CPEB2, A Novel Putative Translational Regulator in Mouse Haploid Germ Cells

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ABSTRACT

Translational control of specific mRNAs by cytoplasmic polyadenylation has fundamental roles in gametogenesis. The cytoplasmic polyadenylation element binding (CPEB) protein regulates cytoplasmic polyadenylation of mRNAs as a trans factor in oogenesis and spermatogenesis. The CPEB protein contains two RNA recognition motifs and a Zn-finger structure. Proteins (KIAA0940 and KIAA1673) with similar structures are predicted from the genome database, but nothing is known about their expression and function. Here, we report another novel member of the CPEB protein family, CPEB2. Comparison of the amino acid sequences of CPEB family members suggests that the family can be divided structurally and, perhaps, functionally into two groups: the CPEB group, and the CPEB2-KIAA0940-KIAA1673 group. The CPEB2 maps to mouse chromosome distal 5B and is abundantly expressed in testis. However, it was detected by reverse transcription-polymerase chain reaction in all tissues that we examined. It preferentially binds to poly(U) and localizes to the cytoplasm in transfected HeLa cells. The CPEB2 is expressed postmeiotically in mouse spermatogenesis, suggesting a possible role in translational regulation of stored mRNAs in transcriptionally inactive haploid spermatids.

spermatid, spermatogenesis, testis

INTRODUCTION

Spermatogenesis is a multistep and highly organized process in which self-renewing precursor cells continuously give rise to mature sperm. In addition to transcriptional regulation, specific posttranscriptional controls, such as germ cell-specific mRNA splicing, mRNA transport between haploid cells through syncytial bridges, and postmeiotic mRNA storage and translational regulation, are essential for the proper progression of spermatogenesis [1, 2]. These regulatory pathways assure the continuous production of mature sperm.

During murine spermatogenesis, the developing haploid genome becomes transcriptionally dormant [3, 4]. Many mRNAs that are required for nuclear shaping and the processes of spermiogenesis during the haploid stage are stored in the cytoplasm of spermatids, until they are recruited for translation days later. This tight translational regulation of specific mRNAs permits changes in protein levels to occur in the absence of new transcription.

One of the mechanisms regulating translational activation or repression of eukaryotic mRNAs involves dynamic changes in the length of their poly(A) tails in the cytoplasm [5]. This phenomenon, known as cytoplasmic polyadenylation, is mediated by cytoplasmic polyadenylation element binding (CPEB) protein, has been well studied during Xenopus oocyte maturation, in which poly(A) addition is associated with translational stimulation [6–9]. In mammalian oogenesis and spermatogenesis, translational regulation is modulated by a change in adenylation. During oogenesis, the poly(A) tail of tissue-type plasminogen activator mRNA is deadenylated in the cytoplasm, resulting in translational silencing [10]. In contrast, poly(A) shortening is associated with the translational activation of protamine mRNA during late spermatogenesis [11]. The specific poly(A) elongation and shortening in the cytoplasm are mediated by interactions of specific cis elements, the cytoplasmic polyadenylation element (CPE), and trans factors, RNA-binding proteins (CPEB). In addition to CPEB, the human and mouse have two additional members of this family of proteins, named KIAA0940 and KIAA1673. Although their precise functions are unknown, they may act as translational regulators, dividing their functions both spatially and temporally. Considering the complexity of posttranscriptional gene regulation during spermatogenesis, many additional germ cell-specific RNA-binding proteins are likely to have important functions in male germ cells.

