

The Cleavage and Polyadenylation Specificity Factor in *Xenopus laevis* Oocytes Is a Cytoplasmic Factor Involved in Regulated Polyadenylation

KIRSTEN S. DICKSON, ANDREA BILGER,[†] SCOTT BALLANTYNE, AND MARVIN P. WICKENS*

Department of Biochemistry, College of Agricultural and Life Sciences,
University of Wisconsin, Madison, Wisconsin 53706

Received 19 March 1999/Returned for modification 23 April 1999/Accepted 7 May 1999

During early development, specific mRNAs receive poly(A) in the cytoplasm. This cytoplasmic polyadenylation reaction correlates with, and in some cases causes, translational stimulation. Previously, it was suggested that a factor similar to the multisubunit nuclear cleavage and polyadenylation specificity factor (CPSF) played a role in cytoplasmic polyadenylation. A cDNA encoding a cytoplasmic form of the 100-kDa subunit of *Xenopus laevis* CPSF has now been isolated. The protein product is 91% identical at the amino acid sequence level to nuclear CPSF isolated from *Bos taurus* thymus. This report provides three lines of evidence that implicate the *X. laevis* homologue of the 100-kDa subunit of CPSF in the cytoplasmic polyadenylation reaction. First, the protein is predominantly localized to the cytoplasm of *X. laevis* oocytes. Second, the 100-kDa subunit of *X. laevis* CPSF forms a specific complex with RNAs that contain both a cytoplasmic polyadenylation element (CPE) and the polyadenylation element AAUAAA. Third, immunodepletion of the 100-kDa subunit of *X. laevis* CPSF reduces CPE-specific polyadenylation *in vitro*. Further support for a cytoplasmic form of CPSF comes from evidence that a putative homologue of the 30-kDa subunit of nuclear CPSF is also localized to the cytoplasm of *X. laevis* oocytes. Overexpression of influenza virus NS1 protein, which inhibits nuclear polyadenylation through an interaction with the 30-kDa subunit of nuclear CPSF, prevents cytoplasmic polyadenylation, suggesting that the cytoplasmic *X. laevis* form of the 30-kDa subunit of CPSF is involved in this reaction. Together, these results indicate that a distinct, cytoplasmic form of CPSF is an integral component of the cytoplasmic polyadenylation machinery.

The dynamic length changes that occur on mRNA 3' poly(A) tails in eukaryotes often lead to regulation of mRNA function. Decreases in length are commonly associated with translational repression, while increases often accompany translational activation (16, 38, 51). Changes in poly(A) tail length also appear to influence mRNA stability, with removal of poly(A) to below a certain length often triggering mRNA decay (5). A variety of sequences within the 3' untranslated region (UTR) of mRNAs have been shown to regulate the rates of both poly(A) addition and removal (51), thereby influencing both translation and mRNA stability. The detailed molecular mechanisms that underlie these alterations in poly(A) tail length are unclear.

Regulated changes in poly(A) length occur throughout development and have been examined in detail in oocytes and early embryos. In the female germline of many species, cytoplasmic polyadenylation first occurs at, or shortly before, fertilization. In *Xenopus laevis*, cytoplasmic polyadenylation is activated during meiotic maturation when oocytes, arrested at first meiosis, proceed through second meiosis prior to ovulation. The ability to induce meiotic maturation in culture with the hormone progesterone allows for biochemical analysis of both the elements and factors involved in cytoplasmic polyadenylation. Only those mRNAs that contain a cytoplasmic polyadenylation element (CPE) and the polyadenylation sequence

AAUAAA within their 3' UTRs undergo cytoplasmic polyadenylation (13, 28, 33). CPE-binding protein (CPEB) binds preferentially to CPE-containing RNAs and appears to be a positive-acting polyadenylation factor (17, 34, 41); immunodepletion of CPEB blocks polyadenylation activity *in vitro* (17, 41), and injection of anti-CPEB antibodies reduces polyadenylation *in vivo* (41). In addition, the cytoplasmic polyadenylation reaction requires a poly(A) polymerase (PAP) (1, 12, 15), which adds successive AMP residues to the 3' end of the mRNA.

In this report we focus on the involvement of a third component, cleavage and polyadenylation specificity factor (CPSF), in cytoplasmic polyadenylation. CPSF was first characterized based on its role in nuclear cleavage and polyadenylation (21, 27, 49). In the nucleus, both the cleavage and subsequent poly(A) addition reactions require the sequence AAUAAA, typically located 10 to 30 nucleotides (nt) 5' of the polyadenylation site (10, 47, 48). CPSF binds directly to this sequence and is required for both reactions (22, 29). In mammalian somatic cells, purified CPSF consists of four subunits, with molecular masses of 160, 100, 73, and 30 kDa (6, 18, 29). The 160-kDa subunit interacts with the AAUAAA sequence and nuclear PAP (19, 22, 29, 30). This interaction brings PAP, which has little or no intrinsic specificity for RNA (43, 46), to the substrate mRNA. The 30-kDa subunit of CPSF may also bind to the AAUAAA sequence (18). In addition, the 30-kDa subunit binds preferentially to U-rich sequences (3, 22). Proteins related in sequence to each CPSF subunit have been identified in *Saccharomyces cerevisiae* (8, 20, 23, 35, 42, 53) and are essential for 3' end processing in that organism.

While some mechanistic similarities are evident, cytoplasmic and nuclear polyadenylation are distinct biologically. Both re-

* Corresponding author. Mailing address: Department of Biochemistry, College of Agricultural and Life Sciences, University of Wisconsin, 433 Babcock Dr., Madison, WI 53706. Phone: 608-262-8007. Fax: 608-262-9108. E-mail: wickens@biochem.wisc.edu.

[†] Present address: McArdle Laboratory, University of Wisconsin—Madison, Madison, WI 53706.

actions require PAP, an enzyme present in both the nucleus and cytoplasm of *X. laevis* oocytes (1, 15). Both reactions require the polyadenylation sequence AAUAAA; however, cytoplasmic polyadenylation requires the additional presence of a CPE (13, 28, 33). Additionally, cytoplasmic polyadenylation affects only a subset of mRNAs and does so at specific times during development (10, 38, 51) whereas nuclear polyadenylation is a nearly universal and constitutive reaction (49). Finally, CPSF in mammalian, somatic cells is predominantly nuclear (18) and therefore is not available for a cytoplasmic event. If a CPSF-like factor is required for cytoplasmic polyadenylation, as proposed previously (7), it must be localized to the cytoplasm, as removal of the nucleus prior to meiotic maturation does not interfere with the reaction *in vivo* (13).

This report demonstrates that a cytoplasmic form of the 100-kDa subunit of CPSF is present in *X. laevis* oocytes. Although it is closely related to its counterpart in mammalian, somatic cells, the oocyte protein is largely cytoplasmic. The 100-kDa subunit of *X. laevis* CPSF is present in CPE-dependent complexes formed *in vitro* and is required for efficient cytoplasmic polyadenylation in egg extracts. A putative homologue of the 30-kDa subunit of CPSF is also present in the cytoplasm of *X. laevis* oocytes and may also be required for this reaction. The data support the hypothesis that a cytoplasmic complex, closely related to CPSF, is required for CPE-dependent polyadenylation.

MATERIALS AND METHODS

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

Oocyte manipulations. Oocyte removal and the induction of meiotic maturation were performed essentially as described in reference 2. Oocyte injections were performed essentially as described in reference 52. Stage VI oocytes were injected with ≈ 50 nl of RNA (final concentrations for labeled, reporter mRNA transcripts and for production of proteins, 100 fmol/ μ l and 1 μ g/ μ l, respectively).

Nuclei were removed from oocytes under mineral oil as described in reference 7. For collagenase treatment, enucleated oocytes were incubated for several hours in Marc's modified Ringer's (MMR) containing 1 mg of bovine serum albumin (Sigma, St. Louis, Mo.) per ml to allow healing. Healthy cells were then incubated in collagenase (Boehringer Mannheim, Indianapolis, Ind.) (0.2% in 100 mM sodium phosphate [pH 7.4]) for 60 to 90 min. To confirm the removal of associated follicle cells, intact enucleated oocytes were stained with Hoechst dye 33258 (Sigma; 1 μ g per ml of solution containing 110 mM NaCl, 2 mM KCl, 1 mM MgSO₄, 2 mM NaHCO₃, 0.5 mM sodium phosphate, 15 mM Tris-Cl [pH 7.6]) and analyzed by fluorescence microscopy (excitation wavelength, 356 nm) (information applies to Fig. 1 only).

Antibody preparation. Antibodies raised against the 100-kDa subunit of *Bos taurus* CPSF were prepared as described previously (18). These antibodies recognize both the native and denatured forms of the 100-kDa subunit of *B. taurus* CPSF. The monoclonal antibody cell line J1/27 (20a) was produced in response to highly purified *B. taurus* CPSF and recognizes the native form of the 100-kDa subunit of *X. laevis* CPSF only. The monoclonal cell line raised against the carboxy-terminal 219 amino acids of p34^{cdc2} (A17) was produced as described previously (31).

