# Protein-only mechanism induces self-perpetuating changes in the activity of neuronal *Aplysia* cytoplasmic polyadenylation element binding protein (CPEB)

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Contributed by Susan Lindquist, December 22, 2010 (sent for review November 12, 2010)

Neuronal cytoplasmic polyadenylation element binding protein (CPEB) plays a critical role in maintaining the functional and morphological long-lasting synaptic changes that underlie learning and memory. It can undergo a prion switch, but it remains unclear if this self-templating change in protein conformation is alone sufficient to create a stable change in CPEB activity: a robust "protein-only" biochemical memory. To investigate, we take advantage of yeast cells wherein the neuronal CPEB of Aplysia is expressed in the absence of any neuronal factors and can stably adopt either an active or an inactive state. Reminiscent of wellcharacterized yeast prions, we find that CPEB can adopt several distinct activity states or "strains." These states are acquired at a much higher spontaneous rate than is typical of yeast prions, but they are extremely stable—perpetuating for years—and have all of the non-Mendelian genetic characteristics of bona fide yeast prions. CPEB levels are too low to allow direct physical characterization, but CPEB strains convert a fusion protein, which shares only the prion-like domain of CPEB, into amyloid in a strain-specific manner. Lysates of CPEB strains seed the purified prion domain to adopt the amyloid conformation with strain-specific efficiencies. Amyloid conformers generated by spontaneous assembly of the purified prion domain (and a more biochemically tractable derivative) transformed cells with inactive CPEB into the full range of distinct CPEB strains. Thus, CPEB employs a prion mechanism to create stable, finely tuned self-perpetuating biochemical memories. These biochemical memories might be used in the local homeostatic maintenance of long-term learning-related changes in synaptic morphology and function.

functional amyloid | molecular memory | synapse maintenance | translational activation

The mammalian prion protein, PrP, can adopt an alternative self-perpetuating conformation that causes transmissible neurodegenerative diseases (1). In fungi, unrelated proteins adopt similar self-perpetuating prion conformations that serve as "proteinonly" elements of epigenetic inheritance (2). Most fungal prion proteins can exist in two structurally and functionally distinct conformations, one soluble and the other a self-templating fibrillar amyloid (2–5). These self-templating amyloid states are passed from mother cells to their daughters via the cytoplasm, resulting in the inheritance of prion phenotypes that do not follow the Mendelian laws of inheritance. Notably, many yeast prions populate an ensemble of related but distinct amyloid states, each of which faithfully self-templates, which thereby produce a series of related phenotypes with distinct intensities.

Prion amyloids are not normally toxic in yeast. Indeed, depending upon the growth conditions, the phenotypes conferred by yeast prions can produce strong growth advantages (5, 6). In the human brain, amyloids are generally associated with fatal neurodegenerative diseases, but it is now widely believed that in these conditions, the amyloid is itself a benign, even protective, state with toxicity caused by other types of misfolded conformers (7). Indeed, amyloids are now known to play beneficial roles in

several normal biological processes in diverse organisms, including cell-adhesion, skin pigmentation, and peptide hormone storage (4, 5, 8–12). A particularly exciting possibility for an amyloid function is one suggested for the neuronal isoform of *Aplysia* CPEB (cytoplasmic polyadenylation element binding protein): that this protein uses a prion-like amyloid switch to create a molecular memory at neuronal synapses, thereby establishing a longlasting mark for synapse maintenance (13–17).

CPEBs are found in many cell types and regulate the translational dormancy and activation of specific mRNAs (18, 19). They bind U-rich cytosolic polyadenylation elements (CPEs) in mRNA 3' UTRs and subsequently recruit the polyadenylation machinery. The neuronal version of CPEB localizes in the presynaptic terminal of *Aplysia* and the dendrites of mice, where it can be activated following synaptic stimulation (15). Active CPEB then elongates polyadenylated tails of CPE-containing mRNAs, which encode structural and regulatory proteins that maintain long-term synaptic growth. The neuronal isoforms of CPEB differ from that found in other cell types in having a glutamine-rich N-terminal extension similar to the prion-determining domains of yeast prions (17).

In yeast, Aplysia CPEB can exist either in an active or an inactive form (17). When CPEB is active, it induces the translation of a reporter mRNA with a CPE element in its 3' UTR. This form of CPEB is in a larger protein/RNA complex than the inactive form and is dominant in crosses, suggesting that it might be a prion form of CPEB. This hypothesis came as a surprise because yeast prions are generally inactive in their amyloid conformations. The notion that the translationally active form of CPEB is prionlike gained support from a recent study in which overexpressed Aplysia CPEB formed self-templating multimers of an amyloidnature in the Aplysia neuron (15). Aplysia CPEB is active at the synapse, where it binds to CPE-containing mRNAs (18). It remains to be determined whether CPEB multimers actually represent translationally active sites in the neuron. However, conversion to the multimeric state is enhanced by the relevant neurotransmitter, and blocking this conversion (with a multimerspecific antibody) interferes with the maintenance of long-term synaptic facilitation. Furthermore, the Drosophila CPEB homolog orb2 is required for long-term conditioning of male courtship behavior. Like Aplysia CPEB, Orb2 carries an N-terminal glutamine-rich sequence. Deletion of this domain impairs long-term memory formation (20), indicating a physiological role for this prion domain-like sequence.

