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A Neuronal Isoform of the *Aplysia* CPEB Has Prion-Like Properties

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Summary

Prion proteins have the unusual capacity to fold into two functionally distinct conformations, one of which is self-perpetuating. When yeast prion proteins switch state, they produce heritable phenotypes. We report prion-like properties in a neuronal member of the CPEB family (cytoplasmic polyadenylation element binding protein), which regulates mRNA translation. Compared to other CPEB family members, the neuronal protein has an N-terminal extension that shares characteristics of yeast prion-determinants: a high glutamine content and predicted conformational flexibility. When fused to a reporter protein in yeast, this region confers upon it the epigenetic changes in state that characterize yeast prions. Full-length CPEB undergoes similar changes, but surprisingly it is the dominant, self-perpetuating prion-like form that has the greatest capacity to stimulate translation of CPEB-regulated mRNA. We hypothesize that conversion of CPEB to a prion-like state in stimulated synapses helps to maintain long-term synaptic changes associated with memory storage.

Introduction

The term "prion" was first applied to the proteinacious infectious agent in a group of mammalian neurodegenerative disorders (transmissible spongiform encephalopathies) (Prusiner, 1998). Transmissibility is widely believed to stem from the ability of the prion form of PrP protein, PrP^{sc}, to promote the conformational change of the normal cellular form, PrP^{c}, to the PrP^{sc} conformation (Collinge, 2001; Prusiner, 1998). Wickner suggested that a similar mechanism might explain the unusual dominant, cytoplasmic inheritance of certain traits in the yeast *Saccharomyces cerevisiae* (Wickner, 1994) and a wide array of genetic and biochemical evidence supports this hypothesis (Glover et al., 1997; Patino et al., 1996; Paushkin et al., 1997; Sparrer et al., 2000; Wickner, 1994; Wickner and Masison, 1996).

Unlike PrP, yeast prions are generally not pathogenic. Rather, they produce changes in phenotype that mimic conventional loss-of-function mutations. The patterns of their inheritance, however, are very different. The loss-of-function phenotypes caused by yeast prions are dominant, rather than recessive. Moreover, they are inherited in a non-Mendelian fashion. After cells containing the prion form of the protein are mated to cells containing the nonprion form and then sporulated, most progeny contain the prion form. The dominant nonnuclear inheritance of prion phenotypes results from two basic factors. First, proteins that are in the prion conformation promote conversion of other proteins of the same type to the prion state. Second, the prion form of the protein is transferred from a mother cell to her daughters and mating partners, creating a dominant change in phenotype that is perpetuated through subsequent generations (Patino et al., 1996; Paushkin et al., 1997; Sparrer et al., 2000). The study of fungal prions has established that stable self-perpetuating conformational changes in proteins can occur in diverse organisms, produce distinct phenotypes, involve molecules with very different physiological functions, and can sometimes be beneficial (Coustou et al., 1997; True and Lindquist, 2000).

In the well-characterized yeast prions [SUP35], [URE3], and [RNQ1], a specific region is responsible for prion behavior (DePace et al., 1998; Glover et al., 1997; King et al., 1997; Masison and Wickner, 1995; Sondheimer and Lindquist, 2000). These regions of 65–250 amino acids show no sequence homology to each other but share four striking characteristics: (1) they are unusually rich in the polar residues glutamine (Q) or asparagine (N) (Masison and Wickner, 1995; Michelitsch and Weissman, 2000; Osherovich and Weissman, 2001; Sondheimer and Lindquist, 2000); (2) by structural-prediction algorithms they score as having a low propensity for any particular secondary structure, an indication of conformational flexibility that likely relates to their ability to switch states; (3) they can exist as soluble species or ordered self-perpetuating aggregates; and (4) they are dispensable for the normal function of their associated protein domains (Tuite, 2000). While studying a neuron-specific isoform of *c*ytoplasmic *p*olyadenylation *e*lement *b*inding protein (CPEB) in *Aplysia californica*, we noted that its N terminus has features reminiscent of a prion-like domain in yeast.

CPEB was initially identified in *Xenopus* oocytes as a translational regulator that activates dormant mRNAs by elongating their poly (A) tails (Hake and Richter, 1994). CPEB serves not only as an activator, but in some cases it is also a repressor (deMoor and Richter, 1999). These dual functions are normally controlled by phosphorylation (Mendez et al., 2002). However, the neuronal isoform of *Aplysia* CPEB lacks these phosphorylation sites. Instead synaptic stimulation with serotonin increases the amount of the CPEB protein (Si et al., 2003 [this issue of *Cell*]). Furthermore, in *Aplysia*, the neuronal CPEB is causally involved in maintaining long-term synaptic facilitation (Si et al, 2003).

We report that N-terminal domain of the *Aplysia* neu-

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ronal CPEB can confer upon another protein, the glucocorticoid receptor, a self-perpetuating change in state with the hallmarks of a yeast prion. Moreover, full-length CPEB itself has prion-like properties, and surprisingly the prion form of the protein is the more active form of the protein. Although we lack direct evidence that the protein exists in a prion-like state in *Aplysia*, our findings suggest a mechanism by which a prion-like state of CPEB could selectively sustain an altered rate of translation locally, at some synapses and not at others, and thereby contribute to the long-term maintenance of a self-sustaining synapse-specific plastic change.

Results

The Prion-Like Region of CPEB Produces Distinct Heritable Functional States

The amino-terminal end of *Aplysia* CPEB has an unusual amino acid composition. The N-terminal 160 amino acids of 44 randomly selected *Aplysia* proteins have an average glutamine + asparagine (Q + N) content of 10%, typical of proteins in other species (see Supplemental Figure S1 available at http://www.cell.com/cgi/content/full/115/7/879/DC1). In contrast, the N-terminal 160 amino acids of ApCPEB have a Q + N content of 48% (Figure 1A). The *Aplysia* CPEB N-terminal domain also resembles yeast prion domains in that it lacks predictable secondary structure when analyzed by standard structural predication algorithms (data not shown). A search of the protein sequence database revealed putative homologs of the *Aplysia* neuronal CPEB in *Drosophila*, mouse, and humans, with N-terminal extensions of similar character (see Supplemental Figure S1 available on *Cell* website). Moreover, the *Drosophila* (Si et al. 2003) and mouse isoforms have been found to be expressed in neurons (Theis et al., 2003). The conservation of such a CPEB neuronal isoform suggests that the N-terminal extension has an evolutionarily conserved function. Since many different rigorous tests for prions are available in yeast cells, we employed them for further characterization of the properties of *Aplysia* CPEB.

One test of a prion determinant is the ability to confer upon another protein, to which it is fused, a capacity to exist in distinct physical and functional states that are interconvertible and heritable. As previously described for the prion-determining region of yeast prions (DePace et al., 1998; Edskes et al., 1999; Li and Lindquist, 2000), we fused the N-terminal 160 amino acids of *Aplysia* CPEB (CPEBQ) to the green fluorescence protein (GFP). GFP is normally soluble in yeast, but the fusion to CPEBQ conferred for the GFP a capacity to exist in distinct states: a few large aggregates, many small aggregates, or soluble protein. Notably, these distinct states were heritable. Mother cells almost always gave rise to daughter cells in which the GFP fusion protein was in the same state: soluble, small aggregates, or large aggregates (Supplemental Figure S2 available on *Cell* website).

Next, we took advantage of the fact that prion domains in yeast are modular and can transfer prion-like behaviors to heterologous proteins (Li and Lindquist, 2000; Sondheimer and Lindquist, 2000). As previously described for the prion domain of Sup35, NM (Li and Lindquist, 2000), we fused the N-terminal domain of CPEB to a constitutively active variant of the rat glucocorticoid receptor (GR^{526}), a transcription factor (Figure 1B). A β-galactosidase gene under the control of a GR response element provided a convenient blue-white colony color assay for GR activity: blue when the protein is active and white when the protein is inactive. Most cells expressing CPEBQ-GR^{526} were blue and remained blue from generation to generation (Figure 1B), indicating that the CPEBQ fusion did not impair the transcriptional activity of GR^{526}. As is the case for most yeast prions and for the NM-GR fusion (Li and Lindquist, 2000), the CPEBQ-GR activity had a metastable character. Blue colonies gave rise to white colonies with a frequency (10^{-5}) that is higher than the typical rate of spontaneous mutation (10^{-6}). These white colonies continued to give rise to white colonies upon restreaking for generation after generation, but occasionally gave rise to blue colonies again (Figure 1B). In contrast, GR^{526} alone rarely (10^{-6}) produced white colonies, and when it did, they never reverted to blue colonies again.

This interconversion between blue and white states could result from either a genetic or an epigenetic change. Analysis of plasmids extracted from blue and white CPEBQ-GR^{526} colonies, and of the cells themselves after loss of the plasmids, demonstrated that the changes in GR activity in blue and white cells were not due to mutations in either the plasmids or the cellular genomes (data not shown). Furthermore, as shown by immunoblotting, the level of GR^{526} produced by white and blue colonies was similar (Figure 1C). Thus, when CPEBQ is fused to GR^{526}, the fusion protein acquires the ability to exist in two functionally distinct states that are heritable and interconvertible at low frequency, in an epigenetic manner.

Distinct Functional States of CPEBQ-GR Are Associated with Distinct Physical States and Are Transmissible

To test directly if the distinct heritable functional states of CPEBQ-GR^{526} were associated with distinct physical states of the protein, we examined the sedimentation behavior and resistance to protease digestion of the two functional states (Figure 2A). When extracts were analyzed on step gradients of 30%–40% sucrose at 140,000 × g the CPEBQ-GR^{526} proteins of blue and white colonies behaved very differently. Protein from blue cells was concentrated in the middle of the gradient, protein from while cells toward the bottom (Figure 2B). In the same experiment, the distribution of two other yeast proteins—a 60S ribosomal protein Tcm1p (Figure 2B, bottom image) and a golgi associated protein Bet3p (data not shown), as well as total Coomassie stainable material—were similar in blue and white cells.