Western blots. Oocytes were homogenized in TE (10 mM Tris-Cl [pH 8] and 1 mM EDTA) plus protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μ g of aprotinin/ml, 10 μ g of pepstatin/ml, 10 μ g of leupeptin/ml, and 10 μ g of chymostatin/ml). In some instances Complete-Mini (Boehringer Mannheim) protease inhibitor cocktail was substituted for these inhibitors. Clarification of oocyte homogenates was performed as described previously (4). Proteins were separated by electrophoresis through sodium dodecyl sulfate (SDS)-6.5 to 10% polyacrylamide gel electrophoresis (PAGE) gels (25) and then transferred electrophoretically to Immobilon-P (Millipore, Bedford, Mass.) in transfer buffer (25 mM Tris, 190 mM glycine, 20% methanol; except for the blots shown in Fig. 1B, where 50 mM Tris, 380 glycine, 0.1% SDS, and 20% methanol was used). Blocking, antibody incubations, and washing were performed according to standard protocols (40). Primary antibody dilutions were as follows: unpurified and Protein A-Sepharose (Sigma)-purified polyclonal anti-CPSF antibodies at 1:2,000 to 1:10,000, affinity-purified polyclonal anti-CPSF antibodies at 1:1,000, monoclonal anti-hemagglutinin (HA) antibodies (Berkeley Antibody Company, Berkeley, Calif.) at 1:667, and monoclonal anti-FLAG M2 antibodies (Eastman Kodak, Rochester, N.Y.) at 1:250. Secondary anti-rabbit and anti-mouse antibody (Amersham, Arlington Heights, Ill., and Kirkegaard & Perry, Gaithersburg, Md.) dilutions were according to the manufacturers' spec-

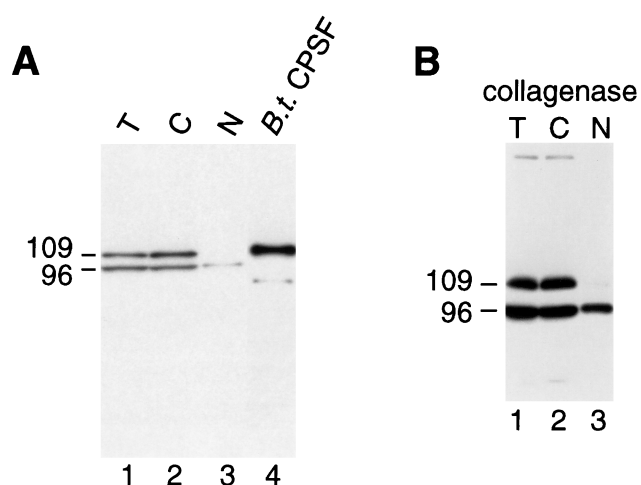


FIG. 1. Western blot of *X. laevis* oocyte extracts obtained by using antibodies raised against the 100-kDa subunit of *B. taurus* CPSF. (A) Total oocyte extracts (lane 1) as well as cytoplasmic (lane 2) and nuclear (lane 3) oocyte extracts were analyzed by using anti-CPSF¹⁰⁰ antibodies. Oocyte cytoplasm and nuclei were manually dissected under mineral oil to reduce nuclear leakage. The equivalent of one oocyte cytoplasm or nucleus was analyzed per lane. For comparison, purified *B. taurus* CPSF (lane 4), corresponding to 3.6 ng of the 100-kDa subunit, was analyzed as well. (B) Oocytes were treated with collagenase to remove tightly associated follicle cells. After removal of follicle cells, oocytes were manipulated as described for panel A, and total oocyte (lane 1), cytoplasmic (lane 2), and nuclear (lane 3) proteins were examined by Western blotting by using anti-CPSF¹⁰⁰ antibodies.

ifications. Detection was performed with the LumiGLO chemiluminescent substrate kit (Kirkegaard & Perry) as described by the manufacturer.

Apparent molecular weights of proteins were determined in duplicate using "Hi" molecular-weight protein standards (BioRad, Hercules, Calif.). Molecular weights of *X. laevis* proteins detected were deduced based on the mobilities of flanking standards. In addition, "Kaleidoscope" (BioRad), "Benchmark" (Gibco-BRL Gaithersburg, Md.), or "Rainbow" (Amersham) standards were included on all blots.

Expression library screen and RACE protocol. Affinity-purified polyclonal antibodies raised against the 100-kDa subunit of *B. taurus* CPSF (2685) were used to screen a lambda ZAP *X. laevis* expression library made with oligo(dT)-selected, maternal mRNA from two-cell embryos (gift from Peter Klein). cDNAs were directionally cloned as *EcoRI-XhoI* fragments. Inserts have an average length of 1 to 2 kb. The library was screened by using a modification of the procedures described in references 40 and 50. Primary antibody diluted in 5% Tris-buffered saline-Tween (1:20,000) was used to screen 100,000 PFU. Positive colonies were detected by using a colorimetric assay reagent (BioRad) as described by the manufacturer. Phagemid DNAs were excised according to the manufacturer's protocol (Stratagene, La Jolla, Calif.). Phagemid DNAs were analyzed by restriction digestion with *EcoRI* (Promega, Madison, Wis.) and *XhoI* (Promega). Phagemid DNAs were sequenced by using fluorescent dideoxynucleoside triphosphates (University of Wisconsin—Madison Biotechnology Center). The largest phagemid DNA, a 3-kb fragment, was used as a substrate for a protocol for rapid amplification of cDNA ends (RACE) (14) to isolate additional 5' cDNA sequence as described by the manufacturer (Marathon cDNA Amplification kit; Clontech, Palo Alto, Calif.). The 3' antisense oligonucleotide used in this protocol was 5'CCAGCATCTTTGGTCCTCC3' (University of Wisconsin—Madison Biotechnology Center).

DNA constructs and preparation of RNAs. A full-length CPSF¹⁰⁰ cDNA was constructed by ligation of the RACE PCR fragment with the 3-kb cDNA. The RACE PCR fragment was cloned in frame, 5' of the 3-kb cDNA, as a *NotI* (Promega)-*NdeI* (Promega) fragment. The upstream open reading frames (uORFs) in this full-length cDNA were removed by digestion with *BglII* (Promega) and extended to a blunt end with the Klenow fragment of DNA polymerase I (Promega) and digestion with *SmaI* (Promega). This construct is referred to as *Xlo* CPSF¹⁰⁰. The *Xlo* CPSF¹⁰⁰ ORF was placed in frame, downstream of an HA tag from pSB33 (1). Both *Xlo* CPSF¹⁰⁰ and pSB33 were digested with *XhoI* (Promega), extended to a blunt end with the Klenow fragment of DNA polymerase I (Promega), and digested with *BglII* (Promega) (HA-CPSF¹⁰⁰). A FLAG (Eastman Kodak) epitope tag was placed at the carboxy terminus of HA-CPSF¹⁰⁰ by using PCR. The FLAG epitope (underlined) was encoded in a 3' antisense oligonucleotide (5'CGTCTAGATCACTTATCGTCATCGTCCTTGTAGTCCACAATGGCATATTGTTCT3') (University of Wisconsin—Madison Biotechnology Center) and encompassed a unique *XbaI* site. The first amino acid

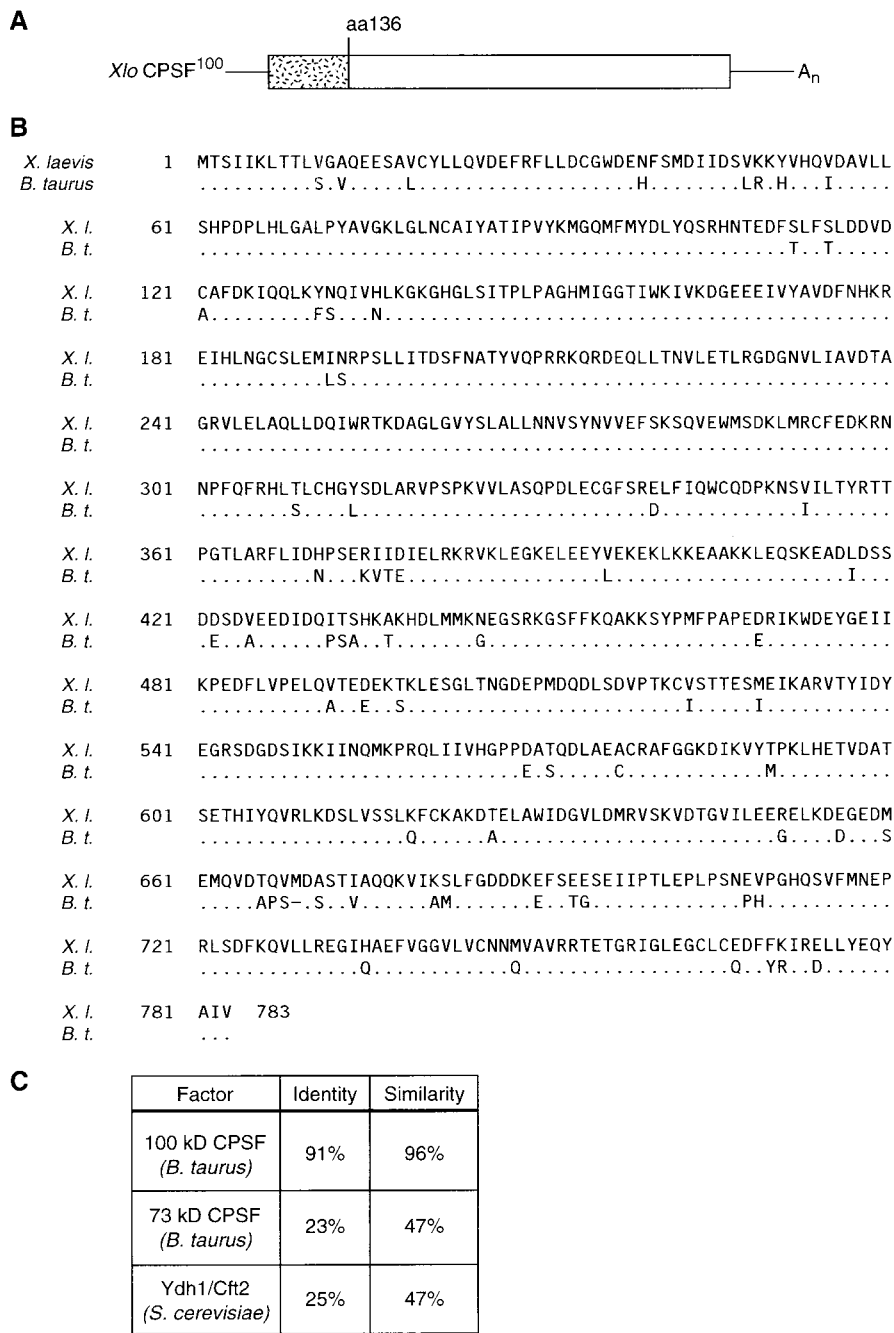


FIG. 2. Schematic of the 100-kDa subunit of *X. laevis* CPSF and alignment with other CPSF homologues. (A) Schematic representation of the *Xlo* CPSF¹⁰⁰ cDNA (GenBank accession no. AF139986). The portion of the cDNA starting from aa136 was obtained via expression library screening (white). An additional 5' sequence upstream of aa 136 was obtained by RACE amplification (stippled). The 5' UTR is 62 nt in length, and the 3' UTR is 820 nt in length and is followed by a poly(A) tail of 50 nt. (B) The *Xlo* CPSF¹⁰⁰ amino acid sequence was aligned with the amino acid sequence from the 100-kDa subunit of *B. taurus* CPSF (EMBL accession no. X75931). Only the amino acids which differ from those for the *X. laevis* sequence are depicted for the *B. taurus* sequence. (C) The identity and similarity of the amino acid sequence of *Xlo* CPSF¹⁰⁰ with the 100-kDa subunit of *B. taurus* CPSF, the 73-kDa subunit of *B. taurus* CPSF (EMBL accession no. X95906), and the 100-kDa subunit of *S. cerevisiae* CPSF (Ydh1/CftII) (GenBank accession no. U53877) were determined by using the Wisconsin GCG program.