Despite these supporting data, the notion that a switch to a self-templating amyloid polymer could serve as biochemical

Author contributions: S.U.H. and S.L. designed research; S.U.H. performed research; S.U.H. and S.L. analyzed data; and S.U.H. and S.L. wrote the paper.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10. 1073/pnas.1019368108/-/DCSupplemental.

memory in the synapse was so unexpected that it continues to be viewed with considerable skepticism. The importance of understanding the mechanisms of synaptic memory demands a high standard of proof. Is a conformational switch in the CPEB protein alone sufficient to create a stable self-perpetuating change in CPEB activity and thereby form a protein-only molecular memory? This question is analogous to the long-standing controversy about whether self-perpetuating conformational changes in mammalian PrP were alone sufficient to create the transmissible agent in the spongiform encephalopathies. Confirming the protein-only hypothesis required generating prion conformers in vitro from purified protein and using this protein to transmit disease (21). Analogous experiments in which recombinant prion conformers transformed yeast cells, transmitting heritable new phenotypes, confirmed the prion mechanism for several endogenous yeast proteins (2, 22-24).

Yeast cells are 1 billion years removed from *Aplysia* evolutionarily and do not have the synaptic environment normally involved in regulating CPEB's neuronal activities. Therefore, yeast cells provide a "living test tube" to investigate the intrinsic capacity of heterologously expressed *Aplysia* CPEB to act as a protein-only molecular memory. Using yeast, we demonstrate that CPEB has an ability to exist in several related but distinct selfperpetuating activity states (strains) that are, indeed, based on a protein-only prion mechanism.

#### Results

**CPEB Can Adopt Distinct Heritable Activity States.** In yeast, the activity of neuronal *Aplysia* CPEB can be readily assayed with a  $\beta$ -gal reporter mRNA carrying a CPE sequence in its 3' UTR (17, 25). When CPEB is active yeast cells turn blue in the presence of the substrate X-Gal. When CPEB is inactive,  $\beta$ -gal is not expressed and cells remain white (Fig. 1*A*). Previous studies in yeast used a 2  $\mu$  vector with a highly variable copy number in cells that also produced a red pigment. To obtain much more uniform levels of CPEB expression and to more clearly visualize its activity state, we used a single copy *CEN* vector in cells without the red pigment. In this case, transformants were either white or blue on X-Gal plates. However, blue colonies were of many different shades (Fig. 1*B*). Such differences in color were not previously remarked upon.

After transformation, the ratio of cells in the CPEB-active versus the CPEB-inactive state was strongly influenced by the growth phase. Transformation of early log-phase cultures produced roughly equal mixtures of blue and white colonies; late log-phase cultures produced mostly blue colonies. This result might reflect some early influence of metabolism, as yeast cells switch from fermentation to respiration during this growth transition, or the effects of chaperone proteins, which are induced in late log. In any case, for both early and late log-phase transformants, blue colonies always exhibited many different shades of color.

To ensure that these differences in colony color reflect differences in CPEB activity, and not some artifactual difference in growth on X-Gal plates or substrate permeability, we assessed  $\beta$ -gal activity with the water-soluble substrate CPRG (chlorophenol red- $\beta$ -D-galactopyranoside) in detergent-permeabilized cells (Fig. 1*B*, *Lower*). Here, the intensity of red color reflects the activity of the enzyme. Many different levels of CPEB activity were observed with suspensions from different individual colonies, and these correlated with the intensities of blue color in the original colonies on X-Gal.

**Distinct CPEB Activity States Are Stably Inherited and Have a Prion-Like Genetic Character.** The stable inheritance of distinct but related phenotypic states, known as strains, over thousands of generations, is a common feature of prions in yeast. We continuously streaked cells with different CPEB activities biweekly over the course of a year or more. Switches in colony color oc-



**Fig. 1.** The activity of *Aplysia* CPEB in yeast. (*A*) Neuronal *Aplysia* CPEB acts as a translational activator in its putative prion conformation. Yeast cells carrying soluble inactive CPEB and a  $\beta$ -gal reporter followed by a CPE are translationally dormant and white in the presence of X-Gal. Cells convert to the active aggregated CPEB state, leading to the translation of  $\beta$ -gal and the generation of blue color. Both  $\beta$ -gal and CPEB are expressed on *CEN* plasmids under the GPD promoter. (*B*) Yeast cultures (w303 MAT $\alpha$ , ADE<sup>+</sup>) freshly transformed with *Aplysia CPEB* and  $\beta$ -gal display a variety of shades on X-Gal plates. The watersoluble  $\beta$ -gal activity in permeabilized cells (*Lower*). White cultures produce yellow color in the CPRG assay, blue cultures produce red. (C) Cytoductants were plated on 2% X-Gal plates or grown in CPRG buffer (*Lower*).

curred in both directions, but they were rare (Fig. S1). Activity states were stable for thousands of generations, with each cell giving rise to millions of progeny, and transitioning continuously through very different metabolic states: from rapid growth with abundant nutrients to stationary phase starvation. Thus, although a variety of CPEB activity states are accessible during the initial transformation, once a particular state is established: it is extremely stable and virtually impervious to changes in metabolism.

Another characteristic of prions in yeast is the dominance of their phenotypes in genetic crosses with nonprion strains. This dominance is because the protein template brought in by the prion-containing partner is equally efficient at templating the protein of both parents. To test the dominance of CPEB states, we mated cells from white colonies to cells from colonies with various shades of blue (hereafter "white cells" and "blue cells"). Matings between two white cells always produced white cells. Matings between white and blue cells always produced blue cells, with a color intensity true to that of the blue parent (Fig. S2).