Next, peak fractions containing CPEBQ-GR^{526} were equalized for total protein concentration and treated with proteinase K (Figure 2C). With proteins derived from blue colonies, all immunoreactive CPEBQ-GR^{526} polypeptide disappeared within 1 min. In contrast, with proteins derived from white cells an immunoreactive fragment of ~22 KD appeared early in digestion and persisted for at least 10 min (Figure 2C left, top and bottom images).

The defining feature of prion-like propagation in yeast

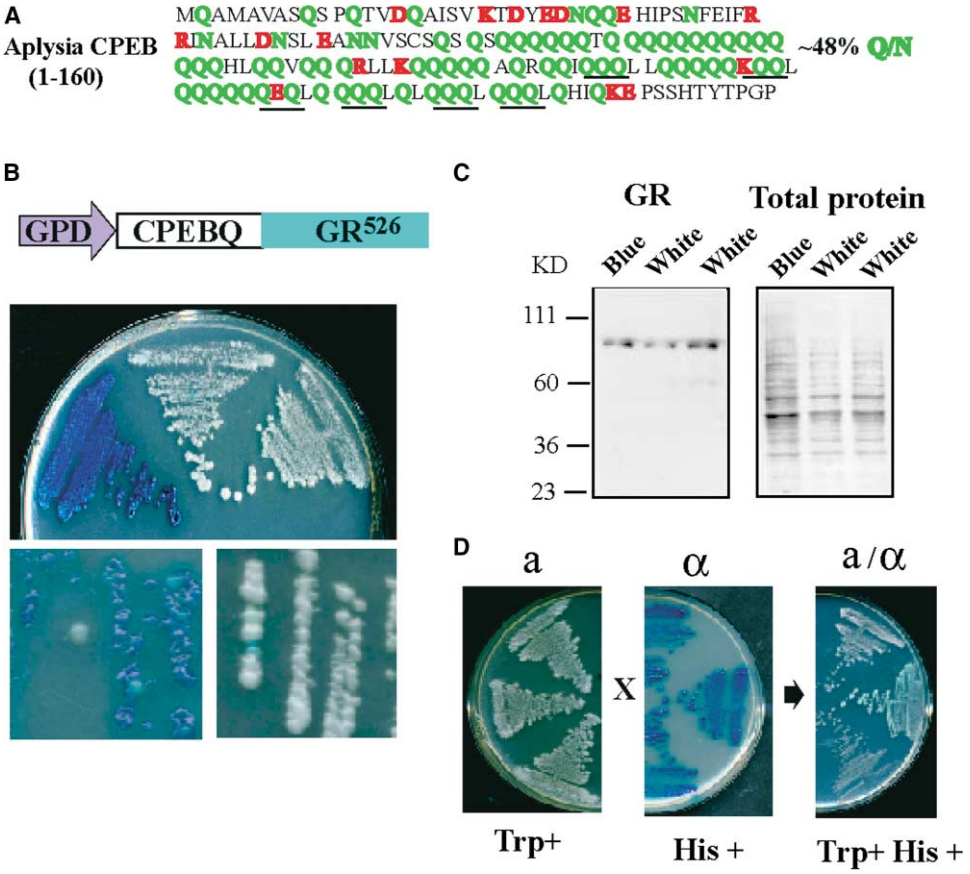

Figure 1. ApCPEB N-Terminal Domain Can Confer Two Distinct Functional States

(A) Sequence of the glutamine (Q)/asparagine (N) rich N-terminal 160 amino acid of ApCPEB. The imperfect repeat sequence QQQL is underlined.

(B) Schematic diagram of the *Aplysia* CPEB N-terminal 160 amino acid and glucocorticoid receptor (CPEBQ-GR^{526}) fusion construct. The fusion construct was expressed under constitutive GPD promoter. CPEBQ-GR^{526} gives rise to metastable blue and white cells. W303a cells were transformed with plasmids bearing CPEBQ-GR^{526} [TRP1] and GRE-β-gal [LEU2]. Leu$^+$ Trp$^+$ double transformants were selected and replica plated onto –Leu-Trp$^+$ 2% x-gal containing plates. Representative CPEBQ-GR^{526} blue and white cells are shown in the top image. The inset shows spontaneous conversion of the white cells to blue and blue to white.

(C) Similar amount of GR protein is present in both cell types. Total cell lysates were prepared from CPEBQ-GR^{526} blue and white cells. 50 μg of total protein was immunoblotted with anti-rat GR monoclonal antibody (left image). The membrane blotted with anti GR antibody was also stained with Coomassie blue to check the total protein content of each sample (right image). Molecular weight standards are indicated in the left.

(D) CPEB N-terminal domain has a dominant conformational state. The CPEBQ-GR^{526} was cloned into two different plasmids, one with HIS3 marker and the other with TRP1 marker. White cells were selected from W303α cells transformed with CPEBQ-GR^{526} [HIS3] plasmid (left image) and blue cells were selected from W303a cells transformed with CPEBQ-GR^{526} [TRP1] plasmid (middle image). After mating, the His$^+$ Trp$^+$ diploids were selected and plated in 2% X-Gal containing plates (right image). The diploids were white in X-Gal plates.

is the ability of a protein in the prion state to induce other proteins carrying the same prion domain to enter the prion state, thereby self-perpetuating the prion form. When the NM-GR fusions protein enters the prion state, it forms large complexes (L. Li and S.L., unpublished data), GR activity is lost, and this loss of activity is dominant in crosses with cells that have active NMGR that are not in a prion state (Li and Lindquist, 2000). The physically distinct, inactive state of CPEBQ-GR^{526} also behaved like a yeast prion; it had the same unusual dominant loss-of-function phenotype characteristic of other yeast prions. When haploid cells producing active CPEBQ-GR^{526} (His$^+$ blue cells) were mated to cells producing inactive CPEBQ-GR^{526} (Trp$^+$ white cells) all Trp$^+$His$^+$ diploids were white and remained white upon replating (Figure 1D) consistent with the expression of an inactive CPEBQ-GR^{526}. We conclude that the N-terminal domain of *Aplysia* CPEB confers upon GR^{526} the ability to exist in distinct functional and physical states that are heritable, interconvertible, and transmissible in the manner of a prion-like protein.

Full-Length *Aplysia* CPEB Protein Can Also Exist in Two Functionally Distinct Heritable States

What is the functional significance of the N-terminal prion-like domain of *Aplysia* CPEB? To determine if the N-terminal region of CPEB could behave like a prion in the context of native full-length CPEB, we developed an assay to score the activity of the full-length protein in yeast, based on the following rationale. The translational activation by CPEB can be broken down into two broad activities: recognition or binding of CPEB to CPE and

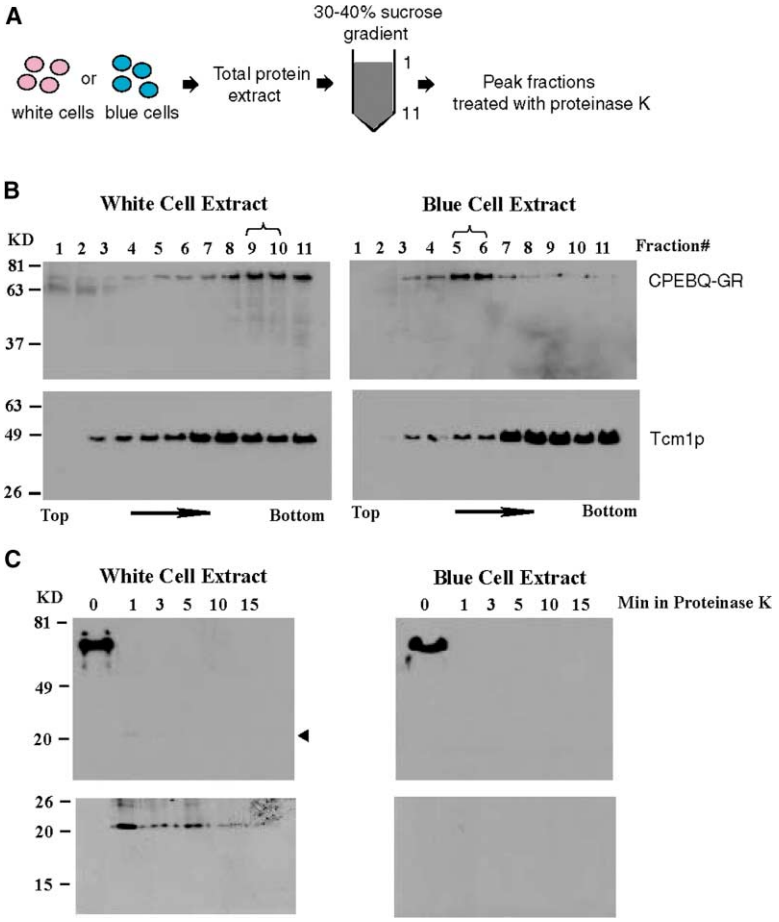

Figure 2. The N-Terminal Domain Has Two Distinct Physical States

(A) Schematic representation of the experimental procedure.

(B) CPEBQ-GR^{526} protein has different sedimentation velocity in blue-white cells. Total cell extracts (1 mg of protein) from blue or white cells were analyzed in a 30%–40% sucrose gradient (weight/volume) by ultra centrifugation at 140,000 × g for 1 hr 45 min at 4°C. Following centrifugation, the gradient was fractionated and immunoblotted for GR (top image) or 60S ribosomal protein Tcm1P (bottom image).

(C) Protease resistance of CPEBQ-GR^{526} in white cells. The peak gradient fractions (tube# 5 & 6 from the blue cell gradient and tube# 9 & 10 from the white cell gradient) for each cell type were pooled, treated with 100 ng of proteinase K for indicated times, and Western blotted for GR with rabbit anti GR polyclonal antibodies: Top image: 10% SDS-PAGE optimized for the separation and transfer of the full-length CPEBQ-GR. Bottom image: 12.5% SDS-PAGE optimized for low molecular weight protease resistance fragment. The proteinase K resistance fragment in the white cell extracts is also detectable in the top image (◀).

the recruitment of the polyadenylation machinery via its interaction with cleavage and polyadenylation specificity factor, CPSF160. There is no known homolog of CPEB in yeast. However, the components of the core polyadenylation machinery in yeast (including CPSF160) are functionally and structurally homologous to those of mammalian cells (Shatkin and Manley, 2000). In view of this structural and functional similarity between the yeast and mammalian polyadenylation machinery, we reasoned that ectopically expressed CPEB might recruit the polyadenylation machinery, which in turn could translationally activate mRNAs containing a CPE.

