CPEB3 and CPEB4 in neurons: analysis of RNA-binding specificity and translational control of AMPA receptor GluR2 mRNA

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CPEB is a sequence-specific RNA-binding protein that promotes polyadenylation-induced translation in oocytes and neurons. Vertebrates contain three additional genes that encode CPEB-like proteins, all of which are expressed in the brain. Here, we use SELEX, RNA structure probing, and RNA footprinting to show that CPEB and the CPEB-like proteins interact with different RNA sequences and thus constitute different classes of RNA-binding proteins. In transfected neurons, CPEB3 represses the translation of a reporter RNA in tethered function assays; in response to NMDA receptor activation, translation is stimulated. In contrast to CPEB, CPEB3-mediated translation is unlikely to involve cytoplasmic polyadenylation, as it requires neither the *cis*-acting AAUAAA nor the *trans*-acting cleavage and polyadenylation specificity factor, both of which are necessary for CPEB-induced polyadenylation. One target of CPEB3-mediated translation is GluR2 mRNA; not only does CPEB3 bind this RNA *in vitro* and *in vivo*, but an RNAi knockdown of CPEB3 in neurons results in elevated levels of GluR2 protein. These results indicate that CPEB3 is a sequence-specific translational regulatory protein.

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Introduction

One widely used mechanism to activate the translation of dormant mRNAs is cytoplasmic polyadenylation. Although this process was first described in invertebrates, it is also important for vertebrate oocyte development (Hake and Richter, 1994; Sheets *et al*, 1995; Stebbins-Boaz *et al*, 1996; Tay and Richter, 2001), cell cycle progression (Groisman *et al*, 2002), and neuronal synaptic plasticity (Alarcon *et al*, 2004). CPEB is the key protein that controls this process; it binds the 3' UTR cytoplasmic polyadenylation element (CPE; consensus UUUUUU) of target mRNAs (Hake and Richter, 1994).

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CPEB also interacts with a number of proteins that are important for polyadenylation and include (i) cleavage and polyadenylation specificity factor (CPSF), which binds the hexanucleotide AAUAAA, another *cis* element in the RNA essential for polyadenylation, (ii) symplekin, a scaffold protein that helps link CPEB to CPSF, and (iii) Gld-2, a cytoplasmic poly(A) polymerase (Barnard *et al*, 2004). CPEB also binds a guanine nucleotide exchange factor (Reverte *et al*, 2003), an RNA helicase (Minshall and Standart, 2004), and amyloid precursor proteins (Cao *et al*, 2005), all of which influence CPEB-dependent polyadenylation. Polyadenylation is initiated when CPEB is phosphorylated by Aurora A, which results in an enhanced interaction between CPEB and CPSF and between CPEB and Gld-2 (Mendez *et al*, 2000a,b; Barnard *et al*, 2004). These events induce Gld-2 to extend the poly(A) tail. Translation of CPE-containing RNAs is most proximally controlled by Maskin, which simultaneously binds CPEB and the cap-binding factor eIF4E. The association of Maskin with eIF4E inhibits assembly of the eIF4F (eIF4E, eIF4G, eIF4A) initiation complex (Stebbins-Boaz *et al*, 1999; Richter and Sonenberg, 2005). Phosphorylation (Barnard *et al*, 2005) and polyadenylation and poly(A)-binding protein (PABP) help Maskin dissociate from eIF4E, thereby allowing translation initiation to proceed (Cao and Richter, 2002; Barnard *et al*, 2005).

In neurons, CPEB promotes the dendritic transport (Huang *et al*, 2003) and polyadenylation-induced translation of CPE-containing mRNAs following synaptic stimulation (Wu *et al*, 1998; Huang *et al*, 2002; Du and Richter, 2005). Because local mRNA translation modulates synaptic efficacy (Steward and Schuman, 2003; Bailey *et al*, 2004), it is not surprising that CPEB knockout (KO) mice display defects in synaptic plasticity (Alarcon *et al*, 2004), as do *Aplysia* neurons treated with an antisense oligonucleotide against CPEB mRNA (Si *et al*, 2003). However, three additional genes encode CPEB-like proteins in vertebrates (Mendez *et al*, 2002) that, at least at the RNA level, are expressed in the brain (Theis *et al*, 2003; Human Unidentified Genome Encoded Large Protein Database). The possibility that these proteins might partially compensate for the loss of CPEB caused us to investigate not only their RNA-binding specificities, but also their involvement in translational control in neurons.

All CPEB-like proteins in both vertebrates and invertebrates have a similar structure in which most of the carboxy-terminal region is composed of two RNA recognition motifs (RRMs) and two zinc-fingers. At least for CPEB, all of these domains are important for binding to the CPE with high affinity (Hake *et al*, 1998). In spite of these structural similarities, however, a sequence comparison of the RNA-binding regions indicated that CPEB is distinct from CPEB2–4 (Mendez and Richter, 2001). Indeed, mouse CPEB is more similar to *Drosophila* CPEB (also known as Orb) than it is to mouse CPEB2–4. This observation suggests that CPEB2–4 might bind a sequence other than the CPE. Using the

RNA-binding region of CPEB4, we now report that SELEX (systematic evolution of ligands by exponential enrichment) analysis has identified a new binding sequence for these proteins. RNA gel shifts using this sequence as well as the CPE demonstrate that although CPEB binds the CPE and CPEB2–4 bind the SELEX sequence with high affinity (K_d of 100–160 nM), CPEB does not bind the SELEX sequence nor do CPEB3–4 bind the CPE. Although CPEB recognition of the CPE does not appear to involve RNA secondary structure, such structure is important for CPEB3–4 interaction with the SELEX sequence. CPEB3–4 are expressed in partially overlapping regions in the brain and are found in dendrites; CPEB3 colocalizes with a synaptic marker whereas CPEB4 does not. Experiments employing reporter RNAs transfected into neurons demonstrate that CPEB3 represses and then stimulates translation in response to NMDA treatment. CPEB3 neither interacts with CPSF nor requires the AAUAAA hexanucleotide for translational activation, implying that, in contrast to CPEB, it regulates translation in a polyadenylation-independent manner. The AMPA receptor GluR2 mRNA is a target of CPEB3 regulation; not only does CPEB3 bind this RNA *in vivo*, but an RNAi knockdown of CPEB3 in neurons results in elevated translation of GluR2 mRNA. Thus, based on RNA-binding specificity and functional regulation of translation, CPEB2–4 form a class of proteins distinct from CPEB.