A large number of RNA-binding proteins share an evolutionarily conserved, 80- to 90-amino acid domain known as an RNA-recognition motif (RRM). This domain includes two ribonucleoprotein identifier sequences, RNP-1 and RNP-2 [12, 13]. In the present study, we have applied a degenerate polymerase chain reaction (PCR) strategy to clone another RRM-containing protein, a CPEB-related protein (CPEB2), from mouse. We report the characterization of CPEB2 below.
TABLE 1. Oligonucleotide primers.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Nucleotide sequence (5’ → 3’)</th>
<th>Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>RNP-1</td>
<td>GGGCGGATCCATTGCTACAGGACGCG</td>
<td>RM1 (amino acids 276–282)</td>
</tr>
<tr>
<td>RNP-2</td>
<td>GGGCGGATCCAGTGTCTCTTGCGG</td>
<td>RM2 (amino acids 411–416)</td>
</tr>
<tr>
<td>CPEB2-MIP2</td>
<td>TGGAGATGATGATGCGC</td>
<td>N-terminal</td>
</tr>
<tr>
<td>CPEB2-M2P1</td>
<td>AGCTCTTGGGGATTTT</td>
<td>C-terminal</td>
</tr>
<tr>
<td>CPEB2A</td>
<td>ATGAATTCTCCTACAGC</td>
<td></td>
</tr>
<tr>
<td>CPEB2B</td>
<td>TTATTCGGCAGGAAGGATGATGAT</td>
<td></td>
</tr>
</tbody>
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MATERIALS AND METHODS

Cloning of CPEB2 cDNA by Degenerate PCR

Total RNA was extracted from male mouse (BALB/c/J) brains. After purification by Oligo d(T)25, total RNA (2 μg) was reverse transcribed with AMV reverse transcriptase (Life Science, St. Petersburg, FL) with d(T)25 primer according to the manufacturer’s protocol. The PCR reaction mixture (10 μl) contained 3 ng of single-stranded cDNA, 1× PCR buffer (Applied Biosystems, Foster City, CA), 1.5 mM MgCl2, 250 μM dNTP, 100 pmol of each primer, and 0.25 unit of AmpliTaq DNA polymerase (Applied Biosystems). To clone RR-M1P2 and CPEB2-M2P1 primers based on an alignment of the eight- and six-amino acid stretches by treatment with 0.1 M triethanolamine/0.25% acetic anhydride for 10 min at room temperature, the sections were subjected to in situ hybridization. The mRNA in situ hybridization histochemistry was carried out on tissue sections as outlined in the DIG System protocol with a hybridization temperature of 58°C in a buffer containing 50% formamide.

Chromosome Mapping of CPEB2 Gene by Fluorescence In Situ Hybridization

The direct R-banding fluorescence in situ hybridization (FISH) method was used for determination of mouse chromosomal localization of the CPEB2 gene. Preparation of R-banded chromosomes and FISH were performed as described by Matsuda et al [15] and by Matsuda and Chapman [16]. The same 1683-bp cDNA fragment of CPEB2 used for mRNA in situ hybridization was labeled by nick translation with biotin-labeled 1683-bp cDNA (Roche). The hybridized biotinylated probes were reacted with goat anti-biotin antibody (Vector Laboratories, Burlingame, CA) and then stained with fluorescent donkey anti-goat immunoglobulin G (Nordic Immunological Laboratories, Tilburg, Netherlands) at 1:500 dilution for 1 h at 37°C. The hybridization signals were visualized with Nikon filter sets B-2A and UV-2A (Nikon Corporation, Tokyo, Japan). Kodak Ektachrome ASA 100 films (Eastman Kodak, Rochester, NY) were used for microphotography.

Northern Blot Analysis and RT-PCR

Total RNA (15 μg) from each tissue sample was separated on a 1.5% agarose-formaldehyde gel and transferred to a Hybond-N+ membrane (Amersham Pharmacia Biotech, Piscataway, NJ) in 50 mM NaOH, 200 mM NaCl, 1 mM EDTA, and 1% BSA. Microfiltration of the product was PCR amplified using CPEB2A and CPEB2B primers with pTU Turbo DNA polymerase (Stratagene). The cycling reaction was performed in a PCR thermal cycler (Applied Biosystems) at 94°C for 1 min, 58°C for 4 min; 30 cycles of 94°C for 30 sec; 58°C for 30 sec; and 72°C for 30 sec.