We therefore made a reporter construct for CPEB activity by fusing a 78 nucleotide fragment from the 3′ UTR of *Xenopus* cyclinB1 mRNA to the 3′ end of β-galactosidase mRNA (β-gal-CPE, Figure 3A). This 78 nucleotide fragment contains a canonical CPE motif, UUUUUAAU, and a polyadenylation element AAUAAA that is sufficient for CPEB-dependent polyadenylation (de Moor and Richter, 1999). When β-gal-CPE RNA was expressed from a single-copy plasmid under a constitutive GPD promoter, colonies remained white on plates containing X-Gal (one blue colony appeared in 5376 transformants after 2 days; Figure 3B2). β-galactosidase activity in these cell extracts was very low. In contrast when we replaced the 78 nucleotide cyclin B1 sequence with other sequences of varying lengths and nucleotide composition, the transformants turned blue within 10–12 hr (Figure 3B1). Although the constructs produced very different levels of β-galactosidase activity, they produced the same levels of RNA at steady state (Supplemental Figure S3 available on *Cell* website). The poor translation of the β-gal RNA containing CPE indicates that the 78 nucleotides of cyclin B1 mRNA acts to make the β-gal mRNA translationally dormant. This poor translation could be due to the binding of some endogenous yeast protein to the U-rich sequence of cyclin B1 3′ UTR or to the formation of stable secondary structures at the 3′ end of the mRNA.

When cells containing β-gal-CPE mRNA were cotransformed with a plasmid encoding *Aplysia* CPEB, 21% of the colonies turned blue in 2 days (228 in 1091 transformants; Figure 3B3), as would be expected if the protein can enter into stable alternative functional states. Blue colonies gave rise to blue colonies upon restreaking and white gave rise to white. Several controls demonstrated that the translation of the β-gal-CPE mRNA in blue colonies depended upon CPEB activity. Cotransformation with an empty plasmid or one expressing the unrelated yeast protein, Ade1p, produced no blue colonies in 2340 and 2136 transformants, respectively (Supplemental Figure S3 available on *Cell* website). Even a plasmid expressing the neuronal isoform of another *Aplysia* RNA binding protein, Staufen, very rarely gave rise to blue colonies (3 in 2821 transformants). Finally, when two conserved residues Cys664

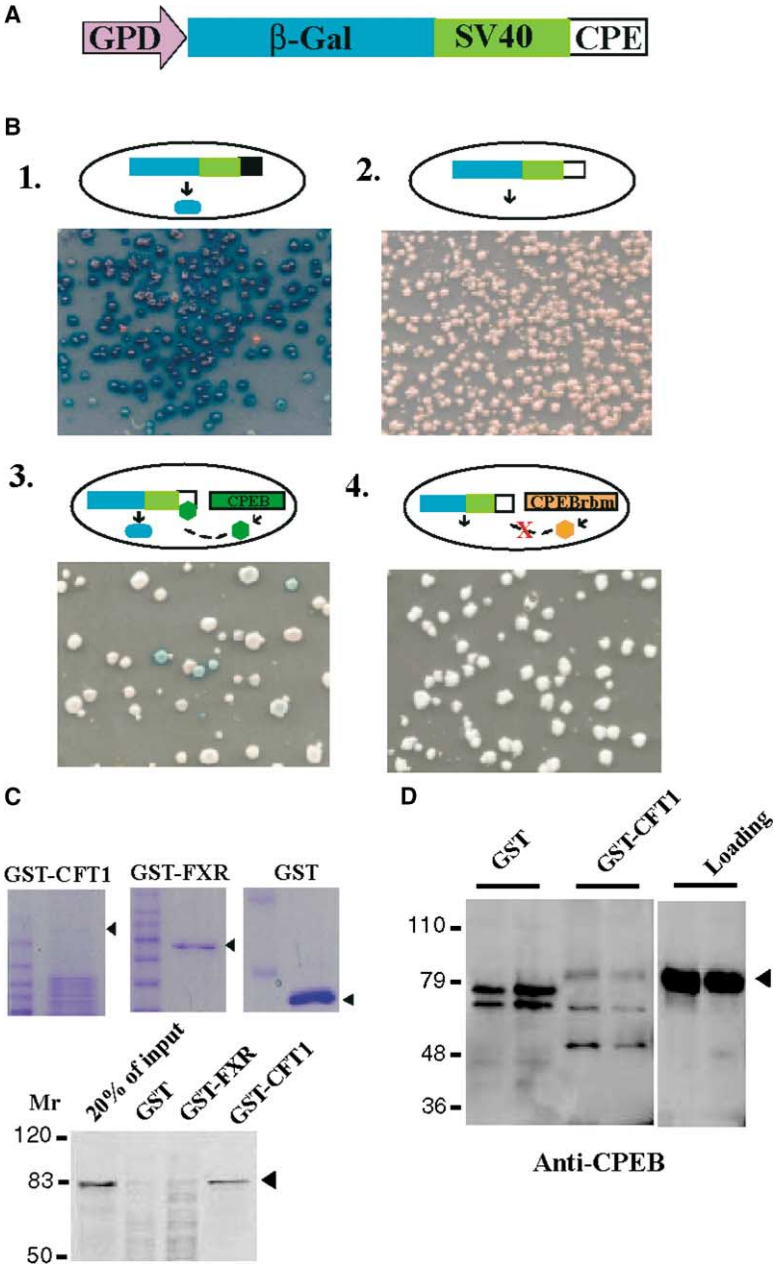

Figure 3. An Assay for *Aplysia* CPEB Activity in Yeast

(A) Schematic diagram of β-galactosidase *Xenopus* cyclin B1 3′ UTR fusion construct (β-gal-CPE). The 3′ UTR of the reporter construct is comprised of 393 ntds of the SV40 3′ UTR and 78 ntds of the cyclin B1 3′ UTR containing a CPE element UUUUUAAU and a hexanucleotide polyadenylation signal AAUAAA.

(B) W303a cells transformed with either β-gal-SV40-SynCAM 3′ UTR (B1), or β-gal-SV40-CPE (B2), or β-gal-SV40-CPE and p9214-ApCPEB (B3) or β-gal-CPE and p9214-ApCPEBrbm (B4). Transformants were plated on a 2% X-Gal plate and photographed after two days.

(C and D) *Aplysia* CPEB interacts with the yeast Cstf1. GST pull-down experiment.

(C) Top image: Coomassie blue staining of recombinant purified GST-Cft1-H_6 (please see Experimental Procedures for details), GST-FXR and GST used in the GST pull-down reaction. The position of the purified protein is marked with an arrowhead (◀). The amount of full-length GST-Cft1-H_6 is significantly less in comparison to GST-FXR or GST. Bottom image: GST-Cft1-H_6, GST-FXR, or GST alone bound to glutathione beads were incubated with ^{35}S-methionine-labeled in vitro translated *Aplysia* CPEB. 1/5th of the lysate used for binding reaction is loaded as input. The ^{35}S-methionine-labeled *Aplysia* CPEB binds specifically to the GST-Cft1, not to a truncated RNA binding protein FXR or to GST alone.

(D) Total cell extracts prepared from *Aplysia* pleural ganglia were incubated with either GST-Cft1-H_6 or GST alone and the glutathione bead bound material was blotted with anti *Aplysia* CPEB antibodies. The position of *Aplysia* CPEB is marked with an arrowhead (◀). The right image shows 1/10th of the total *Aplysia* CPEB used in the binding reaction. The bands around 70 KD in GST lanes are antibody crossreacting polypeptides.

and His672, located in the Zn finger domain of CPEB and critical for RNA binding, (Hake et al., 1998) were mutated to alanine, few blue colonies appeared in transformants (6 blue cells in 1478 transformants) (Figure 3B4 CPEBrbm). These results suggest that the ability of *Aplysia* CPEB to bind mRNA is crucial for the efficient translation of the β-galactosidase reporter RNA.

Next, we asked if *Aplysia* CPEB interacts with the component of yeast polyadenylation machinery. We used a recombinant GST-tagged Cft1 (Dichtl et al., 2002; Stumpf and Domdey, 1996), the yeast homolog of the mammalian cleavage and polyadenylation specificity factor CPSF160. The yeast GST-Cft1 specifically interacted with in vitro translated $^{[35]}$S-methionine-labeled *Aplysia* CPEB (Figure 3C). Moreover, when we incubated the total protein extracts from *Aplysia* central nervous system, the neuronal CPEB specifically interacted with the GST-tagged yeast Cft1 (Figure 3D). We did not detect any significant change in the length of the poly (A) tail of β-gal mRNA (data not shown). The CPEB dependent activation of β-gal mRNA most likely results from the binding of the *Aplysia* CPEB to RNAs containing a CPE and the recruitment of the CPSF complex. Irrespective of its detailed mechanism of activation, the assay in principle recapitulates the activity of CPEB i.e., selective interaction with a poly (A) specificity factor and the selective translational activation of an mRNA carrying a CPE.

