated region located 5′ to the *Pst*I site (see Fig. 1) did not bind any detectable specific proteins (data not shown).

To verify that the p53-p55 factor that was associated with the 3′ untranslated regions of cdk2-Eg1 and Eg5 mRNA was the same as that associated with Eg2 mRNA, u.v. cross-
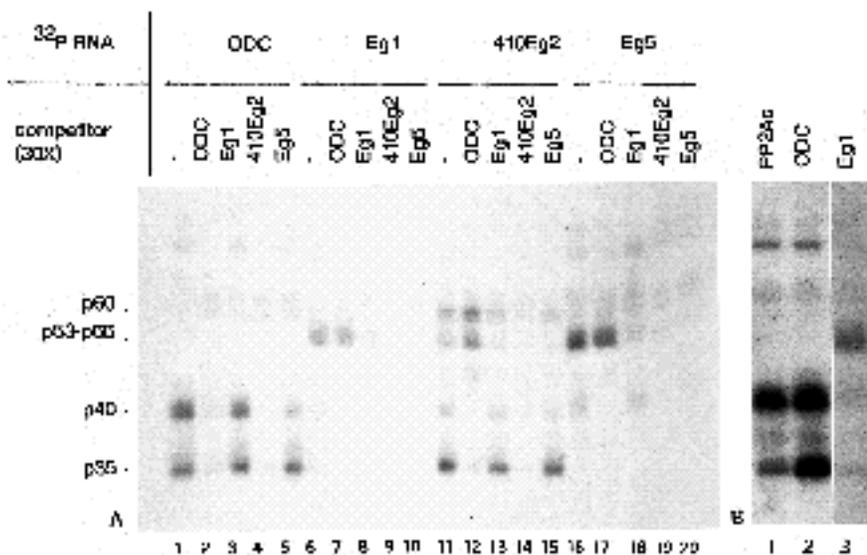
Fig. 5. Identification of the proteins that cross-link to the 3′ untranslated regions of cdk2-Eg1, Eg2 and Eg5 transcripts. (A) ^{32}P-labelled transcripts corresponding to the 3′ untranslated regions of ODC (lanes 1-5), cdk2-Eg1 (lanes 6-10), the 410 terminal nucleotides of Eg2 mRNA (lanes 11-15) and Eg5 (lanes 16-20) were incubated in embryos extracts and processed as described in the legend to Fig. 3. Positions of the major factors that cross-link to the 3′ untranslated region of Eg2 mRNA are indicated on the left. In lanes 2-5, 7-10, 12-15 and 17-20, 30-fold molar excess of unlabelled competitor transcripts was added as indicated. (B) Comparison of the cross-linking pattern obtained with the 3′ untranslated regions of PP2Ac (lane 1), ODC (lane 2) and cdk2-Eg1 (lane 3) mRNAs.

linking experiments were performed in the presence of a 30-fold excess of homologous or heterologous unlabelled transcripts. The cross-linking of the p53-p55 factor to the cdk2-Eg1-derived RNA (lane 6) was not affected by the presence of excess unlabelled ODC 3′ untranslated region (lane 7) but was reduced by that of Eg2, cdk2-Eg1 and Eg5 3′ untranslated regions (lanes 8-10). The same specificity of cross-linking competition was observed when the 410Eg2 transcript (lanes 11-15) or the 3′ untranslated region of Eg5 mRNA (lanes 16-20) were used as labelled transcripts.

That the competition of the Eg transcripts for the p53-p55 is specific is illustrated by the effect of the different competitor RNAs on the binding of the p35 and p40 factors. The binding of the p35 factor to the ^{32}P-labelled ODC and 410Eg2 transcripts was competed by the corresponding unlabelled transcripts (lanes 2, 4, 12 and 14) and not by the 3′ untranslated regions of cdk2-Eg1 and Eg5 (lanes 3, 5, 13 and 15). Similarly the binding of the p40 factor to the 3′ODC, 410Eg2 and 3′ Eg5 RNAs was affected by the same unlabelled transcripts and not by the 3′ Eg1 transcript, which fails to bind this factor. Therefore these experiments allow us to conclude that the same p53-p55 factor associates with the 3′ untranslated regions of cdk2-Eg1, Eg2 and Eg5 mRNAs. As an additional control, proteins that cross-link to the 3′ PP2Ac mRNA were compared to those that bind to the 3′ ODC and 3′ Eg1 RNAs. As shown in Fig. 5B the 3′ untranslated regions of *Xenopus* ODC and PP2Ac mRNA exhibited a very similar pattern of cross-linked factors and both fail to complex with the p53-p55 factor (lanes 1 and 2).

Several RNA-binding proteins have already been identified in *Xenopus* oocytes or embryos, which are thought to play a role in the regulation of maternal mRNA translation. Some of these RNA-binding proteins have apparent molecular masses which are close to p55.

