Autoregulation of GLD-2 cytoplasmic poly(A) polymerase

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ABSTRACT

Cytoplasmic polyadenylation regulates mRNA stability and translation and is required for early development and synaptic plasticity. The GLD-2 poly(A) polymerase catalyzes cytoplasmic polyadenylation in the germline of metazoa. Among vertebrates, the enzyme is encoded by two isoforms of mRNA that differ only in the length of their 3'-UTRs. Here we focus on regulation of vertebrate *GLD-2* mRNA. We show that the 3'-UTR of *GLD-2* mRNA elicits its own polyadenylation and translational activation during frog oocyte maturation. We identify the sequence elements responsible for repression and activation, and demonstrate that CPEB and PUF proteins likely mediate repression in the resting oocyte. Regulated polyadenylation of *GLD-2* mRNA is conserved, as are the key regulatory elements. Poly(A) tails of *GLD-2* mRNA increase in length in the brain in response to neuronal stimulation, suggesting that a comparable system exists in that tissue. We propose a positive feedback circuit in which translation of *GLD-2* mRNA is stimulated by its polyadenylation, thereby reinforcing the switch to polyadenylate and activate batteries of mRNAs.

Keywords: CPEB; GLD-2; PUF; cytoplasmic polyadenylation; poly(A) polymerase

INTRODUCTION

The 3'-untranslated region (3'-UTR) of mRNAs plays a key role in controlling nuclear export, localization, translation, and stability of mRNAs (for review, see Sonenberg 1994; Hentze 1995; Wickens et al. 2002; Kuersten and Goodwin 2003). Cytoplasmic lengthening of the poly(A) tail increases translation and mRNA stability, while its removal can cause translational repression and mRNA decay. These regulated changes in poly(A) length are critical in early development, and participate in pattern formation and cell cycle control (for review, see Richter 2000; Wickens et al. 2000). In the nervous system, repeated stimulation of synapses activates polyadenylation and local translation (Wu et al. 1998; Huang et al. 2002; Si et al. 2003a; Theis et al. 2003), events that are critical in long-term potentiation (LTP) and learning (Alarcon et al. 2004). The widespread anatomical distribution of polyadenylation factors suggests that the same system may operate in many other biological contexts as well.

Meiotic maturation of *Xenopus* oocytes and mitotic timing in embryos require cytoplasmic polyadenylation of cyclin and *c-mos* mRNAs (for review, see Mendez and Richter 2001; Wickens et al. 2002). The polyadenylation of these mRNAs requires elements in their 3'-UTRs, including a uridine-rich cytoplasmic polyadenylation element (CPE)

and the hexanucleotide sequence AAUAAA (Fox et al. 1989; McGrew and Richter 1990). CPE-binding protein (CPEB) binds target mRNAs and elicits their polyadenylation and activation (Hake and Richter 1994). A cytoplasmic form of cleavage and polyadenylation specificity factor (CPSF) complex, a multiprotein complex, binds AAUAAA (Bilger et al. 1994; for review, see Mendez and Richter 2001). Neuronal CPEB is required for polyadenylation of CPE-containing mRNAs (Wu et al. 1998; Si et al. 2003a) and is important for formation of hippocampal-dependent memories (Alarcon et al. 2004; Berger-Sweeney et al. 2006).

CPEB is both an activator and repressor. The inhibitory function is linked to several other translational repressor proteins, including maskin, PUF, and Nanos proteins. Members of each family, along with CPEB, are bound to specific mRNAs in *Xenopus* oocytes, and cause their repression prior to meiotic maturation (Stebbins-Boaz et al. 1999; Nakahata et al. 2001; Cao and Richter 2002; Nakahata et al. 2003; for review, see Richter 2000). These different factors may repress through redundant mechanisms. Maskin directly interacts with CPEB and with eIF-4E, preventing initiation (Stebbins-Boaz et al. 1999; Cao and Richter 2002). PUF proteins can recruit the Ccr4 complex, containing both deadenylases and the inhibitory Dhh1p RNA helicase (Goldstrohm et al. 2006). CPEB and PUF proteins are required for learning (Dubnau et al. 2003) and localize to dendrites (Wu et al. 1998; Ye et al. 2004; Vessey et al. 2006).

GLD-2, a divergent cytoplasmic poly(A) polymerase (PAP), was first identified in *Caenorhabditis elegans* (Wang et al. 2002). The enzyme is related to the Cid1 and Cid13

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PAPs of *Schizosaccharomyces pombe* (Read et al. 2002; Saitoh et al. 2002). *GLD-2* enzyme activity is stimulated by interaction with an RNA-binding protein, *GLD-3* (Wang et al. 2002). Together, *GLD-2* and *GLD-3* are thought to form a novel heterodimeric PAP, in which the RNA-binding component *GLD-3* recruits the catalytic subunit *GLD-2* to specific mRNAs (Wang et al. 2002). *GLD-2* homologs in *Saccharomyces cerevisiae*, *Trf4p* and *Trf5p*, localize to the nucleus. Like *GLD-2/GLD-3* in *C. elegans*, *Trf4p*'s polymerase activity is stimulated by RNA-binding proteins *Air1p* or *Air2p*. *Trf4p*, *Air2p*, and *Mtr4p* comprise the TRAMP complex (Vanacova et al. 2005). In contrast to *GLD-2*, however, polyadenylation by this complex leads to RNA degradation by the nuclear exosome, rather than to activation (LaCava et al. 2005; Vanacova et al. 2005; Wyers et al. 2005).