As with the natural yeast prions and the CPEBQ-GR^{526} fusion protein, the activity states of established colonies expressing full-length CPEB were metastable, but heritable. Upon replating, the majority of blue colonies gave

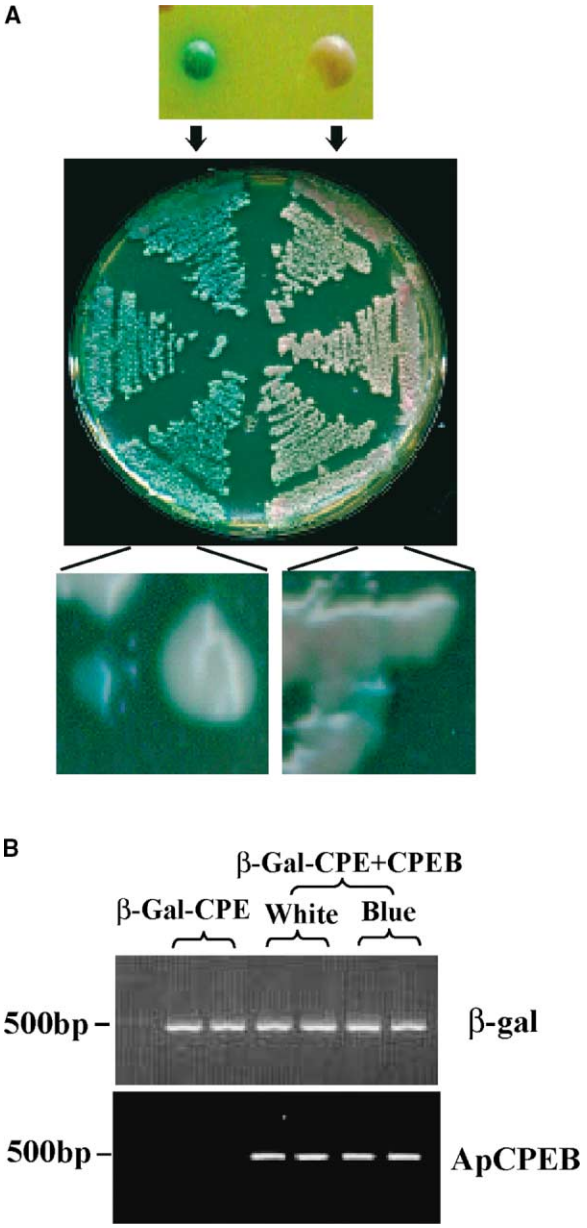

Figure 4. Full-Length *Aplysia* CPEB Can Exist in Two Functionally Distinct States

(A) Top image: representative blue and white cells expressing ApCPEB and β-gal-CPE. Middle image: maintenance of the blue and white phenotype upon restreaking. Bottom image: spontaneous conversion of blue and white cells.

(B) A similar amount of β-galactosidase mRNA and ApCPEB mRNA is present in both blue and white cells. Total RNA was isolated from blue and white cells and 3 μg of RNA was used for RT-PCR. Top image: β-galactosidase mRNA. Bottom image: ApCPEB mRNA.

rise to blue colonies (Figure 4A, middle). However, occasionally blue colonies would give rise to white (Figure 4A, bottom). These too propagated faithfully but occasionally gave rise to blue colonies (Figure 4A, bottom). Notably, the expression levels of β-gal-CPE mRNA and the levels of both CPEB mRNA and protein were the same in blue and white colonies (Figures 4B and 5D). Moreover, the specific activity of the β-galactosidase in white CPEB cells was low but detectable, similar to that of cells expressing β-gal-CPE mRNA alone (data not shown). Thus, white cells made a β-galactosidase mRNA that was capable of producing active enzyme but the mRNA was not efficiently translated.

Distinct CPEB Functional States Correlate with Distinct Physical States

The ability of the yeast prions to exist in distinct physical states is commonly detected by direct visualization of green fluorescent protein (GFP) fusions (Patino et al., 1996; Sondheimer and Lindquist, 2000). To visualize the physical state of the *Aplysia* CPEB in blue and white cells, we fused *Aplysia* CPEB to a variant of GFP (cpEGFP) (Figure 5A). This fusion did not interfere with the CPEB activity: cells expressing the fusion protein gave rise to both blue and white cells when they were cotransformed with the βGal-CPE construct. When we analyzed the fluorescence of ApCPEB-cpEGFP fusion protein in white cells we noticed a diffused fluorescence, similar to the pattern observed for GFP alone (Figure 5A). In contrast in blue cells (in 48 out of 580 cells) there was a striking coalescence of the GFP fluorescence. Moreover, the soluble and aggregated states of the fusion protein seem to be heritable, because the majority of the daughter cells have the same soluble or aggregated state as the mother.

Similar levels of CPEB were detected in total proteins from blue and white colonies by immunoblotting, but the signals were weak (due to low protein concentrations) and insufficient for further analysis. In an attempt to enrich the protein for other assays, we introduced a 3× FLAG tag at the C-terminal end of *Aplysia* CPEB. Tagged CPEB behaved like untagged CPEB in vivo in its ability to form blue and white colonies. Surprisingly, when lysates were passed over an anti-FLAG affinity column, the CPEB-FLAG protein from white colonies but not blue colonies was retained on the column (Figure 5C). Similar results were obtained in the presence of 1% Triton X-100, a detergent that disrupts many protein complexes, but allows efficient binding of anti-FLAG antibody to FLAG tagged proteins. In contrast when FLAG-tagged CPEB was purified by cation-exchange chromatography, similar quantities were obtained from both cell types (Figure 5D). Thus, the CPEB protein in blue cells, but not in white cells is in a physical state that produces small aggregates and limits the accessibility of the C-terminal region of the protein to the antibody. Whether this is solely due to the conformational state of CBEB itself or to the binding of other factors to CPEB is unclear. In either case, the heritable differences in the activity states of full-length CPEB in blue and white cells are associated with distinct heritable physical state of the protein.

The Transmissible State Is the Active Form of the CPEB Protein

Surprisingly, when blue W303a cells were mated to white W303α cells, the diploid cells were blue on X-Gal plates. Thus, unlike CPEBQ-GR, the active form of the full-length CPEB is dominant. This might indicate that the CPEB protein is not capable of producing a transmissible prion-like state or that, unlike CPEBQ-GR and other

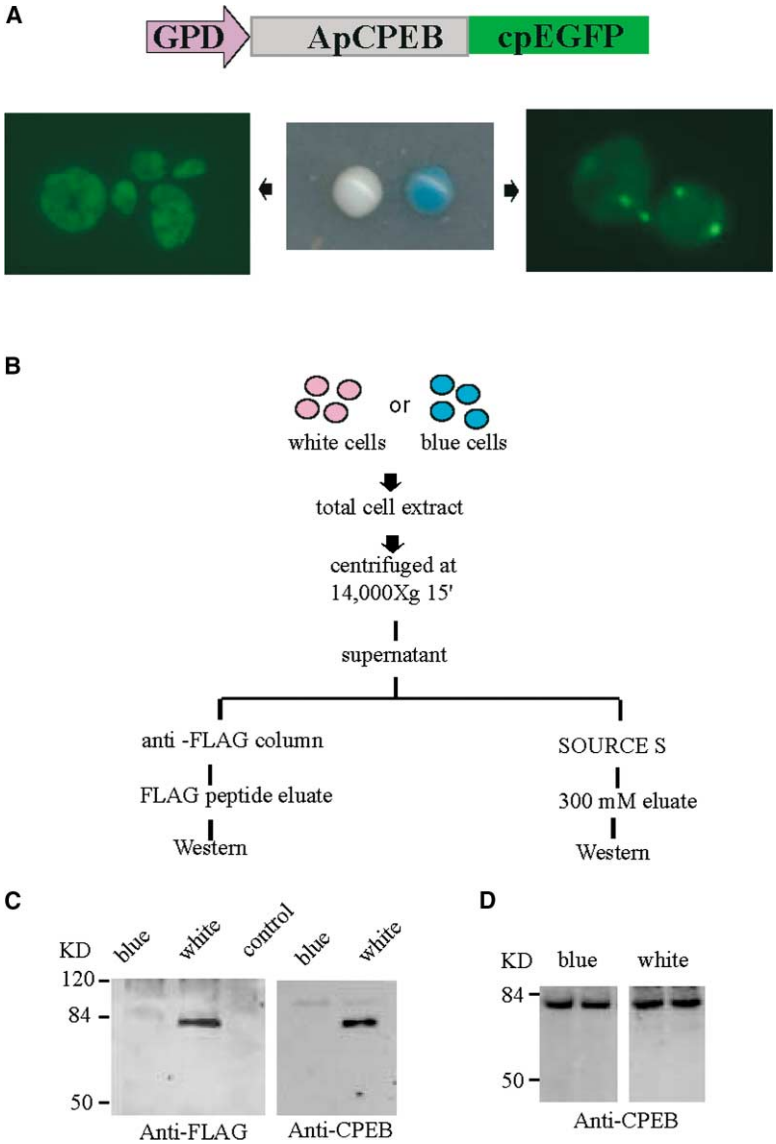

Figure 5. Two Physical States of the *Aplysia* CPEB Protein

(A) Schematic diagram of *Aplysia* CPEB and circularly permutated GFP (cpEGFP) fusion construct and the GFP fluorescence of the blue and white cells. We have noticed that unlike regular GFP the fluorescence intensity of the cpGFP fused to *Aplysia* CPEB is considerably low.

(B) Schematic of the purification of CPEB from blue and white cells. Blue or white transformants were grown in 1 lit of selective media (−Leu-Ura) to log phase and cell extracts were prepared using glass beads. The total cell lysates were centrifuged at 14,000 × g to clarify the extract. Approximately 1 mg of total protein was loaded into an anti-FLAG antibody column and eluted with FLAG peptide. For purification through SOURCE S cation exchanger, approximately 100 mg of total protein was loaded into the column and washed with a buffer containing 150 mM NaCl. *Aplysia* CPEB came out of the column in 300 mM NaCl. The 300 mM fractions were pooled, concentrated, and used in the protease assay.

(C) Western blotting of the 1/5th of the FLAG column elute either with anti-FLAG antibodies or with anti *Aplysia* CPEB antibodies.

(D) Western blotting of the pooled 300 mM fractions of the SOURCE S cation exchanger with anti *Aplysia* CPEB antibodies.

well characterized yeast prions, the active form of the protein is the prion-like state. Though unexpected, this is consistent with two other findings; (1) the protein of the blue cells (CPEB-FLAG) have a C-terminal domain that is altered so as to be inaccessible for binding by the anti-FLAG antibodies, and (2) the protein of the blue cells forms oligomeric species and small aggregates.