As nonchromosomal, protein-based genetic elements, prions can be "donated" to mating partners by cytoduction (25), a process in which cytoplasmic factors (such as organelles or prions) are transferred from one cell to another without nuclear exchange. White, light blue, blue, and dark blue donors (the same strains as in Fig. 1*B*) were mated to white recipients. These recipients had both a defect nuclear fusion defect (Kar $\Delta$ 1–15) (Fig. 1*C*) and a mitochondrial defect ( $\rho^0$ ) that prevented growth on glycerol. Haploid progeny of nuclear fusion-deficient diploids were selected for the nuclear marker of the recipient (Ura<sup>+</sup>) and the cytoplasmic marker of the donor (by growth on glycerol without uracil). All lacked the His<sup>+</sup> marker of the donor, confirming the lack of nuclear transfer. When replicated to X-Gal plates, they exhibited various shades of blue, all faithfully reflecting the distinct CPEB activity state of the donor (Fig. 1*C*, *Right*). Control cytoductions with white donors produced only white progeny, confirming that mating itself did not change CPEB activity. Thus, as for yeast prion strains, the distinct self-perpetuating activity states of CPEB are not because of nuclear polymorphisms but to stable cytoplasmically inherited traits (5). Following yeast nomenclature, we designate these states [*CPEB*<sup>+</sup>], with the brackets indicating non-Mendelian inheritance, and the capital letters and italics designating a dominant heritable element.

[CPEB<sup>+</sup>] Strains Can Template Distinct Self-Perpetuating Amyloid States. A further characteristic of prion proteins is the modular nature of their prion domains. This nature allows them to template their distinct conformational states to other proteins that share the same prion domain, but no other amino acid sequence homology (4). The putative prion domain of CPEB, CPEB-Q, is an N-terminal, 128 amino acids long, and has a glutamine-rich composition reminiscent of yeast prion domains (17). Indeed, a glucocorticoid receptor transcription factor (GR) fused to the CPEB-Q domain spontaneously adopts active or inactive states that self-perpetuate as expected for a modular prion domain (17).

Using the single-copy constitutive expression constructs that allowed us to maintain and monitor distinct [*CPEB*<sup>+</sup>] strains so effectively, we were unable to physically detect the protein, either by Western blotting or immunofluorescence. Instead, to determine whether distinct [*CPEB*<sup>+</sup>] states are associated with distinct physical properties, we asked if they could template those states to a CPEB-Q-EGFP fusion protein. The CPEB-Q domain is very amyloidogenic and occasionally caused off-pathway nonamyloid aggregation in such fusions. Therefore, we included the highly charged M domain of the yeast prion protein Sup35, which counterbalances the aggregation tendency of its own prion domain but is otherwise not involved in prion formation (26). Cells with different CPEB activity states were transformed with a galactose-inducible *CBEB-Q-M-EGFP* on a 2 µ plasmid.

When induced with galactose, the fusion protein never produced foci in cells expressing CPEB-Q-M-EGFP alone (Fig. 2*A*) nor in white cells expressing full-length CPEB protein (Fig. 2*B*). The protein did produce foci in blue cells (Fig. 2 *C* and *D*). The variable copy number of the 2  $\mu$  *CPEB-Q-M-EGFP* vector precluded quantitative assessment and some cells were dark because they had lost the plasmid. However, consistently, large foci were observed in dark blue cells; smaller and fewer foci were observed in light blue cells, with some faint diffuse fluorescence both in cells with and without foci. A galactose-induced control without the putative prion domain, M-EGFP, never formed foci (27).

Most known prion proteins adopt an SDS-resistant amyloid conformation in the prion state. To determine whether these in vivo-templated foci were SDS-resistant amyloids, whole-cell lysates were separated using semidenaturing detergent agarose gel electrophoresis (SDD-AGE) (3, 28) and probed with an anti-GFP antibody (Fig. 2*E*). The CPEB-Q-M-EGFP protein from white cells ran at the position of the soluble nonamyloid monomer; the protein from blue cells ran as an SDS-resistant amyloid polymer. The polymers of light blue and dark blue cells were of similar size, but a greater fraction of the protein from dark blue cells migrated at this position (Fig. 2*E*). Thus, preexisting CPEB conformers in blue strains template an amyloid state to the CPEB-Q domain of newly synthesized protein, with strain-specific efficiencies.

Whole-Cell Lysates Seed Amyloid Formation by Purified CPEB-Q-M in a Strain-Specific Manner. The efficiencies with which cell lysates with well-characterized yeast prions seed the polymerization of soluble recombinant prion protein vary in a strain-specific manner (29). We asked whether whole-cell lysates (0.7% vol/vol) from our white, light blue, and blue strains can seed the aggregation of recombinant CPEB with strain-specific characteristics. The protein was purified under denaturing conditions (6 M guanidine hydrochloride, GdnHCl) to remove any preexisting structure. We used CPEB-Q-M for these studies because full-length CPEB produces amorphous aggregates when so denatured.

Purified CPEB-Q-M diluted into assembly buffer remained soluble in quiescent reactions over the course of 18 h (Fig. 3*A*, first panel). Lysates of cells not expressing CPEB had no effect (Fig. 3*A*, second panel). Lysates of strains expressing CPEB caused a fraction of purified CPEB-Q-M to assemble into SDS-resistant amyloid (Fig. 3*A*, third to fifth panels). This amyloid-templating activity was strongest with lysates from dark blue cells. Assembly was, however, always incomplete; higher concentrations of lysate did not result in more assembly. We note that seeding the polymerization of bona fide yeast prion proteins with whole-cell lysates is also generally incomplete (29, 30). The efficiency we obtained with [*CPEB*<sup>+</sup>] was lower than characteristically obtained



**Fig. 2.** CPEB strains template distinct self-perpetuating amyloid states. (*A*) *CPEB-Q-M-EGFP* is under a galactose promoter on a 2  $\mu$  plasmid. It remains soluble when overexpressed for 6 h in w303 ADE<sup>+</sup> cells lacking full-length CPEB. (*B*) Overexpressed CPEB-Q-M-EGFP remains soluble in white CPEB-expressing cells [in which full-length CPEB protein is soluble (16); data not shown]. In contrast, overexpression of CPEB-Q-M-EGFP in light blue (*C*) or blue (*D*) cells (in which full-length CPEB is aggregated; data not shown) results in fluorescent foci, indicating the aggregation of the protein. (*E*) Lysates of galactose-induced white, light blue, and blue cells expressing CPEB-Q-M-EGFP were separated using SDD-AGE and probed with an anti-GFP antibody. CPEB-Q-M-EGFP protein from white cells does not form SDS-resistant aggregates, whereas CPEB-Q-M-EGFP from light blue and blue cells is mostly SDS-resistant. The protein marker represents size in kilodaltons.



