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Translational Control of Cyclin B1 mRNA during Meiotic Maturation: Coordinated Repression and Cytoplasmic Polyadenylation

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Translational control is prominent during meiotic maturation and early development. In this report, we investigate a mode of translational repression in *Xenopus laevis* oocytes, focusing on the mRNA encoding cyclin B1. Translation of cyclin B1 mRNA is relatively inactive in the oocyte and increases dramatically during meiotic maturation. We show, by injection of synthetic mRNAs, that the *cis*-acting sequences responsible for repression of cyclin B1 mRNA reside within its 3′UTR. Repression can be saturated by increasing the concentration of reporter mRNA injected, suggesting that the cyclin B1 3′UTR sequences provide a binding site for a *trans*-acting repressor. The sequences that direct repression overlap and include cytoplasmic polyadenylation elements (CPEs), sequences known to promote cytoplasmic polyadenylation. However, the presence of a CPE per se appears insufficient to cause repression, as other mRNAs that contain CPEs are not translationally repressed. We demonstrate that relief of repression and cytoplasmic polyadenylation are intimately linked. Repressing elements do not override the stimulatory effect of a long poly(A) tail, and polyadenylation of cyclin B1 mRNA is required for its translational recruitment. Our results suggest that translational recruitment of endogenous cyclin B1 mRNA is a collaborative effect of derepression and poly(A) addition. We discuss several molecular mechanisms that might underlie this collaboration.

INTRODUCTION

During early development, transcription is relatively quiescent. As a result, changes in the pattern of protein synthesis commonly rely on posttranscriptional regulation. In particular, translational control is critical in a variety of diverse processes such as pattern formation, the determination of cell fates, and cell proliferation (reviewed in Curtis *et al.*, 1995; Wickens *et al.*, 1996; Richter, 1996). Indeed, many of the mRNAs that regulate the meiotic and mitotic cell cycles are under translational control (reviewed in Standart, 1992). These mRNAs include the cyclins, cyclin-dependent kinases, and c-*mos*, as well as enzymes required for DNA replication and chromatin assembly.

The 3′ untranslated region (UTR) is a repository of regulatory information affecting mRNA expression during early development (reviewed in St Johnston and Nüsslein-Volhard, 1992; Curtis *et al.*, 1995; St Johnston, 1995; Wickens *et al.*, 1996; Gray and Wickens, 1998). Both negative and positive elements appear to function as binding sites for *trans*-acting factors, relatively few of which have been identified (reviewed in Gray and Wickens, 1998). Repressor elements have been identified in many mRNAs, including *fem-3*, *tra-2*, and *lin-14* in *Caenorhabditis elegans* and *hunchback* and *nanos* in *Drosophila*. 3′UTRs can also contain positive-acting translational control elements, including cytoplasmic polyadenylation elements (CPEs). CPEs promote regulated extension of preexisting mRNA poly(A) tails in the cytoplasm during development (reviewed in Richter, 1996). Cytoplasmic polyadenylation commonly correlates with translational recruitment: lengthening of the poly(A) tail is associated with translational activation and removal of poly(A) with repression. For several mRNAs, the changes in poly(A)

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length are required for the changes in translational activity (reviewed in Richter, 1996; Gray and Wickens, 1998).

This report focuses on translational regulation of cyclin B1 mRNA by elements in its 3′UTR. We concentrate on the period of meiotic maturation, in which oocytes advance from first to second meiosis in response to progesterone. Several lines of evidence demonstrate that cyclin B1 is critical both during this interval and in the early embryo. Cyclin B1 heterodimerizes with a cyclin-dependent kinase (CDK1) to form maturation-promoting factor (MPF), a complex that drives both meiotic and mitotic cell cycles (Gautier *et al.*, 1990). In *Xenopus laevis*, overexpression of cyclin B1 in oocytes is sufficient to induce meiotic maturation (Pines and Hunt, 1987; Westendorf *et al.*, 1989); elimination of specific cyclin B1 mRNAs from embryos disrupts mitoses after fertilization (Weeks *et al.*, 1991). Cyclin B1 appears to be essential during early development, since mice lacking cyclin B1 die *in utero* (Brandeis *et al.*, 1998).

