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Functional Analysis of Proteins Involved in Translational Regulation During Xenopus Oocyte Maturation.

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Cytoplasmic polyadenylation regulates translational activation of mRNA stored in immature Xenopus oocytes. This event is necessary for the beginning of oocyte maturation, and later for critical processes in early embryonic development. A major protein required for polyadenylation is the cytoplasmic polyadenylation element-binding protein (CPEB), which recruits a factor that promotes the interaction between Poly(A) polymerase and the end of the mRNA. Polyadenylation in turn leads to translation through interactions between CPEB and other proteins.

Using a yeast two-hybrid screen, several of these proteins were identified and cloned, including two of note. X295, a zinc-finger containing novel protein, and DEK, which has significant homology with the Homo sapiens DEK involved in certain juvenile leukemias.

Through the cloning of the genes encoding these proteins, transcription of mRNA, and protein overexpression in oocytes, a series of protein-protein interaction binding assays were performed. Immunoblotting of SDS-PAGE analyzed samples shows that GST-CPEB and HA-X295 interact in ovo, and suggests a possible in ovo interaction of endogenous CPEB and endogenous X295. In similar experiments, DEK and CPEB do not interact, suggesting they may not interact in ovo.

INTRODUCTION:

Early development in many systems relies on the selective translation of stored maternal mRNAs rather than new transcription, conserving the time and resources of a rapidly dividing embryo. Events ranging from the control of meiotic and mitotic cell divisions to the establishment of embryonic polarity are dependent on the temporal and spatial activation of these individual mRNAs. Polyadenylation-induced translation, required for Xenopus oocyte maturation, is a major mechanism directing this mRNA activation. Those mRNAs that acquire an elongated poly (A) tail on their 3′ end are translationally activated, while the shortening of the poly (A) tail leads to translational repression.

Translational regulation by this mechanism requires the mRNA to contain a U-rich sequence, the cytoplasmic polyadenylation element (CPE) [UUUUUAU] within the 3′UTR. The CPE is recognized and bound by the specific RNA-binding protein CPEB, which then recruits several intermediate proteins leading to the elongation of the poly (A) tail by poly (A) polymerase, triggering the subsequent translation of the mRNA (Hake and Richter 1996).

To identify additional proteins that influence CPEB function during oogenesis and early maturation, a yeast two-hybrid screen was performed. Through this system, potential CPEB-interacting proteins were isolated, including two of interest: X295 (Figure 1) and DEK (Figure 2). A protein similar to X295 has yet to be identified or characterized in any organism, based on sequence database comparisons. This 370 amino acid (39.8 kD) protein likely contains a putative zinc-finger domain (aa 238 - 306), one or more potential sumoylation sites (aa 176 - 179, 266-269), and a PEST domain (aa 1-22).

DEK, which has significant homology with the Homo sapiens DEK (Figure 2c), has never been characterized in Xenopus, though much is known about its human counterpart. In Homo sapiens, DEK is a major factor in certain juvenile acute myeloid leukemias (von Lindern et al., 1992). DEK also remains bound to the exon-product RNA after splicing, an association requiring the prior formation of a spliceosome, which suggests that DEK may also be a factor in controlling postsplicing steps in gene expression (Le Hir et al., 2000). DEK has recently been shown to introduce constrained positive supercoils into DNA, an induced change that is reversible after the removal of DEK protein (Waldmann et al., 2002). This coiling in nucleosomes serves as a barrier to the entry of proteins involved in transcription, replication, and other DNA processes. Xenopus DEK (314 aa, 35.5 kD) contains a

lysine-rich region (aa 73-256), as well as 33 potential phosporylation sites. Further analysis of the Xenopus DEK coding region showed a high likelihood of nuclear localization, concurrent with the nuclear localization of DEK in Homo sapiens (Fornerod et al., 1995).

