The EMBO Journal Vol.17 No.1 pp.278–287, 1998

EDEN and EDEN-BP, a *cis* element and an associated factor that mediate sequence-specific mRNA deadenylation in *Xenopus* embryos

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During *Xenopus* early development, gene expression is regulated mainly at the translational level by the length of the poly(A) tail of mRNAs. The Eg family and c-mos maternal mRNAs are deadenylated rapidly and translationally repressed after fertilization. Here, we characterize a short sequence element (EDEN) responsible for the rapid deadenylation of Eg5 mRNA. Determining the core EDEN sequence permitted us to localize the c-mos EDEN sequence. The c-mos EDEN confered a rapid deadenylation to a reporter gene. The EDEN-specific RNA-binding protein (EDEN-BP) was purified and a cDNA obtained. EDEN-BP is highly homologous to a human protein possibly involved in myotonic dystrophy. Immunodepleting EDEN-BP from an egg extract totally abolished the EDEN-mediated deadenylation activity, but did not affect the default deadenylation activity. Therefore, EDEN-BP constitutes the first *trans*-acting factor for which an essential role in the specificity of mRNA deadenylation has been directly demonstrated.

Keywords: deadenylation/poly(A) tail/RNA-binding protein/translation/3′ untranslated region

Introduction

Almost all eukaryotic mRNAs have a stretch of adenines at their 3′ ends that is added during their nuclear maturation. Once in the cytoplasm, mRNAs are subject to regulated alterations in the length of their poly(A) tails.

During the early development of many species including *Xenopus* (Fox *et al.*, 1989; McGrew *et al.*, 1989), *Drosophila* (Salles *et al.*, 1994) and mouse (Huarte *et al.*, 1992), the cytoplasmic polyadenylation [elongation of the poly(A) tail] of maternal mRNA is directed by specific *cis* elements and induces the recruitment of untranslated mRNAs into polysomes. These *cis* elements are localized within the 3′ untranslated region (3′UTR) of mRNAs and have been named cytoplasmic polyadenylation elements (CPEs) (Paris and Richter, 1990) or adenylation control elements (ACEs) (Huarte *et al.*, 1992). In *Xenopus* oocytes, mRNAs devoid of a functional CPE are deadenylated by a default process (Fox and Wickens, 1990; Varnum and Wormington, 1990).

Deadenylation [poly(A) shortening] provokes the release of translated mRNAs from polysomes during early development of many species (for review, see Jackson and Standart, 1990). Similarly, in yeast and eukaryotic somatic cells, poly(A)$^-$ mRNAs fail to be translated efficiently (Gallie, 1991). Furthermore, deadenylation is the initial step in the degradation of many eukaryotic mRNAs (for review, see Beelman and Parker, 1995). In *Xenopus* embryos, the deadenylation of a mRNA is sufficient to provoke its degradation at the blastula stage (Audic *et al.*, 1997a). Several factors implicated in mRNA deadenylation have been identified. Two subunits of the catalytic activity [poly(A) nuclease (PAN)] responsible for poly(A) shortening in yeast cells have been cloned (Boeck *et al.*, 1996; Brown *et al.*, 1996). This activity requires the poly(A)-binding protein (Sachs and Davis, 1989). Similar activities were also characterized in mammalian cells (for review, see Virtanen and Astrom, 1997). Lastly, cDNAs encoding factors that specifically bind the A/U-rich elements which direct rapid deadenylation and degradation in mammalian cells have been cloned. These proteins are good candidates for *trans*-acting factors that regulate deadenylation and/or degradation, although this has not been demonstrated directly (for review, see Jarzembowski and Malter, 1997).

The maternal *Xenopus* mRNAs Eg1-cdk2, Eg2, Eg5 and c-mos display a characteristic polyadenylation/deadenylation behavior. These mRNAs, which contain a functional CPE, are polyadenylated and translationally activated during oocyte maturation, and then deadenylated and translationally repressed after fertilization (Paris *et al.*, 1988; Paris and Philippe, 1990; Sheets *et al.*, 1994).

In a previous study (Bouvet *et al.*, 1994), we showed that, in contrast to the default deadenylation, the post-fertilization deadenylation of Eg2 mRNA was a sequence-specific process. The deletion of a 17 nucleotide (nt) fragment from the 3′UTR of this mRNA switched the behavior of a chimeric RNA from deadenylation to adenylation. CPE silencing (Simon *et al.*, 1993; Simon and Richter, 1994), which makes an RNA a substrate for the default deadenylation process, does not account for the deadenylation of Eg mRNAs in embryos (Legagneux *et al.*, 1995). In addition, the Eg-specific deadenylation activity is contained in a multimeric complex that is clearly separable from that which achieves default deadenylation (Paillard *et al.*, 1996). A candidate for a specificity factor that directs the Eg-specific deadenylation activity complex to the Eg mRNAs has been identified (Legagneux *et al.*, 1992). This is the Eg-specific binding factor p53/55 that is detected as a doublet in UV cross-linking experiments. Deletion of the above-mentioned 17 nucleotides of the Eg2 3′UTR abrogated not only the deadenylation of the chimeric RNA in embryos but also the binding of this factor (Bouvet *et al.*, 1994).