**Fig. 3.** Whole-cell lysates seed recombinant CPEB-Q-M amyloid assembly in a strain-specific manner. (*A*) Lysates of light blue and blue strains constitutively expressing CPEB and overexpressing CPEB-Q-M-EGFP seed amyloid assembly of purified his6-tagged CPEB-Q-M. An SDD-AGE gel was run and probed with anti-his6 antibody to identify SDS-resistant aggregates of CPEB-Q-M. Unrotated CPEB-Q-M does not aggregate in the absence of lysate (first panel). Lysates of cells not expressing CPEB or of a white strain incubated with CPEB-Q-M for 3 or 18 h cause little aggregation (second and third panels). In contrast, lysates of light blue and blue strains polymerize CPEB-Q-M (fourth and fifth panels). (*B*) Primary seed (from *A*) templates the aggregation of CPEB-Q-M in a strain-specific manner; 0.7% primary seed from white cells causes very little aggregation after 18 h (second panel). In contrast, primary seed from light blue and blue cell lysates GPEB-Q-M (third and fourth panels). Unrotated CPEB-Q-M remains soluble (first panel).

with the Sup35 prion, but not much lower than obtained with another well-characterized yeast prion, Rnq1 (30).

We asked whether the aggregates generated in such primary seeding reactions could be propagated to purified CPEB-Q-M in a secondary seeding reaction. This result would provide further evidence that the seeding templates have a protein-only nature. The very small amount of aggregation observed with white cells could not be propagated. The aggregation activities from light blue and dark blue cells could be propagated (Fig. 3*B*), but again, reactions did not go to completion. Presumably, the remaining protein spontaneously adopts an off-pathway conformation that does not productively interact with the template. Again, similar results have been reported for Rnq1 (30). In vivo, chaperones and other factors likely act to maintain these proteins in conformations that permit more efficient templating.

**CPEB-Q-M Fibers Are Sufficient to Induce Self-Perpetuating Changes** in **CPEB Activity.** The "gold standard" for establishing that a protein is a prion is to assemble recombinant, purified protein in vitro into a protein-only template that can transform cells from the nonprion to the prion state (31). To generate CPEB prion conformers in vitro solely from recombinant protein, we used purified guanidine-denatured CPEB-Q and CPEB-Q-M. Purified yeast prion domains spontaneously form prion fibers most efficiently under gentle agitation. When purified CPEB-Q and CPEB-Q-M were diluted into assembly buffer and subjected to gentle agita-



CPRG assay

**Fig. 4.** Fibers of CPEB-Q-M are sufficient for templating self-perpetuating change in CPEB activity. (A) Agitated CPEB-Q-M forms SDS-resistant amyloid polymers over time. Transmission electron micrographs show fibers and aggregates of CPEB-Q-M (B) and CPEB-Q (C). (Scale bars, 100 nm.) (D) Preformed CPEB-Q-M amyloid fibers are introduced along with a physiological marker (URA3) into yeast spheroplasts by treatment with polyethylene glycol. Cells with inactive CPEB stay white when transformed with URA3 alone (D1), whereas cells with active CPEB remain blue (D2). Cells also maintain their initial CPEB activity states when soluble CPEB-Q-M is introduced alongside URA3 (D3 and D4). Only when sonicated CPEB-Q-M fibers are transformed, cells turn blue indicating that CPEB switches into its active state (D5). (E) Fiber-transformed yeast tested with the CPRG assay: cells with inactive CPEB remain red (E2). Many fiber-transformed cells switch from yellow to the red CPEB activity state (E3).

tion, both formed insoluble material that was resistant to solubilization by SDS, as expected for an amyloid (Fig. 4*A* and Fig. S3). This material produced the same spectral shift on staining with Thioflavin T as observed with other amyloids. By transmission electron microscopy (TEM) (Fig. 4*B* and *C*), CPEB-Q-M formed fibers that were more homogeneous than CPEB-Q, suggesting that the addition of the solubilizing M domain of Sup35 decreases off-pathway, nonamyloid aggregation and supports more efficient templating. In full-length CPEB, the RNA binding domain likely serves a similar function (perhaps with associated RNA). To determine whether the CPEB-Q and CPEB-Q-M fibers are fully functional prion replication templates, we used them to transform cells stripped of their cell walls (23, 24). Blue and white cells transformed with the control plasmid (Fig. 4 D1 and D2) or with CPEB-Q-M in its soluble form (Fig. 4 D3 and D4) maintained their initial phenotypes: white cells never gave rise to blue, blue never gave rise to white. Complementary results were obtained with CPRG (Fig. 4 E1 and E2). However, blue cells did sometimes shift to a different shade of blue. Thus, although the stress of the protein transformation protocol did not induce or cure [*CPEB*<sup>+</sup>], it could cause cells to shift strain types (Fig. 4 D2 and E2).