To explore the interaction between CPEB and these proteins in ovo, mRNA encoding each was transcribed in vitro and injected into Stage VI Xenopus oocytes, which were subsequently incubated to allow translation. Allowing the translation of these mRNAs in ovo increases the likelihood that post-translational modifications or localization of each protein found during oocyte maturation will be present. Affinity bead binding assays were used to explore the two-hybrid system interactions, in which glutathione agarose beads were used to bind GST-CPEB and to allow separation of HA-X295 or HA-DEK bound to CPEB from the remaining oocyte protein extract. These assays confirmed that X295 and CPEB interact in ovo, while similar experiments with DEK do not show this interaction.

Following these results, research is continuing into the mechanism of this interaction between X295 and CPEB, while binding assay conditions and further sequence information for DEK are being investigated. Further elucidation of the molecular interactions and the roles of these two proteins in meiotic

maturation may lead to a clearer understanding of mechanisms underlying prevalent cancers and early development.

MATERIALS AND METHODS

Supplies and Reagents:

All chemicals were obtained from Sigma-Aldrich. Restriction endonucleases and DNA modifying enzymes were obtained from New

England Biolabs. Primary and secondary antibodies were purchased from Santa Cruz Biotechnology.

Sequencing:

Inserts from the positive clones of X295 and DEK from the yeast two-hybrid screen were sequenced by the Beth Israel Deaconess Medical Center Sequencing Facility using a series of primers (Table 1), and analyzed by BLAST comparison to the National Center for Biotechnology Information (NCBI) Internet nucleotide database (http://www.ncbi.nlm.nih.gov:80/BLAST).

Plasmid Constructs:

In order to amplify the coding regions of DEK and X295 from the pACT2 plasmid, 5′ primers were designed to include a site for the restriction enzyme NdeI, overlapping the predicted start codon. 3′ anti-sense primers were designed to include a site for BglII, and included the stop codons of the original predicted sequences. (Restriction sites in bold.)

The X295 primers used for this amplification were
sense 5′-GTGATA**CATATG**GCAACTTCCTACCCAAGT-3′ and
anti-sense 5′**AATAGAT**CT**T**CACCCGGAAGCTTTTGCCAC-3′.

The DEK primers used for this amplification were
sense 5′-GTGATA**CATATG**AGCGTATCTAAAAAGGAGCT-3′ and

anti-sense 5′-**AATAGAT**CTTCAAGGGTTCATCATCAGAGCTG-3′.

Conditions for the polymerase chain reaction (PCR) were an initial DNA denaturing of 2 minutes at 95°C, followed by 35 cycles each containing 30 second denaturing, 2 minute extension at 54°C (X295) or 57°C (DEK), and 2 minute annealing at 72°C. The products were then extended for 10 minutes at 72°C, and used as below.

The PCR products and an HA-SP6 plasmid were double digested using NdeI and BglII restriction endonucleases, purified through 1:1 phenol-chloroform extraction, and ethanol-precipitated. Using T4 ligase, the PCR product and the digested plasmid were ligated together overnight at compatible sites. All ligations were transformed into E.Coli XL1-blue competent cells (Stratagene) and plated on LB with ampicillin. Potential clones were selected and incubated in 10ml liquid LB Amp overnight, followed by small-scale DNA preparation (Qiagen). A series of digestions with various restrictive enzymes confirmed positive clones, and led to large-scale DNA isolation.

RNA Transcription, Injection, and Protein Translation:

SP6-HA plasmids containing the X295 or DEK insert (pHA-295-SP6, pHA-DEK-SP6, respectively) were linearized overnight using

XbaI. The linear DNA was extracted with 1:1 phenol-chloroform, and ethanol-precipitated. In vitro transcription was performed using the SP6 mMessage Machine (Ambion), and product RNA was analyzed through electrophoresis using a 1% agarose, 8% formaldehyde denaturing gel (Figure 6). GST and GST-CPEB mRNA was also transcribed using the mMessage Machine, following overnight linearization of a stock plasmid (pGST-CPEB, pGST), and confirmed through electrophoresis.