of FLAG replaces the HA-CPSF¹⁰⁰ stop codon. The 5' sense oligonucleotide (5'CAGCAATGAGGTCCCAGGACACC3') (University of Wisconsin—Madison Biotechnology Center) surrounded a unique *Ppu*MI site in HA-CPSF¹⁰⁰. The resulting cDNA contained an in-frame amino-terminal HA tag and an in-frame carboxy-terminal FLAG tag (HA-CPSF¹⁰⁰-FL). *Xlo* CPSF¹⁰⁰ cDNA was linearized with *Xho*I (Promega) and then transcribed with T₃ RNA polymerase (Gibco-BRL and Stratagene). HA-CPSF¹⁰⁰ cDNA was linearized with *Hinc*II (Promega) and then transcribed with SP6 RNA polymerase (Promega). HA-CPSF¹⁰⁰-FL cDNA was linearized with *Clal* (Promega) and

transcribed with T₇ RNA polymerase (Stratagene). All reactions were performed based on the manufacturers' specifications with the exception that GTP (Stratagene) was added 5 min after initial incubation to allow increased incorporation of the m⁷GpppG cap (New England Biolabs, Beverly, Mass.). Plasmids encoding L1 RNA and L1 RNA with UUUUUUUAU were constructed as described (7, 12, 45). To generate the same RNAs with G substituted for U in AAUAAA, DNA encoding the L1 RNAs was subcloned into pGEM3Z (F+) (Promega), altered by site-directed mutagenesis (24), digested with *Afl*III (New England Biolabs), and incubated with T₇ RNA polymerase (Stratagene).

Digestion with *Afl*III (New England Biolabs) and incubation with T₇ RNA polymerase (Stratagene) yielded a 115-nt (L1) or 123-nt (L1 with UUUUUUUAU) RNA.

NS1 constructs, obtained from Robert Krug, were constructed as described in reference 36.

Gel mobility shift and supershift assays. Protein-RNA interactions were detected by using a procedure based on that described previously (12). Egg extract (0.2 μ l of a 49-mg/ml solution, prepared as described in reference 12, containing 6.3 μ l of buffer [100 mM KCl, 150 μ M EDTA, 50 mM Tris, 10% glycerol]) and RNA (1 μ l of a 4- to 5-fmol/ μ l solution) were incubated for 10 min at 25°C and then on ice for 1 h. One microliter of Protein A-Sepharose (Sigma)-purified antibodies (monoclonal antibody J1/27, 0.12 mg/ml; polyclonal antibody 2685, 0.82 mg/ml; preimmune antibody, 29 mg/ml) in buffer (55% glycerol, 25 mM Tris, 50 mM KCl, 1.5 mM MgCl₂) or 1 μ l of buffer alone was then added to some samples, and the mixtures were incubated for 2 h on ice with occasional agitation. One microliter of heparin (1.2 mg/ml) was then added to each sample, and the resulting mixtures were incubated on ice for 5 min before electrophoresis through a nondenaturing 4% acrylamide gel for 2 to 3 h at 185 V in a cold chamber at 4°C. Complexes were detected by exposure to X-ray film (Eastman Kodak) or a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

Depletions. Protein A-Sepharose 4B fast-flow beads (Sigma) were swollen in NET buffer (150 mM NaCl, 50 mM Tris-Cl [pH 8.0], 0.1% [vol/vol] Nonidet P-40 [Sigma]) and equilibrated in IP1 buffer (100 mM Na₂HPO₄, 2 mg of bovine serum albumin/ml, 0.1% NaN₃). Ten to 40 microliters of Protein A-Sepharose (Sigma)-purified monoclonal, polyclonal, or preimmune antibodies was then added to 20 μ l of bead slurry (10 μ l of beads and 10 μ l of IP1 buffer) together with 100 μ l of cold IP1 buffer. Antibodies were allowed to bind to the beads for 2 h on ice with occasional agitation. The beads were washed twice with cold IP1 buffer and then equilibrated in buffer B (100 mM KCl, 150 μ M EDTA, 10% glycerol, 50 mM Tris-Cl [pH 8.5] at 4°C). The beads were then divided into two portions. Buffer was drained from one portion, and a mixture of 12 μ l of egg extract (49-mg/ml solution prepared as described in reference 12) and 48 μ l of buffer B was added. Beads and extract were incubated together 2 h on ice with occasional agitation. The second tube of beads was drained, and extract from the first tube was transferred to the second. The extract was incubated with these beads for 2 h on ice with occasional agitation. Five microliters was transferred to tubes containing 20 U of RNasin (Promega), 6.5 μ g of yeast RNA (Torula type IV; Sigma) (further purified by multiple organic extractions and alcohol precipitations), 12.5 mM dithiothreitol (Boehringer Mannheim), 5 mM ATP, 5 mM MgCl₂, and 37.4 mM creatine phosphate in a total volume of 2 μ l. Either 1 μ l of buffer B or 1 μ l of CPSF at \approx 40 ng/ μ l (\approx 17 U/ μ l) concentration was then added, followed by 1 μ l (2 fmol/ μ l) of RNA. Reaction mixtures (total volume, 9 μ l) were incubated 20 min at 25°C and processed as described (7). Mock depletions were performed in parallel by using the same buffers, volumes, and incubation times.

Nucleotide sequence accession number. The nucleotide sequence for the *Xlo* CPSF¹⁰⁰ cDNA has been deposited with GenBank under accession no. AF139986.

RESULTS

Cytoplasmic homologues of the 100-kDa subunit of CPSF.

Previous work suggested that a cytoplasmic factor, related to nuclear CPSF, participates in cytoplasmic polyadenylation (7, 12). To test whether a homologue of the 100-kDa subunit of CPSF is present in *X. laevis* oocytes, analyses by Western blotting were performed on *X. laevis* oocyte extracts by using affinity-purified polyclonal antibodies raised against the 100-kDa subunit of *B. taurus* CPSF (anti-CPSF¹⁰⁰ antibodies) (18). *X. laevis* whole-cell oocyte extracts (Fig. 1A, lane 1), as well as dissected oocyte cytoplasm and nuclei (Fig. 1A, lanes 2 and 3), were analyzed. Partially purified *B. taurus* nuclear CPSF was probed in parallel for comparison (Fig. 1A, lane 4). Proteins with apparent molecular masses of 109 and 96 kDa were detected in oocyte extracts. The 109-kDa species comigrated with the 100-kDa subunit of *B. taurus* CPSF and is predominantly localized to the cytoplasm of *X. laevis* oocytes. (For simplicity, the 109-kDa *X. laevis* protein is referred to hereafter as the 100-kDa subunit.) The 96-kDa species is present in both oocyte cytoplasm and nuclei. Additionally, the 100-kDa protein is recognized selectively by a monoclonal antibody raised against the 100-kDa subunit of *B. taurus* CPSF; the 96-kDa protein is not (data not shown).

Oocytes are surrounded by a thin layer of somatic cells. To ensure that the two proteins detected were present in the oocyte and not somatically derived, oocytes were treated with

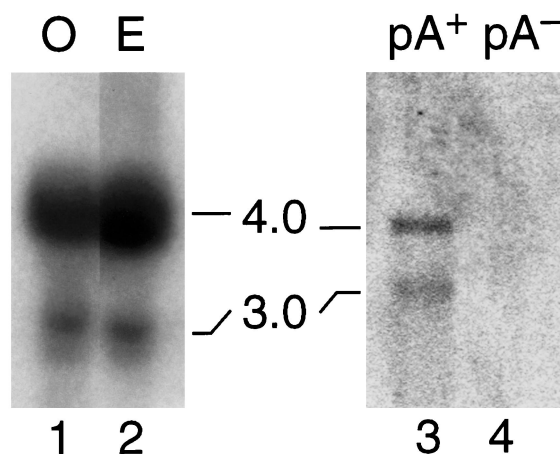


FIG. 3. Northern blot for the *X. laevis* CPSF¹⁰⁰ mRNA. Total RNA from oocytes (lane 1) or eggs (lane 2) was analyzed. Poly(A)-plus RNA (pA⁺) was selected by multiple passes over oligo(dT) cellulose (lane 3). The flowthrough, representing poly(A)-minus RNA (pA⁻), was also analyzed (lane 4). Membranes were incubated with a [³²P]dATP-radiolabeled probe encoding the entire 5' UTR, ORF, and 3' UTR sequences from *Xlo* CPSF¹⁰⁰. RNAs were visualized by autoradiography.

collagenase to remove somatic cells prior to dissection (Fig. 1B). Collagenase-treated and untreated cells contained both proteins in comparable abundance. The presence of the 100-kDa protein in the cytoplasmic fraction of collagenase-treated cells (Fig. 1B, lane 2) suggests that it is truly cytoplasmic in the oocyte, rather than a contaminant from adherent somatic tissue.

