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Poly(A) Polymerase and the Regulation of Cytoplasmic Polyadenylation*

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Translational activation in oocytes and embryos is often regulated via increases in poly(A) length. Cleavage and polyadenylation specificity factor (CPSF), cytoplasmic polyadenylation element binding protein (CPEB), and poly(A) polymerase (PAP) have each been implicated in cytoplasmic polyadenylation in *Xenopus laevis* oocytes. Cytoplasmic polyadenylation activity first appears in vertebrate oocytes during meiotic maturation. Data presented here shows that complexes containing both CPSF and CPEB are present in extracts of *X. laevis* oocytes prepared before or after meiotic maturation. Assessment of a variety of RNA sequences as polyadenylation substrates indicates that the sequence specificity of polyadenylation in egg extracts is comparable to that observed with highly purified mammalian CPSF and recombinant PAP. The two *in vitro* systems exhibit a sequence specificity that is similar, but not identical, to that observed *in vivo*, as assessed by injection of the same RNAs into the oocyte. These findings imply that CPSFs intrinsic RNA sequence preferences are sufficient to account for the specificity of cytoplasmic polyadenylation of some mRNAs. We discuss the hypothesis that CPSF is required for all polyadenylation reactions, but that the polyadenylation of some mRNAs may require additional factors such as CPEB. To test the consequences of PAP binding to mRNAs *in vivo*, PAP was tethered to a reporter mRNA in resting oocytes using MS2 coat protein. Tethered PAP catalyzed polyadenylation and stimulated translation ~40-fold; stimulation was exclusively *cis*-acting, but was independent of a CPE and AAUAAA. Both polyadenylation and translational stimulation required PAPs catalytic core, but did not require the putative CPSF interaction domain of PAP. These results demonstrate that premature recruitment of PAP can cause precocious polyadenylation and translational stimulation in the resting oocyte, and can be interpreted to suggest that the role of other factors is to deliver PAP to the mRNA.

During early embryogenesis in many species, transcription is quiescent, and changes in protein synthesis rely on post-transcriptional controls (1). In particular, cytoplasmic changes in the length of the poly(A) tail regulate translation of a number of mRNAs. Cytoplasmic polyadenylation is generally correlated with translational activation and deadenylation with translational repression (1–3). These changes affect diverse developmental processes, including pattern formation in the *Drosophila* embryo and control of the meiotic cell cycle (1, 3, 4). In yeast and somatic cells, poly(A) enhances translation at least in part through a tripartite protein-protein bridge, consisting of poly(A)-binding protein (PAB),[1] eIF-4G, and the mRNA cap-binding protein, eIF-4E (1, 5–7). In oocytes, PAB stimulates translation (8) and binds eIF-4G (8, 9), and eIF-4G is required for polyadenylation-dependent translation (8–10); these findings suggest that the effects of polyadenylation during early development exploit a mechanism similar to that in somatic cells.

Virtually all mRNAs receive poly(A) in the nucleus through two coupled mRNA processing reactions. Pre-mRNAs are first cleaved, then poly(A) is added to the new 3′ end (reviewed in Refs. 11–14). Nuclear poly(A) addition requires cleavage and polyadenylation specificity factor (CPSF), poly(A) polymerase (PAP), and a *cis*-acting sequence, AAUAAA. CPSF, a complex of four polypeptides (160, 100, 73, and 30 kDa), binds directly to AAUAAA (15–17) and PAP (18–20). The formation of this ternary complex causes the intrinsically nonspecific PAP to polyadenylate AAUAAA-containing RNAs preferentially. Prior to cleavage, binding of CPSF to pre-mRNAs is strengthened via interactions with a third factor, cleavage stimulatory factor, which binds to sequences downstream of the cleavage site, enhancing the sequence specificity of cleavage and polyadenylation (17, 21, 22).

Once mRNAs emerge from the nucleus, their tails can be lengthened or shortened. During early development, specific mRNAs are deadenylated rapidly, causing their repression (23); later, the same mRNAs receive poly(A) and become translationally active (reviewed in Refs. 1–3). This cytoplasmic polyadenylation reaction requires AAUAAA and a nearby U-rich element (cytoplasmic polyadenylation element, CPE) (24, 25). CPSF appears to be important in this reaction (26), since purified CPSF and PAP recapitulate CPE-dependent polyadenylation (27). These findings suggested that a cytoplasmic form of CPSF existed with a preference for CPE-containing mRNAs (27). Indeed, an unusual cytoplasmic form of CPSF, apparently lacking one of the subunits of nuclear CPSF, has since been identified (28).

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[1] The abbreviations used are: PAB, poly(A)-binding protein; CPSF, cleavage and polyadenylation specificity factor; eIF, eukaryotic initiation factor; PAP, poly(A) polymerase; CPE, cytoplasmic polyadenylation element; β-gal, β-galactosidase; PCR, polymerase chain reaction; UTR, untranslated region; CPEB, cytoplasmic element-binding protein.

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CPE-binding protein (CPEB) is also critical in cytoplasmic polyadenlyation (reviewed in Ref. 3). Depletion of CPEB reduces CPE/AAUAAA-specific polyadenylation *in vitro* (29, 30). Moreover, Eg-2-mediated phosphorylation of CPEB is sufficient to cause precocious activation of cytoplasmic polyadenylation in *Xenopus laevis* oocytes (31). However, as CPEB has been proposed to translationally repress mRNAs in oocytes (32–34), relief of this repression could indirectly contribute to the activation of polyadenylation.