For injection of oocytes, equal volumes of GST-CPEB RNA and HA-X295 or HA-DEK RNA (all 400 ng/ul) were mixed, and 46 nL of the resulting solution were injected into Stage VI Xenopus laevis oocytes. In a negative control, GST RNA was substituted for GST-CPEB RNA. The oocytes were incubated in 1X Barth's saline with streptomycin overnight at 23°C to allow protein synthesis.

Protein Interaction Binding Assays:

The protein extracts were prepared from oocytes expressing various fusion proteins by resuspension in 6 μl/oocyte of 1X Phosphate Buffered Saline (PBS) (140mM NaCl, 2.7mM KCl, 10mM Na_2HPO_4, 1.8mM KH_2PO_4) with 0.1% Triton X-100 and 10 ng/μl each protease inhibitors (Leupeptin, Chymostatin, Pepstatin). The oocyte homogenate was separated into distinct layers by two

centrifugations for 15 minutes each at 8000xG. The middle, protein-containing aqueous layer was removed, equivalent to 100 oocytes of protein extract. One oocyte equivalent was removed here and added to B-mercaptoethanol containing SDS-sample loading buffer, to be analyzed as 'Input'.

The remaining protein extract was incubated with 20μl packed glutathione-agarose beads (Pharmacia Biotech), pre-equilibrated with 1X PBS, for 16 hours at 4°C, rotating over end. RNAse A (0.01 ug/ul) was added prior to incubation. Beads were then pelleted (15 minutes at 2000xG) and washed 3 times with 30 volumes of 1X PBS. One oocyte equivalent of 'Unbound' protein extract was removed after pelleting of the beads, but prior to washing. An equal volume of SDS sample loading buffer was then added to the glutathione beads, heated at 95°C for 4 minutes, and analyzed by 10% SDS-PAGE.

Western Blotting:

The proteins were transferred to a nitrocellulose membrane through an electric wet transfer system (Bio-Rad) for 1 hour at 100 V, and the membrane was then incubated with 5% milk-TBST (0.2M Tris, 1.5M NaCl, 0.05% Tween-20) overnight. For subsequent Western blotting, the primary antibody dilutions in TBST were 1:2000 for mouse monoclonal anti-HA, and 1:7000 for goat

polyclonal anti-GST. The secondary antibody dilutions were 1:10,000 for both donkey anti-goat HRP and goat anti-mouse HRP. The HRP-linked secondary antibodies were detected using a chemiluminesence system (Perkin-Elmer) and exposure of BioMax MR film (Kodak).

RESULTS

Identification Of New CPEB-Interacting Proteins, X295 and DEK

In order to identify proteins that could interact with CPEB and affect its role in early development and maturation, a yeast two-hybrid screen was performed (Attfield, Hunker, and Hake, unpublished) using the amino-terminal half of CPEB fused to the lex-A DNA binding domain as bait. A Xenopus laevis oocyte cDNA library was used as prey, and several potential CPEB-interacting proteins were identified, including X295 and DEK. These proteins

were isolated for further analysis of their roles in oocyte maturation.

The nucleotide sequences encoding X295 (Figure 2) and DEK (Figure 3) were determined using sense and anti-sense DNA primers (Table 1) in a series of sequencing reactions. The X295 DNA sequence contained a 1110 nucleotide coding region, with a 32 nucleotide 5'UTR and 147 nucleotide 3' UTR. Comparisons of the protein-coding region to sequence databases show no homology to characterized proteins. Through sequence motif analysis (ExPASy Proteonomics Tools: http://us.expasy.org/tools/), the predicted X295 peptide encodes a potential zinc-finger domain (aa 238 – 306), a PEST domain (aa 1-22), and two or more potential sumoylation sites (aa 176 – 179, 266-269).

The cDNA encoding Xenopus DEK (942 nuc) shows significant homology to Homo sapiens DEK (Figure 2). A lysine-rich region (aa 73-256) was identified, as well as 33 potential phosporylation sites. Further analysis of the DEK coding region through the Reinhardt method (Reinhardt 1998) showed a high likelihood (94% reliability) of nuclear localization, also in agreement with that of Homo sapiens.