Results

All CPEB-like proteins have a carboxy-terminal RNA-binding domain (RBD), which is comprised of two RRM and two zinc-fingers, and an amino-terminal domain, which, in the case of CPEB, stimulates polyadenylation-induced translation once it is phosphorylated on T171 (in the mouse protein, S174 in *Xenopus*) by Aurora A. Although there is no significant identity among the amino-terminal domains of the CPEB proteins within a species (e.g., the mouse) or between species (e.g., mouse and fly), there is strong identity among the RBDs. For example, mouse CPEB and mouse CPEB2 are 45% identical in this region. However, mouse CPEB2, CPEB3, and CPEB4 are >95% identical. Interestingly, mouse CPEB has a higher identity to fly CPEB (also known as Orb) than to mouse CPEB2–4. Moreover, fly CPEB2 is more similar to mouse CPEB2–4 than it is to fly CPEB (Figure 1A). These comparisons imply that CPEB2–4 might interact with a sequence different from that of CPEB. Such a possibility was further suggested by experiments in injected *Xenopus* oocytes. Although mRNA encoding CPEB or CPEB3 had no effect on progesterone-induced oocyte maturation, mRNA encoding a chimeric protein composed of the regulatory domain of CPEB3 fused to the RBD of CPEB inhibited maturation (Figure 1B, left, the right shows Western blots of the resulting proteins). We infer that the chimeric protein acted as a repressor of translation of CPE-containing mRNAs required for maturation because it could not respond to progesterone stimulation; CPEB3 did not repress translation because it could not bind these mRNAs (see below).

SELEX identifies CPEB2–4 binding sequences

To determine whether the RBDs of CPEB2–4 indeed interact with sequences other than the CPE, a SELEX experiment was performed. The RBD of CPEB4 was mixed with *in vitro*-

Figure 1 Structural features and comparison of CPEB-like proteins. (A) All CPEB-like proteins have an amino-terminal region and a carboxy-terminal region containing two RRM and two zinc-fingers (Zif). CPEB T171, which is not conserved in CPEB2–4, must be phosphorylated for polyadenylation to occur. Among the amino-terminal regions of the CPEB proteins, there is little identity (NS, not significant). Among the RBDs, there is considerable identity. However, mouse CPEB (designated CPEB1 for convenience) is closer to *Drosophila* CPEB (Orb) than it is to mouse CPEB2; in addition, *Drosophila* CPEB2 (Orb2) is more similar to mouse CPEB2 than it is to Orb. Mouse CPEB2–4 are nearly identical in the RBDs. (B) *Xenopus* oocytes were injected with water or RNA encoding myc-CPEB or CPEB3, or myc fused with the amino domain of CPEB3 and the RBD of CPEB. The oocytes were incubated with progesterone and scored for oocyte maturation as assessed by germinal vesicle breakdown (GVBD). A Western blot probed with myc antibody shows the level of the myc fusion proteins in oocytes.

synthesized RNA derived from an oligonucleotide library composed of a randomized 25-mer flanked by constant regions for PCR; the mixture was subjected to eight rounds of binding and elution. After the final elution, the RNA was cloned and the sequences of 50 plasmid inserts were determined, some of which are shown in Figure 2A. *In vitro* gel shifts confirmed that CPEB4 RBD bound all cloned RNAs tested (Figure 2B). Two point mutations that disrupted the CPEB4 zinc-fingers abrogated binding to a selected RNA, 1904 (Figure 2C), indicating the importance of this domain for RNA interaction, which is consistent with a previous finding that the zinc-fingers are important for CPEB to bind the CPE (UUUUUAU derived from *Xenopus* mos; Hake *et al*, 1998; de Moor and Richter, 1999). Further analysis showed that while CPEB3 and CPEB4 bound the 1904 sequence, CPEB did not. Moreover, CPEB did bind CPE-containing RNA, as expected, but CPEB3 and CPEB4 did not (Figure 2D). When analyzed kinetically, the binding constant (K_d) of CPEB for the CPE was 130 nM (Hake *et al*, 1998); CPEB did not interact with 1904. In contrast, while the CPEB3 and CPEB4 RBDs (>95% identical; Figure 1) did not bind the CPE, the K_d values for the 1904 sequence were 166 and 100 nM, respec-

A SELEX sequences

1914	GAUCGGUGUUUUUUUUGAGGGCGG
1915	UUGGUUCAUGUUUUUUUUGAGGGCGG
1926	GUGUCCGUGUUUUUUUUGAGGGCGG
1908	GUAUUCGUGUUUUUUUUGAGGGCGG
1901	AUUGAGGCUCGAUGUUUUUCAGGAC
1904	AAAGAGGAUUUGUGUUUUUCAGGAC
1929	UAAAAGGAUUCGUGUUUUUCAGGAC
1912	CUGUGGAUCUUGUGUUUUUCAGGAC
1907	UGCAUGUUUUUCGAGUACAGUCCC
2925-2	CGCAUAAUUUCGAGUAUCAGCCCU
2963-3	CACAUUGUUUCGAGUUCUAGUCCC
1916	CGCAUACUUUCGUGUAUUAGUCCG

E

K_d	CPEB	CPEB3 RBD	CPEB4 RBD
CPE	0.13 μ M*	NDB	NDB
1904	NDB	0.16 μ M	0.10 μ M

*From Hake *et al* (1998)

