

Human fertility protein PUMILIO2 interacts *in vitro* with testis mRNA encoding Cdc42 effector 3 (*CEP3*)

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Received: 5 April 2006; accepted: 15 June 2006

SUMMARY

PUMILIO protein regulates translation of specific mRNAs in morphogenesis and in development of the germ-line of model organisms such as flies and worms. Given that a human homologue (PUMILIO2) was recently identified in the germ-line stem cells, the question was raised whether it regulates translation of fertility mRNAs similarly to *Drosophila* Pumilio. Here, we describe a candidate mRNA encoding Cdc42 effector protein 3 (CEP3), however, a function for this protein in reproduction has previously not been reported. We detected three *CEP3* transcripts in the testis tissue including one which was highly expressed and testis specific by northern blotting. We found that *CEP* mRNA contains GUUGU (A) and AUUGUA (B) motifs (ABB) within the 3' untranslated region (3'UTR), which are also present in mRNA targets of Pumilio in *Drosophila*. Interaction of PUMILIO2 with

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the fragment of *CEP3* transcript containing the ABB array was tested by mobility shift assay and we found that PUMILIO2 binds the 3' untranslated region of the *CEP3* mRNA. These results support the hypothesis that *CEP3* mRNA may be a target of PUMILIO2 protein in the human male gonad and be under translational control mediated by specific nucleotide motifs within the 3'UTR. *Reproductive Biology* 2006 6:103–113.

Key words: germ cells, Cdc42 effector 3, PUMILIO2, translation

INTRODUCTION

From early development, the mechanism of translational regulation is used to precisely dose protein concentration. In the past few years, mRNA specific translational control by *cis*-elements located in the 3' untranslated region (3'UTR) was found to be crucial in body patterning and germ line development of model organisms [8]. One well-studied case is translational regulation of the *hunchback* (*hb*) morphogene during early embryogenesis in *Drosophila*. Two conserved RNA-binding proteins, Pumilio and Nanos, are specifically required for repression of the *hb* translation to promote development of the posterior [1, 2, 9, 13]. The Pumilio RNA-binding domain known as PUF (for Pumilio and FBF – the *C elegans* homologue domain) binds two bipartite 32-nucleotide sites in the 3'UTR of *hb* (Nanos Response Elements – NREs), each of them containing two short nucleotide motifs GUUGU and AUUGUA which are specifically recognized by Pumilio [11, 14, 15, 16]. Since human homologue of this protein (PUMILIO2) was recently identified and was found to be germ cell specific [6, 10], the question was raised whether it binds mRNAs to regulate expression of specific fertility factors similarly to *Drosophila* ancestor. Although it was recently reported by *in vitro* approaches as well as by experiments in yeast cells that human PUMILIO2 protein specifically binds *SDADI* mRNA, the role of this interaction in translational regulation of *SDADI* mRNA was not shown [4].

There are two Pumilio homologous genes in the human genome [12]: besides the germ cell specific *PUMILIO2* there is *PUMILIO1* which is

expressed in all tested tissues [10]. It was previously demonstrated that human PUMILIO1 PUF domain, which is highly similar to PUMILIO2 PUF domain [12], binds *in vitro* *hb* NRE GUUGU and AUUGUA motifs with high affinity [16]. Hence, we assumed that PUF-domains of human PUMILIO1, PUMILIO2 and the one of *Drosophila* Pumilio might all have equivalent nucleotide sequence requirements for RNA-binding. To test this possibility we first screened the GeneBank for genes encoding GUUGU and AUUGUA motifs in their 3'UTR regions.

MATERIALS AND METHODS

Electronic screening of the GeneBank for candidate mRNAs

We designed software to retrieve from the GeneBank DNA sequences containing two short nucleotide motifs GUUGU (A) and AUUGUA (B). From the initial pool of sequences which we obtained using this approach we subsequently selected genes containing both A and B motifs in their 3'UTR regions.

Tissue expression

A 308 bp cDNA fragment encoding 3'UTR of *CEP3* was PCR amplified from Human Testis Marathon-Ready cDNA (BD Clontech) using the following conditions: the forward 5'TGTTGGTAAGACACAGGCAAA, and the reverse 5'TGTGGTGTAGCCCACAGTCT primers, 0.5 μ M each, dATP, dGTP, dCTP, dTTP, 0.05 mM each, 40 mM Tritin-KOH, 15 mM potassium acetate, 1.5 mM magnesium acetate, 3.75 μ g/ml BSA, 0.005% Tween20, 0.005% Nonidet P40 and about 0.5 U Advantage Taq polymerase (DB Clontech). Initial denaturation was at 94°C, 5 min, and 30 PCR cycles were performed with denaturation at 94°C, 45 s, annealing at 65°C, 45 s, synthesis at 68°C, 1 min, final synthesis at 68°C, 4 min. Amplification products were analyzed in 2% agarose gels in TBE buffer (0.045 M Tris pH 8.0, 0.045 M boric acid, 2 mM EDTA). DNA fragment of the expected

size was purified from agarose gel using QIAquick Gel Extraction Kit (Qiagen), and was subjected to digestion with *AccI* restriction enzyme (New England BioLabs) analysis to confirm its identity.