Subcloning of X295 and DEK Into pSP6-HA

Using 5' and 3' DNA primers DEK Fp and DEK Rp (Table 1), the coding region of DEK was amplified from the pACT2 plasmid,

while these primers inserted NdeI and BglI restriction endonuclease sites to the ends of the PCR product. The product was then digested with these enzymes, extracted with phenol-chloroform, ethanol-precipitated, and electrophoresed in a 1% agarose gel. The predicted size of the DEK product is 962 bp., which agrees with the band seen at 1.0 kB (Figure 4a, lane 2).

The X295 coding region was also amplified from the pACT2 plasmid, using 5′ and 3′ primers 295F and 295R (Table 1). The product was digested with NdeI and BglII restriction enzymes, extracted with phenol-chloroform, precipitated with 95% ethanol, and electrophoresed in 1% agarose. The expected PCR product of 1110 bp is seen at roughly 1.1 kB (Figure 4b, lane 2).

Ligation of PCR Products and in vitro Transcription of HA-X295 / HA-DEK mRNA

The digested and purified DEK and X295 PCR products were ligated at compatible sites with an HA-SP6 plasmid construct, previously double digested with NdeI and BglII restriction endonucleases. Ligations were then transformed into XL1-Blue Competent Cells (Stratagene).

The SP6-HA-DEK and SP6-HA-X295 plasmids were linearized using XbaI, and in vitro transcription was done through the mMessage Machine (Ambion). In agarose gel containing

formaldehyde, the HA-DEK transcript (expected to be 962 nucleotides in length) can be seen at roughly 0.8 kB (Figure 6a), while the HA-X295 transcript (expected length of 1110 nucleotides) is seen roughly at 1.3 kB (Figure 6b). It is important to note that these single-stranded mRNAs were electrophoresed using double-stranded DNA as a marker, and in the agarose and formaldehyde gel all samples will be denatured. A DNA marker size of 1.1 kB should correlate with an mRNA size of 2200 nucleotides in the gel. Because the DNA was also denatured in the formaldehyde, it cannot be used as an accurate prediction of mRNA product sizes. Instead, the gel serves to show a single band of intact product mRNA from the in vitro transcription.

<u>CPEB and X295 Interact in ovo</u>

To confirm that X295 interacts with CPEB in Xenopus oocytes, glutathione-S-transferase-tagged CPEB (GST-CPEB) and HA-tagged X295 (HA-X295) mRNA were overexpressed in Stage VI oocytes. Glutathione agarose bead pulldown assays were performed, followed by immunoblotting with anti-HA and anti-GST antibodies (Figure 5). The results of these assays are seen, as HA-X295 binds to beads containing GST-CPEB (Figure 7a, lane 1), but not to beads lacking GST-CPEB (lane 5). Immunoblotting of

the membrane with an anti-GST antibody (Figure 7b) shows equal amounts of GST-CPEB is present only on the beads analyzed in lane 1, while GST is present on beads in lane 5. These results confirm that X295 and GST-CPEB are able to interact in ovo, and imply an interaction in ovo between endogenous CPEB and endogenous X295.

As can be seen in Western blots (Figure 7a), HA-X295 appears through 10% SDS-PAGE to be found in the region of 64 kD. Through the sequence analysis of the predicted HA-X295 protein, a molecular weight of 40 kD was anticipated. There are many possible post-translational modifications in a Xenopus oocyte, such as phosphorylation, or sumoylation. As these experiments were done in vivo, any of this processing that occurs in the oocyte will be seen in the results. One of these modifications is most likely the cause of the shifted band size, and further investigation will continue into the identity and nature of the processing found in these experiments.

Ongoing sequence analysis during binding assays also revealed an important nucleotide mutation at position 399 in the X295 coding region, where an adenine residue was replaced by thymine. In the subsequent transcription and translation of X295, a TTA region coding for the amino acid leucine was instead replaced with a TAA stop codon. If this sequence information is correct, it would lead to the translation of one protein with a

predicted size of 15.8 kD. This size band is not seen, however, through Western blot analysis. Instead, the X295 protein appears in the region of 64 kD. Further work is currently being done to confirm this mutation, by using a series of sequencing reactions more localized to the nucleotide in question.