Isolation of a homologue of the 100-kDa subunit. To isolate cDNAs encoding a homologue of the 100-kDa subunit of CPSF, an *X. laevis* cDNA expression library (gift from Peter Klein) was screened by using anti-CPSF¹⁰⁰ antibodies (18). The largest cDNA isolated was 3.0 kb and encoded a partial ORF corresponding to amino acids (aa) 136 to 783 of the *B. taurus* peptide. The cDNA contained the entire 3' UTR, as judged by the presence of an AAUAAA sequence followed by a poly(A) tail (Fig. 2A). An additional 5' sequence (Fig. 2A) was obtained by using the RACE protocol (14). The assembled full-length cDNA contained the entire ORF, as well as both 5' and 3' UTRs. The 5' UTR contained two small uORFs, one encoding a 9-aa peptide and the other encoding an AUG immediately followed by a stop codon. Both were removed to allow efficient expression of the 100-kDa protein. The ORF of this cDNA, referred to as *Xlo* CPSF¹⁰⁰ (*Xlo* denotes *X. laevis* oocyte), is 78% identical at the nucleotide sequence level and 91% identical at the amino acid sequence level to the 100-kDa subunit of *B. taurus* CPSF. The amino acid sequence of the 100-kDa subunit of *X. laevis* CPSF was aligned with the 100-kDa subunit of *B. taurus* CPSF (Fig. 2B). Comparisons between the 100-kDa subunit of *X. laevis* CPSF and the 73-kDa subunit of *B. taurus* CPSF, which shares extensive sequence similarity with the 100-kDa subunit of *B. taurus* CPSF (20), and the 100-kDa subunit of *S. cerevisiae* CPSF(Ydh1/Cft2) (35, 53) (Fig. 2B) are shown (Fig. 2C).

Analysis by Northern blotting of *X. laevis* mRNA, using a probe spanning the entire ORF, detected two mRNAs of 3.0 and 4.0 kb. Both mRNAs are present in oocytes and eggs (Fig. 3, lanes 1 and 2) and persist throughout early embryogenesis (data not shown). Both are polyadenylated as assessed by oligo(dT) selection (Fig. 3, lane 3 and 4). Mammalian cells also contain two forms of CPSF¹⁰⁰ mRNA (18).

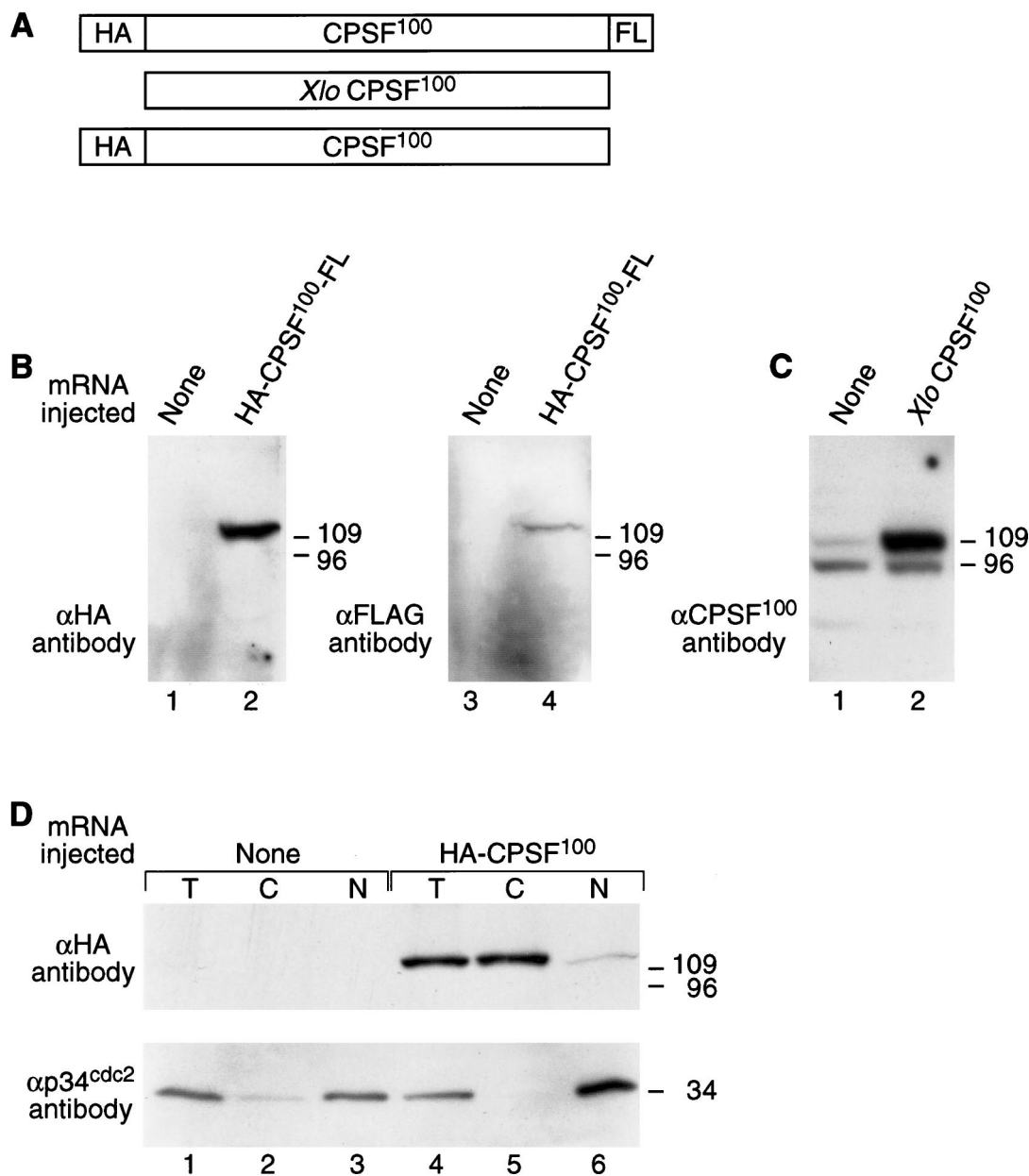


FIG. 4. Western blot of the *in vivo* expression and localization of the *Xlo* CPSF¹⁰⁰ protein product. (A) Schematic representation of the protein products encoded by the three cDNA constructs used in this assay. (B) An mRNA encoding a double-epitope-tagged form of the 100-kDa subunit of *X. laevis* CPSF (HA-CPSF¹⁰⁰-FL) was injected into oocytes. Protein was isolated from both uninjected (lanes 1 and 3) and injected cells (lanes 2 and 4), and production of the HA-CPSF¹⁰⁰-FL protein was visualized by Western blotting using antibodies raised against the HA epitope tag (anti-HA antibodies) (lanes 1 and 2) or the FLAG epitope tag (anti-FLAG antibodies) (lanes 3 and 4). (C) An mRNA encoding an untagged form of *Xlo* CPSF¹⁰⁰ was expressed in oocytes and visualized by Western blotting using anti-CPSF¹⁰⁰ antibodies. Production of the *Xlo* CPSF¹⁰⁰ protein was viewed as an increase in antibody signal in injected cells compared to uninjected cells (compare lanes 2 and 1). (D) mRNA encoding an N-terminal HA epitope-tagged form of the 100-kDa subunit (HA-CPSF¹⁰⁰) was injected into oocytes. Both uninjected (lanes 1 to 3) and injected (lanes 4 to 6) oocytes were manually dissected into cytoplasmic and nuclear fractions. Total oocyte extracts (lanes 1 and 4), cytoplasm (each lane is equivalent to one oocyte) (lanes 2 and 5), and nuclei (each lane is equivalent to one oocyte nucleus) (lanes 3 and 6) were analyzed by Western blotting. The top half of the membrane was probed for the HA-CPSF¹⁰⁰ protein by using anti-HA antibodies. The bottom half was analyzed by using anti-p34^{cdc2} (gift from Tim Hunt) to detect this nuclear protein.

In vivo expression of *X. laevis* CPSF¹⁰⁰. The two CPSF-like proteins detected immunologically in the oocyte cytoplasm could be encoded by one or more mRNAs. To determine whether the isolated cDNA encoded one or both of these proteins, the N and C termini of *Xlo* CPSF¹⁰⁰ were epitope-tagged with HA (39) and FLAG tags, respectively (HA-CPSF¹⁰⁰-FL) (Fig. 4A). This cDNA was transcribed *in vitro*,

and the mRNA was injected into oocytes (Fig. 4B, lanes 2 and 4). Proteins produced by the injected mRNA were identified by using anti-HA (Fig. 4B; compare lanes 2 and 1) or anti-FLAG (Fig. 4B; compare lanes 4 and 3) antibodies. A single protein of constant mobility was detected with each antibody. The production of a protein carrying both the N- and C-terminal epitopes from the HA-CPSF¹⁰⁰-FL mRNA suggests that the

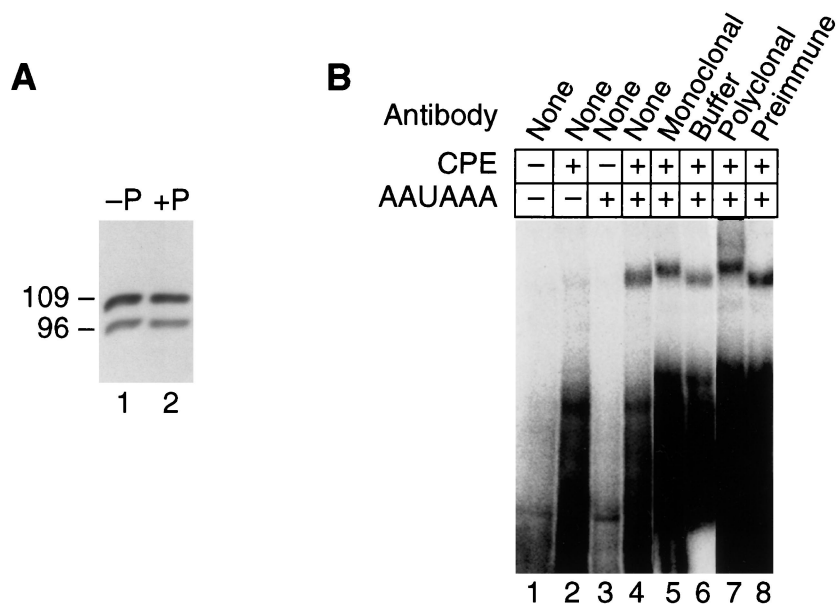


FIG. 5. Electrophoretic gel mobility supershift assay of a CPE- and/or AAUAAA-containing mRNA performed in the presence of *X. laevis* egg extract and anti-CPSF¹⁰⁰ antibodies. (A) Protein was isolated from oocytes (lane 1) and from oocytes induced to undergo meiotic maturation by incubation in the hormone progesterone (lane 2). *Xlo* CPSF¹⁰⁰ protein was visualized by Western blotting by using anti-CPSF¹⁰⁰ antibodies. (B) mRNAs without a CPE or an AAUAAA (lane 1), with only a CPE (lane 2), with only an AAUAAA (lane 3), or with both a CPE and an AAUAAA sequence (lanes 4 through 8) were in vitro-transcribed in the presence of [α -³²P]UTP. Each mRNA was then incubated in the presence of *X. laevis* egg extract alone (lanes 1 through 4). In addition, the CPE- and/or AAUAAA-containing mRNA was incubated in the presence of *X. laevis* egg extract plus either monoclonal anti-CPSF¹⁰⁰ antibodies (lane 5), monoclonal buffer (lane 6), polyclonal anti-CPSF¹⁰⁰ antibodies (lane 7), or preimmune serum antibodies (lane 8). mRNAs were separated on a native polyacrylamide gel and visualized by exposure to film.

translational product does not undergo proteolytic processing at either end.