To provide a more rigorous test of the prion state we turned to cytoduction, which has been used previously to establish the prion properties of yeast [URE3], [PSI], and [RNQ] (Patino et al., 1996; Sondheimer and Lindquist, 2000; Wickner, 1994). The yeast karyogamy deficient strain A3464 (*Kar*Δ*1-15*) *MATa* can undergo normal conjugation with *MAT*α strains but is unable to fuse its nucleus with that of its mating partners. As a result, organelles are mixed and cytoplasmic proteins from the two cells come into contact with each other, but haploid progeny that bud from the mating pair contain nuclear information from just one of the parents. We used blue W303α (His$^+$) cells expressing ApCPEB and β-gal-CPE mRNA as the donors of cytoplasmic material and white A3464 (Trp$^+$) cells expressing ApCPEB-cpEGFP and β-gal-CPE mRNA as the recipients (Figure 6A). To ensure cytoplasmic transfer from donor cells, the cytoplasm of the recipient A3464 cells was marked with a petite mitochondrial mutation [rho^o]. A3464 cells are unable to grow in a nonfermentable carbon source, such as glycerol, unless they received wild-type mitochondria from the donor cytoplasm.

Following cytoduction, haploid progeny were selected that had retained A3464 nuclear markers (Trp$^+$His$^-$) but had received cytoplasmic material from the donor and consequently could grow in glycerol (Figure 6A). Indeed, when plated onto X-Gal plates 33% of the cytoductants turned blue (8 out of 23). The cytoduction of the blue state was specific; when we used blue cells derived from the full-length *Drosophila* neuronal CPEB as a donor, all of the cytoductants remained white after three days in X-Gal containing plates (0 out of 30). This is consistent with well-documented "species

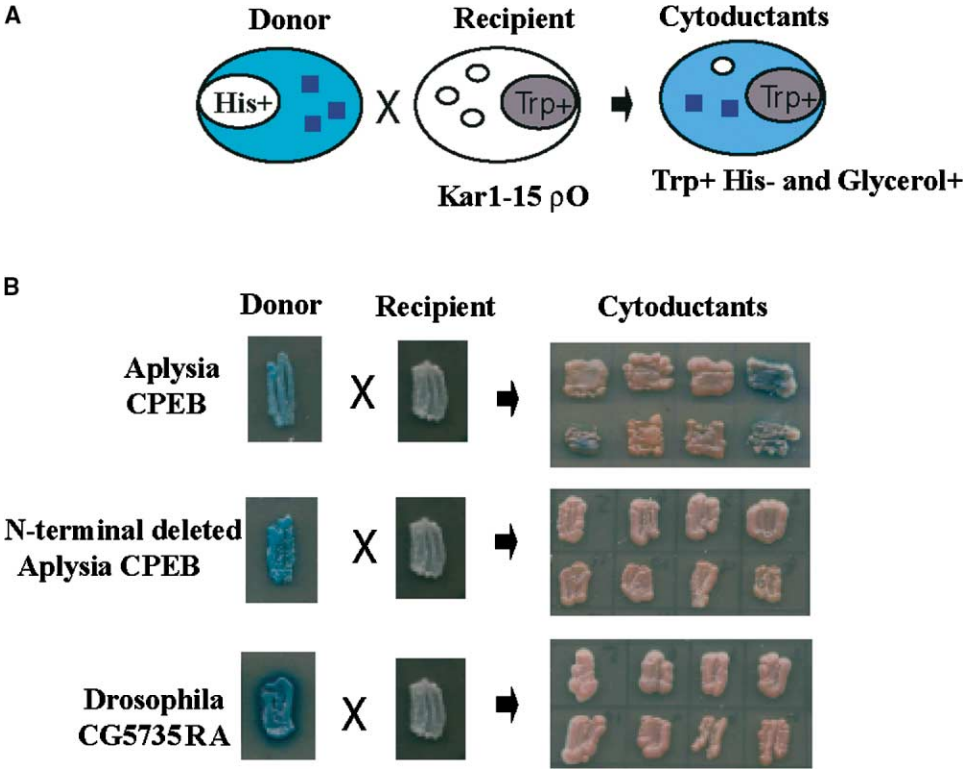

Figure 6. The Dominant Non-Mendelian Inheritance of the Blue Phenotype

(A) Schematic representation of cytoduction: W303 cells (mating type α) were transformed with ApCPEB and β-galactosidase-CPE and blue cells were selected. These cells were cytoduced to A3464 (mating type a) white cells and cytoductants were selected on media lacking tryptophan and had glycerol as the sole carbon source. This selected against W303α, which was Trp- and the noncytoduced A3464, which lacked mitochondrial DNA and unable to grow in glycerol. The haploid cytoductants were plated on 2% X-Gal.

(B) Representative blue and white cells and eight of the resultant haploid cytoduced cells in X-Gal plate.

barrier", wherein a prion from one species either is not capable or very inefficient in introducing conformational change into a prion from another species. To control for the unlikely possibility that the small quantity of β-galactosidase enzyme transferred in the cytoduction would be sufficient to turn colonies blue, we cytoduced unrelated blue cells expressing β-galactosidase to the same white recipients. The cytoduced haploid cells of this mating either did not turn blue at all or exhibited only the very light blue color seen in cells translating β-gal-CPE mRNA at low efficiency (data not shown).

An Aggregated Form of CPEB Binds to CPE Containing RNA

The dominant, cytoducible character of the blue state, together with the formation of small aggregates in blue cells, suggested that CPEB was more active in the prion-like aggregated state. Since the assay depends on the proteins ability to bind RNA, the formation of small aggregates in the blue cells is surprising because it suggests that the protein in its aggregated state can bind RNA. To assay the protein's RNA binding activity directly, we tested the ability of different forms of CPEB to selectively bind RNAs containing CPEs in vitro. To do so, we incubated them with a radiolabeled 300 ntd long RNA derived from the 3′ UTR of *Aplysia* neuronal actin (Si et al., 2003) and resolved by electrophoresis the resulting RNA protein complexes under nondenaturing conditions. *Aplysia* CPEB did not bind RNA when the soluble protein was recovered from *E. coli* (data not shown), nor when it was produced by in vitro translation in reticulocyte lysates (Supplemental Figure S4A available on *Cell* website). The failure to detect any RNA binding activity with soluble *Aplysia* CPEB suggested two possibilities: (1) *Aplysia* CPEB does not have any RNA binding activity even though it is the RNA binding domain that is most conserved between the *Aplysia* neuronal CPEB and other CPEBs or (2) *Aplysia* CPEB binds RNA only under conditions when it forms small aggregates. In separate experiments (K.S. and E.R.K., unpublished data), we found that when a histidine-tagged bacterially expressed *Aplysia* CPEB was purified under denaturing conditions and dialyzed against a buffer containing 2 M urea for 32 hr (see Experimental Procedures) it forms long fibers. A similar treatment allows the yeast prions. Rnq1 and full-length Sup35 to assemble into ordered aggregates (fibers) rather than amorphous aggregates (Glover et al., 1997). When CPEB fibers were sonicated and incubated with the radiolabeled actin RNA, a slow-migrating complex was formed (Figure 7A). Under the same conditions of denaturation and dialysis in urea, the developmental isoform of mouse CPEB (mCPEB1) did not bind to the actin RNA. The interaction with *Aplysia* CPEB aggregates was specific:

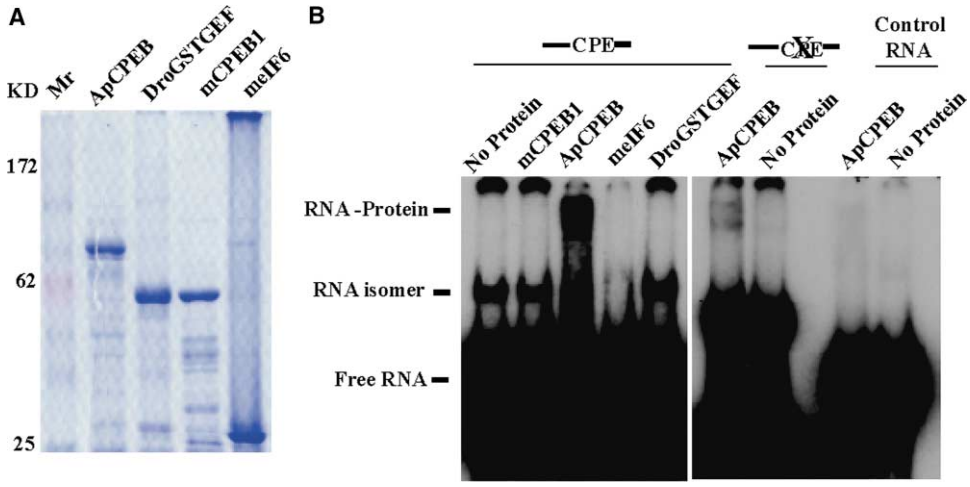

Figure 7. Aggregated *Aplysia* CPEB Binds RNA

(A) Coomassie blue staining of the purified recombinant His_6-mCPEB1, His_6-meIF6, His_6-ApCPEB, and GSTDrogef used in the RNA-gel retardation assay.

(B) Gel retardation assay with recombinant *Aplysia* CPEB. Assays were done with radiolabeled purified RNAs and purified recombinant histidine or GST-tagged proteins. Left image: only oligomeric *Aplysia* CPEB not other proteins produces a slow-migrating RNA-protein complex. Right image: recombinant *Aplysia* CPEB in its oligomeric state binds only to CPE containing RNA. In the actin CPE mutant RNA, there was some residual binding. This might be due to the presence of a putative CPE element UUUUGUU 35 nucleotide upstream of the canonical CPE element UUUUUUAU. The control RNA is a 350 nucleotide long fragment derived from the 3′ UTR of *Drosophila* Ork gene, which does not contain any CPE element. The reason for the slower migration of some of the free actin mRNA is not known (marked as RNA isomer). However, similar isomers have previously been observed for other CPE containing RNA (Stebbins-Boaz et al., 1996).

incubation of the actin RNA with another highly insoluble hexahistidine-tagged, bacterially expressed, protein—mammalian translation factor 6 (meIF6)—did not cause a mobility shift (Figure 7A). Moreover, the mobility shift was not observed when actin mRNA was incubated with a number of histidine- and GST-tagged fusion proteins (Figure 7A and Supplemental Figure S4A available on *Cell* website).