The objective of this study was to characterize the

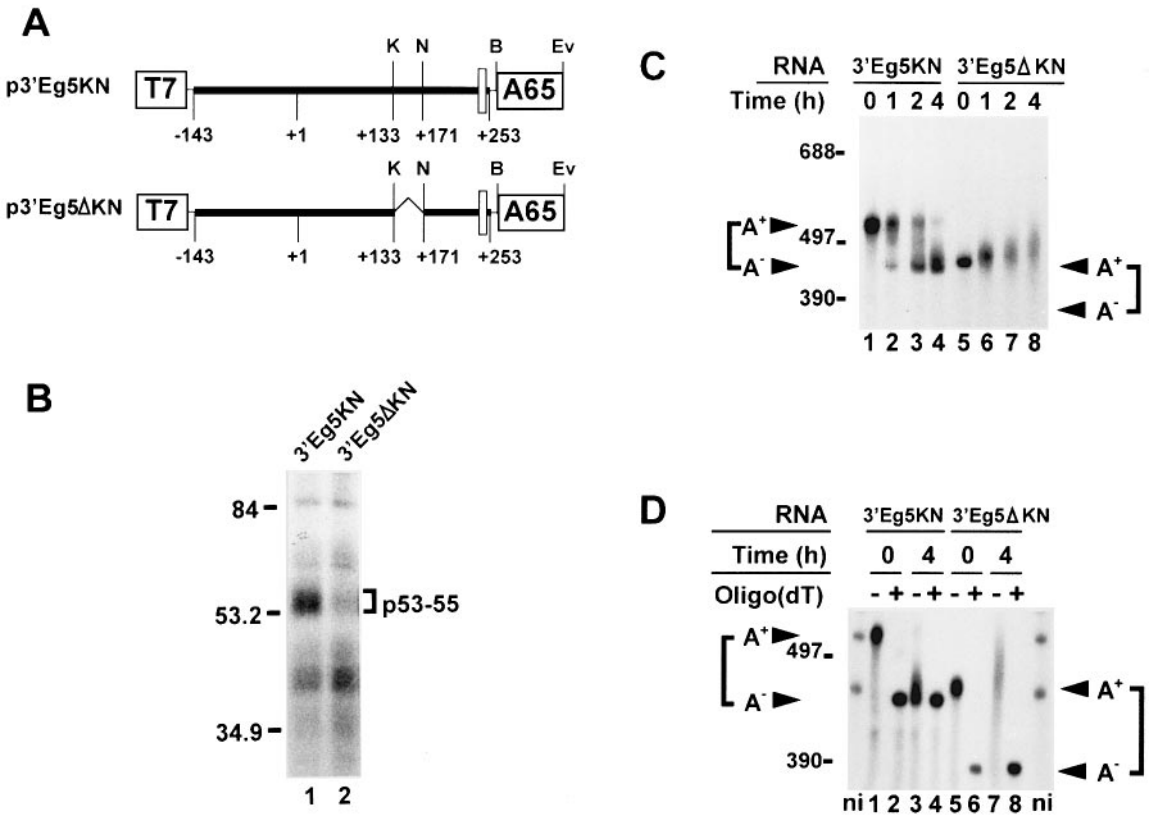

Fig. 1. Effect of a 39 nt deletion on p53/55 binding and on the adenylation/deadenylation behavior. (**A**) Schematic diagram of the p3′Eg5KN and p3′Eg5ΔKN chimeric genes. The Eg5 cDNA is represented by a thick line and the pBluescript KS plasmid sequence by a thin line. The numbering of nucleotides is relative to the stop codon. The T7 promoter and the cloned 65 nt adenosine tail are represented by boxes. The positions of the introduced *Kpn*I (K) and *Nhe*I (N) restriction sites are indicated as well as the positions of the *Bam*HI (B) and *Eco*RV (Ev) restriction sites used to linearize the plasmids before transcription. The AATAAA polyadenylation signal is denoted by an open box. (**B**) Radiolabeled 3′Eg5KN (lane 1) or 3′Eg5ΔKN (lane 2) transcripts were incubated in an egg extract, irradiated with UV light and treated with RNase A. The proteins were resolved on a 10% polyacrylamide gel in the presence of SDS, and radiolabeled proteins were revealed by autoradiography of the dried gel. The positions of the molecular weight markers and of p53/55 are indicated on the left and right, respectively. (**C**) Capped, radiolabeled 3′Eg5KN (lanes 1–4) or 3′Eg5ΔKN (lanes 5–8) transcripts were injected into embryos and incubated for the indicated times. RNAs were then extracted, resolved on a 4% polyacrylamide gel containing urea and revealed by autoradiography of the dried gel. The positions of RNA molecular weight markers are indicated on the left. Arrowheads indicate the positions of the fully adenylated (A^+) and fully deadenylated (A^-) forms of the transcripts. (**D**) Oligo(dT)/RNaseH analysis of the 3′Eg5KN (lanes 1–4) and 3′Eg5ΔKN (lanes 5–8) RNAs. Above each lane is indicated the time after injection of the poly(A)$^+$ RNAs at which the embryo samples were taken and whether the RNase H digestion was performed in the absence (–) or the presence (+) of oligo(dT). In the lanes denoted (ni), the non-injected poly(A)$^+$ RNAs (3′Eg5KN and 3′Eg5ΔKN) were deposited as samples. The positions of RNA molecular weight markers are indicated on the left. Arrowheads indicate the positions of the fully adenylated (A^+) and fully deadenylated (A^-) forms of the transcripts.

mechanism responsible for the post-fertilization deadenylation of Eg and c-mos mRNAs. We have identified the core sequence of a *cis* element that is bound specifically by the p53/55 protein and confers rapid deadenylation on a reporter RNA in *Xenopus* embryos. The p53/55 factor that associates with this element has been cloned and shown to be an essential *trans*-acting factor for sequence-specific deadenylation of mRNAs in *Xenopus* embryos.

Results

Deletion of a 39 nt portion of Eg5 3′ UTR abolishes both p53/55 binding and deadenylation

As previously mentioned (Bouvet *et al.*, 1994), comparison of the 17 nt region required for deadenylation of Eg2 mRNA with the 3′UTRs of several other Eg mRNAs did not allow a conserved sequence motif to be identified. To permit the identification of a consensus sequence, we have localized the sequence information in the Eg5 3′UTR that was required for p53/55 binding and rapid deadenylation. A series of deletion mutants was made that sequentially scanned the 3′UTR of Eg5 mRNA. All the mutations that abolished p53/55 binding, as tested by UV cross-linking, were included in a 39 nt region (data not shown). To test whether this region was also required for deadenylation, two chimeric genes were constructed (Figure 1A). In one, p3′Eg5KN, *Kpn*I and *Nhe*I restriction sites were introduced into the Eg5 3′UTR sequence (Legagneux *et al.*, 1995) on either side of this 39 nt region. In the other, p3′Eg5ΔKN, this 39 nt region was removed by cutting at the introduced sites. A UV cross-linking experiment performed with radiolabeled RNAs transcribed from these matrices indicated that this deletion abolished p53/55 binding (Figure 1B).

The adenylation/deadenylation behavior of these RNAs injected into embryos as capped, poly(A)$^+$ transcripts was determined by electrophoresis on acrylamide/urea gels (Figure 1C). The chimeric RNA 3′Eg5KN (lanes 1–4) underwent the already described (Legagneux *et al.*, 1995) characteristic shortening that is due to *cis* element-depend-