To determine whether the product of *Xlo* CPSF¹⁰⁰ comigrated with the endogenous, cytoplasmic CPSF, mRNA encoding an untagged form of the protein (Fig. 4A) was injected into oocytes and the protein was detected by Western blot analysis using anti-CPSF¹⁰⁰ antibodies (Fig. 4C). Injection of the untagged CPSF¹⁰⁰ mRNA caused a substantial increase in the amount of protein seen at the position of the endogenous 100-kDa CPSF homologue; no increase in the amount of the 96-kDa species was detected (Fig. 4C, lane 2 and 1). Taken together, these results demonstrate that the 100-kDa protein corresponds to the protein encoded by the *Xlo* CPSF¹⁰⁰ cDNA and that the 96-kDa protein is neither derived from *Xlo* CPSF¹⁰⁰ by alternate initiation or termination nor derived from the 100-kDa protein by proteolysis.

To determine whether the cloned *Xlo* CPSF¹⁰⁰ protein was cytoplasmic, an mRNA encoding an N-terminal HA epitope-tagged mRNA (HA-CPSF¹⁰⁰) (Fig. 4A) was injected into oocytes. After overnight incubation to allow translation and transport to occur, oocytes were manually dissected into nuclear and cytoplasmic fractions. The majority of the HA-tagged protein was present in the cytoplasm (Fig. 4D; compare lanes 5 and 6). Effectiveness of the enucleations was analyzed by Western blotting with anti-p34^{cdc2} antibodies (31) for this nuclear protein (Fig. 4D). These data indicate that the cloned *Xlo* CPSF¹⁰⁰ protein is predominantly cytoplasmic.

Specific association of *Xlo* CPSF¹⁰⁰ with a cytoplasmic polyadenylation substrate. To determine whether the 100-kDa subunit of *X. laevis* CPSF was present during meiotic maturation when cytoplasmic polyadenylation is activated, oocyte extracts obtained before (Fig. 5A, lane 1) and after (Fig. 5A, lane 2) maturation were analyzed by Western blotting using anti-CPSF¹⁰⁰ antibodies. Both the 100-kDa subunit of CPSF and

the cross-reacting 96-kDa protein are present after maturation. Neither the abundance nor the electrophoretic mobility of these proteins is altered during maturation (Fig. 5A) or early embryonic development (data not shown).

An RNA-binding activity, specific for CPE/AAUAAA-containing RNAs, is present in *X. laevis* egg extracts competent for cytoplasmic polyadenylation (12, 33). This activity fractionates in a manner similar to that of nuclear CPSF (12) and can be functionally replaced in vitro with purified nuclear CPSF (7). In order to determine whether *Xlo* CPSF¹⁰⁰ was part of this complex, electrophoretic gel retardation assays were performed by using *X. laevis* egg extracts in the presence or absence of anti-CPSF¹⁰⁰ antibodies (Fig. 5B). As neither polyclonal antibodies nor monoclonal antibodies raised against the 100-kDa subunit of CPSF recognize the native form of the 96-kDa protein, no conclusions can be drawn about the presence or absence of this factor in the complex. Radiolabeled RNAs containing both a CPE and the polyadenylation element AAUAAA formed a specific complex when incubated in egg extracts competent for cytoplasmic polyadenylation (Fig. 5B, lane 4) (12). This complex is not formed with RNAs that lack the CPE or contain a point mutation in AAUAAA (AAGA AA) (Fig. 5B, lanes 1, 2, and 3) (12). These results are consistent with previous data (12). When either monoclonal or polyclonal anti-CPSF¹⁰⁰ antibodies were added to the RNA-egg extract mixture, the mobility of the specific complex was reduced (Fig. 5B, lanes 5 and 7). The addition of buffer alone or rabbit preimmune serum had no effect on the mobility of this complex (Fig. 5B, lanes 6 and 8). These data demonstrate that *Xlo* CPSF¹⁰⁰ is present in polyadenylation-specific complexes.

Immunodepletion of *Xlo* CPSF¹⁰⁰ inhibits CPE-dependent polyadenylation. In order to determine whether *Xlo* CPSF¹⁰⁰ was required for cytoplasmic polyadenylation in vitro, both monoclonal (Fig. 6A) and polyclonal (data not shown) anti-

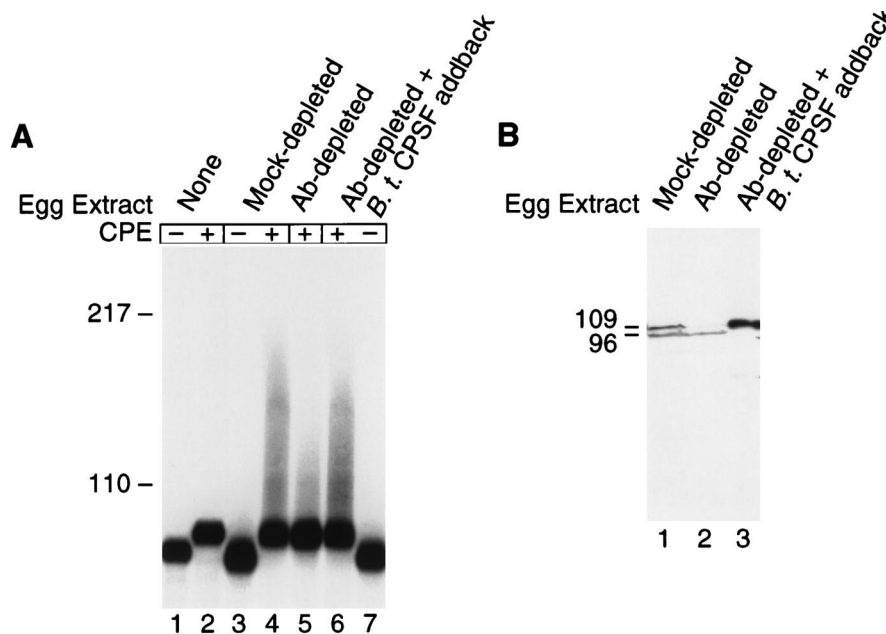


FIG. 6. In vitro polyadenylation assay in *X. laevis* egg extract depleted of the cytoplasmic form of the 100-kDa subunit of CPSF. (A) mRNAs with only an AAUAAA sequence (lanes 1, 3, and 7) or with both a CPE and an AAUAAA sequence (lanes 2, 4, 5, and 6) were in vitro transcribed in the presence of [α - 32 P]ATP. mRNAs were left untreated (lanes 1 and 2) or incubated in *X. laevis* egg extract passed over Protein A-Sepharose (lanes 3 and 4), egg extract washed over Protein A-Sepharose coupled to anti-CPSF¹⁰⁰ antibodies (lane 5), or egg extract washed over Protein A-Sepharose coupled to anti-CPSF¹⁰⁰ antibodies and then supplemented with purified nuclear *B. taurus* CPSF (lanes 6 and 7). mRNAs were separated on a denaturing 6% polyacrylamide gel and visualized by exposure to film. (B) *X. laevis* egg extract washed over Protein A-Sepharose (lane 1), washed over Protein A-Sepharose coupled to anti-CPSF¹⁰⁰ antibodies (lane 2), or washed over Protein A-Sepharose coupled to anti-CPSF¹⁰⁰ antibodies and then supplemented with purified *B. taurus* CPSF (lane 3) was separated by SDS-PAGE. Proteins were visualized by Western blotting using anti-CPSF¹⁰⁰ antibodies.

CPSF¹⁰⁰ antibodies were used to immunodeplete the *Xlo* CPSF¹⁰⁰ protein from egg extracts. Depleted egg extracts were tested for the ability to activate CPE-dependent polyadenylation in vitro. Polyadenylation in antibody-depleted extracts was reduced in comparison to that in mock-depleted extracts (Fig. 6A; compare lanes 5 and 4). Addition of purified nuclear CPSF fully restored activity to the depleted extracts in a CPE-dependent manner (Fig. 6A, lanes 6 and 7).

The quantity of *Xlo* CPSF¹⁰⁰ in control and depleted extracts was assessed by Western blot analysis using anti-CPSF¹⁰⁰ antibodies. *Xlo* CPSF¹⁰⁰ was depleted to near-undetectable levels in antibody-depleted (Fig. 6B, lane 2) but not mock-depleted (Fig. 6B, lane 1) extracts. Addition of purified nuclear CPSF increased the concentration of the *Xlo* CPSF¹⁰⁰ protein to approximately twice the endogenous level as evaluated by Western blot analysis (Fig. 6B, lane 3). CPSF concentrations comparable to endogenous levels also restored full activity (data not shown). The 96-kDa protein was not recognized by either monoclonal antibodies or polyclonal antibodies and remained present in all egg extracts. It is possible therefore that the residual polyadenylation activity is due to the presence of this protein. These data demonstrate that removal of the *Xlo* CPSF¹⁰⁰ significantly reduces cytoplasmic polyadenylation in vitro.