GLD-2 poly(A) polymerases were recently identified in vertebrates, including frogs, mice, and humans (Barnard et al. 2004; Kwak et al. 2004; Rouhana et al. 2005). The mouse (*mGLD-2*) and *Xenopus* *GLD-2* (*XIGLD-2*) proteins are both nuclear and cytoplasmic in oocytes (Rouhana et al. 2005; Nakanishi et al. 2006). Cytoplasmic *XIGLD-2* protein is physically associated with CPEB and CPSF (Barnard et al. 2004; Rouhana et al. 2005), but not with CPEB-protein-bound repressors Pumilio and maskin (Rouhana et al. 2005). Vertebrate *GLD-2* proteins are important for meiotic maturation of oocytes, and likely for activation of many mRNAs throughout early development (Barnard et al. 2004; Rouhana et al. 2005; Nakanishi et al. 2006). In the mouse brain, *GLD-2* mRNA is expressed in anatomical regions necessary for long-term cognitive and emotional learning (Rouhana et al. 2005). In the hippocampus, *mGLD-2* mRNA co-localizes with CPEB1 and Pumilio1 mRNAs, both of which are involved in synaptic plasticity. In the cerebellum, *mGLD-2* mRNA is present within the major neuronal cell populations, coincident with the distribution of other CPEB isoforms.

In this study, we focus on the post-transcriptional regulation of *GLD-2* mRNA. We show that two previously identified *GLD-2* mRNA isoforms [*XIGLD-2A(L)* and *XIGLD-2A(S)*] differ in their regulation during oocyte maturation. In particular, the mRNA with a long 3'-UTR causes repression before maturation, then elicits polyadenylation-dependent activation once maturation begins. This polyadenylation-dependent activation of a poly(A) polymerase's mRNA may comprise a positive-feedback circuit that reinforces a regulatory switch that supports activation of many mRNAs.

RESULTS

XIGLD-2A(L) 3'-UTR causes translational stimulation during oocyte maturation

The *XIGLD-2A* gene encodes two mRNAs, termed *GLD-2A(S)* and *GLD-2A(L)*. These mRNAs differ solely in the length of their 3'-UTRs, due to the use of alternative 3'-end cleavage in the nucleus. The long 3'-UTR extends an

additional 747 nucleotides (nt) beyond that of the short form (Fig. 1A,B). The structures and polyadenylation sites of the two mRNAs were confirmed by isolation of multiple ESTs, directed RT-PCR, and Northern blotting (data not shown; Rouhana et al. 2005). The 3'-termini of both mRNA forms are preceded by polyadenylation hexanucleotides, either AAUACA or AAUAAA. A second *Xenopus* *GLD-2* gene, *XIGLD-2B*, produces a single mRNA with a long 3'-UTR that is 83% identical to that of *XIGLD-2A(L)*. Human (*hGLD-2*) and mouse *GLD-2* genes produce two mRNA isoforms analogous to *XIGLD-2A(L)* and (S); these, too, encode identical proteins. The human and frog *GLD-2* 3'-UTRs diverge substantially. Despite only 3% identity throughout the 3'-UTRs, the human 3'-UTR contains islands of sequence conserved in the two frog mRNAs. For example, the last 32 nt of human and frog mRNAs are 87% identical.

The conserved pattern of isoforms and sequence islands suggested that the 3'-UTRs might have a regulatory role. To test this hypothesis, we prepared mRNAs in vitro bearing the luciferase open reading frame (ORF) followed by either the long or short form of the *XIGLD-2A* 3'-UTR (Fig. 1C). The mRNAs were injected into the cytoplasm of *Xenopus laevis* oocytes. Luciferase activity was determined after incubation in the absence or presence of progesterone, which induces meiotic maturation. A β -galactosidase mRNA carrying 15 adenosines was used as an internal control. Translation of the mRNA bearing the *XIGLD-2A(L)* 3'-UTR was stimulated >50-fold during oocyte maturation (Fig. 1C). The *XIGLD-2B* 3'-UTR stimulated translation only two- to threefold, while the *XIGLD-2A(S)* had no significant effect (Fig. 1C). An mRNA bearing the *cyclin B1* 3'-UTR, used as a positive control, stimulated translation ~40-fold.

The human *GLD-2* gene produces two mRNAs with different 3'-UTRs, analogous in lengths to those of the *Xenopus* *GLD-2A* gene. As mentioned above, the 3'-UTR sequence of the human *GLD-2(L)* 3'-UTR is only 3% identical to that of the frog *GLD-2A(L)* 3'-UTR overall; however, both 3'-UTRs possess putative CPEs and PUF protein-binding sites near their 3'-termini. To test whether the 3'-UTR of *hGLD-2(L)* conferred translational regulation, we injected mRNAs bearing that 3'-UTR into oocytes. The human 3'-UTR enhanced luciferase activity 18-fold after progesterone treatment (Fig. 1D). An mRNA bearing the *XIGLD-2A(S)* 3'-UTR, used as a control, did not increase activity. We conclude that despite the great sequence divergence between the human and frog 3'-UTRs, both specifically stimulate translation during oocyte maturation.

XIGLD-2A(L) is polyadenylated during oocyte maturation

To determine whether endogenous *GLD-2A* mRNA was polyadenylated during oocyte maturation, we determined

FIGURE 1. *GLD-2* mRNA architecture and activation during oocyte maturation. (A) *GLD-2* 3'-UTRs. Diagram of vertebrate *GLD-2* mRNA 3'-UTRs and the position of putative cytoplasmic polyadenylation elements (CPEs; white boxes), PUF binding elements (PBEs; gray diamonds), and cleavage and polyadenylation specificity sequences (black boxes) are shown. The last 90 nt of the *XIGLD-2A(L)* 3'-UTR, analyzed in later figures, is indicated by the black bar over *XIGLD-2A(L)* mRNA. Regions of high homology with *XIGLD-2A(L)* sequence are indicated by the black backbone. (B) *XIGLD-2A* 3'-UTR sequence. The 932-nt sequence of the *XIGLD-2A(L)* 3'-UTR is presented. The *XIGLD-2A(S)* 3'-UTR sequence is underlined; an arrowhead indicates the poly(A) addition site of that mRNA. The elements depicted in A are shown here using the same color key. (C) The *XIGLD-2A(L)* 3'-UTR increases translation of an mRNA during oocyte maturation. Luciferase reporter mRNAs, bearing different 3'-UTRs, were injected into *Xenopus* oocytes and incubated overnight in the absence or presence of progesterone. *LacZ* mRNA was coinjected as an internal control. Luciferase activity was measured and normalized to β -galactosidase activity and is shown for progesterone-treated and untreated oocytes. The ratio of luciferase activity in treated over untreated oocytes is shown under the X-axis. (D) Elements required for translational stimulation by the *XIGLD-2A(L)* 3'-UTR are conserved in the *hGLD-2(L)* 3'-UTR. A luciferase reporter mRNA bearing the *hGLD-2(L)* 3'-UTR was injected and analyzed as in C.