During meiotic maturation and early cleavage divisions, the translation of several cyclins (Koboyashi *et al.*, 1991) and CDK2 (Stebbins-Boaz and Richter, 1994) increases. In particular, cyclin B1 protein levels rise steadily during this time period (Kobayashi *et al.*, 1991). The increase in cyclin B1 mRNA translation is correlated with lengthening of the mRNA's poly(A) tail, from some 30 nucleotides in the oocyte to approximately 250 after maturation is complete (Sheets *et al.*, 1994). The 3′UTR of cyclin B1 mRNA contains several canonical, U-rich CPEs that promote this cytoplasmic polyadenylation reaction (Sheets *et al.*, 1994; de Moor and Richter, 1997).

We demonstrate that repression of cyclin B1 mRNA prior to maturation is mediated by negative regulatory elements in its 3′UTR. Injection of increasing amounts of reporter mRNA relieves this repression, strongly suggesting these elements function through a *trans*-acting repressor. A sequence including the CPEs of the cyclin B1 3′UTR is responsible for this repression. Although mutations that eliminate the CPEs also abolish repression, the mere presence of a CPE in an RNA appears to be insufficient to cause repression. This suggests that unique elements overlapping the CPEs, or the unique arrangement of the CPEs in the cyclin B1 3′UTR, cause translational repression. Relief of repression and activation of cytoplasmic polyadenylation are intimately linked, leading us to suggest that translational recruitment of cyclin B1 mRNA is a collaborative effect of derepression and poly(A) addition.

MATERIALS AND METHODS

Synthetic RNAs

The luciferase/cyclin B1 mRNAs used in Figs. 1–4 have been described previously (Sheets *et al.*, 1994). The luciferase/polylinker mRNA, used in Fig. 1, is identical to luciferase/X_{114} mRNA (Gillian-Daniel *et al.*, 1998).

Luciferase/cyclin B1-$(A)_0$ and luciferase/cyclin B1-$(A)_{65}$ mRNAs, used in Fig. 7, were transcribed from *Bam*HI-digested pAFB62 and *Spe*I-digested pAFB62, respectively. pAFB62 was constructed as follows. First, a polylinker was placed into the *Bgl*II site behind the $(A)_{65}$ sequence in pAFB6 (Barkoff *et al.*, 1998). This was done by first annealing two oligonucleotides, oAFB39 (5′-gatctcttaagtatatgtacgtaactagtctcgaga-3′) and oAFB40 (5′-gatctctcgagactagttacgtacatatgcttaaga-3′). These were then ligated into *Bgl*II-digested pAFB6. This created pAFB61a. Next, the 1-kb band from *Sca*I/*Stu*I-digested pAFB61a was ligated to the 4-kb band from *Sma*I/*Sca*I-digested pLuc/B1 (Sheets *et al.*, 1994). This created pAFB62. It contains 30 nt between the end of the cyclin B1 3′ UTR and the start of the poly(A) tail and 20 nt between the end of the poly(A) tail and the *Spe*I site used to make the $(A)_{65}$ run-off transcript.

The four luciferase/cyclin B1 variant mRNAs used in Fig. 3 were transcribed from four separate plasmids: Variant 1 was transcribed from pAFB65, Variant 2 was transcribed from pAFB66, Variant 3 was transcribed from pAFB67, and Variant 4 was transcribed from pAFB67. Each plasmid was digested with *Bam*HI prior to transcription. To make these plasmids, pairs of oligonucleotides were ordered with the desired sequences, then annealed and inserted into *Bgl*II/*Bam*HI-digested pLuc/B1 (Sheets *et al.*, 1994). The following oligonucleotides were used in the construction of these plasmids: to make pAFB65, oAFB31 (5′-gatcgcccgctgtgcggtgtttttttaatgttttactggttttaataaagctcattttaacatctag-3′) and oAFB32 (5′-gatcctagatgttaaaatgagctttattaaaaccagaaaacattaaaaaacaccgcacag-cgggc-3′); to make pAFB66, oAFB33 (5′-gatctaaatagtgtattgtgggggccgtggggcagttggggaataaagctcatttttaacatctag-3′) and oAFB34 (5′-gatcctagatgttaaaatgagctttattccccaactgccccacggccccccacaatacatattta-3′); to make pAFB67, oAFB35 (5′-gatctaaatagtgtattgtgtttttaatgttttactggttttccgccctagacggggccacgctag-3′) and oAFB36 (5′-gatcctagcgtggccccgtctagggcggaaaaccagtaaaacattaaaaacacaatacactattta-3′); to make pAFB68, oAFB37 (5′-gatctaaatagtgtattgtgggggggaatgggggactgggggggaataaagctcagggaacatctag-3′) and oAFB38 (5′-gatcctagatgttccccctgagctttattccccccagtcccccattcccccacaatacactattta-3′).