Transfection of GFP-CPEB2 Expression Plasmid

The full-length coding sequence of CPEB2 cDNA was subcloned into the pEGFP C1 vector, which produces a fusion protein with the green fluorescent protein at the N-terminus of CPEB2. HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Cells were transiently transfected with the plasmid construct using Polyfect transfection reagent (Qiagen, Hilden, Germany) according to the manufacturer’s protocol. Cells were grown on slide coverglasses (Matsum-ami Glass, Tokyo, Japan) and fixed at 24-h posttransfection using 4% PFA/ PBS, washed of twice in PBS, and mounted. Thirty minutes before fixation, 0.6 μg/ml of Hoechst 33258 was added to the cultures for nuclear staining.
RESULTS

CPEB2 Sequence

One of the cloned CPEB2 amplification products (no. 51-36) of 145 bp in length contained an open reading frame (ORF) whose deduced amino acid sequence revealed the presence of RNP-2 and RNP-1 sequences at the N- and C-termini, respectively, with several identifier sequences between the RNP-1 and RNP-2 motifs [12, 13]. The sequence of no. 51-36 differed from those of similar genes in human and mouse databases. To further establish if no. 51-36 is a novel RRM protein, it was used as a hybridization probe to isolate longer cDNA clones from a mouse seminiferous tubule cDNA library. After screening 4 × 10⁶ phage, we obtained two positive phage clones. Nucleotide sequence analysis of the two cDNA inserts, containing sequences identical to that of no. 51-36, yielded 1771- and 1700-bp sequences, respectively. Both had essentially the same sequence except for the lack of 71 bp at the 5'-terminus.
mRNA in the testis. The initiation ATG codon was located at nucleotides 113–115, which reside in a Kozak consensus sequence [18]. A predicted 0.3-kilobase (Kb) 3′-noncoding sequence included a poly(A) tail and a polyadenylation signal (AATAAA). From our protein database survey against deduced amino acid sequences, the protein showed significant homology to Xenopus, mouse, and human CPEBs, proteins involved in translational regulation of specific mRNAs, and with KIAA0940 and KIAA1673. Therefore, we named the protein CPEB2.

When we compared the amino acid sequence homologies of CPEB family proteins, CPEB2, KIAA0940, and KIAA1673 were much closer to each other than to CPEB. Particularly, amino acid sequences in the RRM-Zn finger region, which might be required for specific RNA binding, were almost identical. Next, we examined genetic distance as calculated by the Kimura corrected distance algorithm among CPEB family proteins from various organisms (Fig. 1d). From the tree, CPEB family proteins can be divided into two subgroups. One includes CPEB2-KIAA0940-KIAA1673, Drosophila CG5735, and Caenorhabditis elegans Cpb-1 and Cpb-2. A second group includes CPEB proteins from Spisula, Aplysia, zebrafish, Drosophila, Xenopus, mouse, and human proteins.

**Chromosome Mapping of the Mouse CPEB2 Gene**

The chromosome location of the CPEB2 gene was determined by R-banding FISH using a 1683-bp cDNA fragment as a probe (Fig. 2). The CPEB2 gene localized to mouse chromosome 5B distal [19]. From the human genome database, human CPEB2 gene was found on chromosome 4q32-33 (GenBank accession no. NT_022941.2).

**Expression of mCPEB2 mRNA**

To determine the expression profile of CPEB2 mRNA, we carried out Northern and RT-PCR analyses. Northern hybridization for samples from various mouse tissues showed that the CPEB2 probe gives rise to a prominent, approximately 2.5-kb transcript in testis exclusively (Fig. 3a). Because we originally cloned CPEB2 from a brain cDNA library, we next examined the expression of CPEB2 in various mouse tissues using RT-PCR. The DNase-treated mRNAs from various mouse tissues were reverse transcribed and PCR amplified using CPEB2-M1P2 and CPEB2-M2P1 primers. Expected size of amplified band was 421 bp.
by RT-PCR, which demonstrated that all tissues examined (cerebrum, cerebellum, salivary gland, thymus, liver, spleen, kidney, intestine, testis, and ovary) expressed CPEB2 mRNA (Fig. 3b). These data suggested that although the expression of CPEB2 is below the detection level by Northern blot analysis in all tissues but testis, it is ubiquitously expressed in mouse tissues.

Next, we determined the cell type(s) expressing the cpeb2 mRNA in testis by mRNA in situ hybridization. The 1683-bp cDNA fragment was used for generating antisense and sense riboprobes. The sense probe did not give any hybridization signals at any stages of spermatogenesis (Fig. 4, b and d). Lower magnification (100×) revealed that the distribution of hybridization signals obtained by antisense probe displayed a stage-specific pattern (Fig. 4a). Higher magnification (200×) revealed that spermatogonia and spermatocytes did not express detectable levels of CPEB2 mRNA and that CPEB2 mRNA was confined primarily to round spermatids of steps 1–7 (Fig. 4c), showing the haploid expression. These cells were identified by their location within the seminiferous tubule, their nuclear morphology, and the germ cell composition of the tubule [20].