CPEB and DEK Do Not Interact in GST-CPEB Binding Assays

Following the same procedure as with X295, glutathione-S-transferase-tagged CPEB (GST-CPEB) and HA-tagged DEK (HA-DEK) were overexpressed in Stage VI oocytes. Glutathione agarose bead pulldown assays were performed, followed by immunoblotting with anti-HA and anti-GST antibodies (Figure 5). HA-DEK binds to beads containing GST-CPEB (Figure 8a, lane 1), as well as to beads lacking GST-CPEB (lane 5). Immunoblotting of the membrane with an anti-GST antibody (Figure 8b) shows equal amounts of GST are present on the beads analyzed in lanes 1 and 5, and a significant binding of GST-CPEB to the beads in lane 1. These results indicate that the binding assay used in these experiments cannot confirm an interaction between DEK and CPEB in ovo. However, these proteins may indeed interact in the Xenopus oocyte, and current work is with improving the binding conditions of the assay, further sequence analysis, and potential alternative interaction experiments.

DISCUSSION:

The complicated nature of CPEB's role in oocyte maturation, and its implications for insight into signal transduction

pathways and molecular mechanisms of cancers, led to interest in identifying potential CPEB-interacting proteins. HA-X295, a novel protein showing no homology to characterized genes, interacts in ovo with CPEB as demonstrated by glutathione-agarose bead pulldown assays. These experiments are continuing, as binding conditions and stringency are improved.

As noted previously, recent sequencing reactions have shown an important mutation in the coding region of HA-X295 at position 399, where an adenine residue was replaced by thymine. In the subsequent transcription and translation of X295, a TTA region coding for the amino acid leucine was instead replaced with a TAA stop codon. If this sequence information is correct, it would lead to the translation a shortened protein of molecular weight 15.8 kD, although this size is not currently seen through Western blotting. Instead, the X295 band appears at roughly 64kD, also in disagreement with the predicted size 39.8 KD. Further work is currently being done with more localized sequencing reactions to confirm this mutation, as well as to identify other key domains in the X295 sequence.

Similar binding assays with HA-DEK are not able to confirm the interaction found through the yeast two-hybrid screen, or that DEK and CPEB interact in ovo. It is still possible that these proteins do interact in the Xenopus oocyte, but this interaction is not seen in the current binding assay. Currently,

work is with improving the binding conditions and oocytes used in the assay, sequence analysis and motif identification, and potential alternative interaction experiments. The continuing elucidation of the molecular roles of these two proteins in meiotic maturation will lead to a clearer understanding of mechanisms underlying prevalent cancers and early development.

FIGURE LEGENDS

FIGURE 1a

Nucleotide Sequence of X295

Shown is the complete X295 nucleotide sequence in the SP6-HA plasmid. Features highlighted include: SP6 promoter, Globin 5' untranslated region, HA-tag, start and stop codons, sequencing primers used, Globin 3' UTR, and poly (A) tail.

FIGURE 1b

Predicted Peptide Sequence of HA-X295

The predicted HA-X295 protein is 39.8 kD with a pI of 9.25. Features highlighted include: potential zinc-finger region, potential sumoylation sites, and possible PEST domain.

FIGURE 2a

Nucleotide Sequence of DEK

Shown is the complete X295 nucleotide sequence in the SP6-HA plasmid. Features highlighted include: SP6 promoter, Globin 5' untranslated region, HA-tag, start and stop codons, sequencing primers used, Globin 3' UTR, and poly (A) tail.

FIGURE 2b

Predicted Peptide Sequence of HA-DEK

The predicted HA-DEK protein is 39.5 kD with a pI of 9.63.

Feature highlighted: highly acidic domain (aa 30-49).

FIGURE 2c

DEK Peptide Sequence Homology Between *Xenopus* and*Homo sapiens*

Using the BoxShade application (http://www.ch.embnet.org/software/BOX_form.html), the significant homology of DEK in these species is seen. Black shading indicates sequence identity, gray shading indicates sequence similarity.