Next, we asked if the aggregated form of the *Aplysia* CPEB binds selectively to RNAs containing a CPE. Mutation of two nucleotides in the CPE of neuronal actin (for details, see Si et al., 2003) virtually abolished the slow-migrating RNA-protein complex (Figure 7B). Moreover, three other radiolabeled RNAs without CPEs produced no slowly migrating RNA-protein complex (Figure 7B and Supplemental Data available on *Cell* website for additional controls). Taken together these results suggest that like other CPEBs *Aplysia* neuronal CPEB binds to CPE containing RNAs. However, its RNA binding activity depends on a physical state of the protein that promotes the formation of multimeric structures and is consistent with the idea that it is the self-perpetuating state of the protein that is the active form of the protein.

The Prion-Like Determinant of CPEB Promotes the Active State of the Protein

One explanation for the difference between the CPEBQ-GR fusion protein (in which the prion state inactivates GR) and full-length CPEB (in which the prion state actives CPEB) is that the prion domain promotes the same type of self-perpetuating change in state in both proteins, but it has a different biological consequence in its native context. If the C-terminal region of CPEB has a relatively weak or unstable capacity to activate CPE-containing mRNAs on its own, the prion-like change in state conferred by the N-terminal domain might be employed to stabilize it in the active state. To test this possibility, we deleted the N-terminal 200 amino acids of CPEB and coexpressed the truncated protein with β-gal-CPE mRNA. Compared to full-length CPEB, fewer of the initial transformants turned blue after two days (22% blue colonies were obtained with the full-length protein; 8% with the N-terminal deletion, 58 in 680 transformants; Supplemental Figure S3 available on *Cell* website).

To further investigate the role of the N-terminal domain we analyzed the rate of interconversion between the blue and white colonies expressing the full-length CPEB or the N-terminal deletion. Starting with established white transformants, cells expressing full-length CPEB gave rise to blue colonies at a frequency of 0.4%. Cells expressing N terminally truncated CPEB gave rise to blue colonies much less frequently (0.046%). Starting with established blue colonies, full-length CPEB gave rise to white colonies at a frequency of 0.55% (29 white colonies versus 5252 blue colonies). N terminally deleted CPEB gave rise to white colonies much more frequently (10.2%; 584 white colonies versus 5725 blue colonies). Moreover, when we used an N terminally truncated CPEB as the donor in our cytoduction assay, it failed to confer the blue phenotype into the full-length protein (Figure 6B). Thus, even without the N-terminal domain, *Aplysia* CPEB can activate an mRNA containing CPEs and can assume alternative active states. However, the N-terminal domain increases the rate at which CPEB assumes the active heritable state and, once active, the N-terminal domain helps to maintain the protein in the active state and to transmit this state to the rest of the protein.

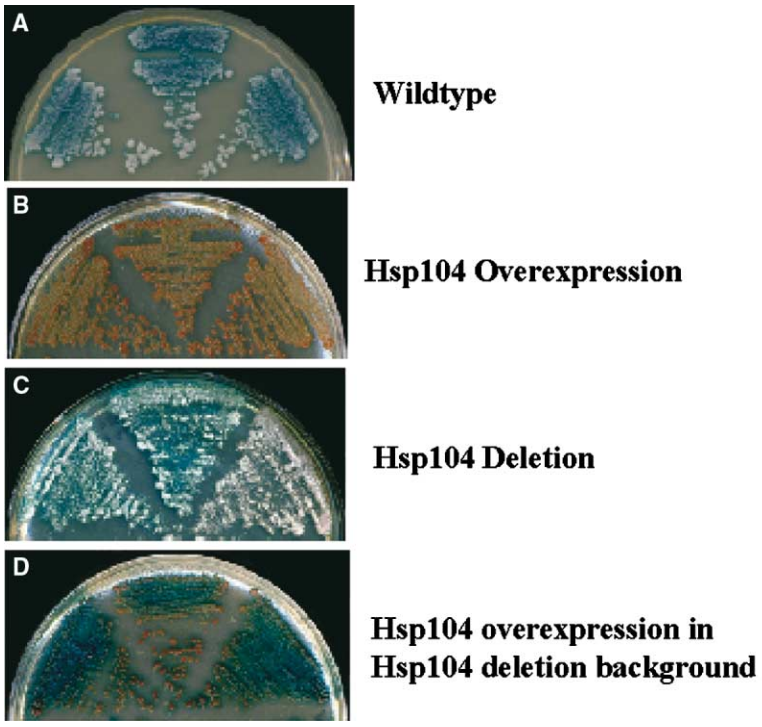

Figure 8. Hsp104 Influence the Activity State of *Aplysia* CPEB

W303a (A and B) or W303aΔ hsp104 (A3224) (C and D) were transformed with ApCPEB [URA3] and β-gal-CPE [LEU2] construct. The Leu^+Ura^+ cells were replica plated on X-Gal plates. The resulting blue colonies were transformed with an Hsp104 overexpressing plasmid (5312) with HIS3 marker (B and D). The $His^+Leu^+Ura^+$ cells were replica plated on X-Gal containing plates. Overexpression of Hsp104 cures the blue phenotype (B). However, in the absence of endogenous Hsp104, the Hsp104 containing plasmid fails to cure the blue phenotype (D).

Hsp104 Overexpression Affects the Propagation of the Blue State

The propagation of most yeast prions depends on the amount and activity of the heat shock protein family members. Among the heat shock proteins, Hsp104, a protein conformation-remodeling factor of the AAA^+ family, has the strongest effects, (Chernoff et al., 1995; Ferreira et al., 2001; Moriyama et al., 2000; Newnam et al., 1999; Patino et al., 1996). When blue cells obtained from full-length *Aplysia* CPEB (Figure 8A) were transformed with a multicopy Hsp104 plasmid, they no longer turned blue on X-Gal plates (Figure 8B). An Hsp104 deletion had very modest effect (Figure 8C). However, the Hsp104 deletion had a significant affect on the N terminally truncated CPEB (Supplemental Figure S5 available on *Cell* website). First, the number of initial transformants that turned blue was reduced by almost 50% (4% of the transformants turned blue compared to 8% under wild-type background). Second, upon replating these blue cells could not propagate the blue phenotype stably. The effects of Hsp104 on the propagation of CPEB activity states are complex, but not unprecedented. Deletion and overexpression of Hsp104 has different effects on different prion proteins. Even in closely related prion domains, Hsp104's effect is influenced by sequences outside of the prion-determining region itself (Crist et al., 2003; Kushnirov et al., 2000; Liu et al., 2002). In any case, the fact that Hsp104 strongly influences the activity states of the CPEB protein provides yet another line of evidence that these activity states are determined by changes in CPEB conformation.

Discussion

We find that *Aplysia* CPEB has properties consistent with it being a prion-like protein. As is the case with other well-characterized prion determinants in yeast, the N-terminal domain of *Aplysia* CPEB is modular and transferable. When fused to GR it produces distinct, heritable, functional states that are associated with distinct physical states; the prion state is inactive and this state is readily transmitted to cells with active protein in a dominant, heritable manner.

As also is the case with conventional prions the full-length *Aplysia* CPEB has a self-perpetuating epigenetic state that is associated with a distinct physical state. However the full-length CPEB differs from other yeast prions in a number of features. In yeast prions that have been characterized to date, the self-perpetuating prion state is biochemically inactive. By contrast, the dominant self-perpetuating state of CPEB is the active state. This is not completely unprecedented: the dominant self-perpetuating form of the fungal prion protein [HetS] also is associated with a genetic gain of function although the biochemical activities associated with [HetS] are still unknown (Coustou et al., 1997). Also, a self-activating protease exhibits prion-like genetic behavior in the active state (Roberts and Wickner, 2003). Our findings also suggest that the CPEB protein forms small aggregates or multimers in its active state. This raises the question: how can an aggregated form of the protein be active? One possibility is that in the aggregated state the protein either retains or acquires certain biochemical activities. To our surprise (but consistent with its prion-like properties), we find that it is only the aggregated state of the protein that binds to CPE containing RNA. The soluble monomeric state of the protein does not bind RNA. However, we do not know if the large aggregates of the *Aplysia* CPEB are the functional state in vivo. The large aggregates we see in vitro and sometimes in vivo are likely to be an expansion of smaller multimeric forms of the protein and it may be the smaller multimeric

forms that are the functional RNA binding unit. As is true for other prions, we also do not yet know if the change in state is self-autonomous or requires other factors. In any case, once the active state is achieved, it is self-perpetuating and transmissible to other CPEB proteins.

Although we lack direct evidence in neurons we speculate that CPEB has at least two conformational states: one is inactive or acts as a repressor (perhaps monomeric), the other is active (perhaps multimeric). Based on these considerations, we propose a model for CPEB, which represents a variant of the conventional prion mechanisms. This variant has features that are particularly relevant in the context of long-term memory storage. Long-term memory in both invertebrates and mice involves long-term synapse-specific modifications. In the invertebrate *Aplysia*, the synapse-specific modifications are mediated by serotonin, a modulatory neurotransmitter released during learning. This long-term synapse-specific modification requires independent mechanisms for both spatial restriction (synapse specificity) and for persistence (duration). We have shown (see Si et al, 2003) that serotonin acts locally partly by increasing the amount of CPEB in a restricted synapse-specific way. If CPEB has prion-like properties in *Aplysia*, this single molecule can achieve both restriction and persistence.