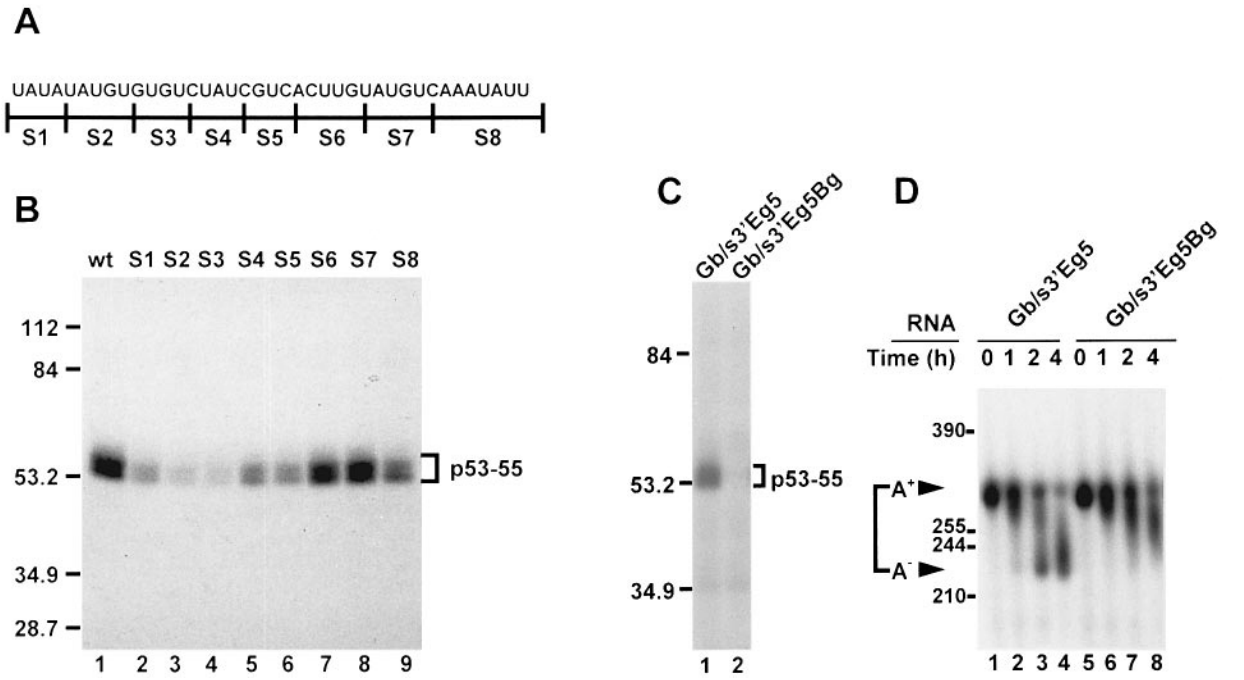

Fig. 2. Characterization of the Eg5 p53/55-binding site and effect of a mutation therein on deadenylation behavior. (**A**) The sequence of the Eg5 39 nt region that is required for rapid deadenylation. S1–S8 correspond to the portions that contain degenerate substitutions. (**B**) Short radiolabeled transcripts corresponding to the wild-type (lane 1) or to the partially degenerated (lanes 2–9) 39 nt region were incubated in an egg extract, and treated for UV cross-linking as described in Figure 1B. The positions of the molecular weight markers and of p53/55 are indicated on the left and right, respectively. (**C**) Radiolabeled Gb/s3′Eg5 (lane 1) or Gb/s3′Eg5Bg (lane 2) transcripts were incubated in an egg extract and treated for UV cross-linking as in Figure 1B. The positions of the molecular weight markers and of p53/55 are indicated on the left and right, respectively. (**D**) Capped, radiolabeled Gb/s3′Eg5 (lanes 1–4) or Gb/s3′Eg5Bg (lanes 5–8) transcripts were injected into embryos, incubated for the indicated times and treated as in Figure 1C. The positions of RNA molecular weight markers are indicated on the left. Arrowheads indicate the positions of the fully adenylated (A^+) and fully deadenylated (A^-) forms of the transcripts.

ent deadenylation. This RNA was shortened to a size corresponding to the non-adenylated RNA, and both deadenylated and the original adenylated (65A) RNAs co-exist. The deadenylation process was almost complete after 4 h (lane 4). A completely different adenylation/deadenylation behavior was observed for the 3′Eg5ΔKN RNA (lanes 5–8). Over the 4 h period studied, this RNA gradually increased in size, indicative of an elongation of the poly(A) tail of the injected RNA.

That these size changes were due to changes in poly(A) tail length was confirmed by oligo(dT)-directed RNaseH digestion (Figure 1D). This *in vitro* deadenylation caused the poly(A)$^+$ 3′Eg5KN RNA in the 0 h sample to be shortened to a size comparable with that of the 3′Eg5KN RNA in the sample taken 4 h after injection. Oligo(dT)/RNaseH treatment of the 3′Eg5KN RNA in the 4 h sample did not cause a decrease in the size of the transcript below that observed for the *in vitro* deadenylation of the 0 h sample (compare lanes 2 and 4). The 3′Eg5ΔKN RNA in the 0 h sample underwent a similar decrease in size when digested with RNaseH in the presence of oligo(dT). In the 4 h sample, these transcripts were of more heterogenous size but they were all reduced to the size of the *in vitro* deadenylated 3′Eg5ΔKN transcript in the 0 h sample, showing that they were polyadenylated prior to this treatment. Hence, the deletion of this 39 nt portion from the Eg5 3′UTR abolished p53/55 binding and switched the polyadenylation/deadenylation behavior of this RNA in embryos from deadenylation to further polyadenylation.

Mutations in the core motif of p53/55-binding site abolish rapid deadenylation in embryos

To identify the sequence information required for p53/55 binding, a set of RNAs was synthesized using oligonucleotide templates (see Materials and methods). First, a ^{32}P-labeled transcript was made that corresponded to the 39 nt region of Eg5 3′UTR (see Figure 2A). When used as a probe in UV cross-linking experiments, this transcript gave a >50 kDa doublet (Figure 2B, lane 1). Competition experiments confirmed that this doublet corresponded to p53/55 (data not shown). Therefore, this 39 nt region of Eg5 3′UTR is both necessary and sufficient for p53/55 binding. To define better the sequence information required for p53/55 binding, a set of ^{32}P-labeled RNAs (S1–S8, Figure 2A) were synthesized using oligonucleotides as matrices that contained degenerate substitutions. Each matrix corresponded to the complete 39 nt region in which short portions (designated S1–S8) were degenerated. Therefore, these matrices produced RNAs with degenerate mutations that sequentially scannned the 39 nt region. These transcripts were tested by UV cross-linking for p53/55 binding (Figure 2B). Mutations within S2 and S3 regions almost totally abolished p53/55 binding (lanes 3 and 4), whereas other mutations had a weaker or no effect. The regions S2 and S3 contain one UA and three UG dinucleotides. The 17 nt portion of the Eg2 3′UTR (UGUCCUUUUAUAUGUAA) whose deletion abrogated both p53/55 binding and the rapid deadenylation behavior (Bouvet *et al.*, 1994) also contains several U(A/G) repeats.

This indicates that a repetition of U(A/G) dinucleotides probably constitutes the core motif of the p53/55-binding site.

To test if a mutation in the S2 + S3 region would abrogate deadenylation, two chimeric genes were made, pGb/s3′Eg5 and pGb/s3′Eg5Bg. In pGb/s3′Eg5, the distal 120 nt portion of Eg5 3′UTR (between the introduced *Kpn*I and the *Bam*HI sites, Figure 1A) was cloned between the globin 5′ untranslated region (5′UTR) and a track of 65 As. In pGb/s3′Eg5Bg, the $(UG)_3$ motif in the S2 + S3 region was replaced by a *Bgl*II site. This mutation, which disrupts a stretch of six contiguous (UR) dinucleotides, abolished p53/55 binding (Figure 2C).

Radioactive capped poly(A)$^+$ RNAs were transcribed from these two chimeric genes and injected into embryos. The adenylation/deadenylation behavior of the injected RNAs was determined by electrophoresis on acrylamide/urea gels (Figure 2D). The chimeric RNA Gb/s3′Eg5 (lanes 1–4) underwent size changes very similar to those observed for the full-length 3′UTR (compare with Figure 1C, lanes 1–4). The Bg mutation that abolished p53/55 binding (Figure 2C) also caused a loss of the rapid deadenylation behavior (Figure 2D, lanes 5–8). The poly(A)$^+$ Gb/s3′Eg5Bg RNA became progressively heterogeneous in size, with a gradual decrease in the average size. However, even at 4 h post-injection, no RNA migrating at the position of the poly(A)$^-$ transcript was observed. Very similar results were obtained with another chimeric RNA (Gb/s3′Eg5C6) in which the UG repeats had been replaced by a stretch of six cytosines (data not shown). The residual size changes observed for the Gb/s3′Eg5Bg (Figure 2D) and Gb/s3′Eg5C6 (data not shown) RNAs were similar to those previously described for an RNA, containing neither a functional CPE nor a deadenylation element, that is a substrate for default deadenylation (Legagneux *et al.*, 1995).

Maternal Eg mRNAs are polyadenylated during oocyte maturation and deadenylated after the fertilization (Paris *et al.*, 1988; Paris and Philippe, 1990). In addition, cytoplasmic extracts made from metaphase II-arrested eggs do not display an Eg-specific rapid deadenylation activity (Legagneux *et al.*, 1995). Therefore, the rapid deadenylation of Eg mRNA is only observed in embryos and activated egg extracts. Consequently, the *cis* element that is required for rapid deadenylation in embryos and the core motif of which is a U(A/G) repeat has been given the name of an embryo deadenylation element (EDEN); the p53/55 factor that recognizes this element has been named EDEN-BP (EDEN-binding protein).