In Fig. 4, luciferase/cyclin A1 and luciferase/cyclin B2 mRNAs were transcribed from *Bam*HI-digested pLuc/A1 and pLuc/B2 (M. Sheets, unpublished). To make these plasmids, an insert containing the last 50 nt of the cyclin A1 and cyclin B2 3′UTRs were generated by annealing two ssDNA oligonucleotides. This insert was then cloned into *Bgl*II/*Bam*HI-digested pT7-Luc[BglII at stop codon] (Gallie, 1991). The following oligonucleotides were used: to make pLuc/A1, o9201-11 (5′-gatctaacttgtgatggtgttaagtgtttttaataaactgactttactcaag-3′) and o9201-12 (5′-gatccttgagtaaagtcagtttattaaaaacacttaacaccatcacaagtta-3′); to make pLuc/B1, o9201-13 (5′-gatctaatgctgtaatgtcagtatgaaataaaacttcacatttttttatttg-3′) and o9201-14, (5′-gatccaaataaaaaatggaagttttatttcatactgacattacagcatta-3′).

Luciferase/c-*mos* mRNA, also used in Fig. 4, was transcribed from *Bam*HI-digested pAFB13. The mRNA contains the luciferase coding region followed by the entire 2-kb 3′UTR from c-*mos*. pAFB13 was made as follows. The c-*mos* 3′UTR was amplified in a standard PCR using pXmos-8 (Sagata *et al.*, 1988) as the template. The primers were up1 (5′-ggtagatctcgtccagaacagggagccaatc-3′) and down1 (5′-ccgttttggatcctctagacaaatcaatttctttattataaaac-3′). The PCR product was then cloned into *Bgl*II/*Bam*HI-digested pT7-Luc[BglII at stop codon] (Gallie, 1991).

Luciferase/L1 and luciferase/L1-CPE mRNAs, also used in Fig. 4, were transcribed from *Afl*II-digested pLuc/L1 and pLuc/L1mut, respectively. These plasmids were made as follows. First, pL1/3Z (Fox *et al.*, 1992) and pL1+CPE/4Z (Verotti *et al.*, 1996) were used in standard PCR to amplify the 3′UTRs of L1 and L1-CPE mRNAs, respectively. The same primers were used in both PCRs. They were usL1 (5′-cgattcccaagctagggaagcagc-3′) and dsL1 (5′-cgggtaccgagctcgaattcc-3′) (S. Thompson, unpublished). The PCR

products were cloned into the *Sma*I site of pLuc/cyclinB1/polylinker. pLuc/cyclinB1/polylinker was made by inserting a multiple cloning site (*Xho*I/*Sma*I/*Spe*I/*Bgl*II) into the *Bgl*II site of pLuc/B1 (Sheets *et al.*, 1994). The multiple cloning insert was created by annealing oligos 9408.01 (5′-gatcctcgagcccgggactagta-3′) and 9408.02 (5′-gatctactagtcccgggctcgag-3′) (J. Coller, unpublished). NS1 and mutant NS1 mRNAs, used in Fig. 8, have been previously described (Qian *et al.*, 1994).

All mRNAs used in this study were transcribed with T7 RNA polymerase. Usually, the transcription protocol was the same as for luciferase mRNAs in Barkoff *et al.* (1998). When the mRNA was to be analyzed following oocyte injection, 100 μCi of [α-^{32}P]UTP rather than 20 μCi was used in the reaction. All mRNAs were purified as described (Barkoff *et al.*, 1998) and resuspended in 88 mM NaCl prior to injection. The integrity of each mRNA was verified by agarose gel electrophoresis prior to injection into oocytes.

Oocyte Injection

X. laevis oocytes were isolated, injected, and incubated as described (Ballantyne *et al.*, 1997). In all experiments, 50 nl of mRNA solution was injected. Progesterone (Sigma) was added to the media to achieve a final concentration of 10 μg/ml, as appropriate. Oocyte maturation was determined by analyzing the oocytes for the presence of a white spot, indicative of germinal vesicle (nuclear) breakdown (GVBD) and completion of first meiosis. In experiments in which samples were taken at the "end of maturation." this was two to three times $GVBD_{50}$ (the time after progesterone addition when half of the oocytes display a white spot). All incubations of oocytes were done at room temperature (20°C). Oocytes from different frogs were used in all experiments, and at least 15 oocytes were used for each data point.