Here, we demonstrate that cytoplasmic CPSF and CPEB interact both before and after meiotic maturation. These findings are consistent with the recent report of a CPSF·CPEB complex (35), but emphasize the presence of CPSF·CPEB complexes in resting oocytes. Systematic comparison of *in vitro* and *in vivo* polyadenylation efficiencies suggests that CPSF and PAP alone are sufficient to mimic *in vivo* cytoplasmic polyadenylation of many, but not all, mRNAs. Together with the findings of Mendez *et al.* (35), these data suggest that the polyadenylation of all mRNAs requires CPSF, but that polyadenylation of certain mRNAs also requires CPEB. Results presented here demonstrate that artificial tethering of PAP to an mRNA in the oocyte causes both polyadenylation and translational stimulation; stimulation does not appear to require an interaction with CPSF, a CPE, or the AAUAAA element. We discuss these findings in light of the dynamics of CPSF, CPEB, and PAP interactions in the cytoplasm.

EXPERIMENTAL PROCEDURES

All chemicals were supplied by Fisher Scientific, Pittsburgh, PA, unless noted otherwise.

Oocyte Manipulations

Oocyte removal, injection, and the induction of meiotic maturation were performed as previously described (36). RNAs were injected at 100 fmol/μl final concentration for L1-derived transcripts, 24 fmol/50 nl for the luciferase reporter mRNAs, 12 fmol/50 nl for the β-gal reporter mRNA, and 1 μg/μl for mRNAs used in fusion protein production.

DNA Constructs and in Vitro Transcription Reactions

pET15b-CPEB—X. laevis CPEB cDNA was PCR amplified from reverse transcribed DNA derived from *X. laevis* egg RNA. The PCR product was ligated into pGEM4Z (Promega) as an *Eco*RI-*Sal*I fragment (pGEM4Z-CPEB). An *Nde*I-*Sal*I fragment from pGEM4Z-CPEB was ligated into pET-15b (Novagen, Germany) from *Nde*I-*Xho*I (pET15b-CPEB).

L1 and L1+CPE Constructs—L1 and L1+CPE were previously described (27, 37). L1+CPE derivatives were created through site-directed mutagenesis to alter surrounding U-rich sequences to C. All mutants were sequenced for accuracy. Transcription reactions were performed as previously described (38).

MS2 Fusions—pET-MS2, pMS2-UIA, and pMS2-PAB were previously described (8). Bovine PAP was PCR amplified from pGM10-hisPAP (39). The PCR product was ligated into pET-MS2 from *Nhe*I-*Xho*I (MS2-PAP). The PAP mutants were created as follows. The D113A mutation was obtained by PCR from pGM10-hisPAPD113 (39). A single nucleotide was altered from A to C creating a new *Bam*HI site without changing the amino acid (amino acid 10). The D113A PCR fragment was ligated into pMS2-bPAP from *Nhe*I-*Bam*HI (MS2-D113A). The deletion of amino acids 488–739 was achieved by substituting inserting an *Afl*II-*Bam*HI fragment from pGM10-hisPAPΔ488 (39) into pMS2-bPAP (MS2-Δ488). All MS2 fusion protein plasmids were linearized with *Hin*dIII and transcribed with T7 RNA polymerase. Radiolabeled luciferase mRNA was synthesized by the addition of [α-^{32}P]UTP to the transcription reaction.

pLG-MS2 and pJK350 (β-Galactosidase—These constructs were previously described (8).

In Vitro Polyadenylation Assays

X. laevis egg extracts were prepared, and *in vitro* polyadenylation reactions performed, as described previously (28, 37). CPSF was highly purified from calf thymus (40) and recombinant calf thymus PAP was purified from *Escherichia coli* (41).

Antibody Preparation

α-CPEB antibodies were generated by *E. coli* overexpression of full-length CPEB from pET15b-CPEB. His-CPEB antigen was purified over Ni-NTA-agarose (Qiagen, Germany). Rabbit polyclonal antibodies (McArdle antibody service, University of Wisconsin, Madison, WI) were affinity purified. α-CPSF antibodies have been previously described (15).

Coimmunoprecipitation Assays/His-tag Affinity Purification

Co-immunoprecipitations—Oocytes were homogenized in 100 μl/oocyte medium salt buffer (150 mM NaCl, 1% Igepal CA-630, and 50 mM Tris-Cl, pH 8) containing a protease inhibitor mixture (Roche Molecular Biochemicals). The homogenate was centrifuged at 4 °C for 10 min at 3000 rpm and the clear lysate was collected. Lysate from 10 to 15 oocytes was used per immunoprecipitation. Lysate was incubated for 1 h at 4 °C with 30 μl of α-CPEB antibody, preimmune serum or monoclonal α-CPSF^{100} antibodies. 50 μl of a 1:1 Protein A-Sepharose: medium salt buffer slurry was then added. This mixture was incubated at 4 °C for 1/2–2 h then centrifuged at 4 °C for 10 min at 3000 rpm. The precipitate was washed 2 times with medium salt buffer and precipitated proteins were examined by Western blotting (42).

His-tag Purification of MS2 Fusion Proteins—Oocytes were injected with mRNAs encoding MS2 fusion proteins and then [^{35}S]Met labeled as previously described (8). Cytosolic proteins were isolated over Ni-NTA-agarose (Qiagen) in N-buffer (43), washed in N-buffer containing 20 then 50 mM imidazole, and eluted in N-buffer containing 500 mM imidazole. Eluted proteins were visualized by fluorography.

Tethered Function, Luciferase, and β-Galactosidase Assays and in Vivo Labeling of Oocytes

These assays were performed as previously described (8).