four T residues at its 3' terminus, and thus would detect only polyadenylated mRNAs. Gene-specific primers in the 3'-UTR of the mRNA of interest were used to prime second-strand synthesis. After PCR, the lengths of poly(A) tails were deduced from the length of DNA fragments.

The poly(A) tails of endogenous *XIGLD-2A(L)* mRNA became progressively longer at successive times during oocyte maturation (Fig. 2B); after 6 h, the tails had been extended by 150–200 adenosines. In contrast, the poly(A) tails of *XIGLD-2A(S)* mRNA did not appear to change in length during maturation (Fig. 2B), consistent with the lack of translational stimulation by that mRNA's 3'-UTR (Fig. 1C). As controls, the poly(A) tails of *cyclin B1* mRNA were extended by ~150 residues, while polyadenylated forms of ribosomal protein-L1 mRNA apparently disappeared, as expected (Fig. 2B). We conclude that polyadenylation of the endogenous mRNAs mirrors the translational properties of the 3'-UTR reporters, and that endogenous *XIGLD-2A(L)* mRNA is polyadenylated during maturation.

Polyadenylation of *XIGLD-2A(L)* mRNA is specific, as it was not observed with other mRNAs, including ones involved in translational control during maturation (Fig. 2C). The poly(A) tails of *CPEB*, *CPSF-160*, *CPSF-100*, *PABP-1*, *Xcat-2*, *maskin*, and *Xp54* mRNAs were not dramatically different before and after maturation. *Xp54*, *maskin*, and *CPSF-73* mRNAs appeared to undergo transient polyadenylation, during or after germinal vesicle breakdown, but were later deadenylated (Fig. 2C). The multiple bands observed with *PABP-1* mRNA reflect different 3'-UTR lengths, consistent with cDNA and EST databases.

the length of poly(A) on endogenous *XIGLD-2A* mRNA using a PCR-based assay (Fig. 2A; Charlesworth et al. 2004). Total RNA was extracted from *X. laevis* oocytes at various times after addition of progesterone. A DNA oligonucleotide was ligated to the 3'-end of the RNA, and a complementary primer was used to initiate cDNA synthesis by reverse transcriptase. The primer possessed

GLD-2 control is mediated by CPE- and AAUAAA-dependent cytoplasmic polyadenylation

To identify the sequence elements in *XIGLD-2A(L)* mRNA that promote its translational activation, we first analyzed the roles of CPEs and the polyadenylation signal AAUAAA. Both of these elements are conserved in the long isoforms

FIGURE 2. Polyadenylation of endogenous *XIGLD-2A(L)* mRNA during oocyte maturation. (A) Measurements of poly(A) length. Total RNA from *Xenopus* oocytes, maturing oocytes, and eggs was ligated to the 5'-end of a modified DNA oligo. Reverse transcriptase was used to synthesize DNA complementary to the mRNA, using a primer anti-sense to the ligated DNA oligonucleotides; this newly synthesized DNA contained the poly(A) tail region. A gene-specific primer, along with a primer anti-sense to the ligated DNA oligo, was used to amplify the 3'-end of the gene of interest by PCR. The lengths of PCR products from gene-specific primers were determined using 2.5% agarose gels. (B) *XIGLD-2A(L)* is polyadenylated during oocyte maturation. mRNA was prepared at the indicated times after addition of progesterone or from unfertilized eggs. To determine poly(A) lengths of each endogenous mRNA, reactions were performed using primers specific for each of the four indicated mRNAs. The lengths of products obtained in resting oocytes were consistent with the known ends of the mRNA. The PCR products of *XIGLD-2A(L)* and *cyclin B1* show slower electrophoretic mobility during maturation, indicating that those mRNAs are polyadenylated during maturation. The PCR products obtained with *XIGLD-2A(S)* and ribosomal protein L1 primers do not increase in length, indicating that these mRNAs are not polyadenylated. (C) Analysis of mRNAs encoding factors involved in translational regulation during oocyte maturation. The poly(A) tail of mRNAs for *CPEB*, *CPSF-160*, *CPSF-100*, *CPSF-73*, *PABP-1*, *Xcat-2*, and *maskin* were analyzed during oocyte maturation, using the same protocol as in A and B. The lengths of the specific 3'-end fragments, as determined by EST analysis, are indicated with arrowheads.

of *GLD-2* mRNA in vertebrates (Fig. 1A). We focused on the last 90 nt of the *XIGLD-2A(L)* 3'-UTR because it stimulated translation during maturation as efficiently as the full-length *XIGLD-2A(L)* 3'-UTR and is highly conserved between *Xenopus* and humans. The conservation includes tandem CPEs and AAUAAA (Fig. 3A), and the spacings between them (data not shown).

Mutant forms of the 3'-UTR linked to the luciferase ORF were injected into oocytes, which were then induced to mature using progesterone (Fig. 3B). mRNAs with mutations in either CPE alone did not perturb translational stimulation; however, simultaneous mutations in both CPEs decreased stimulation substantially, as did a point mutation in AAUAAA (Fig. 3B). We conclude that the two CPEs act redundantly, and in concert with AAUAAA, to promote translational activation.