Influenza virus protein NS1 inhibits cytoplasmic polyadenylation. The influenza virus protein NS1 interacts with the 30-kDa subunit of *B. taurus* CPSF (32). The NS1-CPSF³⁰ interaction prevents CPSF from binding to the pre-mRNA and thereby inhibits the activities of CPSF in the nucleus, blocking both pre-mRNA cleavage and polyadenylation (32). If CPSF is involved in cytoplasmic polyadenylation, then expression of NS1 in an oocyte might also prevent that reaction. To test this hypothesis, various NS1 mRNA constructs were prepared by

transcription in vitro and injected into oocytes. After incubation of the oocytes overnight to permit NS1 protein production, radiolabeled polyadenylation substrates were injected and meiotic maturation was induced by the addition of progesterone.

The wild-type NS1 protein (NS1^{wt}) (36) prevented cytoplasmic polyadenylation (Fig. 7A; compare lanes 4 and 2). An NS1 mutant lacking the RNA-binding domain, which is required for inhibition of mRNA splicing and export (26, 36, 37) but is capable of interacting with nuclear CPSF³⁰ (NS1^{RBDMmut}) (36), prevented cytoplasmic polyadenylation as effectively as the wild-type protein (Fig. 7A; compare lanes 6 and 4). Support for the specificity of this reaction was provided by the use of mutant forms of the NS1 protein, either carrying an amino acid substitution that prevents binding to nuclear CPSF³⁰ (NS1^{C30mut1}) (36) or carrying a deletion of this same region (NS1^{C30mut2}) (36). Neither of these mutant proteins had any effect on cytoplasmic polyadenylation (Fig. 7A, lanes 8 and 10). The lack of inhibition was not due to lack of these mutant proteins in the injected cells, as both NS1^{C30mut1} and NS1^{C30mut2} proteins were present in injected cells as assessed by Western blot analysis (data not shown). The mRNA degradation phenotype seen in NS1-expressing cells after the induction of meiotic maturation (Fig. 7A, lanes 4 and 6) is similar to that seen for mRNAs containing a point mutation within the AAUAAA sequence (AAGAAA) (Fig. 7B, lane 2) which prevents CPSF from binding (22, 29). This phenotype is consistent with the hypothesis that NS1 displaces CPSF from the mRNA.

The results obtained by using NS1 protein support the view that a CPSF-like factor is required for cytoplasmic polyadenylation. In particular, they suggest that a homologue of the 30-kDa subunit is involved in this reaction.

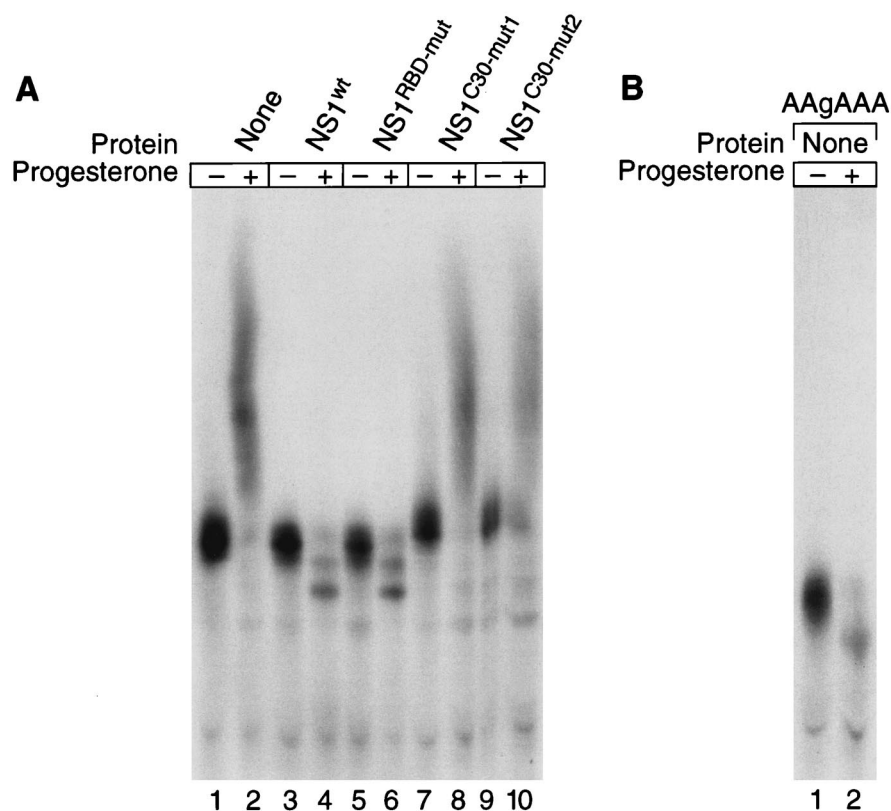


FIG. 7. In vivo polyadenylation assay of injected mRNAs in the presence of NS1. (A) *X. laevis* oocytes were injected with mRNA encoding various forms of the NS1 protein. Protein production was allowed to proceed overnight before subsequent analyses. An mRNA containing both a CPE and the AAUAAA sequence was in vitro-transcribed in the presence of [α - 32 P]UTP. This mRNA was injected into oocytes without NS1 protein (lanes 1 and 2) or oocytes expressing NS1 (lanes 3 and 4) (referred to as NS1-3' ss in reference 36), NS1^{RBDmut} (lanes 5 and 6) (referred to as NS1-RM in reference 36), NS1^{C30mut1} (lanes 7 and 8) (referred to as NS1-EM in reference 36), or NS1^{C30mut2} (lanes 9 and 10) (referred to as NS1- Δ E in reference 36) proteins. After the radiolabeled RNA was injected, meiotic maturation was induced in some cells by incubation in progesterone (lanes 2, 4, 6, 8, and 10). Only cells in which germinal vesicle breakdown had occurred, as assessed by white spot formation, were assumed to have matured. RNA was isolated from cells, separated by electrophoresis through a 6% polyacrylamide gel, and visualized by autoradiography. (B) [α - 32 P]UTP-radiolabeled mRNA containing a point mutation in AAUAAA (AAGAAA) was in vitro-transcribed and injected into oocytes. Total RNAs from both oocytes (lane 1) and oocytes incubated in progesterone (lane 2) were separated by electrophoresis through a 6% polyacrylamide gel and visualized by autoradiography.

Homologues of the 30- and 73-kDa subunits of CPSF. To determine whether a homologue of CPSF³⁰ was present in the cytoplasm of *X. laevis* oocytes, Western blot analysis was performed on oocyte extracts by using affinity-purified polyclonal antibodies raised against the 30-kDa subunit of *B. taurus* CPSF (3). In addition, antibodies raised against the 73-kDa subunit of *B. taurus* CPSF (gift from David Bentley) were used to probe for a putative homologue of this CPSF subunit. Total *X. laevis* oocyte extracts, as well as dissected oocyte cytoplasm and nuclei, were analyzed. Both antibodies cross-reacted with proteins of the approximate size of the *B. taurus* CPSF subunits. The 30-kDa subunit was predominantly localized to the cytoplasm (Fig. 8A, lanes 3 and 4); the presence of this protein in collagenase-treated cells (Fig. 8A, lane 1) suggests that the 30-kDa subunit is a cytoplasmic protein and not a contaminant of adherent somatic tissue. In surprising contrast, the putative homologue of the 73-kDa subunit was predominantly localized to the nucleus (Fig. 8A; compare lanes 4 and 3). Effectiveness of the enucleations was assessed by Western blot analysis using anti-p34^{cdc2} antibodies (31) to detect this nuclear protein (Fig. 8B).

These results indicate that a putative homologue of CPSF³⁰ is present in the cytoplasm of *X. laevis* oocytes, consistent with the hypothesis that NS1 inhibits cytoplasmic polyadenylation

via an interaction with CPSF³⁰. In addition, they illustrate that differences exist between nuclear and cytoplasmic CPSF activities.

DISCUSSION

The work presented here indicates that the *X. laevis* oocyte cytoplasm harbors homologues of both the 30- and 100-kDa subunits of CPSF. The 100-kDa subunit is more than 90% identical in amino acid sequence to its mammalian nuclear counterpart. Both the 100- and 30-kDa subunits appear to be required for efficient cytoplasmic polyadenylation. A putative homologue of the 73-kDa subunit is predominantly nuclear, suggesting that nuclear and cytoplasmic forms of the complex may differ in composition.

CPSF and cytoplasmic polyadenylation. Purified *B. taurus* CPSF directs CPE-specific polyadenylation in vitro when combined with *B. taurus* PAP (7). This circumstantial evidence and related observations first raised the hypothesis that CPSF-like factors are present in the cytoplasm of oocytes and were involved in developmentally regulated polyadenylation (7, 12). The work reported here tests that hypothesis by examining both whether such cytoplasmic factors exist in the oocyte and

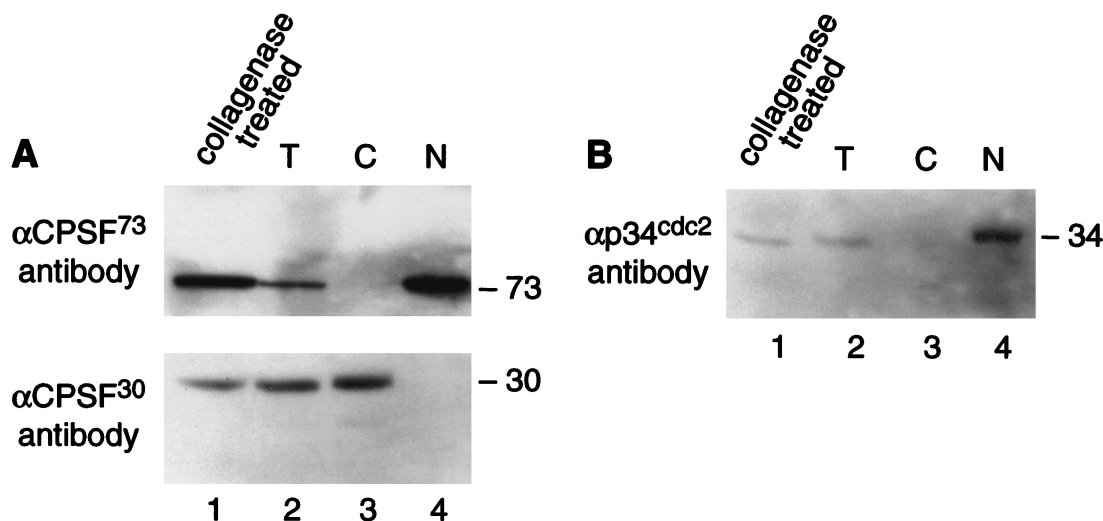


FIG. 8. Western blot of *X. laevis* oocyte extracts obtained by using antibodies raised against the 73- and 30-kDa subunits of *B. taurus* CPSF. Oocyte cytoplasm and nuclei were manually dissected under mineral oil to reduce nuclear leakage. Total oocyte extracts (lanes 1 and 2) as well as cytoplasmic (lane 3) and nuclear (lane 4) oocyte extracts were analyzed. The equivalent of one oocyte cytoplasm or nucleus was analyzed per lane. Some oocytes were treated with collagenase to remove associated follicle cells (lane 1). All blots were generated from the same protein extract. (A) Analysis by Western blotting using anti-73-kDa (top) or anti-30-kDa (bottom) antibodies raised against these subunits of *B. taurus* CPSF. (B) Analysis by Western blotting using anti-p34^{cdc2} antibody to detect this nuclear protein.

whether they are indeed required for CPE-dependent polyadenylation both in vivo and in vitro.