Poly(A) Selection Using an Oligo(dT) Column

For stability and oligo(dT) selection, [α-^{32}P]UTP was incorporated into *in vitro* transcribed luciferase mRNA as previously described (8). After injection, RNA was extracted from oocytes as previously described (28). The precipitated RNA was resuspended in 800 μl of column buffer (0.5 M NaCl, 0.2 M Tris, pH 7.5, 10 mM EDTA, and 0.1% SDS) then boiled for 90 s, quick cooled on ice, and passed over a column packed with oligo(dT) type 7 cellulose (Amersham Pharmacia Biotech). Preheated, then cooled RNA was passed over the column five times and then poly(A)$^-$ flow-through RNA was collected. The column was washed 5 times with 400 μl of column buffer and poly(A)$^+$ RNA was eluted with 800 μl of 65 °C, diethyl pyrocarbonate-treated water. RNA was separated on a denaturing formaldehyde-agarose gel and examined by autoradiography.

RESULTS

Cytoplasmic CPSF^{100} Interacts with CPEB before and after Meiotic Maturation—To determine whether CPEB and CPSF interact *in vivo*, co-immunoprecipitation assays were performed using extracts of *X. laevis* oocytes. Immunoprecipitation of *X. laevis* CPEB, using α-CPEB antibodies, resulted in the co-precipitation of CPSF^{100} from both oocytes and matured oocytes (Fig. 1*A*, *lanes 1* and *2*). The quantity of cytoplasmic CPSF^{100} precipitated was comparable from oocytes and matured oocytes (Fig. 1*A*, compare *lanes 1* and *2*). The interaction was specific in that it did not occur in extracts precipitated with preimmune serum (Fig. 1*A*, *lanes 3* and *4*). The polyclonal α-CPEB antibodies used in this study specifically recognize CPEB from both *X. laevis* oocytes and matured oocytes (data not shown). Polyclonal α-CPSF^{100} antibodies have been previously shown to recognize two proteins by Western blotting: CPSF^{100}, which is present only in the cytoplasm, and a 96-kDa CPSF-like protein that is present in both the cytoplasm and the nucleus; only CPSF^{100} is recognized by immunoprecipitation with α-CPSF^{100} antibodies (28). Results here indicate that α-CPEB antibodies but not the 96-kDa protein interact with CPSF^{100} (Fig. 1*A*, compare *lanes 1* and *2* with *5* and *6*). The decrease in the quantity of CPSF^{100} obtained after maturation was not reproducible (Fig. 1*A*, *lanes 5* and *6*).

Both CPSF and CPEB bind CPE-containing RNAs (27–30, 44), and co-precipitation of CPSF^{100} and CPEB might therefore

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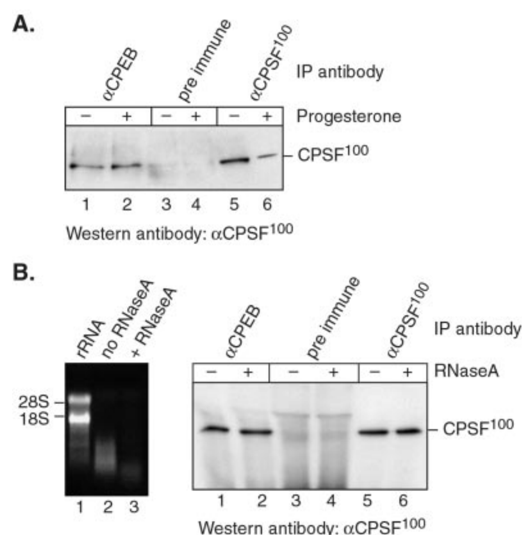

FIG. 1. **Co-immunoprecipitation of $CPSF^{100}$ with CPEB.** *A,* immunoprecipitations were performed on *X. laevis* oocyte extracts (−*progesterone*) or matured oocyte extracts (+*progesterone*) using α-CPEB antibodies (*lanes 1* and *2*), rabbit preimmune serum (*lanes 3* and *4*), or α-$CPSF^{100}$ antibodies (*lanes 5* and *6*). Proteins were detected by Western blot analysis using α-$CPSF^{100}$ antibodies. *B,* immunoprecipitations were performed on *X. laevis* oocyte extracts as in *A* except that the extracts were split in half and either treated (+*RNase A*) or not treated (−*RNase A*) with RNase A. RNA was extracted from the equivalent of one oocyte worth of untreated (*lane 2*) or RNase A-treated (*lane 3*) extract and total RNA was extracted from an intact oocyte as a control (*lane 1*). Ribosomal RNA was then detected by ethidium bromide staining (*left panel*). The remainder of the extracts were used for co-immunoprecipitation as in *A* and proteins were detected by Western blot analysis using α-$CPSF^{100}$ antibodies (*right panel*).

reflect an RNA bridging interaction. To examine this possibility, crude extracts were treated with RNase A prior to immunoprecipitation. The efficiency of RNase A treatment was monitored by examining ribosomal RNA (rRNA), an abundant and relatively nuclease-resistant RNA species. In addition, rRNA represents at least 90% of the total RNA in the oocyte (45) and is bound by protein complexes; degradation of rRNA was therefore taken as indication that all cellular mRNA had been eliminated. Total RNA was extracted directly from an intact oocyte to control for the normal levels of rRNA (Fig. 1*B*, *left panel*, *lane 1*). Without RNase A treatment, rRNA was partially degraded during incubation, presumably due to endogenous ribonucleases (Fig. 1*B, left panel*, *lane 2*). After RNase A treatment, rRNA was nearly undetectable (Fig. 1*B*, *left panel*, *lane 3*), suggesting that the majority of RNA in the extract had been degraded. RNase A-treated and -untreated extracts were then incubated with α-CPEB antibodies or preimmune serum. RNase A treatment did not detectably alter the quantity of cytoplasmic $CPSF^{100}$ co-precipitated with CPEB (Fig. 1*B, right*, compare *lane 2* to *1*). Combined, these results suggest that a protein/protein interaction occurs between cytoplasmic CPSF and CPEB in both the oocyte and the matured oocyte.