Identification and characterization of c-mos EDEN

A test of the generality of the proposed EDEN core motif is that it should allow functionally homologous EDEN elements to be identified in other mRNAs that are also deadenylated after fertilization. The maternal c-mos mRNA is an example of such an RNA (Sheets *et al.*, 1994). Therefore, the sequence of the 3′UTR (2 kb) of *Xenopus* c-mos mRNA (Sagata *et al.*, 1988) was scanned for sequence motifs composed of UR repeats. This revealed several elements containing less than four UR repeats and a single 36 nt region (UAUAUGUAUGUGUUGUUUUA-UGUGUGUGUGUGUGCU) that contained two blocks of six and eight UR repeats (nucleotides 668–703 from the stop codon). Deletion experiments indicated that, amongst these UR repeats, only the 36 nt region was included in a portion of c-mos 3′UTR that was required both for EDEN-BP binding and rapid deadenylation behavior (data not shown). To test whether this region of c-mos 3′UTR was able to confer a rapid deadenylation behavior on a reporter RNA, two chimeric genes (pGbORF and pGbORF/mosEDEN) were made. Both genes contained the 5′UTR, coding sequence, 20 nt of the 3′UTR and the AATAAA polyadenylation signal of *Xenopus* β-globin cDNA. In pGbORF/mosEDEN, the 36 nt putative EDEN-containing region of c-mos 3′UTR was inserted upstream of the polyadenylation signal. UV cross-linking experiments performed with the RNAs transcribed from both of these genes confirmed that EDEN-BP binding was only detected with GbORF/mosEDEN (Figure 3A). The adenylation/deadenylation behavior of these RNAs injected as radioactive, capped poly(A)$^+$ transcripts was determined by electrophoresis on polyacrylamide/urea gels. The results of a representative experiment are given in Figure 3B. As described previously (Audic *et al.*, 1997a), the host poly(A)$^+$ RNA (GbORF) became progressively heterogeneous in size, exhibiting a pattern indicative of a default type deadenylation (lanes 1–4, compare with Figure 2D, lanes 5–8). However, at 4 h post-injection, no signal was detected at the position of the deadenylated RNAs. Indeed, even at 8 h post-injection, the majority of these RNAs are still adenylated (Audic *et al.*, 1997a). A completely different behavior was observed for the injected poly(A)$^+$ GbORF/mosEDEN RNA. After injection, this RNA was shortened and, at 4 h post-injection, the majority of these RNAs migrated at the position of the deadenylated RNA (Figure 3B, lanes 5–8). That this shortening was due to

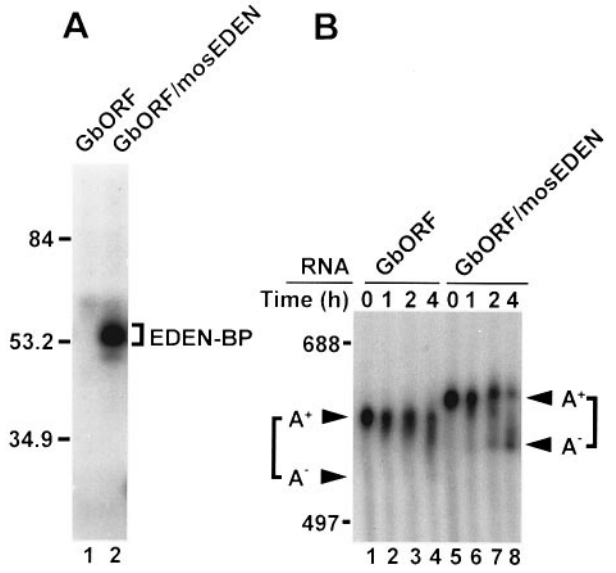

Fig. 3. Identification of the c-mos EDEN. (**A**) Radiolabeled GbORF (lane 1) or GbORF/mosEDEN (lane 2) transcripts were incubated in an egg extract and treated for UV cross-linking as in Figure 1B. The positions of the molecular weight markers and of EDEN-BP are indicated on the left and right, respectively. (**B**) Capped, radiolabeled GbORF (lanes 1–4) or GbORF/mosEDEN (lanes 5–8) transcripts were injected into embryos and treated as in Figure 1C. The positions of RNA molecular weight markers are indicated on the left. Arrowheads indicate the positions of the fully adenylated (A$^+$) and fully deadenylated (A$^-$) forms of each transcript.

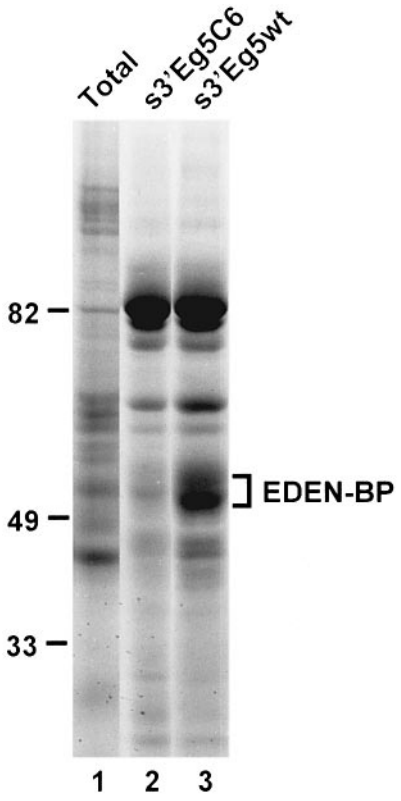

Fig. 4. Analysis of the proteins isolated by affinity chromatography. Total egg extract (lane 1) or proteins eluted from columns made with a s3′Eg5C6 (lane 2) or a wild-type s3′Eg5 (lane 3) RNA affinity ligand were resolved by electrophoresis on a 10% polyacrylamide gel in the presence of SDS. The gel was stained with Coomassie blue. The positions of the molecular weight markers and of EDEN-BP are indicated on the left and right of the gel respectively.

poly(A) removal was confirmed by oligo(dT)-directed RNase H digestion (data not shown). Hence, this 36 nt portion of c-mos 3′UTR is sufficient to bind EDEN-BP and to confer a rapid deadenylation on globin chimeric mRNA in embryos, indicating that it constitutes the c-mos EDEN.

Purification, cloning and characterization of p53/55/EDEN-BP

The results described above imply that EDEN-BP is a candidate for a *trans*-acting factor that mediates rapid deadenylation in embryos. Hence, to analyze the function of this factor, it was purified by RNA affinity chromatography using poly(A)$^+$ s3'Eg5wt or s3′Eg5C6 RNAs bound to a CNBr-activated Sepharose matrix. The s3′Eg5wt transcript contains the distal 120 nt portion of the Eg5 3′UTR. In s3′Eg5C6, the $(UG)_3$ motif in the S2 + S3 region (see Figure 2A) was replaced by six cytosines. A cytoplasmic egg extract was incubated with these RNA affinity beads and, after washing, the bound proteins were eluted by RNase treatment. The eluted proteins were resolved by SDS–PAGE (Figure 4). Amongst the proteins eluted from these columns, a doublet of ~50 kDa was much more concentrated in the eluate from the column made with the wild-type RNA (s3′Eg5wt) than with the mutant RNA (s3′Eg5C6). Therefore, this doublet has the binding specificity of EDEN-BP. The electrophoretic mobility of this doublet was slightly greater

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MNGTMDHPDHPDPDSIKMFVGQVPRSWSEKELRELFEQYGAVYEINVLRD 50
RSQNPPQSKGCCFITFYTRKAALEAQNALHNMKVLPGMHHPIQMKPADSE 100
KNNAVEDRKLFIGMVSKNCNENDIRAMFSPFGQIEECRILRGPDGMSRGC 150
AFVTFTTRSMAQMAIKSMHQAQTMEGCSSPIVVKFADTQKDKEQKRMTQQ 200
LQQQMQQLNAASMWGNLTGLNSLAPQYLALLQQTASSGNLNSLSGLHPMG 250
AEYGTGMTSGLNAIQLQNLAALAAAASAAQNTPSAGAALTSSSSPLSILT 300
SSGSSPSSNNSSINTMASLGALQTLAGATAGLNVNSLAGMAAFNGGLGSS 350
LSNGTGSTMEALSQAYSGIQQYAAAALPSLYNQSLLSQQGLGAAGSQKEG 400
PEGANLFIYHLPQEFGDQDLLQMFMPFGNVVSSKVFIDKQTNLSKCFGFV 450
SYDNPVSAQAAIQSMNGFQIGMKRLKVQLKRSKNDSKPY 489
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Fig. 5. Peptide sequence of EDEN-BP. The peptide sequences obtained from microsequencing are in bold characters. The three RNP-type RNA recognition motifs (Burd and Dreyfuss, 1994) are underlined. The positions of their conserved RNP1 and RNP2 sequences are indicated by double underlining.

than that of cross-linked EDEN-BP (data not shown), which could be due to the short RNA oligonucleotide that remains covalently bound to the UV-cross-linked protein after RNase treatment.