In contrast, when CPEB-Q or CPEB-Q-M was introduced in the amyloid form, many white cells switched to the blue (Fig. 4D5). Importantly, blue cells transformed with the same preparations of CPEB-Q and CPEB-Q-M never gave rise to white. Again, complementary results were obtained with CPRG (Fig. 4E3). Similar results were obtained with several independently assembled fiber preparations, except that the efficiency of transformation varied from 30% to 90%. Transformation efficiencies were somewhat higher with CPEB-Q-M than with CPEB-Q.

We attempted to produce sufficient quantities of pure, strainspecific  $[CPEB^+]$  templates for protein transformation using cell lysates to seed polymerization. Unfortunately, the off-pathway aggregation that takes over in nonagitated assembly reactions (Fig. 3) prevented us from generating sufficient material. In the more efficient rotated reactions, spontaneous assembly took over, again preventing strain-specific template amplification. However, the amyloids produced by spontaneous assembly of both CPEB-Q and CPEB-Q-M gave rise to the full range of blue  $[CPEB^+]$  colors (Fig. 4D5 and E3).

These findings were confirmed to reflect different  $\beta$ -gal activities using the CPRG assay. Mating experiments confirmed the dominance of the transformation-acquired [*CPEB*<sup>+</sup>] state (Fig. S4*A*). Fiber-transformed cells remained blue, and retained their initial diverse color intensities, after continuous restreaking over the course of a full year (Fig. S4*B*). Thus, the diversity of [*CPEB*<sup>+</sup>] strain types are based on a protein-only prion mechanism.

#### Discussion

An intriguing concept emerging in recent years is that cells of both lower and higher organisms can harness prion-based conformational changes to initiate beneficial switches in phenotype with the durability that is normally associated with replicating nucleic acids (2). We demonstrate that protein-only conformational changes in neuronal *Aplysia* CPEB suffice to generate not just one but multiple stable self-perpetuating CPEB activities. As demonstrated previously with yeast prions, amyloid conformers transmit and encode these [*CPEB*<sup>+</sup>] prion states (3, 21–24). Unlike most yeast prions, however, it is the prion form of CPEB that is the active form.

How is it possible for CPEB to function in an assembled amyloid structure? One factor is the modularity of prion domains, which do not propagate their amyloid conformations into adjacent globular domains. In the fusion with CPEB-Q-M, EGFP retains its fold, remaining fluorescent when the prion domain enters the amyloid state. Sup35 and other yeast prion domains behave in the same manner. Indeed, electron microscopy of assembled full-length Sup35 reveals that the C-terminal translation domain forms an orderly array outside of the amyloid fiber core (32) (Fig. S54). Full-length CPEB almost certainly does the same.

However, how could CPEB actually become more active in the prion state? There are at least two, nonexclusive possibilities. First, in its nonamyloid form the prion domain might inhibit the activity of CPEB's RNA binding domain. Assembly would sequester the inhibitor, freeing the C-terminal domain for function. Second, polymerization of CPEB would strongly increase its local concentration, allowing it to more efficiently recruit other factors involved in translational activation, and perhaps even to scaffold their assembly (Fig. S5*B*).

In the yeast system, prion-based conformational switches create heritable and self-perpetuating epigenetic elements. CPEB's prion states confer these same epigenetic properties in this heterologous system. Neurons are nondividing cells that cannot manifest such behaviors. However, the prion conformational switch is a biochemical type of memory that might be exploited for maintaining the structure of newly formed synapses, the essence of neuronal memory. It has long been known that CPEB activity plays a vital part in long-term facilitation in the neuron (15). In addition to promoting long-term maintenance of the active state, the assembly of CPEB into prion polymers would have an additional function at the synapse. Of the hundreds to thousands of connections that neurons make with other neurons, the essential character of memory is the regulation of individual synapses in particular circuitries (33, 34). As previously noted, the nondiffusible nature of CPEB prion assemblies would keep the active form of CPEB local to individual synapses, providing a maintenance function for the local synaptic mark and establishing long-term memory (13).

Like many of the well-characterized yeast prion proteins, CPEB has an intrinsic capacity to exist in several different finetuned activity states, or strains, which are remarkably stable and self-perpetuating. As is the case for strains of bona fide yeast prions, CPEB strains template soluble protein with different efficiencies. It is tempting to speculate that this capacity for finely tuned translational activity, with its associated differences in templating efficiencies, might be used by neurons to create synapses with different strengths and perhaps different durabilities.

In the heterologous yeast system, the appearance of CPEB strains had a stochastic character. However, [CPEB+] appears much more frequently than is typical of yeast prions. That is, this protein has an unusually high propensity to acquire a prion fold. Presumably, a factor that negatively regulates this fold in neurons is missing in yeast. Indeed, given the importance of synaptic memory, it seems certain that CPEB activity states will prove to be tightly regulated by multiple factors in neurons. (Indeed, even in yeast we found that the metabolic state of the cell influenced the frequency of the prion's appearance.) However, once these different states are established, by whatever neuronal factors control them, our findings suggest distinct activity states might be locally maintained by the intrinsic capacity of CPEB to self-perpetuate them. Yeast cells even provide a clue that these states, stable as they are, are subject to modulation. Although CPEB strains were normally very durable, switches between strain types occurred when cells were subject to the stresses of protein transformation. Therefore, the properties of CPEB strains could provide a local, rather than global, mechanism for synaptic homeostasis (35).

In CPEB's natural neuronal regulatory environment it would have been very difficult to unambiguously establish CPEB's intrinsic capacity for acquiring distinct, finely tuned activity states. The absence of neuronal conformation modulators in yeast now provides an ideal system to investigate neuron-specific modulators of CPEB functional status, by introducing them into CPEB-expressing yeast cells.