How might this work? In a naive synapse, the basal level of CPEB is low. Unlike conventional prions in this state the protein might be less active, inactive, or may even inhibit translation of certain CPE-containing mRNAs. Indeed in *Xenopus* oocytes, CPEB acts both as a translation repressor and as an activator for cyclin B1 mRNA (de Moor and Richter, 1999; Mendez et al., 2002). Since synaptic stimulation by serotonin leads to an increase in the neuronal CPEB, serotonin might trigger the conversion to the prion-like state either by itself or in concert with other signals. It is noteworthy that a transient increase in the protein level acts as general trigger for the conversion of all yeast prion proteins to the prion state. The prion-like state of neuronal CPEB, resulting from synaptic stimulation by serotonin, might be more active, have altered substrate specificity, or be devoid of the inhibitory function of the basal state. Once the prion state is achieved in the activated synapse, dormant mRNAs that are made in the cell body and distributed globally to all synapses could be activated locally. The prion-like state would thereby contribute to the long-term maintenance of a self-sustaining, synapse-specific plastic change.

According this model, this variant form of prion mechanism evident in CPEB requires the action of a neurotransmitter for switching the protein to its active self-perpetuating state. This is in a sense equivalent to a posttranslational modification induced by physiological signal. However, a prion-like mechanism introduces an additional feature into signal transduction; once the protein achieves its prion state it is self-perpetuating and no longer requires for maintenance continued signaling either by kinases or phosphatases. Moreover, its activity state is less easily reversed. In principle, mechanisms such as these, where a physiological signal activates a non-Mendelian epigenetic self-perpetuating state of protein, could work in many other biological contexts such as differentiation and transcription (Lindquist, 1997).

Experimental Procedures

Strains and Plasmids

The genotype of yeast strains used are: W303 (*a or* α*; ade2-1; his3-11.15; leu2-3.12; trp1-1; ura3-1*); A3464t (W303*a; ade2-1; his3-11.15; leu2-3.12; ura3-1; can1-100; p0; kar1-*Δ*15*); A3654 (*W303*α; *ade2-1; leu2-3.12; ura3-1; can1-10*,); A3224 (W303a; *ade2-1*; *his3-11.15; leu2-3.12; trp1-1*; *ura3-1*; *can1-100; hso104:kanMX4*); A3933 (W303*a; ade2-1; his3-11.15; leu2-3.12; trp1-1; ura3-1; can 1-100;* Δ*hsp82*). The N-terminal 480 nucleotides were amplified by PCR from the full-length *Aplysia* CPEB cDNA using a 5′-primer CG**GGATCC**ATGC AAGC-CATGGCCGT and a 3′-primer TCC**CCGCGG**TGGACCAGGC GTGTA. To create CPEBQ-EGFP, the PCR product was digested with BamHI and SacII and ligated in-frame to an EGFP coding sequence in 2 μ plasmid p2HG. To make CPEBQ-GR^{526} the plasmid pG1-NMGR526 was digested with BamHI-SacII and the NM fragment was replaced with CPEBQ. To express ApCPEB, the ApCPEB ORF in pGEM7Zf(+) was digested with AatII, blunt ended, and then digested with BamHI. The BamHI/blunt fragment was then cloned into the BamHI/SmaI site of the pRS316-derived vector 9214. The 78 ntds of cyclin B1 3′ UTR construct was made by hybridizing the oligo 5′-GGAATTCGGCACCATGTGCTTCTGTAAATAGTGTATTGTG TTTTTAATGTTGGACTGGTTGGAATAAAGCTCTAGAGC-3′ to its antisense oligo and then cloned into EcoRI/XbaI sites of pGEM7Z(f$^+$) to create pGEM-CPE. The β-gal was cloned into the HindIII/SmaI site of pGEM-CPE to make pGEM-β-gal-CPE. pGEM-β-gal-CPE was digested with HindIII/XbaI and cloned into the same sites of the yeast centromeric plasmid pRS315. To create pRS315-β-gal-CPE, 900 bp SacI (blunt ended)/XhoI GPD promoter fragment was subcloned into ApaI (blunt ended)/XhoI site of pRS315. To overexpress yeast Hsp104, the plasmid 5312 was created by cloning the 3.5 kb Hsp104 coding region under the GPD promoter in a 2 μ plasmid with HIS3 marker. To create a full-length *Aplysia* CPEB-cpGFP construct, the *Aplysia* CPEB was PCR amplified using the primers pairs CGGGATCCATGCAAGCCATGGCCGT and the GGACTAGTGAGAG ACAGAGATGACTTGGT.

Sucrose Gradient of CPEBQ-GR

The velocity gradient analysis was carried out as follows. Blue or white cells expressing CPEBQ-GR^{526} were grown in selective media to an OD600 of 1.0. Approximately 100 ml of the culture was harvested and resuspended in 500 μl of the lysis buffer containing 25 mM Tris-HCl (7.5), 100 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 5% glycerol. Cells were broken with glass beads and centrifuged at 20,000 $\times$ g for 20 min at 4°C and the supernatant was collected. 2 ml step gradients were made using 0.5 ml of 40% sucrose in lysis buffer and 1.5 ml of 30% sucrose in lysis buffer. Approximately 1 mg of total protein (100 μl) was loaded on top of the gradient and centrifuged for 1 hr 30 min at 45,000 rpm at 4°C in an SW65i rotor. 200 μl fractions were collected from the top of the gradient and 1/10th of the fraction was run on a 10% SDS-PAGE gel. The proteins were electrotransferred to a PVDF membrane and incubated with 1:3000 dilutions of rabbit anti-GR polyclonal antibodies (Santa Cruz Biotechnology). The blot was developed with 1:10,000 dilution of antirabbit secondary antibody coupled to HRP. For the ribosomal protein Tcm1P, the blot was incubated with a 1:1000 dilution of monoclonal Anti-Tcm1p antibody and developed with HRP coupled antimouse secondary antibody.

Purification of *Aplysia* CPEB

1 lit of the OD600 1.0 culture was harvested and resuspended in buffer A containing 150 mM NaCl, 50 mM HEPES, NaOH (7.6), 2 mM $MgCl_2$, 1 mM DTT, 5% glycerol and protease inhibitors. Cells were broken in a bead beater and centrifuged twice at 8000 rpm at 4C in a SS34 rotor. Protein concentration of the supernatant was measured and approximately 100 mg of protein was loaded into a 2 ml of SOURCE 30S (1: 50) column preequilibrated with buffer A. The column was washed with 20 column volumes of the loading buffer and eluted with buffer A + 150 mM NaCl. 1 ml fractions were collected and 1/20th of the fraction was blotted with ApCPEB77 antibodies. Fractions containing ApCPEB were pooled and concentrated in a centricon.

3× FLAG Purification

Total cell lysates were prepared from 200 ml of O/N culture in the FLAG buffer containing 50 mM Tris-HCl (7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, and protease inhibitors. Cell lysates were cleared by centrifugation twice at 15,000 × g for 15 min and 1 mg of total protein incubated with FLAG M2-agarose beads for 6 hr at 4°C. The beads were washed 3× with FLAG buffer and the bound material was eluted with 150 μl of FLAG buffer containing 20 μg of FLAG peptide. 30 μl of the eluate was run on an 8% SDS-PAGE gel and blotted with either a 1:500 dilution of affinity-purified ApCPEB77 or a 1:500 dilution of the anti-FLAG polyclonal antibody (Sigma).

Protease Assay

For the proteinase assay for CPEBQ-GR^{526}, 110 μl of the sucrose gradient fractions were incubated with 100 ng of freshly prepared proteinase K at room temperature (23°C). At indicated times, 20 μl of the reaction was added directly into SDS-PAGE gel loading buffer and boiled immediately to inactivate the protease. The gel was transferred to a PVDF membrane and blotted with 1:2000 dilutions of rabbit anti rat GR antibodies (Santa Cruz Biotechnology).

Western Blotting

For the GR western cells were harvested from 3 ml of O/N culture and resuspended in 150 μl ethanol. Cells were broken with 150 μl of glass beads, vacuum dried, and resuspended in 100 μl of SDS-PAGE gel loading buffer. 15 μl of the clear lysates were analyzed on 10% SDS-PAGE and blotted with 1:200 dilution of GR monoclonal antibody.

GST Pull-Down

The yeast Cft1 open reading frame was fused to GST at N-terminal end and six histidine residues were fused at the C terminus and the resulting GST-Cft1-H6 protein was purified as described previously (Dichtl et al., 2002). GST-Cft1-H_6, GST-FXR, and GST were bound to 50 μl of glutathione-Sepharose 4B (Amersham Pharmacia) in phosphate-buffered saline containing 0.01%NP-40, 0.02% Triton X-100, 1 mM DTT, 5% Glycerol and protease inhibitor cocktail set III (Calbiochem). ApCPEB open reading frame was transcribed and translated in the presence of [^{35}S]-methionine in TnT lysate (Promega) in a 50 μl reaction volume. For GST pull-down, 50 μl of the GST beads were incubated with 10 μl of the TnT lysate containing ^{35}S-labeled protein in the presence of 0.2 mg/ml of purified BSA and 100 μg/ml of RnaseA for 1 hr at 4C. The GST beads were washed four times with PBS + 0.01%NP-40 + 0.02% Triton X-100 and the bound proteins were eluted with SDS-PAGE gel loading buffer.