CPE binding proteins are part of the machinery that directs the cytoplasmic polyadenylation of maternal mRNAs in matured oocytes. Interestingly, the poly(A) tract of cdk2-Eg1 mRNA is lengthened during oocyte matura-

tion and the 3′ terminal portion of cdk2-Eg1 mRNA contains several CPE sequence motifs (Paris et al., 1991a). It should therefore bind a CPE-binding factor. Since the previously reported apparent relative molecular mass (58×10^3 M_r) of one of these factors (Paris et al., 1991b) is close to that of the p53-p55 factor specific to Eg mRNAs, it was important to determine whether these two factors are distinct. This was achieved by comparing the cross-linking patterns obtained with 3′ Eg1, GbB4 and GbB4 M6 mutant transcripts. GbB4 transcript consists of the 5′ untranslated region of *Xenopus* β-globin and the 38 terminal nucleotides of *Xenopus* B4 mRNA encompassing the CPE and the polyadenylation signal (Paris et al., 1991b). Like the maternal B4 mRNA (Dworkin and Dworkin-Rastl, 1985), GbB4 is polyadenylated during oocyte maturation. In the GbB4 M6 mutant, the CPE was mutated. The p58 CPE-binding protein was previously identified using extracts of stage VI oocytes and unfertilized eggs (Paris et al., 1991b), therefore extracts made from these same stages were used to compare the proteins cross-linking to the cdk2-Eg1 3′ untranslated region and to the GbB4 RNA (Fig. 6). For comparision, cross-linking experiments were also performed with 4 hour embryo extracts. The electrophoretic profile of the factors cross-linked to the 3′Eg1 transcript shown in this figure is slightly different to that shown in previous figures because a 8% SDS-polyacrylamide gel was used in order to obtain a better resolution of the factors in the $50-60 \times 10^3$ M_r range. In stage VI oocyte and unfertilized egg extracts, a p58 factor cross-linked to both the 3′ Eg1 (lanes 1 and 2) and GbB4 transcripts (lanes 4 and 5). The identity of this p58 factor as the CPE-binding protein was confirmed by using the GbB4-M6 mutant. As previously reported by Paris et al. (1991b), no cross-linking of the p58 factor was detected with this RNA where the CPE has been mutated (lanes 7 and 8). The CPE-binding protein has been shown to be phosphorylated during oocyte maturation leading to a slightly reduced mobility in SDS-PAGE gels (Paris et al., 1991b). Although this change in mobility was not evident for the GbB4 transcript (compare lanes 4 and 5), the p58 factor associated with the cdk2-Eg1
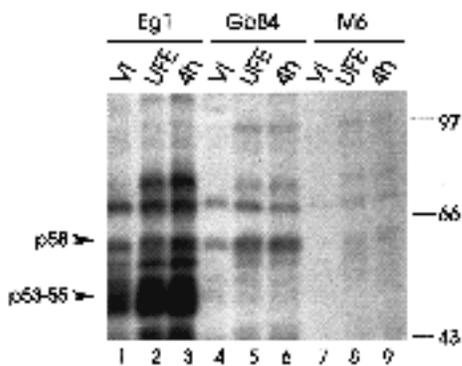
Fig. 6. Verification that the p53-p55 Eg specific proteins and the CPE-binding protein are distinct. ^{32}P-labelled transcripts corresponding to the terminal 187 nucleotides of the cdk2-Eg1 mRNA (lanes 1-3), the GbB4 RNA (lanes 4-6) or to the GbB4 M6 mutant RNA (lanes 7-9) were incubated in stage VI oocyte (VI), egg (UFE) or 4 hour embryo extracts, as indicated in the figure, and processed as described in the legend to Fig. 3 except that the polyacrylamide concentration was 8%. The arrows indicate the expected position of the CPE-binding protein and of the p53-p55 factors. The postion of the molecular weight markers are indicated on the right as $M_r \times 10^{-3}$.

derived transcript in the egg extract was clearly reduced in mobility relative to that in the oocyte extract (compare lanes 1 and 2). No binding of the p53-p55 factor was observed with the GbB4 transcript. Therefore, both these transcripts bind the CPE-binding protein but only the 3 Eg1 transcript binds the p53-p55 factor showing that these factors are distinct. It should be noted that cross-linking of the p58 CPE-binding protein to GbB4 and 3 Eg1 RNAs was detected in 4 hour embryo extracts (lanes 3 and 6). Similarly, cross-linking of p53-p55 to the 3 Eg1 RNA occurred in oocyte and egg extracts (lanes 1 and 2). As these two transcripts have different adenylation behaviours in embryos, the role of the p58 CPE-binding protein, and perhaps also that of p53-p55, in the control of cytoplasmic adenylation may be different in oocytes and embryos.