Analysis of Injected RNAs

Isolation and purification of injected mRNA from oocytes were done essentially as described (Verrotti *et al.*, 1996), except that three cells were homogenized together, using 100 μl of homogenization solution per cell. One oocyte equivalent of RNA was loaded on a 0.8% formaldehyde agarose gel. Following electrophoresis, a photo of ethidium bromide-stained rRNAs was taken, then RNA was transferred to nylon membrane. Electrophoresis and transfer of RNA by capillary action to Biotrans nylon membrane (ICN) were performed as described (Sambrook *et al.*, 1989). The mRNAs were radiolabeled in all cases and so were directly visualized using a PhosphorImager (Molecular Dynamics).

Luciferase Assay

Oocytes were pooled into groups of five and homogenized in 200–1000 μl 1× Cell Lysis Buffer (Promega). The homogenate was next centrifuged for 10 min at 12,000*g* at 4°C and the clear supernatant isolated. The lysate was brought to room temperature before being assayed. The clear lysate (2.5–50 μl) was assayed. The reaction was initiated by adding 100 μl Luciferase Assay Reagent (Promega). Photons were counted with a Monolight 2010 luminometer (Analytical Luminescence Laboratory). Average luciferase activity values were calculated for each type of sample, from at least three pools of five cells.

Polysome Analysis

Pools of 20 oocytes were homogenized in 750 μl of ice-cold buffer PB [0.3 M KCl, 2 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.5), 0.05% deoxycholate] containing 0.15 μg/μl cycloheximide, 2 mM DTT, 0.5 μg/μl heparin, and 0.25 μg/μl RNasin. Samples were centrifuged at 4°C for 10 min at 11,750*g*, and 500 μl of the clarified cytosol was removed and loaded onto an 11-ml gradient (50–10%) in buffer PB containing 0.15 μg/μl cycloheximide, 2 mM DTT, 0.5 μg/μl heparin, and 0.12 μg/μl RNasin. Gradients were centrifuged at 4°C in a Beckman SW 41 rotor at 39,000 rpm for 2 h and were fractionated as previously described (Gray *et al.*, 1994). RNA was extracted from the fractions using TRI Reagent according to the manufacturer's instructions. RNAs were analyzed using Northern blotting and the B1 mRNA was detected using a PCR-derived probe synthesized from plasmid XlB1/GEM (Minshall *et al.*, 1989) using an oligonucleotide containing the T7 RNA polymerase primer and an oligo of the sequence 5′gcatacttgttcttaacagtc 3′.

RESULTS

The Cyclin B1 3′UTR Causes Translational Repression

Sequences in the 3′UTR of cyclin B1 mRNA stimulate its translation during progesterone-induced meiotic maturation by promoting cytoplasmic polyadenylation (Sheets *et al.*, 1994). Thus translation of a luciferase reporter mRNA carrying a 58-nt portion of the cyclin B1 3′UTR increases after progesterone treatment. Surprisingly, we found that the magnitude of the translational increase, measured as the ratio of luciferase activity with and without progesterone, was elevated when lower mRNA concentrations were injected (see below) compared to previous studies (Sheets *et al.*, 1994). In principle, the elevation could be due either to a decrease in the level of translation in the absence of progesterone or to a greater increase in the level of translation after progesterone was added.

To determine whether the 58-nt portion of the cyclin B1 3′UTR contains sequences that can direct translational repression prior to meiotic maturation, we compared the translation of this mRNA to that of a control construct containing polylinker sequence fused downstream of the luciferase coding sequence. mRNAs encoding these constructs were injected into stage VI oocytes at concentrations (0.1 fmol per oocyte) comparable to that of endogenous B1 mRNA (Kobayashi *et al.*, 1991; data not shown). Expression of the luciferase/cyclin B1 reporter mRNA was reduced sevenfold compared to that of the control mRNA (Fig. 1A). The decrease in luciferase activity was not due to differential stability of the reporter mRNAs, as assessed by gel electrophoresis and autoradiography (Fig. 1B). We conclude that the cyclin B1 3′UTR contains sequences that cause translational repression in oocytes.