EDEN-BP was eluted from a preparative gel, digested with endolysine-C and four peptides were microsequenced. Sense and antisense degenerated oligonucleotides were derived from these peptide sequences, and various combinations were used as PCR primers with reverse transcribed *Xenopus* ovary mRNA. The longest PCR product was subcloned and used as a probe to screen a *Xenopus* ovary cDNA library. A clone with the longest insert (2 kb) was sequenced. This cDNA contained an open reading frame (ORF) of 489 amino acids with a deduced mol. wt of 52.4 kDa (Figure 5). The four endolysine peptides were present in the ORF, indicating identity of the purified protein and the protein encoded by the cloned cDNA. This protein contains two adjacent putative RNP-type RNA recognition motifs (Burd and Dreyfuss, 1994) in the N-terminal region and a third one in the C-terminal region. The best homology scores in a BLAST database search were obtained with *elav*-like RNA-binding proteins (Good, 1995), making EDEN-BP a putative new member of this family. The protein with the highest identity (88.4%) to EDEN-BP was human hNab50/CUG-BP (Timchenko *et al.*, 1996) (see Discussion).

To confirm that the cloned cDNA encodes a protein with the same RNA-binding specificity as EDEN-BP from egg extract, recombinant EDEN-BP was synthesized in a nuclease-treated rabbit reticulocyte lysate. The lysate was tested by UV cross-linking using various probes. The GbORF/mosEDEN and Gb-Eg2-410 transcripts both contain an EDEN (Figure 3 and Legagneux *et al.*, 1992). The GbORF transcript was used as a control. A strong UV cross-linking signal migrating at ~53 kDa was detected in the EDEN-BP-programed lysate using probes containing an EDEN (Figure 6A, lanes 4 and 6) but not with a probe devoid of an EDEN (lane 5). This signal was specific for EDEN-BP-programed lysate (compare with lanes 1–3). Therefore, the strong EDEN-specific UV cross-linking signal detected in EDEN-BP-programed lysate is due to the neotranslated recombinant EDEN-BP.

Next, rabbit and guinea pig antisera were raised against a recombinant fragment of EDEN-BP produced in bacteria.

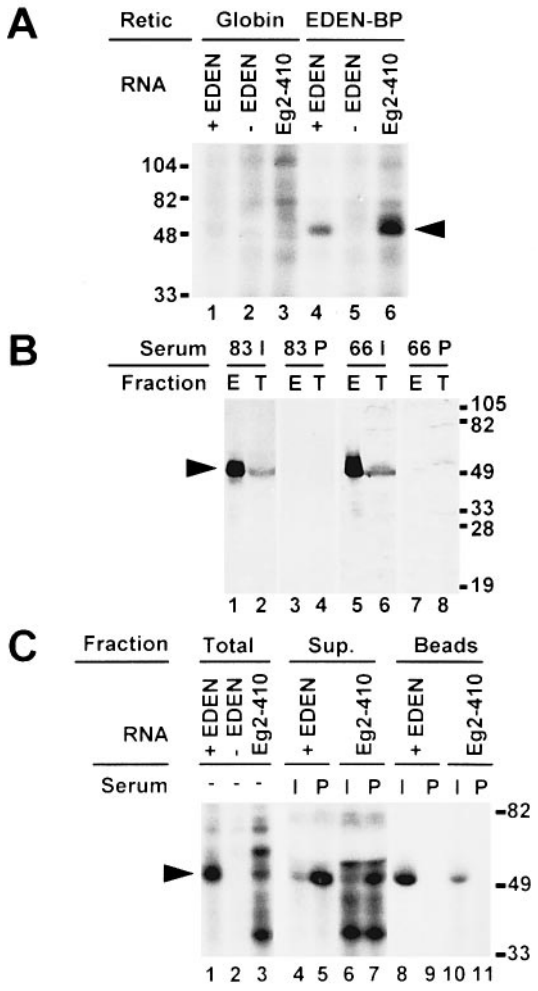

Fig. 6. Characterization of EDEN-BP. (**A**) Radiolabeled GbORF (lanes 2 and 5), GbORF/mosEDEN (lanes 1 and 4) and Gb-Eg2-410 (lanes 3 and 6) RNAs were incubated in rabbit reticulocyte lysates that had been programed with either globin mRNA (lanes 1–3) or EDEN-BP mRNA (lanes 4–6). Samples were then treated for UV cross-linking as described in Figure 1B. The positions of molecular weight markers are indicated. The position of EDEN-BP is indicated by an arrowhead. (**B**) Total egg extract (T, even lanes) or EDEN-BP eluted from an affinity chromatography column (E, uneven lanes) were resolved by electrophoresis on a 10%, SDS-containing polyacrylamide gel and transferred onto a nitrocellulose membrane. The membrane was probed with immune α83 serum (lanes 1 and 2), pre-immune α83 serum (lanes 3 and 4), immune α66 serum (lanes 5 and 6) or pre-immune α66 serum (lanes 7 and 8). The positions of molecular weight markers are indicated on the right. The position of EDEN-BP is indicated by an arrowhead. (**C**) Radiolabeled GbORF (lane 2), GbORF/mosEDEN (lanes 1, 4–5 and 8–9) and Gb-Eg2-410 (lanes 3, 6–7 and 10–11) transcripts were incubated in an egg extract, irradiated with UV light and treated with RNase A. Samples were then directly loaded (lanes 1–3) or incubated with pre-immune (lanes 5, 7, 9 and 11) or immune (lanes 4, 6, 8 and 10) α66 serum and adsorbed on protein A beads. Input (lanes 1–3), supernatant (lanes 4–7) and bound fractions (lanes 8–11) were resolved by electrophoresis on a 10% polyacrylamide gel in the presence of SDS. Proteins were revealed by autoradiography of the dry gel. The positions of molecular weight markers are indicated. The position of EDEN-BP is indicated by an arrowhead.

Both rabbit (α83) and guinea pig (α66) antibodies recognized purified *Xenopus* EDEN-BP in a Western analysis (Figure 6B, lanes 1 and 5). They also recognized a co-migrating doublet in a cytoplasmic egg extract (lanes 2 and 6). Pre-immune antisera did not recognize this doublet (lanes 3–4 and 7–8). The pre-immune and immune α66 sera also reacted weakly with several other proteins in an egg extract, one of which migrated just above EDEN-BP (compare lanes 6 and 8).