RNA Sequence Specificity of Purified CPSF and PAP Mirrors that in X. laevis Egg Extracts, and Overlaps with That Observed in Vivo—Polyadenylation by CPSF and PAP is enhanced by insertion of UUUUUAU, a canonical CPE, near AAUAAA (27). However, it is unclear whether the sequence specificity of polyadenylation by CPSF and PAP directly parallels that seen in egg extracts or *in vivo*, instances where CPEB is present. In light of this, the behavior of a range of related RNA substrates were compared using three different approaches: *in vitro* polyadenylation using purified CPSF and PAP, *in vitro* polyadenylation using unfractionated *X. laevis* egg extract, and *in vivo* polyadenylation of injected of RNAs into oocytes.

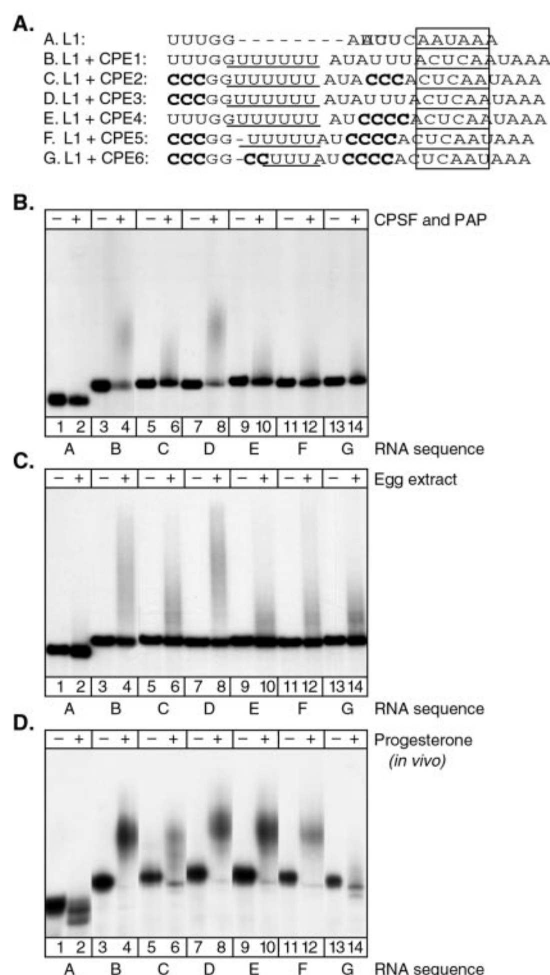

FIG. 2. **Polyadenylation of multiple RNA substrates.** *A,* CPE and surrounding sequences of the RNAs used are shown. CPEs are *underlined* and the AAUAAA is *boxed*. Nucleotides altered from L1 +CPE1 are in *boldface*. *B,* [α-^{32}P]UTP radiolabeled RNAs were used to perform polyadenylation reactions *in vitro* using purified CPSF (7.5 units) and PAP (100 units) (+ *lanes*). − *lanes* are without purified CPSF and PAP added. *C,* polyadenylation reactions were performed on radiolabeled RNAs *in vitro* using unfractionated *X. laevis* egg extract (+ *lanes*). − *Lanes* are without extract. *D,* polyadenylation reactions were performed *in vivo* by injection of radiolabeled RNAs. Untreated oocytes are in *odd lanes* (−*progesterone*) while oocytes exposed to progesterone to induce maturation are in *even lanes* (+*progesterone*).

Seven different RNAs were examined (Fig. 2*A*). The 3′-UTR of L1 mRNA (L1, RNA "A") served as a negative control; it lacks a CPE and does not support cytoplasmic polyadenylation (37, 46). L1+CPE RNA (RNA "B") has a canonical (UUUUUUAU) CPE; all other RNAs vary from L1+CPE only near the CPE (Fig. 2*A*). The variant RNA sequences alter the context or identity of the CPE, but were not designed to copy the 3′-UTRs of specific, natural mRNAs. Polyadenylation of each RNA was examined *in vitro,* either in the presence or absence of purified calf thymus CPSF and recombinant PAP (Fig. 2*B*), or in unfractionated *X. laevis* egg extracts (Fig. 2*C*). The relative behavior of the RNAs was very similar in these two assays, although the efficiency of polyadenylation of all RNAs was higher in the extract.