In addition to serving as a living test tube to investigate *Aplysia* neuronal CPEB activity, it is notable that yeast has a functional homolog of *CPEB*. This homolog, Hrp1, is part of the yeast polyadenylation machinery (19). Hrp1 also meets many of the experimental criteria for a yeast prion described in a comprehensive screen for new prion proteins (3). Indeed, several proteins of the polyadenylation complex (including Hrp1) carry domains enriched in glutamines and asparagines that resemble prion domains. Through its potential interaction with other putative prions in the polyadenylation machinery CPEB/Hrp1 may be part of an ancient prion-based mechanism of regulating mRNA activities in eukaryotic cells. Many newly identified yeast proteins with prion domains (3) interact, as does neuronal CPEB, with nucleic acid, either as RNA binding proteins (e.g., Pub1, Puf2) or

transcription factors (e.g., Mot3, Swi1, Cyc8, Sfp1), which positions them, as well, to act as self-perpetuating regulators of gene expression. Thus, prion proteins may form a new system for durable epigenetic regulation of diverse cellular functions operating in a wide variety of biological contexts.

#### **Materials and Methods**

**Constructs, Yeast Strains, and Media.** *Aplysia CPEB* was codon-optimized for expression in yeast (BioBasic, Inc.) and introduced into the Gateway entry vector *pENTRID-TOPO*. Gateway LR reactions with *413GPD-ccdB*, *413GPD-ccdB-EGFP*, or *416GPD-ccdB-EGFP* destination vectors produced *GPD-ApCPEB* and *GPD-ApCPEB-EGFP* (HIS<sup>+</sup> or URA<sup>+</sup>). The N domain of *pAED4-ScNM-his7* (yA7111) was replaced by codon-optimized *CPEB-Q* to produce his6-tagged *CPEB-Q-M*, and introduced into *pENTRID-TOPO*. Gateway LR reactions with *426GAL-ccdB-EGFP* produced *GAL-CPEB-Q-M-EGFP*. The *βgal-CPE* reporter, his6-tagged *CPEB-Q* constructs and noncodon-optimized CPEB were kindly provided by K. Si (Stowers Institute, Kansas City, MO) (16). CPEB-expressing yeast strains w303 MAT a or  $\alpha$ , ADE<sup>+</sup> (y4436 and y4437), BY4741 (Euroscaff) for fiber transformation and A3464 for cytoduction were grown on standard synthetic media lacking particular amino acids/bases, with D-glucose or D-galactose as a carbon source; details in *SI Materials and Methods*.

X-Gal and CPRG Assays. X-Gal assays on plates were performed as described (16). For CPRG assays, overnight cultures were grown (starting OD<sub>600</sub> 0.2) in YPD buffer for 1h and an equal volume of CPRG assay buffer [100 mM hepes, pH 7.25, 150 mM NaCl, 5 mM L-aspartate, 1% BSA, 0.05% Tween, 0.5% SDS, 1.2 mM CPRG (884308; Roche)] was added.

**Light and Electron Microscopy.** Overnight cell cultures with *GPD-CPEB* (*CEN*),  $\beta$ gal-CPE, and *GAL-CPEB-Q-M-EGFP* (2  $\mu$ ) plasmids were grown (starting

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OD<sub>600</sub> 0.2) in galactose without uracil, histidine, and leucine to midlog phase and examined with an Axioplan microscope with a 100× objective (Zeiss). Photoshop was used for linear adjustments of brightness and contrast. For TEM, 5  $\mu$ L of protein solution was applied to 200 mesh carbon-coated copper grids, stained with uranyl acetate and imaged as detailed in *SI Materials and Methods*.

**Protein Preparation, Assembly, and Analysis.** SDD-AGE analysis of whole cell lysates or in vitro assembly reactions was performed as described (3, 28, 29). His6-tagged CPEB-Q-M and CPEB-Q proteins were purified under denaturing conditions with Ni+2-Agarose columns as described in *SI Materials and Methods.* CPEB-Q-M was used to test the seeding efficiency of various whole cell lysates in assembly buffer (0.2 M GdnHCl, 250 mM NaCl, 5 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5, 5 µg/mL aprotinin and leupeptin, 5 mM EDTA, 5 mM TCEP) for the indicated times using SDD-AGE and visualized with anti-his6 antibody (Invitrogen). For spontaneous assembly, purified his6-tagged CPEB-Q-M or CPEB-Q proteins were diluted into assembly buffer (250 mM NaCl, 5 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.5, 5 mM EDTA, 5 mM TCEP) to a final concentration of 2 µM in 0.5 M or 1 M GdnHCl. Samples were rotated and 20-µL aliquots were removed at indicated times for analysis with SDD-AGE.

For fiber transformation, amyloid fibers of CPEB-Q-M and CPEB-Q were sonicated and transformed into a white or blue BY4741 strain expressing CPEB and carrying the  $\beta$ gal-CPE reporter gene using methodology previously described (23).

ACKNOWLEDGMENTS. We thank K. Allendoerfer, K. Matlack, R. Halfmann, J. Goodman, J. Dong, L. Pepper, and D. Jarosz for critical reading of the manuscript and Thomas DiCesare for designing Fig. S5. We are especially grateful for the help and advice of Kausik Si and Eric Kandel. This work was supported by the G. Harold and Leila Y. Mathers Foundation (S.L.) and National Research Service Award Fellowship AGO30269 (to S.U.H.).