To assess polyadenylation of these RNA sequence variants directly, we injected short, radiolabeled RNAs encoding a 21-amino acid-long micro-ORF linked to each terminal 3'-UTR segment. The ^{32}P -labeled mRNAs, extracted from oocytes after overnight treatment with or without progesterone, were analyzed by electrophoresis (Fig. 3C). RNAs containing the wild-type sequence of the *XIGLD-2A(L)* 3'-UTR increased by ~ 150 nt in length after oocyte maturation (Fig. 3C, lanes 1–3), consistent with the length of poly(A) added to the endogenous mRNA (Fig. 2B). mRNAs that lacked both CPE1 and CPE2 were much less active as substrates (Fig. 3C, lanes 4–6). Mutation of AAUAAA essentially abolished polyadenylation (Fig. 3C, lanes 7–9). Thus the sequence specificities of translational activation and polyadenylation are similar.

GLD-2 protein is bound to *XIGLD-2A(L)* mRNA in *Xenopus* oocytes

The *GLD-2* enzyme catalyzes polyadenylation of certain mRNAs during oocyte maturation and acts in collusion with CPEB and CPSF (Barnard et al. 2004; Rouhana et al. 2005; Nakanishi et al. 2006). We reasoned that *GLD-2* protein might polyadenylate *GLD-2* mRNA. To test whether *XIGLD-2A(L)* mRNA was bound to *GLD-2* protein, we incubated oocyte lysates with anti-*GLD-2* antibodies, extracted immunoprecipitated RNAs, and analyzed them by semiquantitative RT-PCR (Fig. 4). *XIGLD-2A(L)* mRNA was readily detected in immunoprecipitates obtained with α -*GLD-2*, but not with control, pre-immune antibodies (Fig. 4). *cyclin B1* mRNA, used as a

CPEs and PUF-binding elements are required for repression of *GLD-2* mRNA

The last 90 nt of *XIGLD-2A(L)* contains four putative PUF-protein-binding elements (PBEs), as does *GLD-2* mRNA in *Xenopus tropicalis* (Fig. 5A). Mouse and human *GLD-2(L)* mRNAs possess a single PBE in the same location as PBE2 (Fig. 5A). To determine whether PBE2 bound *Xenopus* Pumilio, we performed gel retardation assays using purified recombinant GST-Xl Pumilio (Fig. 5B). The protein bound to both PBE2 and the NRE (derived from *hunchback* mRNA). Binding was specific, since it was reduced by at least an order of magnitude by mutation of the UGU to ACA, as expected for a PUF interaction (Fig. 5B; e.g., Bernstein et al. 2005).

Both CPEB and PUF proteins can inhibit translation of mRNAs to which they bind. To test whether the CPEs or PBEs of *XIGLD-2A(L)* mRNA repressed its translation, we prepared luciferase mRNAs linked to mutant and wild-type 3'-UTRs (Fig. 6A). For repression studies, we prepared mutant mRNAs containing mutations in either all four putative PUF-binding elements (PBE1-4) or both CPEs and a third degenerate CPE. The degenerate CPE was included because it partially overlaps PBE2 and because residual polyadenylation was detected with a 3'-UTR carrying mutations in the first two CPEs (Fig. 3C, lanes 4-6). These mRNAs were injected into resting oocytes, without progesterone treatment. The mRNA that carried CPE mutations was translated ninefold more efficiently than a wild-type mRNA, suggesting that these CPEs repress translation of *GLD-2* mRNA prior to maturation. Repression by the CPEs was dose-dependent: it was observed at

FIGURE 4. GLD-2 protein is bound to *XIGLD-2A(L)* mRNA. Oocyte extracts were incubated with α -GLD-2 or pre-immune guinea pig serum and bound to Protein A-Sepharose. RNA was extracted and used as a template for reverse transcription. mRNAs were detected by semiquantitative PCR on cDNA with gene-specific primers. *XIGLD-2A(L)* mRNA is detected in RNAs immunoprecipitated by α -GLD-2, but not by pre-immune serum. The lengths of products are consistent with the known 3'-termini of the mRNAs.

FIGURE 5. *Xenopus* Pumilio binds to PBEs in the *GLD-2(L)* 3'-UTR. (A) A highly conserved region of the *GLD-2(L)* 3'-UTR contains multiple PBEs. Putative PBEs (PBE1-4 for *Xenopus GLD-2*; gray boxes) and cleavage and polyadenylation hexanucleotides (black boxes) in *GLD-2(L)* mRNAs. The chemically synthesized RNA used for xPum/RNA-binding assays is indicated by the black rectangle on the *XIGLD-2A(L)* sequence. (B) Binding to purified PUF protein. Various concentrations of purified, recombinant GST-xPum were incubated with labeled RNAs in vitro and analyzed by electrophoretic mobility shift assays. The RNAs correspond to the NRE of hunchback mRNA, PBE2, and a mutant form of PBE2 in which the UGU has been changed to ACA. The protein concentrations were 10 nM, 25 nM, 50 nM, 100 nM, 270 nM, and 810 nM.

The CPEs in the *XIGLD-2A(L)* 3'-UTR also are required for translational activation once progesterone is added. mRNAs that lacked CPEs (injected at 2–3 fmol per oocyte) did not increase in activity significantly during maturation (Fig. 6D,E). In contrast, PBE mutant mRNAs did increase in activity in the same period (Fig. 6D,E). The magnitude of stimulation (the ratio of activities with and without progesterone) was less with the PBE mutant than with the wild type; this difference arises because the PBE mutations increased the “basal” level of translation prior to maturation. We conclude that both CPE- and PBE-dependent mechanisms repress *XIGLD-2A(L)* mRNA prior to maturation. The CPEs are required to stimulate expression during maturation, while the PBEs are not.