NDB, no detectable binding at 1 μ M

F

Figure 2 Analysis of CPEB and CPEB4 interaction with RNA. (A) The CPEB4 RBD was mixed with RNA synthesized *in vitro* from oligonucleotides containing a randomized 25-mer central domain flanked by constant regions used for transcription and PCR. The RNA-protein complexes were collected on filters, eluted, and the cycle repeated for eight rounds before cloning. Representatives of 50 clones are shown. (B) Several SELEX RNAs were synthesized *in vitro* with 32 P-UTP and used for gel shifts with 500 nM CPEB4RBD. (C) The CPEB4 RBD, containing or lacking the zinc-finger (ZF), was used in gel shifts with SELEX sequence 1904. (D) RNA gel shifts were performed with CPEB, CPEB3, or CPEB4 with CPE-containing RNA or the 1904 sequence. (E) A kinetic analysis of CPEB3 and CPEB4 RBD interactions with the 1904 sequence or the CPE was used to calculate equilibrium dissociation constants (K_d). Similar experiments were performed with CPEB binding to the 1904; the binding of CPEB to the CPE was taken from Hake *et al* (1998). (F) Various regions of the RBDs of CPEB and CPEB3 were fused to MBP, expressed in bacteria, and used for RNA gel shifts and UV crosslinks with the CPE or the 1904 sequence. The bottom panels show that equal amounts of the MBP fusion proteins were used on all experiments.

tively. Thus, CPEB and CPEB2–4 have different RNA-binding specificities.

To identify the origin of these different RNA-binding specificities, chimeric molecules composed of RRM1, RRM2, and the zinc-fingers (Zif) from CPEB and CPEB3 were fused to maltose-binding protein (MBP) and subjected to RNA gel shifts and UV crosslinking analysis with the CPE and the 1904 SELEX sequence (Figure 2F). Although the entire CPEB RBD was the most efficient at binding the CPE, RRM1 was essential for this binding (gel shift and UV crosslink). Similarly, the entire CPEB3 RBD was the most efficient at binding the 1904 sequence (compare gel shift and UV crosslink), but in this case, both RRMs were important for binding.

Exchange of the zinc-fingers had no effect on RNA binding. Thus, while certain domains within CPEB (RRM1) and CPEB3 (RRMs 1 and 2) are important for binding specificity, the zinc-fingers of both proteins (see also Hake *et al*, 1998), while important for RNA interaction, do not confer specificity.

CPEB3–4 RBD recognizes RNA secondary structure

One of the goals of the SELEX experiments was to identify endogenous mRNAs that are bound by the CPEB3–4 RBD. To make a search more specific, we delineated the nucleotides necessary for binding. Using 1904 as a substrate, mutations in several regions of the 25-mer destroyed or reduced RNA binding (Figure 3A). Surprisingly, deletion of the 3' but not

Figure 3 The CPEB4 RBD recognizes RNA secondary structure. **(A)** Nucleotide changes were introduced into the 1904 sequence and the resulting RNAs were used for gel shifts with the CPEB4 RBD; the relative amount of binding is indicated. **(B)** Compensatory mutagenesis of M14 RNA. The mfold program generated a predicted secondary structure of M14 RNA. The blue line denotes the nucleotides derived from the constant sequence, the black box indicates the part of the stem used for the mutagenesis, and the red nucleotides indicate the mutated sequences. Gel shifts of these RNAs using the CPEB4 RBD is shown at right. **(C)** Structure mapping and RNA footprinting. 5' end-labeled RNA was untreated (lane 1), or subjected to partial alkaline hydrolysis (lane 2), digested with RNase T1 (lane 3), subjected to lead acetate cleavage in the absence or presence of MgCl₂ (lanes 4–6, left panel), or digested with the double-strand-specific nuclease RNase V1 (lane 7, left panel). For RNA footprinting, RNA samples either lacked added protein (lane 4, right), or were 0.25, 0.5, 1, 2, or 4 μ M CPEB4 RBD and were treated with the hydroxyl radical (lanes 5–9, right panel). The products were then analyzed on a sequencing gel. The protected region is denoted by a black bar. **(D)** Predicted RNA secondary structure and nucleotides protected from hydroxyl radical cleavage by CPEB4 RBD; the red box indicates the protected nucleotides. (For colour figure see online version.)

5' constant region destroyed binding; the 25-mer alone was not bound by the RBD (Figure 3A). Using the mfold program (Zuker, 2003), the 5' constant region deleted in RNA M14 is predicted to fold into a secondary structure with poly(U) in the loop; similar structures were predicted for most of the other selected RNAs (Figure 3B and data not shown). To assess whether such a possible structure (ΔG of -21.3 kcal/mol) could be important for CPEB4 binding, three bases were mutated in the bottom stem (CAC for GUG), which completely abrogated CPEB4 binding (Figure 3B). Compensatory changes in the complementary sequence (denoted REV for reverse, GUG for CAU) restored binding by CPEB4; however, substitution of the GUG for CAU alone did not restore binding. These results suggest that RNA secondary structure could be necessary for CPEB4 binding.

To further assess this hypothesis, 5' end-labeled RNA was cleaved with the single-strand-specific lead acetate (Darnell *et al.*, 2005), and RNase V1, which cleaves double-stranded regions (Lockard and Kumar, 1981). These samples were resolved on a sequencing gel and compared to parallel

lanes containing untreated RNA, RNA partially hydrolyzed with NaOH, and RNA cleaved with RNase T1 to locate the guanosines (Figure 3C). Lane 4 shows that the uridine residues predicted to reside in a loop structure (panel B) were susceptible to lead cleavage, which was reduced by inclusion of $MgCl_2$, a competitor of the lead ion (lanes 5 and 6). These uridine regions were also resistant to RNase V1 cleavage, further demonstrating that they are not base-paired with other residues. To determine the region of the RNA bound by the CPEB4 RBD, RNA footprinting was performed. An increasing amount of CPEB4 RBD was mixed with 5' end-labeled RNA; the RNA was then cleaved by the hydroxyl radical generated from a mixture of Fe/EDTA (Wang and Padgett, 1989). Compared to a sample digested with hydroxyl radical only (no protein) (lane 4), CPEB4 protected two regions of the RNA, one was the single-stranded uridines together with the 5' proximal stem and the other was an adjacent double-stranded region (lane 9). The binding of CPEB4 to the 5' proximal stem is consistent with the compensatory mutagenesis result and further suggests the

significance of RNA structure for CPEB4 binding. Two other SELEX clones gave similar RNA footprinting patterns (data not shown). Figure 3D depicts a revised secondary structure of the minimal RNA required for CPEB4 RBD binding based on the data in panels B and C. The residues protected by the CPEB4 RBD are also indicated.