The same RNAs were then examined *in vivo* by injection into *X. laevis* oocytes. Oocytes were either left untreated, or treated with progesterone to induce meiotic maturation and activate polyadenlyation (Fig. 2*D*). All substrates polyadenylated more efficiently *in vivo* than *in vitro*; however, it was apparent that substrates "A," "B," "C," "D," and "G" behaved similarly in all three assays. In contrast, substrates "E" and "F" were more

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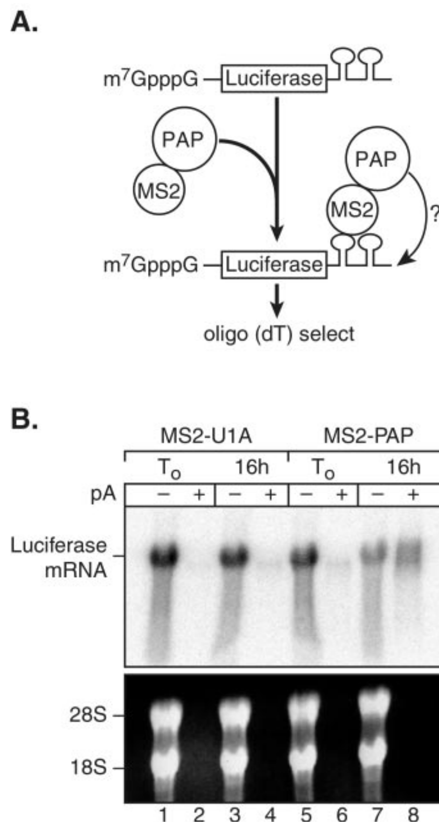

FIG. 3. **Tethered PAP adds poly(A) *in vivo.*** *A,* a depiction of the assay is shown. *B,* [α-^{32}P]UTP radiolabeled luciferase mRNA was injected into oocytes expressing either MS2-U1A or MS2-PAP as indicated. Oocytes were either collected immediately after luciferase mRNA injection (T_0) or after a 16-h incubation at 18 °C (16 h). Total RNA was extracted from the oocytes and passed over an oligo(dT) column. RNA that did not bind the oligo(dT) resin is indicated as pA$^-$ (*odd lanes*). RNA that bound the oligo(dT) resin is indicated as pA$^+$ (*even lanes*). Luciferase RNA was detected by autoradiography (*top panel*). Ribosomal RNAs were examined by ethidium bromide staining (*bottom panel*).

efficiently polyadenylated *in vivo* than either *in vitro* assay; this could reflect either a higher concentration of active CPSF and/or PAP *in vivo* or reflect concentration differences in other components (*e.g.* CPEB) that are either missing entirely (Fig. 2*B*) or are present at greatly reduced concentrations (Fig. 2*C*) in the cell-free systems (see "Discussion").

Tethered PAP Polyadenylates mRNAs in Vivo—Data presented thus far suggest that CPSF and PAP alone can exhibit a polyadenylation activity that mirrors that seen during meiotic maturation. However, both CPSF and PAP are present in *X. laevis* oocytes prior to maturation, yet CPE-dependent polyadenylation does not occur at that stage. Moreover, PAP purified from oocytes is enzymatically active (37). These results suggest two nonexclusive possibilities: PAP may be prevented from interacting with substrate mRNAs in the oocyte, or PAP activity may normally be repressed in the oocyte via a repressor that is removed during fractionation. If binding of PAP to mRNA was the limiting step in activation of polyadenylation *in vivo*, then tethering PAP artificially to the 3′-UTR of an mRNA should cause precocious polyadenylation.

PAP was tethered to an mRNA *in vivo* by creation of a chimeric protein in which MS2 coat protein was fused to the amino terminus of bovine PAP (MS2-PAP) (depicted in Fig. 3*A*). *X. laevis* oocytes were injected with mRNAs encoding either this protein, or a control fusion between MS2 coat protein and U1A (MS2-U1A) (8). An [α-^{32}P]UTP radiolabeled luciferase reporter mRNA containing MS2-binding sites in its 3′-UTR (Luc-MS2) (8), was then injected into *X. laevis* oocytes expressing either MS2-PAP or MS2-U1A. The 3′-UTR of Luc-MS2 mRNA lacked both potential CPEs (oligo(U) tracts) and the AAUAAA sequence. Poly(A)$^+$ mRNA (+) was separated from poly(A)$^-$ (−) mRNA by oligo(dT) chromatography. Luciferase mRNA isolated from MS2-U1A expressing oocytes was not retained on the oligo(dT) cellulose column (Fig. 3*B*, *lane 4 versus 3* and *2 versus 1*). In contrast, RNA isolated from MS2-PAP expressing oocytes was retained: approximately 50% of the RNA bound after incubation in the oocytes, while less than 5% bound at the start of the experiment (Fig. 3*B*, *lane 8 versus 7* and *6 versus 5*). These results suggest that the lack of endogenous cytoplasmic polyadenylation activity in resting oocytes may be due to the inability of PAP to associate with mRNAs. Furthermore, once brought to the mRNA, polyadenylation by PAP does not require a CPE or AAUAAA sequence.

Tethered PAP Stimulates Translation—Upon injection into oocytes, mRNAs with a poly(A) tail show increased translation over mRNAs without a poly(A) tail (47). To examine whether tethered PAP stimulated translation, luciferase activity was assayed in MS2-PAP expressing cells. Oocytes were first injected with mRNAs encoding MS2-PAP, MS2-XlPAB (a fusion containing *X. laevis* PAB (8)), or MS2-yPAB (a fusion containing yeast PAB (8)) to allow expression of these fusion proteins. Two mRNAs were then co-injected to assay translational activity: Luc-MS2, which carries MS2 sites, and β-gal mRNA, which lacks them (Fig. 4*A*); the β-gal reporter encodes β-galactosidase and is used as an internal control (8). To control for the specificity of the effects of the fusion proteins, MS2-U1A was included as its effects on the translation of Luc-MS2 in oocytes are negligible (8).

MS2-PAP stimulated expression of luciferase 42-fold (Fig. 4*B*). This level of stimulation was significantly greater than that of MS2-XlPAB, but less than that of MS2-yPAB. The effect on Luc-MS2 translation was specific in that MS2-PAP did not significantly stimulate a luciferase reporter lacking MS2 sites (Luc-ΔMS2 (8)) (Fig. 4*C*). Furthermore, the increase in luciferase activity was due to enhanced translation, not mRNA stabilization, as Luc-MS2 mRNA was not stabilized in MS2-PAP *versus* MS2-U1A-expressing oocytes (Fig. 4*D*). Combined, the data indicate that tethered PAP stimulates translation efficiently.