The expression pattern of the *CEP3* gene in the adult human tissues was analyzed using northern blotting. For that purpose the 308 bp PCR product corresponding to 3'UTR of *CEP3* and encompassing the ABB array was radioactively labeled at the presence of ^{32}P -dCTP using Multiprime Labeling kit (Roche), purified and used as the probe to hybridize with a series of human RNA samples from different adult human tissues immobilized on nylon blot (BD Clontech). Hybridization was performed in Expresshyb solution (BD Clontech) 1 h at 68°C. Filters were washed at the room temperature in 2xSSC, 0.05% SDS, followed by wash at 50°C in 0.1xSSC, 0.1% SDS 30 min, and exposed for autoradiography at -80°C.

***In vitro* transcription**

The transcription template of 263 bp was synthesized from 308 bp PCR product by amplification using *CEP3* TRF 5'GGGGTAATACGACTCACTATAGGGTGCCTTTGGCTTT-TTATTGCAGAG and *CEP* TRR 5'G TGGTGTAGCCCACAGTCTCTGCAG nested primers. The *CEP3* TRF forward primer was significantly longer since it contained the promoter for T7 RNA polymerase (5'TAATACGACTCACTATAGG; fig. 1). This nested pair of primers was encompassing GUUGU (A) and AUUGUA (B) motifs within 3'UTR of *CEP3* mRNA, and as little as possible of the flanking sequences (fig. 1). Transcription template was synthesized at the presence of 0.5 μM nested primers *CEP3* TRF and TRR, 0.05 mM each dNTP, 10 mM Tris-HCl pH 8.8, 50 mM KCl, 0.08% Nonidet P40, 1.5 mM MgCl_2 and 1U of Advantage Taq polymerase (BD Clontech). The initial denaturation was at 95°C, 5 min, followed by 15 amplification cycles: (denaturation at 95°C, 45 s, annealing of the primers 58°C, 1 min, synthesis at 68°C), 20 cycles: (denaturation at 95°C, 45 s, annealing of the primers at 68°C, 1 min, synthesis at 68°C, 2 min, 30 s). *In vitro* transcription was performed at the presence of the 263 bp template, ATP, GTP and CTP 25 mM each, 10 mM UTP, 40 mM Tris pH 7.5, 24 mM MgCl_2 , 2 mM

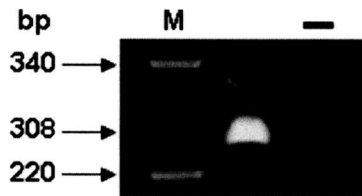
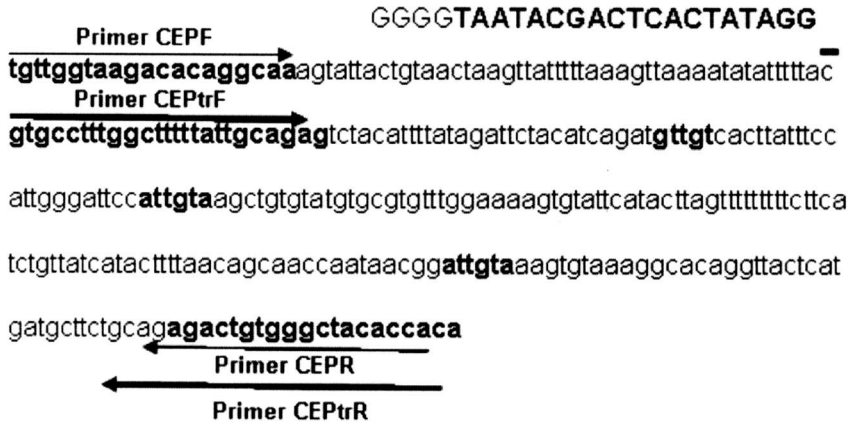