CPEB3 and CPEB4 in the brain

Western blotting shows that CPEB3 and CPEB4 are present in many tissues including the brain (Figure 4A). Immunohistochemistry demonstrates that although both proteins were evident in the hippocampus and granule cells of the cerebellum, only CPEB4 was detected in Purkinje cells of the cerebellum. In contrast, only CPEB3 was detected in mitral cells of the olfactory bulb and interneurons of the cerebellum (Figure 4B). In hippocampal neurons cultured *in vitro* and stained for Map2 to identify dendrites (not shown), CPEB3 often colocalized with synaptophysin, a synaptic marker (Figure 5A). Although CPEB4 appeared to be adjacent to synaptophysin immunoreactivity, both proteins were strongly detected in the post-synaptic density (PSD) fraction (Figure 5B). CPEB4, the only one tested, colocalized with RNA as assessed by Syto-RNaselect staining (Figure 5A). Finally, both CPEB3 and CPEB4, when fused to GFP, were detected in dendrites, often as puncta (Figure 5C). Taken together, these data show that CPEB3 and CPEB4 are expressed in only partially overlapping regions of the brain; within hippocampal neurons, where both are expressed, they appear to be synaptic.

CPEB-like proteins and translation

To investigate whether the CPEB3 could be involved in translational control, we employed a tethered function assay in hippocampal neurons that were transfected with several sets of reporter RNAs (Figure 6A). They encoded the dimeric MS2 coat protein (MS2CP) fused to CPEB3 or mutant CPEB3 proteins that lacked the amino- or carboxy-terminal regions, or as a control, MS2CP fused to GFP. These RNAs were mixed with RNA encoding firefly luciferase that contained or lacked the stem-loops recognized by MS2CP. The mix also contained RNA encoding *Renilla* luciferase, which served as an internal control. In transfected neurons, MS2CP-CPEB3 and MS2CP-GFP were synthesized (Figure 6B, left) and gel shifted a probe containing the MS2CP stem-loops (Figure 6B, right). Figure 6C shows that relative to MS2CP-GFP, MS2CP-CPEB3 and MS2CP-CPEB3 amino terminus reduced firefly luciferase expression by ~30–40%, which was statistically significant ($P < 0.01$). This reduction was not due to a general repression, as the substitution of the MS2CP moiety with myc had no effect on translation. Moreover, removal of the MS2 stem-loop from firefly luciferase abrogated the NMDA-induced translation by MS2CP-CPEB3 (Figure 6D). Thus, CPEB3 can reduce translation when tethered to a specific mRNA.

To assess whether CPEB3 can stimulate translation and if so, whether it requires AAUAAA, a firefly luciferase containing or lacking this sequence was transfected into neurons together with MS2CP-CPEB3 as before and then stimulated with NMDA. MS2CP-CPEB3 enhanced translation by 20–30% irrespective of whether the AAUAAA was present (Figure 6D). The MS2 stem-loops were required for MS2CP-CPEB3 translation. This change in translation occurred even though the

Figure 4 CPEB3 and CPEB4 in the brain. (A) Western blots of several mouse tissues probed for CPEB3 and CPEB4. The blot also shows that the antibodies for CPEB3 and CPEB4 do not crossreact and do not recognize CPEB. The calculated molecular sizes of CPEB3 and CPEB4 are ~74 and 78 kDa, respectively. However, on SDS gels, their apparent molecular sizes are ~100 and ~98 kDa, respectively. (B) Immunocytochemistry for CPEB3 and CPEB4 in rat hippocampus, cerebellum, and olfactory bulb. The arrows point to specific regions of immunoreactivity in interneurons (IN) and Purkinje cells (PC) of the cerebellum and Mitral cells (MC) of the olfactory bulb.

luciferase RNA levels were unchanged (panel E). In injected *Xenopus* oocytes, CPEB was co-immunoprecipitated with CPSF, as shown previously (Mendez *et al.*, 2000b), but CPEB2–4 were not (panel F). Finally, luciferase mRNA was appended with a 3' UTR containing the 1904 SELEX sequence and analyzed for translation when cotransfected with myc-CPEB3 or, as a control, β -galactosidase. CPEB3 reduced translation of the reporter RNA by ~25%. Although NMDA had no effect on luciferase activity when β -galactosidase was cotransfected, it stimulated luciferase activity by nearly 25% when CPEB3 was cotransfected (panel G). These data, as well as those in Figure 1B, suggest that although CPEB3 repressed and then activated translation in response to NMDA, it probably does not do so by changing poly(A) tail length because it does not require the *cis*-acting AAUAAA and does not bind CPSF.

CPEB3 controls *GluR2* mRNA translation

Because RNA secondary structure appears to be important for binding by CPEB3, BLAST searches for endogenous RNAs based on SELEX sequence alone would not be fruitful.

Consequently, we considered several neuronal RNAs whose translation might be regulated and whose 3' UTR could contain a stem-loop structure similar to that shown in Figure 3B. The mRNA encoding the AMPA receptor GluR2 is dendritically localized and may be subject to translational control (Kacharmina *et al*, 2000); the mfold program also predicts several stem-loop structures that resemble those predicted to form in the 1904 SELEX sequence (data not shown). To assess whether CPEB3 might bind this mRNA, the 3' UTRs of GluR2 and Arc (a control) mRNAs were subjected to UV crosslinking *in vitro* with the RBDs of CPEB and CPEB3 fused to MBP. Figure 7A shows that the RBD of CPEB3, but not of CPEB, strongly crosslinked to the 3' UTR of GluR2 but not of Arc, suggesting that GluR2 RNA could be a direct binding substrate of CPEB3. To identify the region of GluR2 3' UTR bound by CPEB3, a deletion series was constructed and used for *in vitro* crosslinking to CPEB3 RBD (Figure 7B). With the exception of nucleotides 1–474,

CPEB3 RBD bound to multiple regions throughout the length of the 3' UTR. Additional deletions were constructed that were used for RNA gel shifts (Figure 7C). Again, CPEB3 bound to multiple regions of the 3' UTR. The binding to one of the regions that was chosen, L4, was specific, as the shift was competed away when the 1904 SELEX sequence was added to the mix but not when 1904-M1 was added. Moreover, a 202-base fragment (S4) derived from the L4 RNA was bound by CPEB3, which again was competed by the 1904 sequence but not by the 1904-M1 sequence (Figure 7D).