Translational Activation by MS2-PAP Does Not Require Interaction with CPSF, But Does Require the Catalytic Core of PAP—In principle, activation by PAP could be entirely a consequence of poly(A) addition, or could require recruitment of other factors to the mRNA to facilitate poly(A) addition. CPSF is the most obvious factor; while an interaction between cytoplasmic CPSF and PAP has not been demonstrated, nuclear CPSF and PAP are known to interact (18–20). To examine whether CPSF binding was required for full PAP catalytic activity, MS2 fusions were created with two different PAP mutant enzymes (Fig. 5*A*). MS2-Δ488 has the C terminus of PAP deleted from amino acid 488; this deletion eliminates the region thought to be responsible for interaction with nuclear CPSF (48), but has been previously shown to not appreciably effect polymerase function *in vitro* (39, 48, 49). MS2-D113A contains an alteration of one of the catalytic aspartic acid residues (amino acid 113) to an alanine; this mutation reduces the catalytic activity of PAP to less than 1% of wild-type (39, 49).

Deletion of the CPSF-interaction domain (MS2-Δ488) did not prevent translational stimulation of Luc-MS2 (Fig. 5*B*). However, mutation of the catalytic core of PAP (MS2-D113A) reduced the ability of this protein to stimulate translation by at least 25-fold (Fig. 5*B*). The increased stimulation by MS2-Δ488

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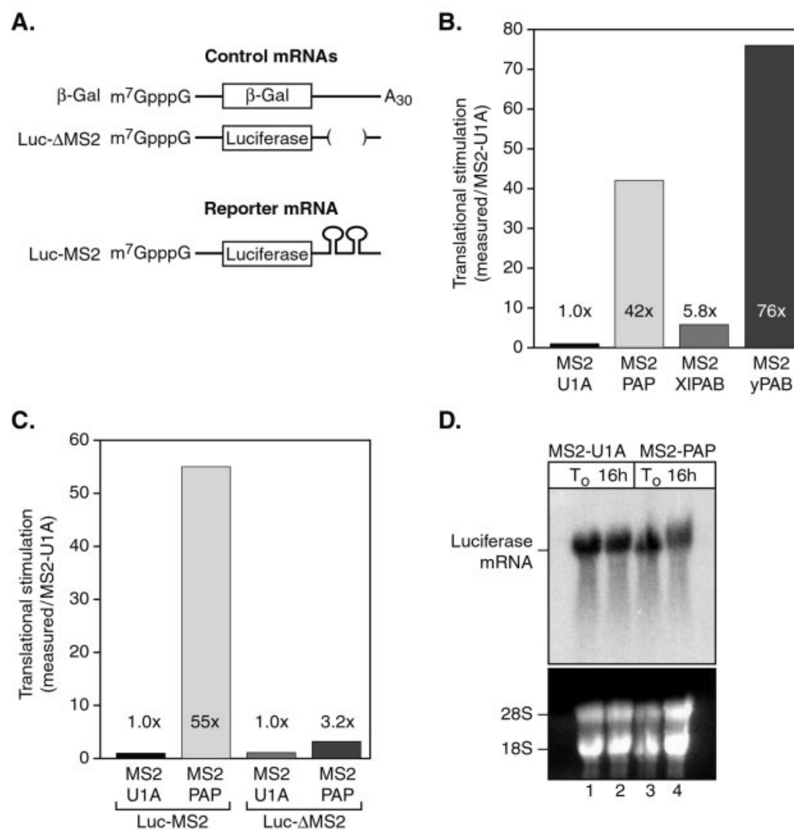

FIG. 4. **Tethered PAP stimulates translation in *cis*.** *A,* reporter mRNAs used in this study are indicated. *B,* MS2 fusion proteins between U1A (MS2-U1A, a negative control), bovine PAP (MS2-PAP), *X. laevis* PAB (MS2-XlPAB, a positive control), and yeast PAB (MS2-yPAB, a positive control) were expressed in oocytes as indicated. Luc-MS2 and β-gal reporter mRNAs were co-injected into these oocytes as a mixture. Translational activity was measured by luciferase and β-galactosidase assays. Luciferase activity (normalized for differences in β-gal activity) is shown as activity over that measured in oocytes expressing MS2-U1A. *C,* assays were performed as in *B* except that in some instances a luciferase mRNA lacking MS2-binding sites (Luc-ΔMS2) was used to control for the specificity of stimulation seen by MS2-PAP. The luciferase activity reported is relative to that calculated for MS2-U1A in each case. *D,* to examine luciferase mRNA stability, total RNA was extracted from oocytes expressing either MS2-U1A or MS2-PAP (as indicated) immediately after injection of the luciferase mRNA (T_0) or after a 16-h incubation at 18 °C (16 h). Luciferase mRNA was examined by autoradiography (*top panel*). Ribosomal RNA was examined by ethidium bromide staining to control for RNA loading (*bottom panel*).

may simply reflect accumulation of this protein in the cytoplasm, since the deletion eliminates two nuclear localization signals (48). The extent of translational stimulation reflected the ability of PAP mutants to polyadenylate the reporter mRNA: MS2-Δ488, but not D113A, resulted in the polyadenylation of approximately half of the Luc-MS2 reporter mRNA molecules, as judged by oligo(dT) chromatography (Fig. 5*C*). Furthermore, each fusion protein was expressed at similar levels, as assessed by [^{35}S]methionine labeling of oocytes and subsequent His-tag purification of the MS2 fusion proteins (Fig. 5*D*). Thus differences in translational stimulation reflect differences in protein activity rather than abundance.