To address the question of whether CPSF subunits are present in the cytoplasm of *X. laevis* oocytes, antibodies against the *B. taurus* factor were used to analyze oocyte nuclear and cytoplasmic fractions. The oocyte cytoplasm contains a protein closely related to the 100-kDa subunit of mammalian, nuclear CPSF (Fig. 1). Similarly, a 30-kDa protein detected by anti-CPSF³⁰ antibodies (3) is also cytoplasmic (Fig. 8). In contrast, the *X. laevis* 73-kDa subunit (Fig. 8) and all identified CPSF subunits cloned from *B. taurus* (18–20, 22, 29, 30) and *S. cerevisiae* (8, 20, 23, 35, 42, 53) are predominantly nuclear at steady state. These data confirm a critical prediction of the earlier hypothesis (7, 12): that cytoplasmic CPSF-like factors do exist in the oocyte cytoplasm. In addition, they raise new questions about the overall composition of this cytoplasmic complex.

Three functional tests support the conclusion that CPSF is an integral and required component of the cytoplasmic polyadenylation machinery. First, CPSF specifically associates with CPE/AAUAAA-containing RNAs (Fig. 5). Previous work suggested that a CPE/AAUAAA-specific RNA-binding activity in *X. laevis* egg extracts was required for cytoplasmic polyadenylation (12). The data presented here indicate that the 100-kDa CPSF protein is part of this RNA-binding complex. Second, depletion of the 100-kDa subunit of CPSF from egg extracts results in a decrease in CPE-specific polyadenylation in vitro (Fig. 6). The fact that activity is only partially lost may reflect either the involvement of complexes that do not contain CPSF or an involvement of the 96-kDa protein that cannot be effectively immunodepleted. Third, overexpression of the influenza virus protein NS1 inhibits cytoplasmic polyadenylation in vivo (Fig. 7).

The recent observation that poly(A)-binding protein II (PABII) and NS1 interact (9) could be interpreted to suggest that NS1 prevents cytoplasmic polyadenylation via an effect on PABII rather than CPSF³⁰. In the nuclear reaction, the interaction of NS1 with PABII results in the nuclear accumulation of stable, cleaved mRNAs with short (ca. 10 nt) poly(A) tails

(9). Two lines of evidence presented here suggest that this is not the case in the oocyte cytoplasm. First, overexpressed NS1 and NS1^{RBD-mut} proteins prevent cytoplasmic polyadenylation in a manner that yields products very similar to those observed with substrates with a point mutation in AAUAAA (AAGAA A). In both cases, the RNAs did not receive any detectable poly(A) and were largely unstable (Fig. 7). Second, the NS1-inhibited cytoplasmic polyadenylation products differ from nuclear polyadenylation products (9) in that they lack the short poly(A) tail. These results suggest that NS1 exerts an effect through an interaction with CPSF³⁰ and not PABII.

CPSF, CPEB, and cytoplasmic polyadenylation. The biochemical events that initiate early cytoplasmic polyadenylation during meiotic maturation, prior to nuclear breakdown, are unknown (see "Nuclear and cytoplasmic CPSF," below). However, modifications of identified cytoplasmic polyadenylation factors are unlikely to trigger this event. Quantitative changes in the amount or mobility of the three CPSF subunits identified thus far are not detected during meiotic maturation (Fig. 1 and unpublished data). Similarly, activation appears to require neither an increase in nor hyperphosphorylation of CPEB (2, 41). Additionally, although PAP is hyperphosphorylated during meiotic maturation (1, 15), the form of PAP found prior to maturation is active in vitro (12). These results suggest that an as yet unidentified factor may be required to activate cytoplasmic polyadenylation during meiotic maturation.

Nuclear and cytoplasmic CPSF. The high degree of sequence similarity between the cytoplasmic *X. laevis* and the nuclear *B. taurus* 100-kDa CPSF subunits suggests that the nuclear and cytoplasmic factors are closely related and could perform similar functions (Fig. 2). The presence of putative homologues of the 30- and 73-kDa subunits of CPSF in *X. laevis* extracts (Fig. 8) suggests conservation of these subunits as well. The ability of nuclear CPSF to recapitulate CPE-specific polyadenylation in vitro indicates that nuclear CPSF can function in the regulated cytoplasmic polyadenylation event (7). Conversely, the ability of enucleated oocytes to undergo cytoplasmic polyadenylation (12), as well as the fact that poly(A) tail length changes occur on a number of mRNAs

prior to nuclear breakdown (2, 11), argues that nuclear and cytoplasmic polyadenylation require distinct machineries. The absence of a detectable 73-kDa subunit in the cytoplasm suggests that at least one important distinction exists between these two activities and may underlie the requirement for an additional factor (e.g., CPEB) in the cytoplasmic event.

The distinct subcellular localization of the 100- and 30-kDa subunits in relation to the 73-kDa subunit could be exploited to regulate polyadenylation activity. For example, it might underlie the existence of two classes of cytoplasmic polyadenylation reactions (2, 11). As described previously, polyadenylation of certain mRNAs is independent of *c-mos* polyadenylation and maturation-promoting factor activation (class I), whereas polyadenylation of other mRNAs requires both *c-mos* and MPF activities (class II) (2, 11). Polyadenylation of *c-mos* mRNA and subsequent production of Mos protein ultimately results in nuclear breakdown during meiosis. In one model, class I polyadenylation, which occurs prior to nuclear breakdown, should require only those CPSF subunits that are cytoplasmic in the oocyte (i.e., the 30- and 100-kDa subunits), while class II polyadenylation should require the 73-kDa subunit in addition and therefore occurs only when that subunit is released after nuclear breakdown. This hypothesis is consistent with the observation that the nuclear 73-kDa subunit can be coimmunoprecipitated with the cytoplasmic 100-kDa subunit from oocyte extracts prepared after nuclear breakdown (7a). Viewed in this manner, the increase in cytoplasmic polyadenylation activity may be analogous to the increase in nuclear 3' end formation activity in B cells, in which elevation of the level of the 64-kDa subunit of cleavage stimulatory factor increases cleavage and polyadenylation at a specific site (44). Regardless, the existence of distinctly compartmentalized CPSF subunits raises key questions as to the mechanism of cytoplasmic polyadenylation. It is now critical to identify components with which cytoplasmic CPSF subunits interact and to determine how cytoplasmic CPSF contributes to regulated polyadenylation in the oocyte and embryo. In particular, the question of whether the activity or recruitment of CPSF to specific mRNAs is regulated through additional components is central.

ACKNOWLEDGMENTS

We thank Walter Keller, Elmar Wahle, Andreas Jenny, and Silvia Barabino for providing advice, purified CPSF, and antibodies to CPSF¹⁰⁰ and CPSF³⁰. David Bentley is thanked for providing antibodies to CPSF⁷⁰. We thank Peter Klein for the *X. laevis* expression library and Claire Walczak for advice on library screening. We thank Xiaoyan Qian, Martin Nemeroff, and Robert Krug for supplying the NS1 constructs and antibodies and for communicating results prior to publication. We thank Tim Hunt and Julian Gannon for providing antibodies to p34^{cdc2}. We thank the Wickens lab, in particular Nicola Gray and Donald Gillian-Daniels for helpful discussion and comments on the manuscript. We especially thank Laura Vanderploeg and Adam Steinberg for their extreme patience in assembling the figures.

This work was supported by NIH research grants GM31892 and GM50942 to M.P.W. and by University of Wisconsin Molecular Biosciences training grant predoctoral fellowships to S.B. and K.S.D.

K.S.D. and A.B. contributed equally to this work.