CPEB2 Binds to poly(U)

Despite much effort to express recombinant CPEB2 protein in Escherichia coli, we were unable to produce a soluble form of the protein. Therefore, to examine the RNA-binding ability of CPEB2, we used denaturing conditions and Northwestern blot analysis (Fig. 5). CPEB2 binds to poly(U), but not to poly(A), poly(G), or poly(C), in the presence of 300 mM NaCl. The binding of CPEB2 to poly(U) requires renaturation with 100 μM ZnSO₄. The binding after incubation with ZnSO₄ suggests that CPEB2 might coordinate Zn²⁺ into its Zn-finger motif in its C-terminus. The binding is reduced as the salt concentration is increased. We conclude that CPEB2 binds to poly(U) preferentially and that the two RRM domains and Zn finger are sufficient for binding.

CPEB2 Protein Localizes in Cytoplasm

To determine the subcellular localization of CPEB2, transfections were performed in HeLa cells using a construct producing a fusion protein of CPEB2 and green fluorescent protein (GFP). Cell and nuclear shapes were identified through nuclear staining by Hoechst 33258 and phase-contrast microscopy. Fluorescent microscopy revealed that the CPEB2-GFP fusion protein was exclusively localized in the cytoplasm of the HeLa cells (Fig. 6). Control transfections with GFP alone did not give any localization.

DISCUSSION

Cloning of the CPEB2 Gene

The deduced amino acid sequence of CPEB2 indicates that it is a novel member of the CPEB protein family. Originally, CPEB was described as a protein that binds the CPE sequences in the 3′ untranslated region (UTR) of c-mos mRNA serving as a key regulator of Xenopus oocyte maturation and is involved in translational regulation [7]. The CPEB contains two RRM domains and a Zn-finger motif as an RNA-binding domain. Both of the RRM domains and the Zn finger are required to bind CPE [21]. Similarly, CPEB2 may form a Zn-finger structure, because ZnSO₄ is facilitated in the renaturation of recombinant CPEB2 protein in the Northwestern blotting analysis with poly(U) (Fig. 5). In addition, recombinant CPEB2 protein binds to Zn-chelate...
FIG. 5. Analysis of the RNA-binding properties of CPEB2 by North-western blot analysis. Purified recombinant CPEB2 protein was run in 12% SDS-PAGE gels and transferred to polyvinylidene fluoride membranes. The membranes were incubated with renaturation buffer and further incubated with 32P-labelled RNA homopolymers in binding buffer at 300–500 mM NaCl. After extensive washing with binding buffer including various concentrations of NaCl, the membranes were dried and exposed.

columns (data not shown), as described by Meetei and Rao [22].

Most of the sequence of the human genome is available in the database. Searching the nucleotide sequence of CPEB2 against this database, we found that the human and mouse genomes contain four CPEB-related protein genes, which are on human chromosome 4 (hCPEB2), chromosome 5 (hKIAA1673), chromosome 10 (hKIAA0940), and chromosome 15 (hCPEB). The mouse CPEB2 is on chromosome 5. By comparing amino acid sequences among this family of proteins, we found that the amino acid sequences of N-termini were less conserved than those of the C-termini, which contain the RNA-binding domains (Fig. 1c). In particular, the RNA-binding domains of CPEB2, KIAA0940, and KIAA1673 are highly homologous, suggesting that they might share a similar recognition sequence. In contrast, the N-termini, where the sequences are more divergent, may provide specific functions.