RNA Gel-Shift Assay

The ^{32}P-labeleled capped mRNAs were generated by transcribing the linearized DNA templates in the presence of 20 μCi of α-^{32}P CTP and 20 μCi of α-^{32}P UTP using message machine (Ambion). The radiolabeled RNAs were precipitated with LiCl/ethanol and resuspended in nuclease-free water. The *Aplysia* CPEB, mouse CPEB1 (mCPEB1), and mammalian translation initiation factor 6 (meIF6) open reading frames were cloned into the hexahistidine vector pRSETA and the expression of the recombinant protein was induced in *E. coli* BL21 by adding 0.5 mM IPTG. The hexahistidine-tagged proteins were purified in Ni^{+2} NTA resin using manufactures protocol (Qiagen). The following buffers were used for purification: lysis and binding buffer, 6 M guanidine hydrochloride; 100 mM $NaPO_4$ (8.0); and 10 mM Tris base; wash buffer, 8 M urea; 100 mM $NaPO_4$ (8.0); 10 mM Tris base; 10 mM imidazole; elution buffer, 8 M urea; 100 mM $NaPO_4$ (8.0); 10 mM Tris base; and 250 mM imidazole. The purified proteins were dialyzed against a buffer containing 2 M urea, 100 mM KCl, 10 mM Na-HEPES (7.6), 1 mM DTT, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$ and 5% glycerol at 4C for 24–32 hr. Following dialysis the *Aplysia* CPEB protein is sonicated for 1 s with microtip and used in the binding reaction. The mouse CPEB did not produce a turbid solution when dialyzed against 2 M urea. The GST-tagged *Drosophila* GEF and *Aplysia* Cdc42 were purified in glutathione Sepharose 4B column following manufacturers protocol (Pharmacia) and the purified proteins were dialyzed against the same 2 M urea containing buffer. For RNA gel shift assay, ~500 ng of purified protein was incubated with 1 μl of purified RNA (5 × 10^4 cpm) in a 20 μl reaction containing 100 mM KCl, 10 mM Na-HEPES (7.6), 5 mM DTT, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, 5% glycerol, 0.1 mg/ml BSA and 50 μg/ml *E. coli* t-RNA. The reaction mixture was incubated at room temperature for 20 min and 1 μl of 50 mg/ml Na-Heparin was added. After incubating for additional 10 min at room temperature, the 4 μl of RNA gel loading buffer was added into the reaction and loaded into a 4% polyacrylamide (37.5:1), 0.5× TBE gel and run at room temperature at 150 mV for 30 min and subsequently at 200 mV until xylene cyanol was at the bottom of the gel. Following electrophoresis, the gel was dried and exposed to autoradiographic films.

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References

Chernoff, Y.O., Lindquist, S.L., Ono, B., Inge-Vechtomov, S.G., and Liebman, S.W. (1995). Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor. Science *268*, 880–884.

Collinge, J. (2001). Prion diseases of humans and animals: their causes and molecular basis. Annu. Rev. Neurosci. *24*, 519–550.

Coustou, V., Deleu, C., Saupe, S., and Begueret, J. (1997). The protein product of the het-s heterokaryon incompatibility gene of the fungus Podospora anserina behaves as a prion analog. Proc. Natl. Acad. Sci. USA *94*, 9773–9778.

Crist, C.G., Nakayashiki, T., Kurahashi, H., and Nakamura, Y. (2003). [PHI+], a novel Sup35-prion variant propagated with non-Gln/Asn oligopeptide repeats in the absence of the chaperone protein Hsp104. Genes Cells *8*, 603–618.

de Moor, C.H., and Richter, J.D. (1999). Cytoplasmic polyadenylation elements mediate masking and unmasking of cyclin B1 mRNA. EMBO J. *18*, 2294–2303.

DePace, A.H., Santoso, A., Hillner, P., and Weissman, J.S. (1998). A critical role for amino-terminal glutamine/asparagine repeats in the formation and propagation of a yeast prion. Cell *93*, 1241–1252.

Dichtl, B., Blank, D., Sadowski, M., Hubner, W., Weiser, S., and Keller, W. (2002). Yhh1p/Cft1p directly links poly(A) site recognition and RNA polymerase II transcription termination. EMBO J. *21*, 4125–4135.

Edskes, H.K., Gray, V.T., and Wickner, R.B. (1999). The [URE3] prion is an aggregated form of Ure2p that can be cured by overexpression of Ure2p fragments. Proc. Natl. Acad. Sci. USA *96*, 1498–1503.

Ferreira, P.C., Ness, F., Edwards, S.R., Cox, B.S., and Tuite, M.F. (2001). The elimination of the yeast [PSI+] prion by guanidine hydrochloride is the result of Hsp104 inactivation. Mol. Microbiol. *40*, 1357–1369.

Glover, J.R., Kowal, A.S., Schirmer, E.C., Patino, M.M., Liu, J.J., and Lindquist, S. (1997). Self-seeded fibers formed by Sup35, the protein

determinant of [PSI+], a heritable prion-like factor of S. cerevisiae. Cell *89*, 811–819.

Hake, L.E., and Richter, J.D. (1994). CPEB is a specificity factor that mediates cytoplasmic polyadenylation during Xenopus oocyte maturation. Cell *79*, 617–627.

Hake, L.E., Mendez, R., and Richter, J.D. (1998). Specificity of RNA binding by CPEB: requirement for RNA recognition motifs and a novel zinc finger. Mol. Cell. Biol. *18*, 685–693.

King, C.Y., Tittmann, P., Gross, H., Gebert, R., Aebi, M., and Wuthrich, K. (1997). Prion-inducing domain 2–114 of yeast Sup35 protein transforms in vitro into amyloid-like filaments. Proc. Natl. Acad. Sci. USA *94*, 6618–6622.

Kushnirov, V.V., Kochneva-Pervukhova, N.V., Chechenova, M.B., Frolova, N.S., and Ter-Avanesyan, M.D. (2000). Prion properties of the Sup35 protein of yeast Pichia methanolica. EMBO J. *19*, 324–331.

Li, L., and Lindquist, S. (2000). Creating a protein-based element of inheritance. Science *287*, 661–664.

Lindquist, S. (1997). Mad cows meet psi-chotic yeast: the expansion of the prion hypothesis. Cell *89*, 495–498.

Liu, J.J., Sondheimer, N., and Lindquist, S.L. (2002). Changes in the middle region of Sup35 profoundly alter the nature of epigenetic inheritance for the yeast prion. Proc. Natl. Acad. Sci. USA *99* (*Suppl 4*), 16446–16453.

Masison, D.C., and Wickner, R.B. (1995). Prion-inducing domain of yeast Ure2p and protease resistance of Ure2p in prion-containing cells. Science *270*, 93–95.

Mendez, R., Barnard, D., and Richter, J.D. (2002). Differential mRNA translation and meiotic progression require Cdc2-mediated CPEB destruction. EMBO J. *21*, 1833–1844.

Michelitsch, M.D., and Weissman, J.S. (2000). A census of glutamine/asparagine-rich regions: implications for their conserved function and the prediction of novel prions. Proc. Natl. Acad. Sci. USA *97*, 11910–11915.

Moriyama, H., Edskes, H.K., and Wickner, R.B. (2000). [URE3] prion propagation in Saccharomyces cerevisiae: requirement for chaperone Hsp104 and curing by overexpressed chaperone Ydj1p. Mol. Cell. Biol. *20*, 8916–8922.

Newnam, G.P., Wegrzyn, R.D., Lindquist, S.L., and Chernoff, Y.O. (1999). Antagonistic interactions between yeast chaperones Hsp104 and Hsp70 in prion curing. Mol. Cell. Biol. *19*, 1325–1333.

Osherovich, L.Z., and Weissman, J.S. (2001). Multiple Gln/Asn-rich prion domains confer susceptibility to induction of the yeast [PSI(+)] prion. Cell *106*, 183–194.

Patino, M.M., Liu, J.J., Glover, J.R., and Lindquist, S. (1996). Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. Science *273*, 622–626.

Paushkin, S.V., Kushnirov, V.V., Smirnov, V.N., and Ter-Avanesyan, M.D. (1997). In vitro propagation of the prion-like state of yeast Sup35 protein. Science *277*, 381–383.

Prusiner, S.B. (1998). Prions. Proc. Natl. Acad. Sci. USA *95*, 13363–13383.

Roberts, B.T., and Wickner, R.B. (2003). Heritable activity: a prion that propagates by covalent autoactivation. Genes Dev. *17*, 2083–2087.

Shatkin, A.J., and Manley, J.L. (2000). The ends of the affair: capping and polyadenylation. Nat. Struct. Biol. *7*, 838–842.

Si, K., Giustetto, M., Etkin, A., Hsu, R., Janisiewicz, A.M., Miniaci, M.C., Kim, J.H., Zhu, H., and Kandel, E.R. (2003). A neuronal isoform of CPEB regulates local protein synthesis and stabilizes synapse-specific long-term facilitation in aplysia. Cell *115*, this issue, 893–904.

Sondheimer, N., and Lindquist, S. (2000). Rnq1: an epigenetic modifier of protein function in yeast. Mol. Cell *5*, 163–172.

Sparrer, H.E., Santoso, A., Szoka, F.C., Jr., and Weissman, J.S. (2000). Evidence for the prion hypothesis: induction of the yeast [PSI+] factor by in vitro-converted Sup35 protein. Science *289*, 595–599.

Stebbins-Boaz, B., Hake, L.E., and Richter, J.D. (1996). CPEB controls the cytoplasmic polyadenylation of cyclin, Cdk2 and c-mos mRNAs and is necessary for oocyte maturation in Xenopus. EMBO J. *15*, 2582–2592.

Stumpf, G., and Domdey, H. (1996). Dependence of yeast pre-mRNA 3′-end processing on CFT1: a sequence homolog of the mammalian AAUAAA binding factor. Science *274*, 1517–1520.

Theis, M., Si, K., and Kandel, E.R. (2003). Two previously undescribed members of the mouse CPEB family of genes and their inducible expression in the principal cell layers of the hippocampus. Proc. Natl. Acad. Sci. USA *100*, 9602–9607.

True, H.L., and Lindquist, S.L. (2000). A yeast prion provides a mechanism for genetic variation and phenotypic diversity. Nature *407*, 477–483.

Tuite, M.F. (2000). Yeast prions and their prion-forming domain. Cell *100*, 289–292.

Wickner, R.B. (1994). [URE3] as an altered URE2 protein: evidence for a prion analog in Saccharomyces cerevisiae. Science *264*, 566–569.

Wickner, R.B., and Masison, D.C. (1996). Evidence for two prions in yeast: [URE3] and [PSI]. Curr. Top. Microbiol. Immunol. *207*, 147–160.