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# **Supporting Information**

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### SI Materials and Methods

Constructs, Yeast Strains, and Media. Sequence for codon-optimized Aplysia cytoplasmic polyadenylation element binding protein (CPEB): ATGCAAGCCATGGCCGTTGCTTCACAAAGCCC-TCAAACTGTTGATCAGGCAATTTCAGTAAAGACGGA-TTATGAGGACAACCAACAGGAACACATCCCGAGCAAT-TTCGAAATTTTTCGACGCATAAACGCTCTCCTGGACA-ACTCTTTGGAAGCAAACAACGTGAGCTGTTCCCAGTC-TCAATCACAGCAACAGCAGCAGCAGACTCAACAGCA-GCAGCAGCAACAGCAGCAACAGCAGCAGCAGCAACA-TTTACAGCAAGTTCAGCAACAGCAGCTATTGAAACAG-CAGCAGCAACAGGCTCAGCAGCAGCAAATTCAGCAG-CAACTTCTGCAGCAGCAGCAGCAAAAGCAGCAGTTAC-AACAGCAACAGCAGCAAGAGCAGTTACAGCAGCAAC-AGCTGCAGCTGCAGCAACAGTTACAGCAACAGTTGCA-GCATATTCAGAAAGAACCCAGCAGCCACACCTACACG-CCTGGTCCATCCCCGGAACTTCAGTCCGTGCTCAACTA-CGCCAATGTTCCGCTGTCCAAGTCTGCCGCCTTCAACT-GCAACAACAGCAGCAGCTACAGCGTGGGTCCGACCCC-GGTGCAGTCTCCGGTCACCCCGAGCCCGGCTGCGAGT-GCTGTGACAGTTAACAGCCCAAGCTATGGAAATTTTC-AGTTGTTCGGTGAGAATGCTTTCGACAGTACGACTCC-GTTTCAGAGTGACGGGACCAGCCAGAGCCACAGTCGA-AGGACCCAATGGTTGTGATGAGCCCAGGGCGAGATA-GTATTATTCCTCTCTCTCCCACCGAGAAGATCCTGTAT-CAGAATTTCTTGCTGTCGAAGCAGGCGCAAGGAGAGA-ACACAGCACTGCCCCCGAGCCCTCCCCACGAAATAAT-GCCCTTGTCGCCCCTTGAAAAGAAGCTGTACTCGAAC-CTGCTCTCCAAGCACACTCAGGGCATGCGCGCTATCA-ACTCGACCAGCCCGCTGCAGACGCCACTCACGCCACC-GAGATCCCCTCAGGAAGTGCTGTACGCCTCTATGCCA-GCTGCTCAAGTGGGCGAGAGCTCTTCTGTCATTGATA-TGATGAGTCGAATGGACTTGAGTGGTCGTAACCAGCA-GGCGGACTACTCTGGTACCCTGGCTTTCCTCGACGCTC-ACAATGTGCTCCGCAGAAGAACCCCTAGTTCCTCGCG-TTCTCGGTCTGTGATGGAGCGCTCTGCCCCTTCCTCGT-ATTTTGCAAACCTTGACCCTTACGCCATCGACAGGGCA-GCTCGTTTGCACAGAAACGCCGCAGCTGTAGGCGAGG-CCAGTTGTACTTGGAGTGGTCACCTGCCCCCAGAAA-CCACGAGAACCCGGTCTACTCGCCCAAGGTCTTTCTGG-GAGGCGTGCCCTGGGATATAACTGAATCTGGTTTGCA-GGCGGCATTCAGCAAGTACGGACATCTCAAGATTGAA-TGGCCAGGAAAAGATGGCTACGTACACCTCCTGTTTG-ACGTGGAGAAGTCTGTGCGCTCTTTGCTGCAGGCTTG-CACCCACGACTTCAGCAATGGGGGATTATTTCTACAAA-ATTTCTTCCAGGAGGATGCGTTCTAAAGAGGTGCAGG-TCATCCCGTGGGTGTTGGCCGGACAGTAACCATGTGTT-CCAGCCCTCGCAGCGTCTGGAGTCGAACAAGACAGTG-TTTGTCGGGGCCCTGCACGGGATGATCACGGCCGAGG-CTCTGGGCCGAATCATGAGCGACCTGTTTGGGAACGT-CGTCTACGCAGGCATCGACACGGATAAACACAAGTAC-CCCATTGGCTCTGGCCGTGTTACGTTCAGCAGTCGCA-AAAGCTACATGAAAGCCGTGCAAGCTGCATTTGTGGA-AATCAAGACGCCCAAGTTTACAAAAAGCTGCAAGTG-GACCCATATCTCGGCGATGCCATCTGCAGCTTGTGTAA-

CTCGCACCAGGGCAACTATTTCTGCCGGGACCTGCTCT-GTTTCAAGTACCTTTGTCGCTCCTGCTGGTACTGGCAG-CACGCCCCGGACTCGATGAGACAGCACCGGCCACTCA-CACGCAACACCAAGTCATCTCTGTCTCTC. Design of entry clone for *Aplysia CPEB* was as follows: Codon-optimized *CPEB* was PCR-amplified with *CPEBcoforfl* 5' CACC-ATGCAAGCT-ATGGCAGTTGCC and *CPEBcorevfl* 5' TAGTGATAAAGA-ACTCTTAG and introduced into the Gateway (Invitrogen) entry vector *pENTR/p-TOPO* using a BP Clonase II enzyme mix following the manufacturer's protocol.

The genotypes of yeast strains used are: w303 (a or  $\alpha$ ; ade2-1:: Ade2; his3-11.15; leu2-3.12; trp1-1; ura3-1; can1-100); A3464 (W303a; ade2-1; his3-11.15; leu2-3.12; trp1-1; ura3-1; can1-100;  $\rho$ 0; kar1-15).