The CPEB-related proteins are expressed in many metazoans as well as vertebrates. To determine the sequence relationship of CPEB2 to other members of CPEB family, we employed the Kimura corrected distance algorithm to construct an unrooted phylogenetic tree based on the RRM and Zn finger (Fig. 1d). Amino acid comparisons demonstrated that the family can be subdivided into two groups. One is CPEB and its orthologues that primarily participate in oogenesis. Another contains CPEB2, KIAA0940, KIAA1673, Cpb-1, and Cpb-2. Both Cpb-1 and Fog-1 are members of CPEB-related proteins, which control key steps during spermatogenesis in C. elegans [23] and are not es-

FIG. 6. GFP-CPEB2 recombinant protein localizes to the cytoplasm (a) following transient expression in HeLa cells. Nuclear staining by Hoechst 33258 (b) and phase-contrast microscopy (c) are also shown for the same cells in a. Twenty-four hours after the transfection, HeLa cells were fixed and examined by fluorescent and phase-contrast microscope. Fluorescence signals were observed exclusively in cytoplasm of the cells expressing GFP. Magnification ×1000.
sential for oogenesis. The high expression of CPEB2 in postmeiotic cells of testis suggests a primary role of CPEB2 during late stages of spermatogenesis.


Possible Function of CPEB2

In addition to the similarity of the primary sequences, CPEB2 shares many similar features with CPEB. In transfections, CPEB2 is localized to the cytoplasm and binds to poly(U) RNA oligomers, as seen for CPE [21], suggesting that CPEB2, like CPEB, may have similar translational regulation functions.

The translational regulation of specific mRNAs is critical in oogenesis and early embryogenesis. Although a number of different mechanisms are probably responsible for the translational control of different mRNAs, one of the best-studied mechanisms is cytoplasmic polyadenylation [8, 9]. Most mRNA is polyadenylated in the nucleus, and a poly(A) tail of approximately 200 nucleotides assures efficient translation in the cytoplasm. In addition, deadenylation of the poly(A) tail of some of mRNAs occurs in the cytoplasm by an uncharacterized mechanism, which likely is different from that regulating the degradation of mRNAs [10]. The mRNAs with short poly(A) tails of approximately 20 nucleotides become translationally quiescent in oocytes. Responding to stimulation, the poly(A) tails are extended again up to approximately 200 nucleotides, and translation is activated. Cytoplasmic polyadenylation requires two cis-acting elements in the 3' UTR of specific mRNAs: a ubiquitous hexanucleotide AAUAAA, which is also necessary for nuclear polyadenylation; and a CPE with a general sequence of UUUUUAU or UUUUUAAU. The protein CPEB functions as a CPE-binding protein in the cytoplasm [6, 7].

In spermatogenesis, the length of poly(A) in mRNAs also correlates with translational activity. Kashiwabara et al. [24] has shown poly(A) elongation of acrosin, sc32, sc38, and actin mRNAs in round spermatids. These mRNAs contain CPE or CPE-like sequences in their 3' UTRs, suggesting that CPE-dependent translational regulation occurs during spermatogenesis as well as oogenesis. Recently, Tay and Richter [25] reported that CPE-deficient mice lack mature oocytes and, interestingly, that most spermatocytes arrest at the pachytene stage of meiosis. In male germ cells, CPEB mRNA is expressed in pachytene spermatocytes and round spermatids, and CPEB regulates translation of two synaptonemal complex protein mRNAs (SCP1 and SCP3) during meiosis. This finding argues that translational regulation by cytoplasmic polyadenylation is essential during spermatogenesis. Because CPEB2 is abundantly expressed in testis and its expression is restricted to haploid spermatids, we propose that CPEB2 regulates the translation of stored mRNAs during spermiogenesis.

The *C. elegans* genome contains three CPEB-related protein genes, Cpb-1, Cpb-2, and Fog-1 [23, 26]. Both Cpb-1 and Fog-1 have key functions at different stages of spermatogenesis. Fog-1 has two isoforms, L (long form) and S (short form). Fog-1(L) specifies the sperm cell fate, whereas Fog-1(S) seems to be inactive. Cpb-1 is essential for the progression of spermatogenesis. These CPEB proteins may regulate different mRNAs at different stages of spermatogenesis. Similarly, the expression of CPEB and CPEB2 is regulated both temporally and spatially. The CPEB is known to function during meiosis, and it controls translation of mRNAs encoding synaptonemal complex proteins. Because CPEB2 mRNA is expressed at a later stage of spermatogenesis in postmeiotic male germ cells, we propose that CPEB2 regulates translation of mRNAs necessary for spermiogenesis.

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REFERENCES