FIGURE 3

Schematic of Cloning Protocol

An outline of the cloning strategy is shown, representing methods from initial PCR through ligation.

FIGURE 4

Polymerase Chain Reaction Products of DEK and X295 Coding Regions from pACT2 Plasmid

Results of PCR were analyzed through electrophoresis on 1% agarose. Lane 1 of each gel contains 1 μg phage lambda DNA digested with PstI, serving as a molecular size marker. Lane 2 of each gel contains 4 μl of the DEK or X295 PCR product. The DEK

product (a) can be seen at roughly 0.9 kB, which agrees with the predicted size of 962 bp. The X295 product (b) appears at 1.3 kB, while its predicted molecular size is 1.1 kB.

FIGURE 5

Outline of Binding Assay

The protocol for the binding assays used to confirm the interactions between X295/DEK and CPEB is shown.

FIGURE 6

Analysis of DEK and X295 in vitro Transcribed mRNA

The RNA products were electrophoresed on a 1% agarose, 8% formaldehyde denaturing gel. In both the DEK and X295 gels, lane 1 contains 1 μg phage lambda DNA digested with PstI, serving as a molecular size marker. Lane 2 of each gel contains 4 μl of the in vitro transcription product. The DEK mRNA transcript is seen at 0.8 kB, while the X295 transcript is seen at 1.1 kB.

FIGURE 7

CPEB and X295 Interact in ovo

For injection of oocytes, equal volumes of GST-CPEB RNA and HA-X295 RNA (400 ng/ul) were mixed, and 46 nL of the resulting solution were injected into Stage VI Xenopus laevis oocytes. In

a negative control, GST RNA was substituted for GST-CPEB RNA. After overnight incubation, oocyte protein extracts were prepared, then incubated with glutathione-S-transferase agarose beads for 8 hours. Bead-bound (Beads), the original protein extract (Input), and the unbound protein extract after bead incubation (Unbound) were analyzed by 10% SDS-PAGE and immunoblotting with either anti-HA (top) or anti-GST (bottom) antibodies.

FIGURE 8

<u>DEK and CPEB Do Not Interact in the Current Glutathione-Agarose Pulldown Assay</u>

For injection of oocytes, equal volumes of GST-CPEB RNA and HA-DEK RNA (400 ng/ul) were mixed, and 40 nL of the resulting solution were injected into Stage VI Xenopus laevis oocytes. In a negative control, GST RNA was substituted for GST-CPEB RNA. After overnight incubation, oocyte protein extracts were prepared, then incubated with glutathione-S-transferase agarose beads for 8 hours. Bead-bound (Beads), the original protein extract (Input), and the unbound protein extract after bead incubation (Unbound) were analyzed by 10% SDS-PAGE and immunoblotting with either anti-HA (top) or anti-GST (bottom) antibodies.

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ACKNOWLEDGEMENTS

First and foremost I would like to thank the continuing support and education I received from Dr. Laura Hake, as well as the members of my lab (Brian Keady, Carlos Garcia-Reverte, Charlemagne Lacza, Lei Yuan, Susana Martinez, and Heather Ransom) for their critical insights and assistance. I would also like to acknowledge the support of my family (Thomas, Grace, and Matthew Raher), and close friends who provided constructive advice. I am extremely grateful for the dedication of Leominster public school teachers David Caporello, Mark Siemaszko, and William Trembley, for their fostering of my early scientific interests, as well as those of thousands of other future scientists.

I feel I must also recognize those who played a pivotal role in setting the foundations for my education, specifically Mr. Fred Rogers, 'Mr. Wizard', and the writers of the television programs 'Sesame Street' and 'Reading Rainbow'. Without their

incredible gifts for stimulating the intellectual interests of young children, and the countless hours I spent enjoying and learning from them, I would not be in the position that I am in today.

Finally, I would like to thank the Department of Biology at Boston College for its support, through the advice of various professors and graduate students, as well as the Beckman Foundation, for its financial generosity toward my research.

La fin est seulement le commencement…