Polyadenylation in resting oocytes

A

Wild type
3xCPe⁻
4xPBE⁻

Wild type
3xCPe⁻
4xPBE⁻

B

Luciferase / β -galactosidase

WT 3xCPe⁻ 4xPBE⁻

C

Luciferase / β -galactosidase

WT 3xCPe⁻ 4xPBE⁻

D

Luciferase +P/-P

Time in progesterone (hour)

● WT
▲ 3xCPe⁻
■ 4xPBE⁻

E

Luciferase / β -galactosidase

WT 3xCPe⁻ 4xPBE⁻

-P +P -P +P -P +P

specifically detect polyadenylated mRNAs, we used a primer complementary to the ligated oligonucleotide plus 15 3'-terminal thymidine residues. Surprisingly, in resting oocytes, both wild-type and PBE mutant mRNAs were polyadenylated. Roughly 40 adenosines were added to the wild-type mRNA and 50 to the PBE mutant mRNA (Fig. 7, lanes 1,2). The length of poly(A) on endogenous *XIGLD-2A(L)* mRNA is comparable (see Fig. 2B). This polyadenylation reaction was largely CPE dependent (Fig. 7, lane 3). As a control, we used a primer that was complementary to the ligated primer, but without an oligo(dT) segment. With this primer, the products obtained correspond in majority to nonadenylated mRNAs: wild-type and mutant mRNAs yielded identical distributions of PCR products, as expected. Together, these observations suggest that *XIGLD-2A(L)* mRNA, prior to maturation, receives a poly(A) tail of limited length. This polyadenylation event is detected on a minority of molecules. We conclude that CPE-mediated polyadenylation occurs in oocytes prior to maturation. This "resting polyadenylation" reaction is counteracted in part by PBEs, presumably acting through the *Xenopus* PUF protein.

GLD-2 expression and polyadenylation in the brain

In the nervous system, translation of dendritically localized mRNAs is enhanced upon synaptic stimulation and is thought to require cytoplasmic polyadenylation (Si et al. 2003a). We reasoned that *GLD-2* mRNA itself might be regulated by polyadenylation in the brain. The components of the regulatory machinery—GLD-2, CPEB, and PUF proteins—co-localize at the mRNA level in the hippocampus, cerebellum, and olfactory bulb of the mouse brain (Rouhana et al. 2005). To detect the tissue distribution of

FIGURE 7. CPE-dependent polyadenylation in resting oocytes. Wild-type, 4xPBE⁻, and 3xCPE⁻ mutant mRNAs were injected into resting oocytes. mRNA from oocytes injected with the reporters shown in Figure 6A was analyzed using the PCR-based poly(A) length assay (Fig. 2A). Reverse transcription and PCR were performed with a primer selective for poly(A) tails [oligo+p(dT)₁₅; lanes 1–3] revealed longer tails for the 4xPBE⁻ mRNA than for wild-type mRNA (lanes 1,2). The poly(A) tail length of 3xCPE⁻ mRNA is minimal (lane 3). The background signal from oocytes not injected with reporter mRNA is shown (lane 4). The polyadenylation difference is not observed when using an oligo that is not selective for mRNAs with a poly(A) tail [oligo+p(dT)₀; lanes 5–7].

GLD-2 protein in *Xenopus*, we extracted total protein from various tissues, including dissected subregions of the brain, and detected GLD-2 by Western blotting with affinity-purified α -GLD-2 antibodies. GLD-2 was most abundant in medulla, spinal nerve, cerebellum, and cerebrum (Fig. 8A). It was present at lower levels in other somatic tissues, including liver, heart, and muscle. α -Actin antibodies served as a loading control.

To determine whether brain *XIGLD-2A(L)* mRNA was polyadenylated after neuronal stimulation, we examined its polyadenylation state after treating intact animals with kainic acid. Kainic acid, a seizure-inducing agent, dramatically stimulates neuronal activity (Watkins and Evans 1981). Male frogs were injected with kainic acid, which induced tremors, as expected; injections of saline were used as controls. After 45 min, the cerebellum was dissected. The lengths of poly(A) on *GLD-2* mRNA were determined using the PCR-based assays described previously. As expected, the lengths of poly(A) on *XIGLD-2A(L)* mRNA were heterogeneous both with and without kainic acid, ranging from ~50 to 200 nt (Fig. 8B,C). However, the average poly(A) tail length for *XIGLD-2A(L)* mRNA increased in response to kainic acid and included a new discrete species of ~220 residues (Fig. 8B,C). The poly(A) tails of *PABP-1* mRNA, used as a control, were homogeneous (~30 adenosines) and were unaffected by kainic acid treatment (Fig. 8B).

To ensure that the heterogeneous PCR products were derived from *XIGLD-2A(L)* and contained poly(A), we exploited a BsrG1 restriction enzyme site located at the 3'-most end of the *XIGLD-2A(L)* 3'-UTR (Fig. 8D, diagram). Treatment of the GLD-2 PCR products with BsrG1 transformed the heterogeneous population into a homogeneous-sized DNA of the predicted length, showing that the PCR products were, indeed, derived from the *XIGLD-2A(L)* 3'-UTR (Fig. 8D). To determine whether the heterogeneity was due to heterogeneity in poly(A) tails, we performed primer extension analysis of the GLD-2 PCR products in the presence of dATP, dTTP, dCTP, or dGTP (Fig. 8E). The primer, which was complementary to the ligated oligonucleotide, was extended only in the presence of dTTP, corresponding to poly(A) tails in the mRNA (Fig. 8E). We conclude that the poly(A) tails of *XIGLD-2A(L)* mRNA in the brain increase in length after kainic acid treatment. These data suggest that *GLD-2* mRNA is selectively polyadenylated after neuronal stimulation, as in oocytes; however, it remains possible that the increase in poly(A) length after stimulation reflects new transcription of mRNAs with longer tails, or inhibition of deadenylation.