Figure 1. Amplification from human testis tissue of cDNA sequences encoding 3'UTR of *CEP3* mRNA and *CEP3* 308 bp PCR. Primers used to detect *CEP3* transcript in human testis tissue are designated with a fine line (Primer CEPF and CEPR, respectively), while primers used for generation of the transcription template are in bold (Primer CEPtrF and CEPtrR, respectively). The sequences added to the transcription forward primer to introduce the T7 promoter into the transcription template are presented at the top (in bold). Short nucleotide motifs **gttgt** and **attgta** are in bold. The panel at the bottom represents the amplification result of the *CEP3* 3'UTR, M – indicates marker of DNA size, the second lane *CEP3* PCR product from the testis while the last lane the negative control (no template added).

spermidin, 0.01% triton x-100, 10 mM DTT, 1U RNAsin (Promega), 120U T7 RNA polymerase (Promega) and ^{32}P -UTP 3000 Ci/mmol at 37°C, 20 h. DNA template was subsequently digested at the presence of 2.5U of RNase free DNase (Promega). Transcript was purified on Sephadex G-25^{FINE} column and run in 9% polyacrylamide gels containing 7 M urea in TBE buffer. After autoradiography exposure transcripts were excised

from gel and eluted in buffer containing 0.225 M sodium acetate pH 5.2 phenol saturated with 0.1 M Tris pH 4.0, at 37°C, overnight. RNA was ethanol precipitated. Transcripts were analyzed in 9% polyacrylamide 7 M urea gel and their specific activity was estimated in scintillation counter. Transcript encoding A and B motifs (ABAB) of *Drosophila* hunchback 3'UTR (*hb* 3'UTR) was used as positive, and a random RNA with neither A nor B motifs (nonspecific – NSP) as negative controls for PUMILIO2 binding in mobility shift assay. These control transcripts were prepared according to Fox et al. [4].

Overexpression and purification of PUMILIO2 protein

Fragment of PUMILIO2 cDNA encoding the C-terminal RNA-binding PUF domain was cloned in fusion with INTEIN-tag (N-terminal fusion) in TYB12 vector using the IMPACT-CN system (New England Biolabs). This construct was used to transform bacteria strain BER 2566. T7 promoter for synthesis of the fusion protein was induced by IPTG at the 0.3 mM final concentration, and the culture was continued overnight at 15°C. Bacteria were harvested and sonicated in buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 7.5, 0.5% triton x-100, and a mixture of protease inhibitors: 0.003 mg/ml pepstatin A, 0.001 mM leupeptin, 5.08 mM benzamidin, 0.013 mg/ml aprotinin, and 0.26 mg/ml AEBSF. Fusion protein was bound to chitin conjugated agarose beads due to the presence of CBD (*Chitin Binding Domain*) within INTEIN-tag. Beads were washed and PUMILIO2 RNA-binding domain was harvested after 40 h incubation at the presence of 0.1 M DTT at 4°C.

Mobility shift assay

Radioactively ³²P labeled 3'UTR *CEP3* transcript, and control transcripts were denatured at 90°C, 3 min and incubated on ice with purified PUMILIO2 protein (900 nM down to 50 nM) in 0.02% Tween 20, 1 mM EDTA, 10 mM HEPES, 50 mM KCl, 2 mM DTT, 0.1 mg/ml BSA, 5% glycerol, and competitors: 0.01 µg/µl yeast tRNA, 0.1 µg/µl synthetic polyU and 0.03 µg/µl heparin. After 1 hour incubation samples were loaded on 4% native

polyacrylamide gel containing 2.5% glycerol and run in TBE buffer at 4°C, 5 W, 2 hours. Gels were dried and autoradiography was performed overnight at -80°C.

RESULTS

In the search for human PUMILIO2 mRNA targets we assumed that they contain A and B motifs within 3'UTR regions identical to Pumilio motifs in *Drosophila* mRNAs targets. By electronic screening of the GeneBank for DNA sequences with A and B motifs we identified human gene encoding Cdc42 effector protein 3 (*CEP3*; Acc NM006449) which is also known as Borg 2 (binder of Rho GTPases) [7]. The *CEP3* mRNA contains one A (GUUGU), and two B (AUUGUA) motifs which are ordered as ABB array. The key finding was that this ABB array is localized within 3'UTR region of the *CEP3* mRNA. Moreover, this region is noteworthy for its high uridine content (fig. 1) which is reminiscent to *Drosophila* NREs of the Pumilio mRNA targets in [3].

The important question was whether the candidate *CEP3* mRNA is expressed in the testis tissue given that PUMILIO2 protein functions in the male reproductive system. To address this issue we performed PCR amplification on testis cDNA library as the template using primers complementary to the *CEP3* 3'UTR and encompassing ABB motifs. As it is shown in Figure 1 we obtained a product of the expected size 308 bp. This result indicates that *CEP3* mRNA is present in the human male gonad.