The S4 RNA was used for structure mapping as in Figure 3. Multiple regions were cleaved by lead acetate, including one containing multiple uridine residues (Figure 7E, lane 4). This region was not cleaved by the double-strand-specific RNase V1 (lane 7). Sequences including these single-stranded uridines were protected by CPEB3 RBD from cleavage by the hydroxyl radical (Figure 7F, lanes 4–8). Thus, the CPEB3

Figure 5 Localization of CPEB3 and CPEB4 in neurons. (A) Co-staining of CPEB3 or CPEB4 with synaptophysin in 21-day-old cultures of hippocampal neurons. CPEB4 is also co-stained with the RNA marker Syto-RNaselect after UV crosslinking and fixation. (B) Detection of CPEB3 and CPEB4 in the PSD fraction. P-CPEB4 refers to putative phosphorylated CPEB4, B refers to the brain extract, S refers to the synaptosome fraction, and syn refers to synaptophysin. (C) Detection of transfected GFP-CPEB3 and GFP-CPEB4 proteins in dendrites of hippocampal neurons co-stained with MAP2.

Figure 6 Translational repression in neurons. (A) Hippocampal neurons were transfected with mRNAs encoding (1) dimeric MS2CP fused to CPEB3 or amino- or carboxy-terminal truncations of this protein, or MS2CP fused to GFP, and (2) firefly luciferase whose 3' UTR contained or lacked the MS2 stem-loop, and (3) *Renilla* luciferase. The right panel shows an RNA gel shift of transfected proteins binding to RNA containing the MS2 stem-loops. (C) The ratio of firefly/*Renilla* luciferase activity in neurons (normalized to the MS2CP-GFP control) transfected with the RNAs noted in panel A. Neurons were transfected with mRNA encoding myc-CPEB3 in place of the MS2CP fusions. All the firefly luciferase RNAs contained the MS2 stem-loop in the 3' UTR. (D) Luciferase values calculated as those in panel C from neurons transfected with RNA encoding firefly luciferase that contained or lacked the MS2 stem-loop and that was treated with NMDA. (E) Semiquantitative RT-PCR of luciferase RNA containing or lacking the MS2 stem-loops following RNA transfection into neurons; some of the neurons were treated with NMDA. (F) *Xenopus* oocytes were injected with mRNAs encoding fusions between myc and CPEB or CPEB2–4. The CPEB proteins were then immunoprecipitated with myc antibody and probed for myc, to note the level of expression, and the 100 kDa subunit of CPSF. (G) Hippocampal neurons were transfected with firefly luciferase mRNA appended with a 3' UTR containing the 1904 SELEX sequence, *Renilla* luciferase mRNA, and either myc-CPEB3 or β -galactosidase. Some of the neurons were also treated with NMDA. Firefly luciferase, normalized to *Renilla* luciferase, was then determined. Probability (*p*) values were derived from the Student's *t*-test.

RBD may interact with a sequence and structure in GluR2 similar to the sequence and structure identified by SELEX. We also note that S4 contains a CPE-like sequence, which interacts with CPEB *in vitro* (data not shown).

To determine whether CPEB3 binds GluR2 mRNA *in vivo*, living cultures of hippocampal neurons were irradiated with UV light, which was followed by cell homogenization in

detergent-containing buffer to reduce nonspecific adsorption followed by immunoprecipitation with CPEB3 antibody or IgG. The precipitates were then deproteinized and subjected to RT-PCR for Arc, Map2, neurofilament (NF), or GluR2 RNAs. Although all the RNAs were clearly amplified from input material, only GluR2 RNA was amplified from the CPEB3 co-immunoprecipitate. No RNAs were amplified

Figure 7 CPEB binds the GluR2 3' UTR. (A) Maltose-binding fusion proteins containing the RBD of CPEB or CPEB3 were expressed in bacteria and used for *in vitro* UV crosslinking with ³²P-labeled 3' UTRs of Arc and GluR2 mRNAs. (B) Deletions of the GluR2 3' UTR were used for UV crosslinking with His-tagged CPEB3 RBD. (C) Additional deletions of GluR2 3' UTR were used for RNA gel shift reactions with His-tagged CPEB3 RBD. (D) Some of the gel shift mixtures also contained unlabeled 1904 or 1904-M1 RNAs as competitors for CPEB3 RBD binding. (E) Structure mapping of GluR2 3' UTR fragment, S4. 5' end-labeled S4 RNA was either untreated (lane 1), subjected to partial alkaline hydrolysis (lane 2), RNase T1 digested (lane 3), treated with 100 mM lead acetate in the absence or presence of 10 mM or 100 mM MgCl₂ (lanes 4-6, left panel), or digested with nuclease V1 (lane 7, left panel) and analyzed on a sequencing gel. The sequence corresponding to the uridine-rich region is shown. (F) RNA footprinting of S4. 5' end-labeled S4 RNA was cleaved by hydroxyl radical in the absence or presence of 0.25, 0.5, 1.0, 2.0, and 4.0 μM CPEB3 RBD (lanes 4-8). Lanes 1-3 is the same as in panel E.

from the control IgG immunoprecipitate (Figure 8A). Thus, GluR2 mRNA is an *in vivo* substrate of CPEB3.