DISCUSSION

Data presented here support three main findings. First, CPSF and CPEB interact in an RNA-independent fashion, both before and after meiotic maturation. Second, the sequence specificity of polyadenylation by purified CPSF and recombinant PAP is similar to that observed in egg extracts and *in vivo*; however, polyadenylation *in vivo* is more robust than in either *in vitro* system, and differs subtly in sequence requirements. These findings suggest that CPSF and PAP are sufficient to promote the polyadenylation of some, but not all, mRNAs *in vivo*. Third, tethered PAP causes both polyadenylation and translational stimulation. The activity of tethered PAP operates only in *cis*, but is independent of the AAUAAA and CPE sequences. Both polyadenylation and translational stimulation require the catalytic center of PAP, but not the region of PAP predicted to interact with CPSF. These results suggest that recruitment of PAP to an mRNA is sufficient to induce cytoplasmic polyadenylation and translation in resting oocytes.

The CPSF/CPEB Interaction—The CPSF/CPEB interaction occurs both before and after meiotic maturation. Our data do not indicate a dramatic alteration in the amount of CPSF·CPEB complex during this time (Fig. 1). Another recent study also demonstrated a CPSF/CPEB interaction in the oocyte, and suggested that the amount of complex increases 4-fold after meiotic maturation due to phosphorylation of CPEB (35). The source of the quantitative difference between our findings is unclear, but could reflect the preparation of extracts for immunoprecipitation analyses; the extracts used here are simply clarified homogenates, while those used in the other work involved additional preparative steps. Although the quantity of complex could itself be an important variable in activating polyadenylation, as emphasized by Mendez *et al.* (35), both analyses clearly show that unphosphorylated CPEB can interact with CPSF. In principle, binding of CPEB to CPSF in oocytes could keep CPSF inactive; if so, inhibition is likely to require additional components as unphosphorylated CPEB does not inhibit CPSF *in vitro* (35). The CPSF·CPEB complex in oocytes could also participate in CPE-dependent translational repression, in addition to any subsequent effects on polyadenylation.

PAP, CPSF, and CPEB—PAPs, purified from oocytes before or after meiotic maturation, are equally active (37), yet polyadenylation activity is not seen *in vivo* until maturation is induced. This lack of polyadenylation activity may simply be a result of the inability of PAP to bind substrate mRNAs in the oocyte. Alternatively, endogenous PAP could be repressed in the oocyte, in a manner that is lost during fractionation. Our results support the first possibility, as PAP, brought to the mRNA artificially, adds a poly(A) tail and increases translation of the mRNA in the oocyte. However, the hypothesis that endogenous PAP is bound to mRNAs in the oocyte, but repressed, cannot be formally excluded as our studies used mammalian PAP. Oocytes possess a cytoplasmic PAP nearly identical to the mammalian PAP used here (50), as well as a shortened form equivalent to the C terminus of bovine PAP used here (Fig. 5, Δ488 form) (51). The polyadenylation activity of tethered PAP is independent of CPEs or AAUAAA in the substrate, and does not require the CPSF interaction domain of PAP. Thus the

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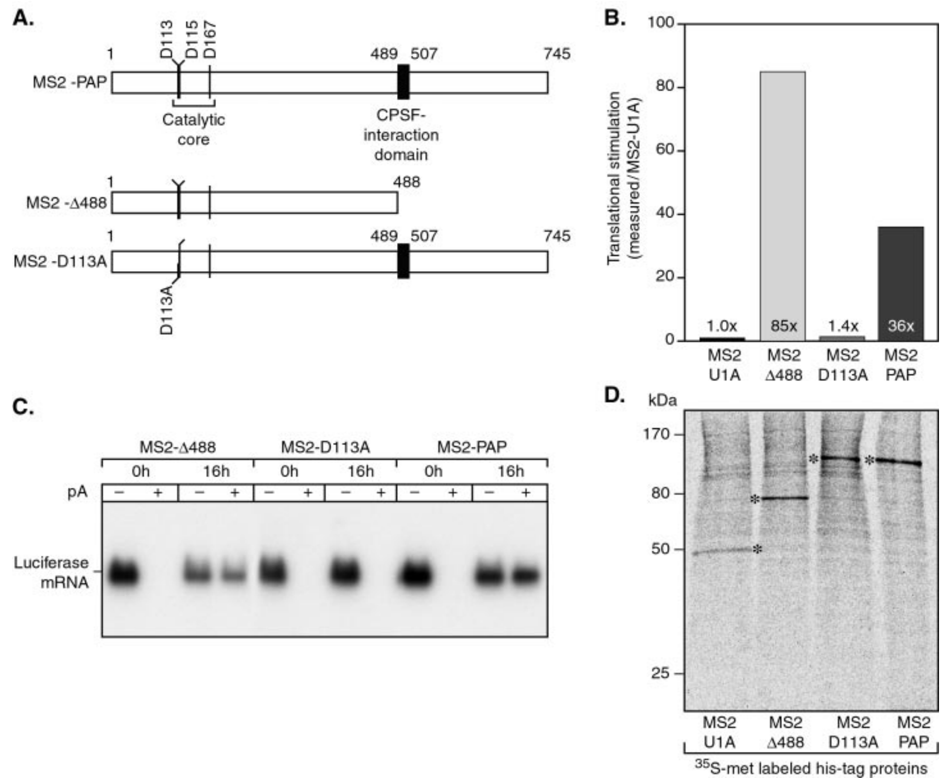