Other RNA-binding proteins have been described (Darnbrough and Ford, 1981; Dearsly et al., 1985; Crawford and Richter, 1987; Cummings and Sommerville, 1988) in *Xenopus* oocytes, whose apparent relative molecular masses are 54×10^3 and 56×10^3 and which are thought to bind untranslated maternal mRNAs. Interestingly, these RNA-binding proteins are identical to the oocyte-specific transcription factors FRG Y1 and FRG Y2 (Tafuri and Wolffe, 1990; Deschamps et al., 1991; Murray et al., 1992). A major characteristic of these proteins is their solubility at high temperatures (up to 80°C) (Deschamps et al., 1991). This provided an easy test for determining if these proteins and the p53-p55 protein that we observed were related. In one set of experiments, the embryo extract was heated (10 minutes at 80°C) and centrifuged (10 minutes at 12000 **g**) before adding the ^{32}P-labelled RNA to the supernatant (Fig. 7A, lanes 2, 7 and 12). In a second set of experiments, the samples were heated after the cross-linking had been performed and, in this case, both supernatants (lanes 3, 8 and 13) and pellets (lanes 4, 9 and 14) were analysed. Similar results were obtained with both protocols. Most of the proteins that cross-link to the 410Eg2, 3 ODC and 3 Eg1 RNAs (lanes
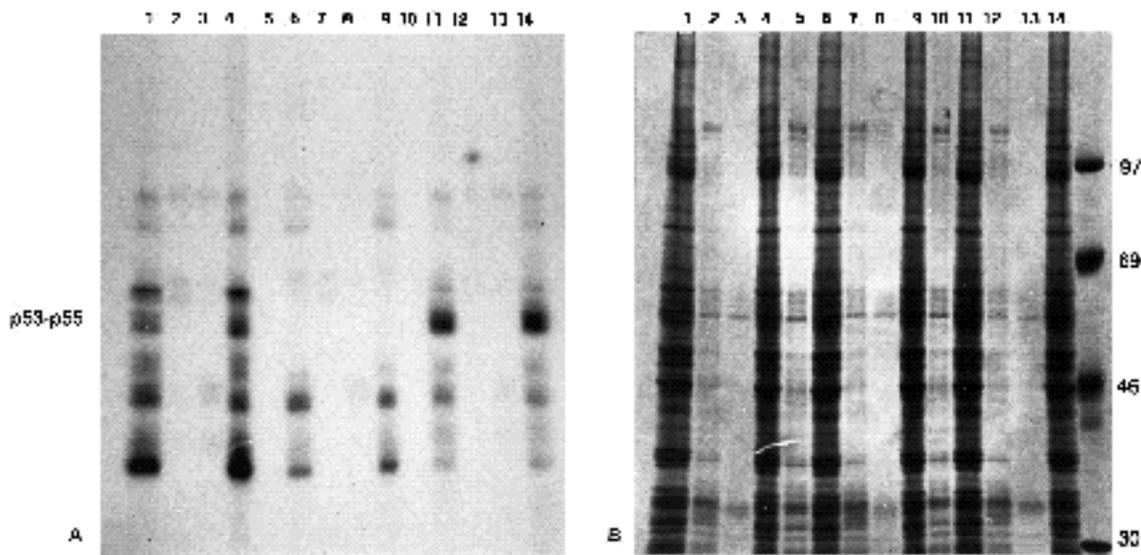

Fig. 7. Evidence that the RNA-binding proteins which cross-link to Eg and ODC mRNAs are precipitated in heated extracts. ^{32}P-labelled transcripts corresponding to the 410 nucleotide 3 terminal portion of Eg2 mRNA (lanes 1-4), the 3 untranslated region of ODC mRNA (lanes 6-9) and the 3 terminal portion of cdk2-Eg1 mRNA (lanes 11-14) were incubated in embryo extracts and processed as described in the legend of Fig. 3. Lanes 1, 6 and 11: standard conditions. Lanes 2, 7 and 12: cross-linking was performed with supernatants (12 000 **g**, 10 minutes) of extracts heated 10 minutes at 80°C. Lanes 3, 4, 8, 9, 13 and 14: cross-linking and RNAase treatment was performed with extracts which were then heated (10 minutes, 80°C) and centrifuged (12 000 **g**, 10 minutes). Lanes 3, 8 and 13: analysis of the 12000 **g** supernatants from heated samples. Lanes 4, 9 and 14: analysis of the 12000 **g** pellets from heated samples disolved in sample buffer. Lanes 5 and 10: 12 000 **g** supernatants of extracted heated 10 minutes at 80°C to which no ^{32}P-labelled transcripts had been added. (A) Autoradiogram; (B) Coomassie blue staining.