We next sought to get more insight into the expression profile of the *CEP3* gene in the human reproductive tissues. Although expression study of this gene in the human tissues was previously reported, and variety of transcripts was identified, *CEP3* expression in gonads was not shown so far [7]. We performed northern blot hybridization on RNA samples representing testis and ovary among other adult tissues. In this experiment, as the hybridization probe we used the 308 bp fragment of the *CEP3* 3'UTR. By this approach we detected three distinct testicular transcripts of about 6.0, 3.5 and 2.4 kb

respectively, corresponding to the *CEP3* gene. Two among them, 6.0 and 2.4 kb were present in all tested tissues, while the most prominent, the 3.5 kb transcript was testis specific (fig. 2). Both ubiquitous transcripts probably

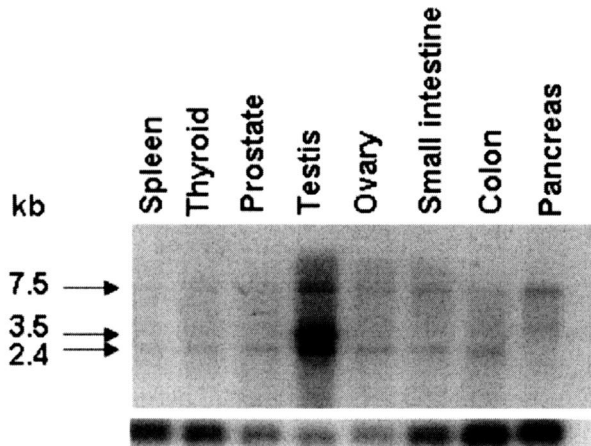


Figure 2. Northern blot to detect tissue expression of *CEP3* gene. RNA samples on the blot originated from different human tissues of an adult (Clontech). A cDNA fragment representing the 3'UTR of *CEP3* was used as the probe for detection of *CEP3* transcripts (upper panel) and actin cDNA for RNA quantification in each lane (bottom panel).

correspond to the transcripts detected in previous study [7]. However, the authors did not precise which portion of *CEP3* cDNA was used as hybridization probe. In the GeneBank of the NCBI homepage there are five cDNA clones (Acc. NM006449, AL136842, AK128735, BC019270 and AF104857) corresponding to the human *CEP3* gene. All of them encode the same amino acid sequence indicating that they all originate from the same gene, and are not products of alternative splicing. Given that there is no information about completeness of these five transcripts it is not possible to assign them to the transcripts which were detected in this study using the 308 bp 3'UTR probe. However, two transcripts AL136842 (isolated from testis) and BC019270 (isolated from lung small cell carcinoma) seem to be complete on the 3' since they both contain a poly(A) tail. Interestingly, these tails starts at different sites indicating that alternative polyadenylation

DISCUSSION

Here, we report that human fertility protein PUMILIO2 *in vitro* recognizes 3'UTR of the *CEP3* mRNA. This mRNA is particular by the presence of specific GUUGU and AUUGUA motifs (ABB) within 3'UTR. These motifs are identical with A and B motifs previously found within 3'UTR NRE of *hb* (ABAB), *bicoid* (ABB), and *cyclin B* (ABB) mRNAs, the known Pumilio targets in *Drosophila* [3].

The CEP3 protein belongs to the family of downstream effector proteins of CDC42, a small Rho GTPase involved in cytoskeleton formation, cell adhesion, cell spreading, motility and cytokinesis [5, 7]. Although a role of CEP3 in fertility was not reported so far, presence of several transcripts in the testis tissue, as well as prominent expression of a one testis specific transcript indicates that CEP3 may be involved in germ cell development. Although the study of ribonucleoprotein complex formation presented here is preliminary, and requires confirmation by *in vivo* tests, it is likely that interaction of PUMILIO2 with *CEP3* 3'UTR by *in vitro* approach may reflect important posttranscriptional regulatory pathway in the human germ cells. Interestingly, analysis of multiple *CEP3* transcripts which was performed in this study indicates that due to differential polyadenylation some of the *CEP3* transcripts contain ABB array in 3'UTR while some other do not. This finding could imply that sequestration of *CEP3* mRNA by PUMILIO2 protein may depend on alternative polyadenylation of the *CEP3* transcripts, and that these two processes are tightly coordinated.

Altogether, our results support hypothesis that *CEP3* mRNA may be a target of PUMILIO2 in the male gonad. Further studies are underway to test this possibility and to find out whether the function of Pumilio as translational regulator is evolutionarily conserved including humans.

ACKNOWLEDGMENTS

We thank Dr. Robert P. Erickson for critical reading of the manuscript. This study was supported by State Committee for Scientific Research as a Solicited Project PBZ-KBN-084/PO6/2002 (2003 to 2005).

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