**Plasmid Transformation.** Overnight cultures of the desired yeast strain were diluted to  $OD_{600}$  0.2 and grown to 0.5 to 0.8 OD at 30 °C, centrifuged, resuspended in 0.1 M LiCH<sub>3</sub>COO, and incubated for 10 min at 30 °C. After centrifugation, yeast pellets were resuspended in 0.1 M LiCH<sub>3</sub>COO, 33% PEG, 3 mg/mL boiled salmon sperm DNA, and 200 ng of the plasmid of interest. After incubating at 30 °C for 30 min, cells were heat-shocked at 42 °C for 20 min and plated on SD plates lacking the appropriate amino acids.

**Mating.** The two desired strains of opposite mating type were streaked out on YPD plates and grown overnight. They were mixed on an YPD plate, grown for 8 h at 30  $^{\circ}$ C and then transferred to SD plates lacking the appropriate amino acids.

**Purification of CPEB-Q and CPEB-Q-M.** One liter of an OD<sub>600</sub> 0.7 culture of BL21(DE3)pLysS competent cells (Stratagene) expressing his6-tagged CPEB-Q-M or CPEB-Q in 2×YT (with ampicillin and chloramphenicol) was induced with 1 mM IPTG and harvested after 1 h of incubation. Pellets were resuspended in 20 mL 6 M GdnHCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH8, and centrifuged for 20 min at 3,500 × g in a Sorvall SS-34 rotor at room temperature. The cleared supernatant was applied to a Ni+2-Agarose column, the column washed five times (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 8, 10 mM Tris-HCl, and 40 mM imidazol), and eluted with 2 mL elution buffer (8 M urea, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 2.5, 10 mM Tris-HCl, and 40 mM imidazol), and protein in the eluate precipitated with ice-cold methanol.

**SDD-AGE.** The protein marker used is Precision Plus Protein Dual Color Standards (BIO-RAD).

**Transmission Electron Microscopy.** For negative-staining, 5  $\mu$ L of protein solution was applied to 200 mesh carbon-coated copper grids (Pella) for 1 min and excess solution was removed. The grid was washed with five drops of 2% (wt/vol) aqueous uranyl acetate solution and then stained with one drop for 2 min. Excess stain was removed and the grids air-dried. Images were obtained with a FEI Technai Spirit Transmission Electron Microscope and recorded on DCC camera (ORCA-HR; Hamamatsu).



Fig. S1. CPEB activity states switch rarely. Typical yeast prions are metastable, and are occasionally lost or gained. This was also true for the distinct CPEB activity states. These rare switches in activity state (red arrows) were more common in older colonies.



**Fig. S2.** [*CPEB*<sup>+</sup>] states are dominantly inherited. Mating experiments establish the dominance of the prion state [*CPEB*<sup>+</sup>]. Haploid white colonies mated with haploid blue colonies always result in blue colonies (light blue × blue and blue × white). Matings between two white colonies always produced white colonies. The results were confirmed with the CPRG (chlorophenol red- $\beta$ -p-galactopyranoside) assay (*Lower*).



Fig. S3. CPEB-Q assembles into an amyloid in vitro. Mildly agitated CPEB-Q forms SDS-resistant amyloid polymers in 1 M GdnHCl. In contrast to CPEB-Q-M, CPEB-Q cannot be transferred to a nitrocellulose membrane after running an SDD-AGE gel. Aggregation of CPEB-Q can be monitored by the disappearance of the band of monomeric CPEB-Q and the concomitant appearance of SDS-resistant aggregates in the wells of an SDS/PAGE gel. Boiling the samples (100 °C) almost completely reduces the aggregation of CPEB-Q material in the wells and restores the monomeric CPEB-Q bands.



**Fig. S4.** The mating of the protein-transformed blue cells to white haploids demonstrates the dominance of the CPEB-Q-M-induced blue CPEB activity. (*A*) Haploid white cells mated with haploid blue cells always result in blue cells (*Upper Quadrant*). White cells mated with mock-transformed (plasmid only) cells result in white cells (*Lower Quadrant*). Blue cells mated with mock-transformed white cells always result in blue cells (*Lower Quadrant*). Blue cells mated with mock-transformed white cells always result in blue cells (*Right Quadrant*). Cells that were transformed with CPEB-Q-M seed that turned blue, mated with white cells, result in blue cells (*Left Quadrant*). (*B*) Fiber-transformed strains were streaked out continuously on X-Gal plates. The strain color was maintained over many generations.

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**Fig. S5.** Model for functional CPEB amyloid assembly. (A) Electron microscopy of full-length Sup35 fibers provides a prototype of prion assembly [Reprinted from *Cell*, Vol 89, Glover JR, et al., Self-seeded fibers formed by Sup35, the protein determinant of [PSI+], a heritable prion-like factor of *S. cerevisiae.*, pp. 811-819, Copyright (1997), with permission from Elsevier]. The N-terminal prion domain forms the spine of the amyloid. The C-terminal translation domain of Sup35 protrudes from the ordered amyloid fiber, which is visualized most effectively in these fibers spread onto grids in the presence of high salt. Full-length CPEB would be expected to behave similarly. For the Sup35 prion [*PSI*<sup>+</sup>] assembly leads to a loss of function because it sequesters the protein from the ribosome, where it normally functions. However, for CPEB, and other prions as well, this might simply provide a scaffold upon which they can function. (*B*) Model of CPEB activity in the amyloid form. Amyloid polymerization of the prion domain CPEB-Q (light blue) releases the sequestered mRNA-binding C terminus (green) of CPEB for function, resulting in a high local concentration of CPEB and scaffolding the recruitment of factors involved in translational activation (hexagons) of mRNA (blue), with CPE consensus sequences (red).