We next performed RNAi knockdown experiments to examine whether CPEB3 regulates GluR2 mRNA translation in neurons. Hippocampal neurons cultured for 4 days were infected with lentivirus containing or lacking a short hairpin sequence for CPEB3 under the control of the U6 promoter. After a further 4 days of culture, the cells were harvested and the extracted protein was probed on Western blots. In two independent experiments, CPEB3 protein was reduced by 80-99% (Figure 8B). In contrast, GluR2 levels increased by three-fold whereas αCaMKII and synaptophysin were unaffected. Because the CPEB3 knockdown had little effect on the level of GluR2 RNA level (Figure 8B), we infer that the translation of GluR2 mRNA is under negative regulation by CPEB3. It is possible that GluR2 mRNA localization could also be affected. Finally, we note that GluR2 levels were identical in wild-type and CPEB KO hippocampus, indicating that the expression of GluR2 RNA is controlled by CPEB3 and not CPEB.

Discussion

In this study, we demonstrate that in contrast to CPEB, CPEB proteins 2-4 do not avidly bind the CPE, but instead strongly interact with a U-rich loop within a stem-loop structure.

While the zinc-fingers are necessary for RNA binding, it is RRM1 and 2 that confer the binding specificity. These results imply that CPEB2-4 probably do not functionally substitute for CPEB, as they interact with RNAs with different binding specificities. There could, however, be RNAs that are bound by CPEB and CPEB2-4. CPEB3 represses translation of a reporter RNA in transfected neurons and stimulates translation in response to NMDA. Although the mechanism of translational control by CPEB3 is not yet known, it does not bind CPSF nor does it require an AAUAAA *cis* element, implying that unlike CPEB, it does not promote cytoplasmic polyadenylation. Most importantly, CPEB3 interacts with GluR2 mRNA *in vivo* and a knockdown of CPEB3 in neurons stimulates the translation of this mRNA. Thus, CPEB3 is a sequence-specific translational repressor that governs the synthesis of the AMPA receptor GluR2.

Unlike CPEB, the CPEB3-4 RBD recognizes a secondary structure and interacts with uridines that are single-stranded as well as double-stranded stems. Although the zinc-fingers of the RBD are necessary for stable RNA binding, they do not confer binding specificity. In other proteins, zinc-finger domains have been shown to bind double-stranded RNA (Yang *et al*, 1999; Mendez-Vidal *et al*, 2002), and we would hypothesize that this could also be the case with the CPEB2-4 RBD.

Figure 8 CPEB3 controls GluR2 mRNA translation. (A) Living cultures of hippocampal neurons were irradiated with UV light, homogenized, and subjected to immunoprecipitation with CPEB3 antibody or IgG in the presence of SDS-containing buffer. The precipitated RNA was extracted after proteinase K digestion and subjected to RT-PCR for Arc, Map2, NF, or GluR2 mRNAs. (B) Endogenous CPEB3 knockdown by RNAi. Cultured hippocampal neurons were infected with a control lentivirus or one expressing a short hairpin RNA against CPEB3 under the control of the U6 promoter. Extracts were then prepared from the cells and analyzed for levels of CPEB3, GluR2, α -CaMKII, and synaptophysin. From other cultures, the RNA was extracted and analyzed for GluR2 mRNA by real-time PCR. The bottom panel shows the level of GluR2 in wild-type and CPEB KO hippocampus. Tubulin served as a loading control.

CPEB2–4 are functionally distinct from CPEB and may also be distinct from one another. Although they bind the same *cis* element, the fact that CPEB3 and CPEB4 reside in only partially overlapping regions (i.e., both are in the hippocampus, but only CPEB4 is in Purkinje cells of the cerebellum and only CPEB3 is in mitral cells of the olfactory bulb) indicates that they may interact with at least some unique RNAs *in vivo*. In addition, the large amino-terminal regions of CPEB2–4 proteins are only 33–43% identical. This relatively low identity suggests that these proteins could respond to different signaling pathways and/or interact with different sets of proteins to modify their activities such as translation repression, stimulation, or RNA transport.

Translational control of GluR2 mRNA

The use of SELEX to identify CPEB2–4 binding sites in RNA was not particularly useful for recognizing endogenous RNA targets, as secondary structure was important for RNA–protein interactions. Consequently, we examined a number of neuronal mRNAs that might form similar secondary structures as determined by the mfold program; the mRNA encoding the AMPA receptor GluR2 was able to do so and was immunoprecipitated with CPEB3 following UV irradiation of living neurons.

An RNAi knockdown of CPEB3 stimulated GluR2 levels while having little effect on GluR2 mRNA, suggesting that CPEB3 is a specific translational repressor protein. We do note the caveat that our attempts to examine the translation of a reporter RNA appended with the GluR2 3' UTR in transfected hippocampal neurons was variable (data not shown), perhaps because the RNA was not properly localized or because it exceeded the amount of the endogenous CPEB3. Nonetheless, the cumulative data in this report do indicate that CPEB3 is at least one factor that mediates GluR2 mRNA expression.

The molecular mechanism by which CPEB3 modulates translation is unknown; perhaps it interacts with an eIF4E-binding protein such as Maskin, competes with eIF4E for binding to the cap (Cho *et al*, 2005), or modulates ribosomal subunit joining (Ostareck *et al*, 2001). Irrespective of how CPEB3 controls translation, the observation that GluR2 is an endogenous target has important implications for AMPA receptor regulation. For example, GluR1 mRNA is present in dendrites (Miyashiro *et al*, 1994) and is regulated at least in part at the translational level (Kacharmina *et al*, 2000; Ju *et al*, 2004). AMPA receptors are also controlled at the protein localization level, as they are trafficked to the membrane in response to activity (Malinow and Malenka, 2002). Thus, both cell soma and local synthesis of GluR1 and GluR2 could contribute to the formation of functional AMPA receptors.