1, 6 and 11) disappeared from the supernatants. Particularly, the Eg mRNA-specific p53-p55 protein was not detectable in heated extract supernatants. It should be noted, however, that the rare proteins that remained detectable were not the same when one or the other of these protocols was used. In addition, all the proteins that disappeared from the supernatant were recovered in the pellet when the second protocol was used, indicating that the cross-linked proteins, including the Eg mRNA-specific p53-p55 protein were insolubilized and therefore probably denatured during the heat treatment. As shown in Fig. 7B, a coloration of the gel visualised the proteins that remained soluble in heated extracts, including a group of 54-56×10^3 M_r proteins. From these experiments, it can be concluded that the p53-p55 factor is not thermoresistant and therefore is probably not related to the already described p54 and p56 RNA-binding proteins of *Xenopus* oocytes.

Discussion

Eg mRNAs represent a family of maternal transcripts that are deadenylated and released from polysomes after fertilization, and are degraded in post-MBT embryos. We have previously shown that the 3′ untranslated region of Eg2 mRNA contains *cis* sequences that are able to direct the post-MBT degradation of CAT coding chimeric RNAs, whereas CAT RNAs that contained either no additional sequences or the 3′ untranslated region of the ODC mRNA remained stable (Bouvet et al., 1991). Here we show that the 3′ untranslated region of Eg2 mRNA also specifically confers deadenylation to the chimeric CAT coding RNAs in fertilized eggs. In addition, the 410 nucleotide distal portion of the Eg2 3′ untranslated region was necessary to confer postfertilization deadenylation of a chimeric transcript. This portion of the Eg2 3′ untranslated region is distinct from that previously shown to confer post-MBT instability (Bouvet et al., 1991). Therefore, in *Xenopus* embryo, not only is the deadenylation of maternal mRNAs temporally dissociated from their degradation (Duval et al., 1990), but also the sequence informations that control these processes are spatially separated on the RNA molecules.

From these results, one can reasonably postulate that the control of Eg mRNA adenylation and stability is exerted by specific *trans* factors that interact with the 3′ untranslated region of Eg mRNAs. The existence of RNA-binding proteins that specifically associate with the 3′ untranslated region of Eg mRNAs was therefore investigated. Such proteins having relative molecular masses of 53×10^3 and 55×10^3 were detected by u.v. cross-linking experiments.

If the p53-p55 protein doublet plays a role in the specific behaviour of Eg mRNAs (i.e. their post-fertilization deadenylation and their degradation in post-MBT embryos), it would most likely be involved in the deadenylation process. Indeed, this doublet binds to the 410 nucleotide 3′ terminal region of Eg2 mRNA, which is able, by itself, to direct the deadenylation of chimeric RNAs. It is worth noting that the immediately upstream 497 nucleotide region, which was shown to direct the post-MBT degradation of chimeric RNAs (Bouvet et al., 1991), is able neither to bind detectable specific factors nor to direct deadenylation.

Cytoplasmic polyadenylation during *Xenopus* oocyte maturation appears to be almost exclusively controlled by the CPE sequence motif (Fox et al., 1989; McGrew et al., 1989; Varnum and Wormington, 1990) and the associated CPE-binding protein (McGrew and Richter, 1990; Paris et al., 1991b). Several of the maternal Eg mRNAs are adenylated during oocyte maturation and the relative molecular mass of one of the CPE-binding protein (p58) is similar to that of the Eg-specific proteins described here. However, u.v. cross-linking experiments using the Eg transcripts and the CPE containing GbB4 RNA showed that both these proteins are distinct.

Eg mRNAs, once deadenylated in the embryos, are no longer translated (Paris and Philippe, 1990). In *Xenopus* oocytes, untranslated mRNAs are associated with RNA-binding proteins that migrate on SDS-PAGE as a doublet of apparent relative molecular mass 54-56×10^3 (Darnbrough and Ford, 1981; Cummings and Sommerville, 1988). These oocyte 'storage particle proteins', which were recently shown to be identical to the transcription factors FRG Y1 and FRG Y2 (Tafuri and Wolffe, 1990; Deschamps et al., 1991; Murray et al., 1992), have particular solubility properties at high temperature (Deschamps et al., 1991). The Eg-specific binding proteins described here are not soluble at high temperature, implying that these two sets of proteins are probably unrelated.

The p53-p55 Eg-specific RNA-binding proteins described here appear therefore to be novel *Xenopus* RNA-binding proteins. Since they associate with the portion of Eg2 mRNA that confers deadenylation to chimeric RNAs in embryos, they may be associated with this aspect of the post-transcriptional regulation of Eg mRNAs. However, determining the function of these p53-p55 proteins must await the precise identification of their binding site.

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