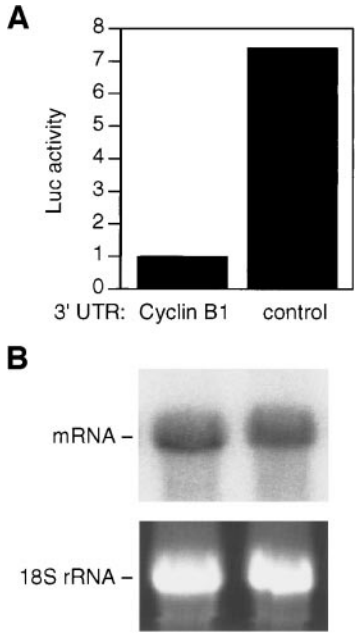

FIG. 1. The cyclin B1 3′UTR causes translational repression. Oocytes were injected with 0.1 fmol/cell of radiolabeled luciferase reporter mRNAs, an amount equal to the amount of endogenous cyclin B1 mRNA (Kobayashi *et al.*, 1991). Following incubation for 4 h, oocytes were homogenized and prepared for either luciferase assays or recovery of RNA. The two mRNAs used differ only in the 3′UTR. One mRNA contains the cyclin B1 3′UTR and the other contains an equivalent length of polylinker as its 3′UTR. The experiment was repeated four times, and a representative experiment is shown. (A) Histogram of luciferase activity, normalized to the luciferase activity directed by luciferase/cyclin B1 mRNA. (B) PhosphorImager scan of a denaturing agarose gel, showing the radiolabeled reporter mRNAs following isolation from oocytes. Ethidium-stained 18S ribosomal RNA, providing a control for RNA loading, is shown below.

A Titratable Factor Is Responsible for Translational Repression of Cyclin B1

To determine whether the factor(s) responsible for 3′UTR-mediated repression could be titrated, we injected different concentrations of the luciferase/cyclin B1 reporter mRNA into oocytes. Concentrations spanning a range of three orders of magnitude were tested. Translational efficiency was calculated as the quantity of luciferase activity produced per femtomole of injected mRNA (Fig. 2A). At low concentrations of mRNA (0.01 to 0.1 fmol), the translational efficiency of the mRNA carrying the cyclin B1 3′UTR was low and nearly constant (Fig. 2A). However, as higher concentrations (0.5 to 10 fmol per cell) were injected, its translational efficiency increased progressively (Fig. 2A). In contrast, the control mRNA carrying an artificial sequence as its 3′UTR displayed no significant change in translational efficiency from 0.1 to 10 fmol injected mRNA (Fig. 2B). The shape of the curve in Fig. 2A suggests that the cyclin B1 3′UTR mediates repression at low concentrations, which is progressively relieved beyond a threshold concentration of approximately 0.1 fmol per oocyte. For simplicity, we refer to this form of repression as "low-dose repression." The magnitude of low-dose repression cannot be rigorously calculated from a concentration curve with a single mRNA (e.g., Fig. 2A) since translational activity continues to rise even at the highest doses tested (i.e., the putative repressor has not been fully titrated). However, comparison of mRNAs with cyclin B1 or control 3′UTRs suggests that the magnitude of repression is at least 10-fold (Fig. 2A vs 2B; see also Figs. 3 and 4).

From our results we infer that at least one factor required for low-dose repression can be saturated. In one simple model, a titratable *trans*-acting factor causes repression of the reporter mRNA. At low concentrations of an mRNA carrying the cyclin B1 3′UTR, essentially all of the mRNA is repressed. At higher concentrations, the factor is titrated, and more of the mRNA is translated.

Relief of Low-Dose Repression during Maturation

To examine whether repression persists during meiotic maturation, we injected the same range of mRNA concentrations into oocytes, then induced meiotic maturation by adding progesterone. After maturation was complete, we assayed luciferase activity and again determined translational efficiency. Translational efficiency was higher in progesterone-treated oocytes (note the difference in the scale of *y* axes in Figs. 2A and 2C) and did not change significantly as a function of concentration (Fig. 2C). Translational efficiency was also unaltered for the control mRNA in both oocytes and matured oocytes (Fig. 2B vs 2D). However, the control mRNA was translated less efficiently than the luciferase/cyclin B1 mRNA following maturation, presumably due to polyadenylation of luciferase/cyclin B1 mRNA (see below). The data in Fig. 2 demonstrate that the apparent magnitude of translational regulation during meiotic maturation varies with the concentration of mRNA injected; in this particular experiment, it ranged from 50- to 10-fold, between 0.01 and 10 fmol mRNA, respectively.