Translational control and synaptic plasticity

Intense interest has focused on local (dendritic) mRNA translation, as it was shown nearly a decade ago to be important for maintaining long-term changes in synaptic strength (Kang and Schuman, 1996). Although many studies have confirmed and extended these results (reviewed by Klann and Dever, 2004; Sutton and Schuman, 2005), in some ways local translation remains enigmatic. For example, although it has become almost axiomatic that activity-induced synthesis of new proteins helps distinguish experienced from naïve synapses (Steward and Schuman, 2001), a demonstration that specific proteins involved has not emerged. The synthesis of several proteins increases upon synaptic stimulation, but only ~2–4-fold (e.g., Kelleher *et al*, 2004; Schrott *et al*, 2004). Such increases could certainly be physiologically significant, especially if they are concentrated at particular synapses. Moreover, relatively modest changes in many proteins could be essential for plasticity. Alternatively, perhaps the synthesis of less abundant proteins, while substantially stimulated by synaptic activity, is obscured by the general but low-level increase. Such a possibility is particularly intriguing, as it is known to occur in other cells. In *Xenopus* oocytes, progesterone stimulation of M-phase progression is accompanied by an ~2-fold increase in general protein synthesis. In contrast, proteins such as Mos and cyclin B1, which are necessary for M-phase progression, increase from nearly undetectable levels to easily observed amounts when assayed by, for example, Western blots. However, because these proteins are relatively rare compared to the bulk of the newly made proteins, they are not readily detected by metabolic labeling unless they are specifically immunoprecipitated. Thus, critically important proteins could be synthesized at synapses, but because they are not abundant, they are difficult to detect. In contrast to specific protein synthesis, the productive capture of certain

newly made proteins by stimulated synapses may be responsible for regulating synaptic efficacy (Frey and Morris, 1997; Kelleher *et al*, 2004).

Although one way to investigate the relationship between protein synthesis and plasticity is obviously to identify mRNAs that are translated in response to activity, an alternative approach is to first identify translational control proteins in the brain and then determine which mRNAs are bound and/or regulated by them. For example, a number of CPE-containing RNAs have now been identified that undergo activity-dependent polyadenylation (Wu *et al*, 1998; Du and Richter, 2005), presumably because they are bound by CPEB. In this study, we have shown that CPEB3 and CPEB4 are components of the PSD and have identified one mRNA that is bound by and under the translation control of CPEB3. By defining the precise binding site in GluR2 mRNA, we may be able to deduce additional RNAs that are regulated by this and the other CPEB-like proteins.

Materials and methods

Plasmids and protein expression

Escherichia coli strain BL21(DE3)pLysS (Novagen) was transformed with expression plasmids (pET28a) encoding CPEB and the RBDs of CPEB3–4. The cells were cultured to OD₆₀₀ 0.3–0.6 before the addition of 1 mM IPTG for 30 min. His-tagged proteins were purified using Ni-NTA agarose resin (Qiagen) and dialyzed against 1X GR buffer (10 mM HEPES, pH 7.6, 50 mM KCl, 1 mM MgCl₂, 0.1 mM ZnCl₂, 10% glycerol, 1 mM DTT) for 2 h. His-CPEB was denatured with 6 M urea and then renatured by stepwise lowering of the urea concentration in the wash buffer to 2 M urea before elution and dialysis against 1X GR with 2 M urea. The CPEB4RBD used for SELEX was further purified by FPLC (AKTA, Amersham Pharmacia Biotech) in a HiLoad 16/60 Superdex 200 column in 1X GR buffer; protein concentrations were determined by BCA protein assay reagent (Pierce).

For other experiments, DH5 α cells transformed with plasmids encoding MBP fused to CPEB proteins were grown to OD₆₀₀ ~0.6 and then induced with 1 mM IPTG for 3 h. The bacterial pellet was resuspended in buffer A (20 mM HEPES, pH 7.6, 500 mM NaCl, 1 mM MgCl₂, 0.5 mM DTT, 0.1% Triton X-100, 10% glycerol, 1 mM PMSF) and incubated for 30–40 min on ice with lysozyme (1 mg/ml). The cells were sonicated to loss of viscosity and clarified by centrifugation at 12 000 g for 15 min. The resulting supernatant was incubated with amylose resin (NEB) and washed with 100 $\times$ volume of buffer A. The protein was eluted with buffer A (100 mM NaCl, no Triton X-100) with 10 mM maltose. Gel shift assays typically employed 100 ng of fusion protein.

SELEX

For SELEX, three oligonucleotide primers were synthesized: PO-67, GGGAGAATTCCGACCAGAAGN₂₅TATGTGCGTCTACATGGATCCTCA; PO-69, TGAGGATCCATGTAGACGCA; PO-71, TAATACGACTCACTA TAGTGGGAGAATTCCGACCAGAAG. To generate templates for a SELEX library, these three primers were used in a PCR reaction at a ratio of PO-67:PO-69:PO-71 = 1:1:3. The PCR products were then used for *in vitro* transcription with T7 RNA polymerase. Free nucleotides from the *in vitro* transcription reaction were removed by FPLC using HiPrep 26/10 desalting column before further purification by denaturing TBE PAGE. For the SELEX, FPLC-purified CPEB4RBD was mixed with heat-denatured RNA library in 1 ml of 1X GR buffer containing 160 μ g tRNA and 5 mg heparin. RNA-protein mixtures were kept on ice for 10 min and at room temperature for 10 min before being filtered through nitrocellulose membranes on a porous plate by gentle suction. The membranes were washed with 5 ml of 1X GR with tRNA (0.5 mg/ml). The membranes were cut into small pieces and mixed with Trizol for RNA extraction as described (Invitrogen). The extracted RNA was used for reverse transcription to generate cDNA by Superscript Reverse Transcriptase II (Invitrogen) and PCR amplification using PO-69 and PO-71. The amplified PCR products were used for

transcribing RNA for the next round of SELEX. To increase the binding specificity of selected RNA to CPEB4RBD, the amount of this protein used in each cycle was reduced by half from 2 μ M for the first cycle to 25 nM for the seventh and eighth cycles. The amount of purified RNA library used for each SELEX cycle was 20 μ g except the first cycle, which was 60 μ g.