To demonstrate that the protein revealed by these antisera in the egg extract corresponded to the endogenous EDEN-BP, a series of immunoprecipitation experiments was performed with samples where the endogenous EDEN-BP was radiolabeled by UV cross-linking. Figure 6C shows the results obtained with α66 sera. The majority of the EDEN-BP signal revealed with GbORF/mosEDEN (lane l) was depleted from the egg extract treated with α66 antiserum (lane 4), and the radiolabeled EDEN-BP was recovered in the bound fraction (lane 8). The pre-immune serum did not decrease the EDEN-BP signal in the supernatant (lane 5), and no EDEN-BP signal was recovered from the pellet (lane 9). The specificity of the immunoprecipitation was demonstrated using GbEg2-410 as a UV cross-linking probe. As previously described (Legagneux *et al.*, 1992), this probe was bound by at least three proteins in addition to EDEN-BP (lane 3). Amongst the proteins that bound to the GbEg2-410 RNA, a differential signal between immune- and pre-immune-treated extracts was only obtained for EDEN-BP (compare lanes 6 and 7). In addition, only EDEN-BP was recovered in the pellet of an extract treated with the immune serum (lane 10). Therefore, these antibodies to a fragment of recombinant EDEN-BP specifically recognized endogenous EDEN-BP, and were able to deplete an egg extract from EDEN-BP. Together, these results confirm that the cDNA obtained from library screening encodes EDEN-BP.

EDEN-BP is required for sequence-specific deadenylation

To investigate the function of EDEN-BP in the EDEN-mediated deadenylation activity, immunodepletion experiments were performed. The test of EDEN-BP function was made by incubating capped, radiolabeled Gb-Eg2-410 and Gb-Eg2-410A transcripts in immunodepleted or mock-depleted extracts. This pair of transcripts was used because Gb-Eg2-410 contains an EDEN and a CPE, and therefore allows EDEN-mediated deadenylation to be measured without interference from the default deadenylation activity; Gb-Eg2-410A is a substrate for the default deadenylation activity as it does not contain either an EDEN or a CPE (Legagneux *et al.*, 1995).

A deadenylation-proficient egg extract was treated with either α83 (Figure 7A) or α66 sera (Figure 7B). In both cases, the EDEN-mediated deadenylation activity, as measured with the Gb-Eg2-410 transcript, was completely abolished by depletion of EDEN-BP with the immune sera (lanes 4–6), as compared with treatment with pre-immune sera (lanes 1–3). Unlike the EDEN-mediated deadenylation, the default deadenylation activity, as measured with Gb-Eg2-410A, was not affected by depletion of EDEN-BP. In addition, depletion of EDEN-BP did not cause a sequence-specific change in RNA stability, since the ratio of Gb-Eg2-410 to Gb-Eg2-410A transcripts, quantified from Instant Imager data, was not affected. Therefore, immunodepleting EDEN-BP from an egg extract specifically abolished the EDEN-mediated deadenylation.

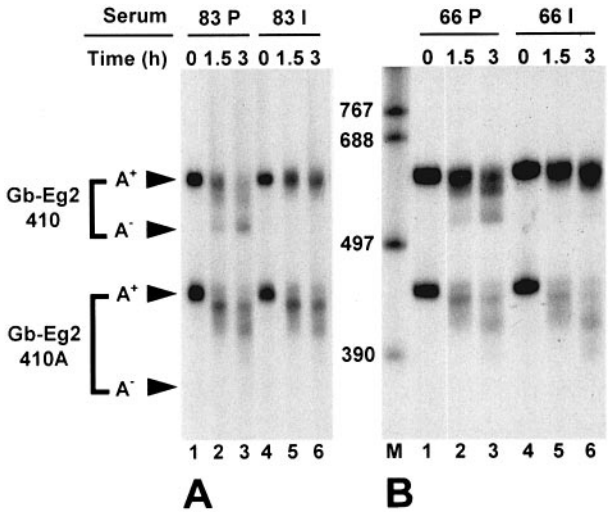

Fig. 7. EDEN-BP is required for the EDEN-specific deadenylation activity. A deadenylation-proficient egg extract was incubated with either pre-immune (lanes 1–3) or immune (lanes 4–6) α83 (**A**) or α66 (**B**) serum absorbed to protein A beads. Capped, radiolabeled Gb-Eg2-410 and Gb-Eg2-410A transcripts were added to the supernatant fractions and incubated for the indicated times. RNAs were then extracted and treated as in Figure 1C. The positions of RNA molecular weight markers (M) and of the fully adenylated (A^+) and fully deadenylated (A^-) forms of the transcript are indicated.

Discussion

In the study presented here, we have identified, as a UR repeat, the core motif of the *cis*-acting element required for rapid deadenylation in *Xenopus* embryos. This deadenylation element, named EDEN, is contained respectively within a 39 and a 36 nt region of Eg5 and c-mos 3′UTR. Deleting or mutating the EDEN sequence of Eg5 3′UTR abolished both rapid deadenylation and the binding of the Eg-specific factor p53/55/EDEN-BP (Figures 1 and 2). Furthermore, the c-mos EDEN inserted into the 3′UTR of β-globin mRNA conferred a rapid deadenylation on this reporter mRNA (Figure 3).

Deleting the EDEN from the complete Eg5 3′UTR led to a polyadenylation of the transcript (Figure 1). In contrast, mutating the core EDEN sequence of the distal portion of Eg5 3′UTR led to a poly(A) removal with kinetics indicative of default deadenylation (Figure 2). This suggests that the Eg5 CPE is localized in a proximal position of Eg5 3′UTR, and that, in the absence of an EDEN, this CPE is still functional after fertilization. A similar switch in the adenylation/deadenylation behavior was obtained previously with chimeric RNAs derived from Eg2 3′UTR (Bouvet *et al.*, 1994), indicating that, in both cases, the EDEN functionally overrides the CPE after fertilization.

A direct implication of the above results is that the EDEN-specific RNA-binding protein, EDEN-BP, could be the *trans*-acting factor that targets EDEN-containing mRNAs to the complex responsible for EDEN-mediated deadenylation. To demonstrate this, the cDNA corresponding to EDEN-BP was cloned and antibodies against a recombinant fragment of the protein were used to deplete the endogeneous EDEN-BP in an activated egg extract. This depletion completely abolished the EDEN-mediated activity (Figure 7). The default deadenylation activity was not affected by depletion. Therefore, EDEN-BP has the characteristics of a *trans*-acting factor that is required for the rapid and sequence-specific deadenylation of EDEN-containing RNAs in embryos. We propose that the binding of EDEN-BP to a target sequence transactivates a PAN activity. This transactivation could be either direct via the interaction of EDEN-BP with a PAN catalytic subunit or involve cofactors in the deadenylation complex. Alternatively, EDEN-BP could mediate deadenylation by acting as a targeting factor that recrutes the PAN to EDEN-containing RNAs.

EDEN-mediated deadenylation has not been detected in oocytes (our unpublished data) or in unfertilized egg extracts (Legagneux *et al.*, 1995), and is therefore activated at fertilization. However, a signal for the cross-linking of EDEN-BP to Eg mRNAs is clearly observed in oocyte extracts (Legagneux *et al.*, 1992). It would appear, therefore, that the activation of EDEN-mediated deadenylation after fertilization is achieved by a post-translational modification of EDEN-BP and/or the synthesis or activation of an associated cofactor. Relative to the developing embryo, the EDEN-mediated deadenylation that leads to translational arrest could be required to inhibit the translation of certain mRNAs whose products would be toxic for the embryo. An example of such a protein is c-mos that inhibits cell divisions during oocyte maturation. Overexpression of c-mos protein in embryos is sufficient to block the cell divisions (Sagata *et al.*, 1989). Therefore, the EDEN-mediated deadenylation of c-mos mRNA could be a way to ensure a complete shut-down of c-mos expression after fertilization.