DISCUSSION

XIGLD-2A(L) mRNA is repressed in the oocyte, then polyadenylated and activated during maturation. This process is conserved, since the human *GLD-2(L)* 3'-UTR behaves comparably (Fig. 1) and mouse *GLD-2* mRNA is

FIGURE 8. Poly(A) tail length of neuronal *GLD-2(L)* mRNA increases upon stimulation. (A) Western blot analysis of GLD-2 protein in tissues of frog brain, spinal nerve, liver, heart, and muscle. GLD-2 protein was enriched in tissues of the nervous system (top), but was also seen expressed in other tissues after longer exposures (middle). Actin was also analyzed as a loading control (bottom). (B) The *XIGLD-2A(L)* poly(A) tail is increased in response to kainic acid in frog brain. PCR-based poly(A)-length assay (performed as in Fig. 2) on cerebellar RNA from kainic acid treated and untreated frogs. High resolution was obtained by ^{32}P -end-labeling of DNA products and electrophoresis in a 6% polyacrylamide gel. (C) Poly(A)-length distribution for *XIGLD-2A(L)* was created from PhosphorImager scans for the kainic acid treated and untreated PCR-based poly(A)-length measuring assay. (D) Heterogeneous *XIGLD-2A(L)* PCR products from poly(A) length analysis due to different lengths at 3'-ends. *XIGLD-2A(L)* DNA products from the poly(A)-length assay were digested with BsrG1, whose substrate sequence is present downstream from the AAUAAA hexanucleotide on *XIGLD-2A(L)*. The BsrG1 digest creates a homogeneous 200-nt product, as is expected from the *XIGLD-2A(L)* UTR sequence. (E) Primer extension analysis confirms heterogeneity of *XIGLD-2A(L)* poly(A) lengths. A ^{32}P -labeled DNA oligonucleotide anti-sense to the oligonucleotide ligated to the end of the mRNA was used for primer extension analysis of *XIGLD-2A(L)* and *PABP-1* PCR products. Primer extensions were performed in the presence of one of dATP, dTTP, dCTP, or dGTP. Primers were extended only in the presence of dTTP, indicative of poly(A) at the end of both *XIGLD-2A(L)* and *PABP-1* substrates. The *XIGLD-2A(L)* products were extended by 50–300 residues in the presence of dTTP, while *PABP-1* products were extended by 20–30 residues. These lengths are consistent with the measurements in B.

polyadenylated during oocyte maturation (Nakanishi et al. 2006). CPEs and AAUAAA are required for polyadenylation of *GLD-2* mRNA and for the increase in translational activity during maturation; this implies that GLD-2 polyadenylates its own mRNA, presumably via protein–protein interactions with CPSF and CPEB (Barnard et al. 2004; Rouhana et al. 2005). Indeed, GLD-2 protein is bound to its own mRNA in the resting oocyte (Fig. 4). A similar circuit may exist in the brain, since the poly(A) tails of *XIGLD-2A(L)* mRNA increase in length in response to the brain in response to neuronal stimuli.

We propose that a positive feedback circuit activates and sustains GLD-2 protein, both during oocyte maturation and in response to synaptic stimulation (Fig. 9). This circuit would enhance translation not only of *XIGLD-2A(L)*, but also of its other, CPE-containing mRNA targets on which

GLD-2 acts. In the nervous system, GLD-2 autoactivation may complement the CPEB prion switch (Si et al. 2003b).

The *GLD-2(L)* mRNA isoform is particularly abundant in mouse and human brains (Rouhana et al. 2005). Other tissues, such as pituitary gland, heart, placenta, ovary, testis, liver, spleen, and kidney; possess abundant *GLD-2(S)* as well as *GLD-2(L)* mRNA (Rouhana et al. 2005). The preponderance of the long form in the brain may reflect the requirement for regulation in that tissue.

Alternative 3'-UTRs in certain other mRNAs also mediate developmental, physiological, and pathological controls (for review, see Hughes 2006). For example, an alternative 3' exon in the *Drosophila elav* gene generates a longer 3'-UTR that is autoregulated by ELAV protein (Borgeson and Samson 2005). Alternative 3'-end formation is often conserved and tissue-specific (for review, see Edwalds-Gilbert

FIGURE 9. Working model: a positive feedback loop activates *GLD-2* mRNA. In the “off” state, translation of *GLD-2* mRNA is repressed via PUF and CPEB, bound to specific sites in the long 3′-UTR isoform of *GLD-2A* mRNA. The bound PUF protein promotes removal of the tail, as suggested by the measurements of poly(A) length on PBE mutant mRNAs. *GLD-2* protein also is bound to the mRNA and modestly promotes poly(A) lengthening prior to maturation. The net result is that the poly(A) tail of the mRNA is short. The PUF and CPEB proteins may also repress by deadenylation-independent mechanisms not depicted in the figure. After stimulation, *GLD-2* mRNA is de-repressed and becomes a substrate for CPE-mediated polyadenylation by *GLD-2* cytoplasmic PAP. The ability of *GLD-2* to polyadenylate its own mRNA creates a positive feedback mechanism for the polyadenylation of this (and other) CPE-encoding mRNAs.

et al. 1997; Tian et al. 2005; Zhang et al. 2005). Longer brain-specific 3′-UTRs have been reported for the Huntington disease gene, *beta-adducin*, and *mCPEB3* (Lin et al. 1993; Theis et al. 2003; Costessi et al. 2006). Recently, a genome-wide study suggested differential repression of transcripts with alternative 3′-UTRs by microRNAs (Legendre et al. 2006). The differential control imposed by alternative 3′-UTRs, as seen with *GLD-2* mRNA, may be common.

The addition of poly(A) in resting oocytes, observed here for the first time, may be catalyzed by previously reported complexes containing CPEB, CPSF, and *GLD-2* (G complexes) (Rouhana et al. 2005). Indeed, this polyadenylation process is CPE dependent and partially counteracted by PBEs. Only a short tail is added prior to maturation, suggesting that either the addition process is blocked or that a deadenylase compensates and balances the tail length, as suggested during oocyte maturation (Fox et al. 1989; Varnum and Wormington 1990).