REFERENCES

- Ballantyne, S., A. Bilger, J. Astrom, A. Virtanen, and M. Wickens. 1995. Poly(A) polymerases in the nucleus and cytoplasm of frog oocytes: dynamic changes during oocyte maturation and early development. *RNA* **1**:64–78.
- Ballantyne, S., D. L. J. Daniel, and M. Wickens. 1997. A dependent pathway of cytoplasmic polyadenylation reactions linked to cell cycle control by *c-mos* and CDK1 activation. *Mol. Biol. Cell* **8**:1633–1648.
- Barabino, S. M. L., W. Hubner, A. Jenny, L. Minvielle-Sebastia, and W. Keller. 1997. The 30-kD subunit of mammalian cleavage and polyadenylation specificity factor and its yeast homolog are RNA-binding zinc finger proteins. *Genes Dev.* **11**:1703–1716.
- Barkoff, A., S. Ballantyne, and M. Wickens. 1998. Meiotic maturation in *Xenopus* requires polyadenylation of multiple mRNAs. *EMBO J.* **17**:3168–3175.
- Beelman, C. A., and R. Parker. 1995. Degradation of mRNA in eukaryotes. *Cell* **81**:179–183.
- Bienroth, S., E. Wahle, C. Suter-Crazzolara, and W. Keller. 1991. Purification of the cleavage and polyadenylation factor involved in the 3'-processing of messenger RNA precursors. *J. Biol. Chem.* **266**:19768–19776.
- Bilger, A., C. A. Fox, E. Wahle, and M. Wickens. 1994. Nuclear polyadenylation factors recognize cytoplasmic polyadenylation elements. *Genes Dev.* **8**:1106–1116.
- Bilger, A., and M. Wickens. Unpublished data.
- Chanfreau, G., S. M. Noble, and C. Guthrie. 1996. Essential yeast protein with unexpected similarity to subunits of mammalian cleavage and polyadenylation specificity factor (CPSF). *Science* **274**:1511–1514.
- Chen, Z., Y. Li, and R. M. Krug. 1999. Influenza A virus NS1 protein targets poly(A)-binding protein II of the cellular 3' end processing machinery. *EMBO J.* **18**:2273–2283.
- Colgan, D. F., and J. L. Manley. 1997. Mechanism and regulation of mRNA polyadenylation. *Genes Dev.* **11**:2755–2766.
- De Moor, C. H., and J. D. Richter. 1997. The Mos pathway regulates cytoplasmic polyadenylation in *Xenopus* oocytes. *Mol. Cell. Biol.* **17**:6419–6426.
- Fox, C. A., M. D. Sheets, E. Wahle, and M. Wickens. 1992. Polyadenylation of maternal mRNA during oocyte maturation: poly(A) addition *in vitro* requires a regulated RNA binding activity and a poly(A) polymerase. *EMBO J.* **11**:5021–5032.
- Fox, C. A., M. D. Sheets, and M. P. Wickens. 1989. Poly(A) addition during maturation of frog oocytes: distinct nuclear and cytoplasmic activities and regulation by the sequence UUUUUU. *Genes Dev.* **3**:2151–2162.
- Frohman, M. A., M. K. Dush, and G. R. Martin. 1988. Rapid amplification of full-length cDNAs from rare transcripts: amplification using a single gene-specific oligonucleotide primer. *Proc. Natl. Acad. Sci. USA* **85**:998–9002.
- Gebauer, F., and J. D. Richter. 1995. Cloning and characterization of a *Xenopus* poly(A) polymerase. *Mol. Cell. Biol.* **15**:1422–1430.
- Gray, N. K., and M. Wickens. 1998. Control of translation initiation in animals. *Annu. Rev. Cell Dev. Biol.* **14**:399–458.
- Hake, L. E., and J. D. Richter. 1994. CPEB is a specificity factor that mediates cytoplasmic polyadenylation during *Xenopus* oocyte maturation. *Cell* **79**:617–627.
- Jenny, A., H. P. Hauri, and W. Keller. 1994. Characterization of cleavage and polyadenylation specificity factor and cloning of its 100-kilodalton subunit. *Mol. Cell. Biol.* **14**:8183–8190.
- Jenny, A., and W. Keller. 1995. Cloning of cDNAs encoding the 160 kDa subunit of the bovine cleavage and polyadenylation specificity factor. *Nucleic Acids Res.* **23**:2629–2635.
- Jenny, A., L. Minvielle-Sebastia, P. J. Preker, and W. Keller. 1996. Sequence similarity between the 73-kilodalton protein of mammalian CPSF and a subunit of yeast polyadenylation factor I. *Science* **274**:1514–1517.
- Jenny, A., and W. Keller. Unpublished data.
- Keller, W. 1995. No end yet to messenger RNA 3' processing! *Cell* **81**:829–832.
- Keller, W., S. Bienroth, K. M. Lang, and G. Christofori. 1991. Cleavage and polyadenylation factor CPF specifically interacts with the pre-mRNA 3' processing signal AAUAAA. *EMBO J.* **10**:4241–4249.
- Kessler, M. M., J. Zhao, and C. L. Moore. 1996. Purification of the *Saccharomyces cerevisiae* cleavage/polyadenylation factor I. *J. Biol. Chem.* **271**:27167–27175.
- Kunkel, T. A. 1985. Rapid and efficient site-specific mutagenesis without phenotype selection. *Proc. Natl. Acad. Sci. USA* **82**:488–492.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**:680–685.
- Lu, Y., X. Qian, and R. M. Krug. 1994. The influenza virus NS1 protein: a novel inhibitor of pre-mRNA splicing. *Genes Dev.* **8**:1827–1828.
- Manley, J. L. 1995. A complex protein assembly catalyzes polyadenylation of mRNA precursors. *Curr. Opin. Genet. Dev.* **5**:222–228.
- McGrew, L. L., E. Dworkin-Rastl, M. B. Dworkin, and J. D. Richter. 1989. Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. *Genes Dev.* **3**:803–815.
- Murthy, K. G. K., and J. L. Manley. 1992. Characterization of the multisubunit cleavage-polyadenylation factor from calf thymus. *J. Biol. Chem.* **267**:14804–14811.
- Murthy, K. G. K., and J. L. Manley. 1995. The 160-kD subunit of human cleavage-polyadenylation specificity factor coordinates pre-mRNA 3'-end formation. *Genes Dev.* **9**:2672–2683.
- Nebreda, A. R., J. V. Gannon, and T. Hunt. 1995. Newly synthesized protein(s) must associate with p34^{cdc2} to activate MAP kinase and MPF during progesterone-induced maturation of *Xenopus* oocytes. *EMBO J.* **14**:5597–5607.
- Nemeroff, M. E., S. M. L. Barabino, Y. Li, W. Keller, and R. M. Krug. 1998. Influenza virus NS1 protein interacts with the cellular 30 kDa subunit of

- CPSF and inhibits 3' end formation of cellular pre-mRNAs. *Mol. Cell* **1**:991-1000.
33. Paris, J., and J. D. Richter. 1990. Maturation-specific polyadenylation and translational control: diversity of cytoplasmic polyadenylation elements, influence of poly(A) tail size, and formation of stable polyadenylation complexes. *Mol. Cell. Biol.* **10**:5634-5645.
 34. Paris, J., K. Swenson, H. Piwnica-Worms, and J. D. Richter. 1991. Maturation-specific polyadenylation: in vitro activation by p34^{cdc2} and phosphorylation of a 58-kD CPE-binding protein. *Genes Dev.* **5**:1697-1708.
 35. Preker, P. J., M. Ohnacker, L. Minvielle-Sebastia, and W. Keller. 1997. A multisubunit 3' end processing factor from yeast containing poly(A) polymerase and homologues of the subunits of mammalian cleavage and polyadenylation specificity factor. *EMBO J.* **16**:4727-4737.
 36. Qian, X.-Y., F. Alonso-Caplen, and R. M. Krug. 1994. Two functional domains of the influenza virus NS1 protein are required for regulation of nuclear export of mRNA. *J. Virol.* **68**:2433-2441.
 37. Qui, Y., and R. M. Krug. 1994. The influenza virus NS1 protein is a poly(A)-binding protein that inhibits nuclear export of mRNAs containing poly(A). *J. Virol.* **68**:2425-2432.
 38. Richter, J. D. 1996. Dynamics of poly(A) addition and removal during development, p. 481-503. In J. W. B. Hershey, M. B. Mathews, and N. Sonenberg (ed.), *Translational control*. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
 39. Roof, D. M., P. B. Meluh, and M. D. Rose. 1992. Kinesin-related proteins required for assembly of the mitotic spindle. *J. Cell Biol.* **118**:95-108.
 40. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
 41. Stebbins-Boaz, B., L. E. Hake, and J. D. Richter. 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.
 42. Stumpf, G., and H. Domdey. 1996. Dependence of yeast pre-mRNA 3' end processing on CFT-1: a sequence homolog of the mammalian AAUAAA binding factor. *Science* **274**:1517-1520.
 43. Takagaki, Y., L. C. Ryner, and J. L. Manley. 1988. Separation and characterization of a poly(A) polymerase and a cleavage/specificity factor required for pre-mRNA polyadenylation. *Cell* **52**:731-742.
 44. Takagaki, Y., R. L. Seipelt, M. L. Peterson, and J. L. Manley. 1996. The polyadenylation factor CstF-64 regulates alternative processing of IgM heavy chain pre-mRNA during B cell differentiation. *Cell* **87**:941-952.
 45. Verrotti, A. C., S. R. Thompson, C. Wreden, S. Strickland, and M. Wickens. 1996. Evolutionary conservation of sequence elements controlling cytoplasmic polyadenylation. *Proc. Natl. Acad. Sci. USA* **93**:9027-9032.
 46. Wahle, E. 1991. Purification and characterization of a mammalian polyadenylate polymerase involved in the 3' end processing of messenger RNA precursors. *J. Biol. Chem.* **266**:3131-3139.
 47. Wahle, E., and W. Keller. 1992. The biochemistry of 3'-end cleavage and polyadenylation of messenger RNA precursors. *Annu. Rev. Biochem.* **61**:419-440.
 48. Wahle, E., and W. Keller. 1996. The biochemistry of polyadenylation. *Trends Biochem. Sci.* **21**:247-250.
 49. Wahle, E., and U. Kuhn. 1997. The mechanism of 3' cleavage and polyadenylation of eukaryotic pre-mRNA. *Prog. Nucleic Acid Res. Mol. Biol.* **57**:41-71.
 50. Walczak, C. E., T. J. Mitchison, and A. Desai. 1996. XKCM1: a *Xenopus* kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. *Cell* **84**:37-47.
 51. Wickens, M., P. Anderson, and R. Jackson. 1997. Life and death in the cytoplasm: messages from the 3' end. *Curr. Opin. Genet. Dev.* **7**:220-232.
 52. Wickens, M. P., and J. B. Gurdon. 1983. Post-transcriptional processing of simian virus 40 late transcripts in injected frog oocytes. *J. Mol. Biol.* **163**:1-26.
 53. Zhao, J., M. M. Kessler, and C. L. Moore. 1997. Cleavage factor II of *Saccharomyces cerevisiae* contains homologues to subunits of the mammalian cleavage/polyadenylation specificity factor and exhibits sequence-specific, ATP-dependent interaction with precursor RNA. *J. Biol. Chem.* **272**:10831-10838.