EDEN-BP was identified originally as a protein doublet (p53/55) that cross-links to Eg mRNAs (Legagneux *et al.*, 1992). A protein doublet is also detected in Western blots made from *Xenopus* egg extracts (Figure 6A). The human protein hNab50/CUG-BP whose sequence is 88.4% identical to that of EDEN-BP (Timchenko *et al.*, 1996) also exists in two biochemically separable forms that migrate as a doublet in polyacrylamide gels. When the two components of EDEN-BP were excised separately from an SDS–polyacrylamide gel, they gave quasi-identical HPLC profiles of the tryptic peptides (our unpublished data). Consequently, this doublet could be indicative of a post-translational modification of EDEN-BP. This doublet may also arise by the presence of two isoforms of EDEN-BP generated by alternative splicing as reported for several RNA-binding proteins (Burd *et al.*, 1989; Funke *et al.*, 1996, and references therein).

The data presented here show that an EDEN is sufficient to target a chimeric RNA for rapid deadenylation. This does not exclude that certain mRNAs contain other *cis*-acting deadenylation elements. A particular case is that of Eg1-cdk2. The 3′UTR of this mRNA binds EDEN-BP (Legagneux *et al.*, 1992). Therefore, the rapid post-fertilization deadenylation of Eg1-cdk2 is very likely to be at least in part EDEN mediated. However, Stebbins-Boaz and Richter (1994) identified within the 3′UTR of this mRNA two sequence elements that act synergistically both to prevent the polyadenylation and to provoke the deadenylation of a poly$(A)^+$ chimeric RNA in embryos. These elements appear to act independently of the EDEN-mediated deadenylation process, since the chimeric RNAs used by these authors do not bind EDEN-BP (Stebbins-Boaz and Richter, 1994) due to a deletion of a region of

the Eg1-cdk2 3′UTR containing the EDEN (F.Omilli, unpublished data). Analogous deadenylation elements, acting independently of EDEN, were not detected in Eg2 or Eg5 3′UTRs.

The EDEN-mediated deadenylation machinary described here could have a counterpart in somatic cells. *Xenopus* EDEN-BP shares 88.4% identity with human hNab50/CUG-BP. This protein, that is expressed in several somatic cell lines including HeLa and lymphoblast cells, is an RNA-binding protein reported to be specific for CUG repeats. Implication of this protein in the pathogenesis of myotonic dystrophy was therefore suggested (Timchenko *et al.*, 1996). Myotonic dystrophy is a genetic disease due to an expansion of CTG repeats in the myotonin protein kinase (Mt-PK) gene leading to an expansion of CUG repeats in the 3′UTR of Mt-PK mRNA. Little is known about the cellular functions of hNab50/CUG-BP, but the very high sequence conservation between *Xenopus* EDEN-BP and human hNab50/CUG-BP suggests that they might have analogous functions. Indeed, a decrease in the concentration of poly(A)$^+$, but not of total Mt-PK mRNA was described in myotonic dystrophy patients (Wang *et al.*, 1995). This is consistent with a role for hNab50/CUG-BP in mRNA deadenylation.

Materials and methods

Plasmids and mutagenesis

p3′Eg5KN was obtained by introducing the *Kpn*I and *Nhe*I sites flanking the EDEN into p3′Eg5-A65 (Legagneux *et al.*, 1995) by site-directed mutagenesis (Kunkel, 1985). The following oligonucleotides were used: (*Nhe*I) 5′ CTACTTTAGAAAGTGCTAGCAATATTTGACATAC 3′ and (*Kpn*I) 5′ GACACACATATATAGGTACCTAACCAAGATAAG 3′. p3′Eg5ΔKN was obtained by first digesting p3′Eg5KN with *Eco*RI and recircularizing, so as to remove a portion of the polylinker containing a *Kpn*I site, then digesting the resulting plasmid with *Kpn*I and *Nhe*I and recircularizing after blunt ending.

To construct pGb/s3′Eg5, the Eg5 EDEN-containing sequence was amplified by PCR using the oligonucleotide 5′ GAGCTCGAGGTACCTATATATGTGTGTCTATCGTCACTTGTATGTCAAATATTGCTAGCAATAAATATTGG 3′ as template and the oligonucleotides 5′ GACAGAGCTCGAGGTACC 3′ and 5′ GATTGGATCCAATATTTATTGCTAG 3′ as 5′ and 3′ primers, respectively. The amplified fragment was digested with *Xho*I and *Bam*HI and cloned into pGb-PP2Ac-700-A65 (Legagneux *et al.*, 1995) also digested with *Xho*I and *Bam*HI. This created pGbEg5EDEN in which the Eg5 EDEN-containing region is situated downstream of the globin 5′UTR. Next, pGbEg5EDEN was digested with *Nhe*I and *Nde*I and the 800 bp fragment was cloned between the *Nhe*I and *Nde*I sites of p3′Eg5KN thereby creating pGb/s3′Eg5. The pGb/s3′Eg5Bg and pGb/s3′Eg5C6 were similarly constructed using the same 5′ and 3′ oligonucleotide primers and the oligonucleotides 5′ GAGCTCGAGGTACCTATATATAGATCTTATCGTCACTTGTATGTCAAATATTGCTAGCAATAAATATTGG 3′ and 5′ GAGCTCGAGGTACCTATATATCCCCCCTATCGTCACTTGTATGTCAAATATTGCTAGCAATAAATATTGG 3′ respectively as templates.

ps3′Eg5wt and ps3′Eg5C6 were obtained by digesting pGb/s3′Eg5 and pGb/s3′Eg5C6 with *Kpn*I, which excised the globin 5′UTR, and recircularizing the plasmids.

The pGbORF plasmid was described by Audic *et al.* (1997a). The pGbORF/mosEDEN was constructed as follows. A DNA fragment consisting of the 36 nt c-mos EDEN-containing region, followed by a 22 nt portion of β-globin 3′UTR that contained the AATAAA polyadenylation signal and terminated by a *Bam*HI restriction site, was amplified by PCR. The oligonucleotide 5′ CTAGAGTATATGTATGTGTTGTTTTATGTGTGTGTGTGTGCTAGCGTATCTGCTCCTAATAAAAAGAGG 3′ was used as template and the oligonucleotides 5′ GCGGCCTCTAGAGTATATG 3′ and 5′ GTAATGGATCCTCTTTTTATTAG 3′ were used as 5′ and 3′ primers respectively. The amplified fragment was digested with *Xba*I and *Bam*HI and cloned into the pGbORF plasmid (Audic *et al.*, 1997a) digested with the same restriction enzymes. The pGbEg2-410 and pGbEg2-410A plasmids were described by Legagneux *et al.* (1995).

The pT7T vector (a kind gift of David Lepetit) was constructed by cloning the *Hin*dIII–*Eco*RI fragment of pSP64T (Krieg and Melton, 1984) into the same sites of pT3/T7α19 (Life Technologies), so that RNAs starting with the globin 5′UTR can be transcribed from the T7 promoter. The EDEN-BP ORF was amplified by PCR with primers containing an additional *Bgl*II site in the untranslated portions. This fragment was subcloned into the *Bgl*II site of the pT7T vector, giving pT7T EDEN-BP.

The cDNA fragment encompassing the fragment of EDEN-BP from S26 to L411 (see Figure 5) was amplified by PCR and subcloned into the *Bam*HI and *Sma*I sites of PQE30 (Qiagen). The resulting plasmid was named PQE-sEDEN-BP.