The presence of two comparable isoforms of *GLD-2* mRNA in frog, mice, and humans suggests a conserved difference in the function of short and long *GLD-2* mRNAs. Here we have shown that in *Xenopus*, the long isoform of *XIGLD-2A* is polyadenylated during oocyte maturation; the short isoform is not. CPEs, presumably acting through CPEB, inhibit translation of *XIGLD-2A(L)* (Cao and Richter 2002); PBEs may effect poly(A) tail length through a PUF–deadenylase interaction (Goldstrohm et al. 2006). Both mechanisms would prevent translation of *XIGLD-2A(L)* mRNA

until stimulation occurs. Once polyadenylation did occur, *GLD-2* protein would then polyadenylate its own *GLD-2(L)* mRNA, creating a positive feedback cascade (Fig. 9). The short isoform of mRNA may contribute a low level of latent poly(A) polymerase, whose activation initiates the cascade.

The level of total *GLD-2* protein detected by α -*GLD-2* antibodies does not change significantly during maturation (data not shown). This might be due to the fact that *GLD-2A(L)* mRNA is repressed in the resting oocyte, while *GLD-2A(S)* and *GLD-2B* mRNAs are readily translatable (Fig. 1C). It is possible that the protein accumulated from *GLD-2A(S)* and *GLD-2B* mRNAs during oogenesis obscures the newly synthesized protein produced from activation of *GLD-2A(L)* mRNA during oocyte maturation, as analyzed by Western blotting. Direct measurements of the relative contributions of *GLD-2A* and *GLD-2B* proteins to the total *GLD-2* in an oocyte have not been possible. The *GLD-2* polyadenylation circuit may be conserved in part because of its role in other tissues. The initial switch in the circuit may involve activation of latent *GLD-2* protein, perhaps produced from the *GLD-2A(S)* or *GLD-2B* isoforms. It is unclear why the 3′-UTR of *XIGLD-2B* is largely inactive, despite its high sequence similarity to that of *XIGLD-2A(L)*.

The positive feedback circuit may be reinforced at multiple levels to achieve a sustained and robust switch in polyadenylation activity. Many mRNAs that undergo cytoplasmic polyadenylation during oocyte maturation encode factors involved in protein phosphorylation cascades (e.g., protein kinases *Cdk-2*, *c-mos*, *Wee1*, *FGFR1*, and *Eg2*; and cyclins *A1*, *B1*, *B2*, and *B4*) (Sheets et al. 1994; for review, see Wormington 1994; Stebbins-Boaz et al. 1996; Charlesworth et al. 2004). Polyadenylation of *GLD-2* mRNA presumably serves as the upstream activator of the cascade, turning on the translation of these mRNAs, as well as its own. Interestingly, *GLD-2* protein activity is regulated in part by the targets it controls, as *Eg2*, whose mRNA is regulated by polyadenylation (Charlesworth et al. 2004), phosphorylates CPEB and thereby enhances polyadenylation (Mendez et al. 2000a,b). At synapses, *CaMKII* mRNA undergoes CPEB-protein-mediated polyadenylation (Wu et al. 1998) and *CaMKII* protein phosphorylates CPEB (Atkins et al. 2004). The robustness of the switch may be critical both in development and in the nervous system. In oocytes, once polyadenylation is activated, the system maintains that state and leads to polyadenylation of batteries of mRNAs. *GLD-2*, CPEB, and PUF proteins act in concert to establish that circuit.

MATERIALS AND METHODS

DNA constructs

pLG-CB1

pLG-MS2 (Gray et al. 2000) was digested with *SpeI* and *BglII*, in order to remove the MS2 sequence, blunt ended with Klenow

DNA polymerase (Promega), and re-ligated. This construct is used for transcription of the luciferase-*cyclinB1* 3'-UTR.

pLuci-XIGLD-2A(L)

The *XIGLD-2A(L)* 3'-UTR was amplified from IMAGE:6638921 using primers CAGCACTAGTACTAGTACTGGAATCTGCAGAGAAATAC and ACAGGATCCGGATCCGTACATACATTTTAT TCCATTCAACATCAT, digested with SpeI and BamHI, and ligated into SpeI- and BamHI-digested *pLG-MS2*. This construct is used for transcription of the luciferase-*XIGLD-2A(L)* 3'-UTR.

pLuci-XIGLD-2A(S)

The *XIGLD-2A(S)* 3'-UTR was amplified from IMAGE:6638921 using primers CAGCACTAGTACTAGTACTGGAATCTGCAGAGAAATAC and ACAGGATCCGGATCCGGTCAATATTTGTATTTAAAC, digested with SpeI and BamHI, and ligated into SpeI- and BamHI-digested *pLG-MS2*. This construct is used for transcription of the luciferase-*XIGLD-2A(S)* 3'-UTR.

pLuci-XIGLD-2B

The *XIGLD-2B* 3'-UTR was amplified from IMAGE:6643643 using primers CAGCACTAGTACTAGTACTGGAATCTGCAGAGAAATAC and ACAGGATCCGGATCCGGTACATACATTTTATTTCCATTCAACACC, digested with SpeI and BamHI, and ligated into SpeI- and BamHI-digested *pLG-MS2*. This construct is used for transcription of the luciferase-*XIGLD-2B* 3'-UTR.

pLuci-hGLD-2(L)

The *hGLD-2(L)* 3'-UTR was amplified from IMAGE:4824607 using primers CAGCACTAGTACTCTGGCCTCTATTTCTTAA TAAATTCCTCC and CGGATCCGGATCCGTCCATATATAAAT TTTATTGCATTTGACATC, digested with SpeI and BamHI, and ligated into SpeI- and BamHI-digested *pLG-MS2*. This construct is used for transcription of the luciferase-*hGLD-2(L)* 3'-UTR.

pLR9 series

The wild-type 3'-most 90-nt sequence from *XIGLD-2A(L)* (as well as mutations describe in Figs. 3A, 6A), were oligo-cloned into SpeI- and BamHI-digested *pLG-MS2*. This series of constructs is used for transcription of the luciferase last 90 nt of the *XIGLD-2A(L)* 3'-UTR (and mutations thereof).

pT7-LR9 series

The pT7-LR9 series is a series of clones based on the *pLR9* series, but with removal of most of the luciferase ORF, created by blunt-end ligation and cloning. This series of constructs is used for transcription of a mini-ORF with the last 90 nt of the *XIGLD-2A(L)* 3'-UTR (and mutations thereof; see Fig. 3A).

pJK350 (β -galactosidase)

This construct was previously described (Gray et al. 2000).