FIG. 5. **Stimulation by MS2-PAP requires the catalytic center of the enzyme, but not the CPSF interaction domain.** *A,* depiction of the MS2-PAP fusion proteins used in this study. *B,* assays were performed as in Fig. 4*B*. Oocytes expressing MS2-U1A, MS2-PAP, or MS2 constructs containing mutations in PAP (MS2-Δ488 or MS2-D113A as indicated) were injected with Luc-MS2 and β-galactosidase reporter mRNAs. Luciferase and β-galactosidase activities were measured as in Fig. 4*B*. Translational activities are all relative to that calculated for oocytes expressing MS2-U1A. *C,* [α-^{32}P]UTP radiolabeled luciferase mRNA was injected into oocytes expressing the protein indicated. Oocytes were either collected immediately after luciferase mRNA injection (0 h) or after a 16-h incubation at 18 °C (16 h). RNA was extracted from the oocytes and passed over an oligo(dT) column. RNA that did not bind the oligo(dT) resin is indicated as pA$^-$; RNA that bound the oligo(dT) resin is indicated as pA$^+$. Luciferase RNA was detected by autoradiography. *D,* after injection of mRNAs encoding MS2-fusion proteins (as indicated), oocytes were incubated in [^{35}S]methionine to permit incorporation of radiolabel into newly synthesized proteins. Whole cell extracts were passed over Ni-NTA resin and the bound proteins eluted, separated by SDS-polyacrylamide gel electrophoresis, and examined by fluorography. The MS2-fusion proteins are indicated by *asterisks* (*).

specific RNA sequences and CPSF may normally function to recruit PAP to specific mRNAs. This hypothesis is consistent with PAP's lack of intrinsic sequence specificity (11) and with the absence of a CPSF-like RNA binding activity in resting oocytes (27, 37). CPEB, by augmenting CPSF binding to certain mRNAs (35), would enhance PAP recruitment. In this respect, CPEB and cleavage stimulatory factor may serve analogous functions (26, 35).

Tethered PAP adds poly(A) in a sequence-independent fashion. In the oocyte, this results in a dramatic increase in translational activity. In somatic cells, a tethered cytoplasmic PAP might be expected to compensate for deadenylation, and thereby stabilize mRNAs that are degraded through the deadenylation dependent decay pathway, and increase the translational efficiency of bound mRNAs.

The sequence specificity of polyadenylation by highly purified CPSF and PAP mimics that in egg extracts (Fig. 2, *B* and *C*). *In vivo*, the reaction is more efficient with all substrates (Fig. 2*D*). In addition, two RNA substrates (*E* and *F* in Fig. 2*D*) are disproportionately active *in vivo*. These data suggest CPSF and PAP are sufficient to account for some, but not all, of the sequence specificity of polyadenylation *in vivo*. CPEB was recently shown to enhance the activity of purified PAP and CPSF (35), and can cause precocious polyadenylation in its phosphorylated form (31). This could underlie the global difference in polyadenylation efficiency observed *in vivo versus* in egg extracts, as egg extracts contain relatively little CPEB (29).

CPSF and PAP alone promote CPE-dependent polyadenylation (this report), yet CPEB can promote this reaction as well (31). These findings prompt the hypothesis that mRNAs differ in their requirements for polyadenylation: all mRNAs may require CPSF, but a subset may also require CPEB. What might distinguish these mRNAs from one another? We consider two possibilities. The first concerns the dependence of polyadenylation on MPF activity. mRNAs that receive poly(A) during maturation can be separated into two classes: those whose polyadenylation requires MPF activity (Class II), and those that do not (Class I) (36, 52). mRNAs whose polyadenylation is MPF-independent can be polyadenylated earlier in maturation, prior to nuclear breakdown. The precise sequence of the CPE largely determines whether an mRNA is Class I or II (36, 52). In one hypothesis, phosphorylated CPEB, via interactions with CPSF, promotes polyadenylation of one class of mRNAs; CPSF, alone or in conjunction with novel factors, is responsible for the other. The findings that phosphorylation of CPEB can cause polyadenylation of a Class I mRNAs *in vivo* (c-*mos* (31)) and stimulate polyadenylation of Class II mRNAs *in vitro* (35), appears to argue against this model. A second hypothesis suggests that the subcellular distribution of the mRNAs is critical. After maturation and during early cleavage, CPEB is largely associated with the mitotic spindle, while CPSF is more disperse (53). Thus CPSF might act on certain mRNAs on its own, while being recruited to those at the spindle by CPEB.

Both CPSF and CPEB show preference for CPEs (27–30, 37, 44). CPEs themselves have dual roles, promoting both repression and subsequent activation (24, 25, 32, 33, 54, 55), and CPEB has been suggested to mediate both of these effects (33, 34, 54). It is possible that the character of the unphosphorylated CPEB·CPSF complex in resting oocytes is critical for repression, or for keeping CPSF silent. Once maturation begins, variations among 3′-UTR sequences may result in differential recruitment of CPSF and CPEB to specific mRNAs. Regardless, our results demonstrate that recruitment of PAP to an mRNA is sufficient to cause precocious polyadenylation and translational stimulation, in the absence of any additional specific sequence information. The simplest interpretation of these findings is that the consummation of CPE-dependent activation is recruitment of PAP. Dissecting the dynamics of the interactions among CPSF, CPEB, and PAP *in vivo* therefore is a critical challenge.

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