RNA synthesis

^{32}P-labeled and capped or uncapped transcripts were made using the Promega Riboprobe transcription kit as previously described (Legagneux *et al.*, 1992). Large-scale uncapped RNA synthesis used for protein purification and reticulocyte programing were performed using the Promega Ribomax kit. The poly(A)$^+$ RNAs were synthesized from the T7 promoter of their respective plasmids after linearizing the plasmids with *Eco*RV. EDEN-BP RNA was synthesized from the T7 promoter after linearizing the plasmids with *Eco*RI.

Transcription from oligonucleotide templates was performed as described by Kohli and Temsamani (1993) with a phosphorylated T7 promoter oligonucleotide (5′ TCGTATTACGCTTTTGCGTAATACGACTCACTATAG 3′) ligated to the different template oligonucleotides. The oligonucleotide templates used were: Eg5, 5′ AATATTTGACATACAAGTGACGATAGACACACATATATACTATAGTGAG 3′ or degenerate substitution mutants of this oligonucleotide. In these substitution mutants, mutated nucleotides, whose positions are indicated on Figure 2A, were replaced by any nucleotide except the wild-type.

Biological and analytical methods

Embryos were obtained by standard procedures and incubated in F1 solution (Paris *et al.*, 1988). Embryos at the two-cell stage were injected with 18.4 nl of *in vitro* transcript (50 000 c.p.m./μl, 7.5–15 fmol/μl) as described by Legagneux *et al.* (1992). Embryos were allowed to develop at 22°C. Alternatively, the *in vitro* transcripts were added to cytoplasmic activated egg extracts that are functional for deadenylation (Legagneux *et al.*, 1995). For each time point, the RNA from five injected embryos or 5 μl of egg extract was extracted as described by Harland and Misher (1988). Samples were solubilized in sequencing stop buffer and analyzed by electrophoresis on polyacrylamide/urea gels followed by autoradiography. When required, the data were quantified by direct counting of the dried gel with an Instant Imager (Packard). For oligo(dT)-directed RNaseH digestion, the RNA extracted from five embryos was resuspended in water and treated as previously described (Osborne *et al.*, 1991). RNA size markers were synthesized *in vitro* using the pBluescript-based plasmid containing the phosphatase 2A cDNA linearized with different restriction enzymes (Audic *et al.*, 1997b). The analysis of proteins cross-linked by UV irradiation to ^{32}P-labeled transcripts was performed as previously described (Legagneux *et al.*, 1992) using cytoplasmic egg extracts or rabbit reticulocyte lysates diluted 10 times in XB100 buffer (100 mM KCl; 10 mM HEPES pH 7.5; 50 mM sucrose; 1 mM $MgCl_2$; 0.1 mM $CaCl_2$). Protein molecular weight markers were purchased from Biorad Laboratories.

EDEN-BP purification and cDNA cloning

Sixty nmol of s3′Eg5 or s3′Eg5C6 transcript were prepared from *Eco*RV-linearized matrices and bound to a CNBr-activated Sepharose matrix (Kaminski *et al.*, 1995). Sixty ml of cytoplasmic egg extracts (Legagneux *et al.*, 1992) were diluted twice in XB50 buffer (50 mM KCl; 10 mM HEPES pH 7.5; 50 mM sucrose; 1 mM $MgCl_2$; 0.1 mM $CaCl_2$) supplemented with 5 mg/ml heparin (Sigma) and 1 mg/ml yeast tRNA (Boehringer) and loaded onto the extensively washed affinity matrix. After 1 h incubation at 4°C, the matrix was washed with 20 column volumes of XB50 containing 0.1% NP-40 and eluted by 0.1 mg/ml RNase A in XB50. The eluted proteins were resolved by SDS–PAGE. After copper staining of the gel, the strip containing EDEN-BP was eluted at 37°C in 20 mM Tris–HCl (pH 7.9), 0.01% SDS and 1 mM $CaCl_2$ (Prussak *et al.*, 1989). The eluted proteins were concentrated by centrifugation on a Centricon 10 (Amicon), lyophilized and sent to Toplab (Martinsried, Germany) for digestion with endolysin-C and microsequencing of the generated peptides.

Degenerate oligonucleotides were derived from the microsequence

data with inosines at positions of maximal degeneracy. These were used as primers for PCR amplification with oligo(dT)-primed reverse-transcribed ovary RNA. The longest PCR fragment obtained was subcloned in pGEM-T (Promega), partly sequenced and used as a probe to screen at high stringency a Uni-ZAP XR *Xenopus* ovary cDNA library (Stratagene). A Bluescript phagemid was excised *in vivo* from the uni-ZAP vector containing the longest insert, as determined by PCR on phage suspensions, and the insert was totally sequenced in both orientations using the T7 sequencing kit (Pharmacia). Sequence analysis were performed using the facilities of the Genome server (Villejuif, France).

Recombinant protein and antibody methods

TG1 bacteria transformed with PQE-sEDEN-BP plasmid produced a peptide of ~40 kDa after induction at 37°C by 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The 40 kDa peptide was purified under denaturing conditions on a nickel–agarose column following the manufacturer's instructions (Qiagen) and sent to Eurogentec (Seraing, Belgium) for immunization of two rabbits and two guinea pigs.

Full-length recombinant EDEN-BP was produced in nuclease-treated rabbit reticulocyte lysate (Promega). A standard reaction (50 µl) contained 35 µl of lysate, 1 µl of RNasin (Promega), 7 µl of a mix of amino acids (1 mM each) and a quantity of transcript corresponding to one-fifteenth of a standard (20 µl) Ribomax transcription reaction. The lysate was incubated for 5 h at 30°C.

Western blots were performed following standard procedures with 1000-fold diluted sera and alkaline phosphatase-coupled secondary antibodies (Sigma).

For immunoprecipitation and immunodepletion experiments, sera were filtered on 0.22 µm cellulose acetate filters (Amicon) before use. For the immunoprecipitation experiments, cross-linking of EDEN-BP to radiolabeled RNAs was performed as described (Legagneux *et al.*, 1992). Then 100 µl of UV- and RNase-treated diluted extract were supplemented with 10 µl of serum and incubated for 1 h at room temperature. Ten µl of pre-washed protein A–Sepharose 4B beads (Pharmacia) were added and incubated for an additional 1 h at 4°C. The supernatant was collected and supplemented with Laemmli buffer. The beads were rinsed four times in XB100 buffer containing 1% NP-40 before elution with Laemmli buffer. For the depletion experiments, 100 µl of serum were first incubated with 50 µl of beads overnight at 4°C and extensively washed with XB100 buffer. Twenty µl of these beads were incubated with 100 µl of undiluted, deadenylation-proficient extract for 90 min at 4°C.

Database accession number

The DDBJ/EMBL/GenBank accession No. for the sequence reported in this paper is AF003923.

Acknowledgements

We thank Ann Kaminski and Richard J.Jackson for sharing results before publication, and David Lepetit for the gift of the pT7T vector. This work was supported by grants from the Institut National de la Santé et de la Recherche Médicale (CRE 91-0112), the European Economic Community DG XII Biotechnology Program (BIO4-CT95-0045), the Ministère Chargé de la Recherche (ACC-SV4), the Association pour la Recherche sur le Cancer (Contract 6788) and the Ligue Nationale Contre le Cancer. V.L. is a staff member of the Institut National de la Santé et de la Recherche Médicale.

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Received September 8, 1997; revised October 16, 1997; accepted October 17, 1997

Note added in proof

After acceptance of this manuscript, the sequence of the *Drosophila* protein Bruno was published (P.J.Webster *et al*., *Genes Dev.*, **11**, 2510–2521). This protein, which is implicated in the translational repression of the maternal mRNA oskar, is 50% identical to EDEN-BP.