In vitro transcription

The *pLG-CB1*, *pLuci-XIGLD-2A(L)*, *pLuci-XIGLD-2A(S)*, *pLuci-XIGLD-2B*, *pLuci-hGLD-2(L)*, *pLR9 series*, and *pT7-LR9 series* constructs were cleaved with BamHI and transcribed with T7 RNA polymerase. Transcriptions were performed in the presence of 6 mM ^{m7}GpppG. In some cases, [α -³²P]UTP was incorporated during in vitro transcription as described (Dickson et al. 2001).

mRNA injection and manipulation of *Xenopus* oocytes

Oocyte removal, injection, and the induction of meiotic maturation were performed as previously described (Ballantyne et al. 1997). mRNAs were injected at either 2–3 fmol/50 nL, or 20–30 fmol/50 nL for the luciferase reporter mRNAs, along with 12 fmol/50 nL for the β -gal reporter mRNA.

Extraction and analysis of mRNAs from oocytes

Total RNA was extracted from frog tissues using 10 oocytes, or eggs, per 1 mL of TRI reagent (Sigma) following the manufacturer's protocol. After ethanol precipitation, the RNA was solubilized in 10 μ L of DEPC-treated water, and either used for the PCR-based poly(A)-length assay, or loaded to a 6% polyacrylamide gel (short ³²P-labeled RNAs) and analyzed by autoradiography.

Luciferase and β -galactosidase measuring assay

These assays were performed as described by Gray et al. (2000).

PCR-based poly(A)-length measuring assay

This technique was performed as described by Charlesworth et al. (2004). Briefly, 4 μ g of oocyte (or 2 μ g of brain) total RNA was ligated to 0.4 μ g of a 3'-amino-modified DNA anchor primer (Operon). The ligated products were then reverse-transcribed using a primer anti-sense to the anchor primer plus four thymidine residues on its 3'-end, using Superscript II (Invitrogen). Two microliters of this reaction were used as a template for gene-specific PCR, using the primers listed in Table 1, and the primer anti-sense to the anchor primer, used in the reverse-transcriptase reaction (unless specified differently). The annealing temperature was 56°C for all genes except for *CPSF-73*, for which the annealing temperature was 48°C. DNA products from the

TABLE 1. Oligonucleotides used in PCR-based poly(A)-length assay

gld2aL_2485	CAATTAAGGCACAATCTGGTGTCTTTG
gld2aS_post-stop SpeI	CAGCACTAGTACTAGTACTGGAATCTGCAGAGAAATAC
cycB1_1072	GGTCCCTGTTATGCAGCATATGGCC
L1_1033	GCTGAACCCATATGCAAGACCGC
cpeb_2975	CTTCCTAGTGCCTAAGCAG
maskin_2896	GGAGAAGATCTGACCCACTGAGGTGG
Xcat2_2661	GGACATTCCAGCTCTCAAGAGACGC
pabp1_2281	GTGCCAACTGCTTAAGATGCATGC
cpsf100_3084	TGCAGCCTCCCTCATAGGGG
cpsf73_1993	GAGGAAGGAATCGATGAGG
cpsf160_D	CTGTTACCCCAACATGCCGGACTCAACCCC
Luciferase polylinker	ACCTCTCTCTCTCTCAGGGCTGATTACTAG

brain *XIGLD-2A(L)* PCR-based poly(A)-length measuring assay, were end labeled with T4 Polynucleotide Kinase (Promega) and [γ - 32 P]ATP.

RNA coimmunoprecipitation

Coimmunoprecipitation and extraction of RNA with α -GLD-2 antibodies were performed as described previously (Rouhana et al. 2005). cDNA was made from total and coimmunoprecipitated oocyte RNA by oligo-coupled reverse transcription with Superscript II (Invitrogen). One-twentieth of the reverse-transcription reaction was used as a template for gene-specific PCR. Oligos GCAGGTTTGCCTTGAGAAAATGTCAC and GGAGGAAGCA GCTGCTATTTGGGAAGGCG were used as primers for *cyclin B1*; GCTGCCTATTTAATACTTATCCATGAG and CAAAGACAC CAGATTGTGCCTTAATTG for *XIGLD-2A(L)*; and CATTGA CCGCTCATCAGTTGTC and CAGAGCGACTTCAGAAAATCCA ACC for *Xfin*.

Electrophoretic mobility shift assays

GST-fused *Xenopus* Pumilio (amino acids 824–1185) was purified as described for GST-FBF-2 (Bernstein et al. 2005) and combined with 100 fMol of 32 P-end-labeled RNA oligoribonucleotides (Integrated DNA Technologies). The shift conditions and binding constants were calculated as described by Hook et al. (2005).

Kainic acid treatment and brain RNA extraction

Kainic acid was administered to males (15 mg/kg) via subcutaneous injection in the lymph sac. Sterile saline (pH 7.4) was administered as a negative control. Frogs were monitored for 45 min, made lethargic through hypothermia, and decapitated. Brain tissue was then dissected for cerebellum RNA extraction and analysis by PCR-based poly(A)-length assay.

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