Electrophoresis mobility shift assay

RNA probes used for mobility shift assays were labeled by *in vitro* transcription with α -³²P-UTP. Mutants of 1904 RNA were transcribed from PCR products using oligonucleotides with the T7 promoter. For gel shifts, 20 μ l reactions in 1X GR buffer included probe RNA (various concentrations, ~10³ c.p.m.), protein (e.g., CPEB4 RBD), 1 μ g tRNA, 50 μ g heparin, and 12 U RNasin. The reactions were kept on ice for 10 min and then at room temperature for 10 min, before being resolved by TBE PAGE.

Immunohistochemistry and RNA transfection in neurons

Two-month-old male mice were anesthetized and perfused with 4% formaldehyde. The fixed brains were embedded in paraffin, sectioned at 10 μ m thickness, and treated with antigen retrieval procedure (Tay *et al*, 2003) before incubation with the affinity-purified CPEB3 and CPEB4 antibodies. Hippocampal neurons were cultured and immunostained as described (Huang *et al*, 2002). Other neuronal cultures were UV irradiated in a Stratagene 1800 Crosslinker, fixed, and stained with Syto-RNaselect (Molecular Probes).

Hippocampal neurons cultured for 9–10 days in Neurobasal medium with B27 supplement (Invitrogen) at a cell density of 30 000–40 000/cm² were cotransfected (TransMessenger Transfection reagent, Qiagen) for 3 h with ~12 pmol of Ms2CP-CPEB RNA, 1.7 pmol of firefly luciferase RNA appended with various 3' UTRs, and 1 pmol of *Renilla* luciferase RNA. The transfected neurons were stimulated with 50 μ M NMDA for 3 h before lysis in 100 μ l of buffer for dual luciferase assay (Promega). To quantify the amount of firefly and *Renilla* luciferase RNAs, total RNA was extracted from transfected neurons, reverse transcribed, and subjected to real-time PCR amplification (Huang *et al*, 2003). The specific primers used were as follows:

firefly sense 5'-GAGATGTATTACGCAAAGTAC and
antisense 5'-CCAGTATGACCTTTATTGAGC;
Renilla sense 5'-GTTGTGTTCAGCAGCTGG and
antisense 5'-CCAGTGACTAAAGGTGACAG.

Lentivirus infection of cultured neurons

To knock down rat CPEB3 (rCPEB3), the coding region of rCPEB3 was RT-PCR amplified from total RNA isolated from rat hippocampal neurons and cloned to pcDNA3.1+ plasmid. Five shRNA sequences designed against the mRNA were cloned into the lentiviral vector PLL3.7-Syn (gift of M Sheng); one that was particularly efficacious when tested in transfected 293T-17 cells corresponded to nucleotides 2320–2337 (CCGTACGTGCTGGATGAT) of rCPEB3. This particular construct was used to produce lentivirus using the viralpower packaging system (Invitrogen) according to the manufacturer's protocol. Generally, hippocampal neurons (4 days *in vitro* (DIV)) were infected with the virus (1–2 MOI) for 24 h. The infected neurons were cultured for another 3–4 days before RNA isolation or protein extraction.

Oocyte injection and immunoprecipitation

A 25 ng portion of RNA encoding myc-tagged CPEB, CPEB3, or chimeric CPEB was injected to *Xenopus* oocytes that were cultured for 14 h before stimulation with progesterone. For immunoprecipitation, 40 injected oocytes were homogenized in 200 μ l of immunoprecipitation buffer (20 mM HEPES, pH 7.6, 150 mM NaCl, 1 mM MgCl₂, 0.5 mM DTT, 0.1% Triton X-100, 100 μ g/ml RNaseA) and centrifuged at 12 000 g for 5 min at 4°C. The supernatant was incubated with myc antibody for 1 h and then immunoprecipitated with Dynabeads conjugated with antibody raised against mouse IgG. After several washes, the co-immunoprecipitated proteins were eluted and analyzed on Western blots. For UV crosslinking and immunoprecipitation, three plates of hippocampal neurons (~6–7 million cells, 21 DIV) were each covered with 200 μ l of immunoprecipitation buffer and UV irradiated on ice for 30 min. The cells were collected and centrifuged at 1000 g for 5 min to remove nuclei. One-twentieth of the resulting supernatant was saved for total RNA isolation. The remaining solution was equally divided for IgG and

CPEB3 antibody immunoprecipitation using Dynabeads conjugated with antibody against rabbit IgG. After 2 h incubation, the beads were washed 4 × with RIPA buffer and 1 × with genomic DNA lysis buffer (50 mM Tris, pH 7.4, 10 mM EDTA, 500 mM NaCl, 2.5 mM DTT, 0.5 mM spermidine, 1% Triton X-100). Approximately 300 µl of proteinase K solution (1 mg/ml in genomic DNA lysis buffer and 0.4 U/µl RNase inhibitor) was added to the total lysate and beads and incubated at 37°C for 30 min. The digested mixtures were used for RNA isolation and subsequent RT-PCR. The primer sequences are as follows:

Map2 sense 5'-GACAATTGGGTACCTTGCAAC and antisense 5'-GGAGAAGGCCAGCTGTAG;
NF sense 5'-GAGATGTATTACGCAAAGTACC and antisense 5'-CCAGTATGACCTTTATTGAGC;
GluR2 sense 5'-CAGAGCTCAGTCTTAGGCAG and antisense 5'-GTTTGTCTCCTTGGAGTACG.

RNA structure probing and footprinting

Methods for RNA alkaline hydrolysis, RNase T1 digestion, and lead acetate-mediated RNA cleavage have been described (Darnell *et al*, 2005). 5' end-labeled RNA was suspended in HEPES-SBB buffer (25 mM HEPES, pH 7.6, 200 mM KOAc, 5 mM Mg(OAc)₂, heat

denatured and cooled on ice, and digested in the presence of 20 µg tRNA with 0.035 U RNase V1 at 37°C for 5 min. The methods for RNA footprinting have been described (Hartmuth *et al*, 1999; Lee *et al*, 2003). The entire rat GluR2 3' UTR can be found in accession number NM_017261.

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