We conclude that low-dose repression mediated by the cyclin B1 3′UTR is relieved during meiotic maturation. A simple model follows that the *trans*-acting factors responsible for low-dose repression in oocytes are inactivated during that period.

CPEs Are Required for Translational Repression

To identify the specific sequences within the 58-nt region of the cyclin B1 3′UTR that cause low-dose repression, we constructed variants of the luciferase/cyclin B1 reporter mRNA (Fig. 3A). Variants 1 to 3 contain clusters of transversion mutations within the 58-nt cyclin B1 3′UTR and collectively alter every nucleotide (Fig. 3A). All mRNAs were injected at identical, low concentrations (0.1 fmol per

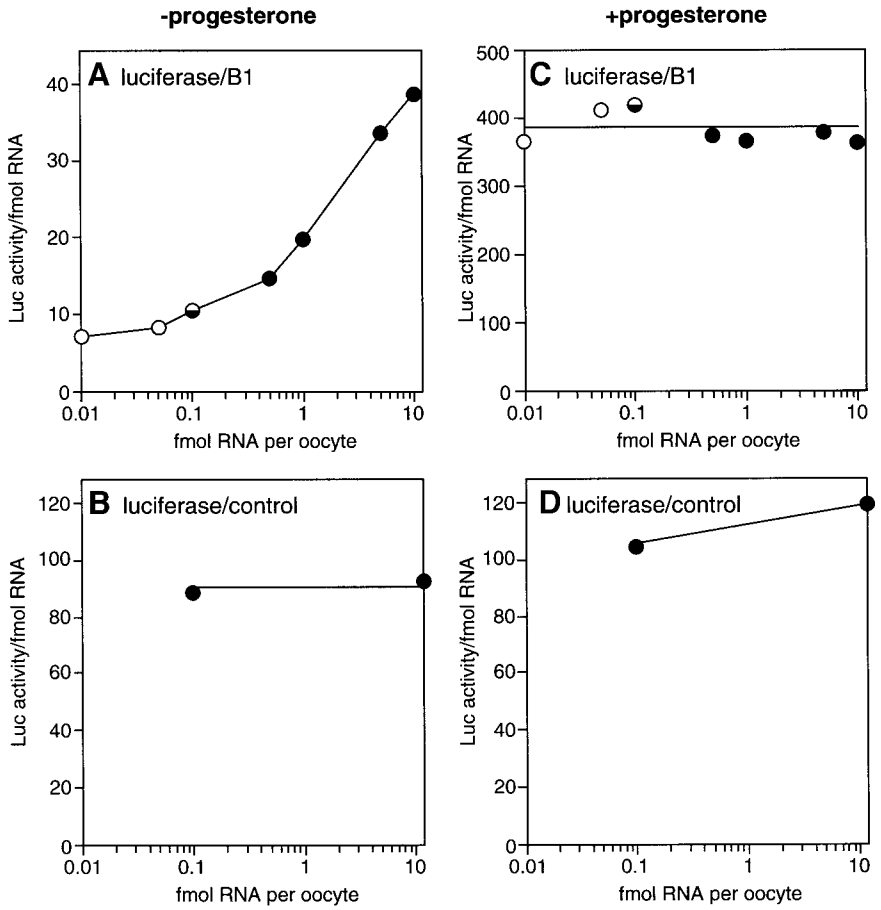

FIG. 2. A titratable factor is responsible for translational repression of cyclin B1. Oocytes were injected with seven different concentrations of luciferase/cyclin B1 reporter mRNA (0.01, 0.05, 0.1, 0.5, 1.0, 5.0, or 10.0 fmol/cell; A and C) or with two different concentrations of luciferase/polylinker reporter mRNA (0.1 or 10 fmol/cell; B and D). Half of each batch of oocytes was treated with progesterone. Oocytes were collected at the end of maturation. This experiment was repeated five times. A representative experiment is shown. Each plot graphs "translational efficiency," defined as luciferase activity per femtomole of mRNA injected, versus the amount of mRNA injected (fmol). In A and C, results of experiments using oocytes from two different frogs are shown in the same graph, indicated by differently shaded symbols. Luciferase activity from cells injected with 0.1 fmol RNA/cell was the same in the two experiments and is shown as a hybrid symbol. (A) Luciferase/cyclin B1 mRNA injected, no progesterone; (B) luciferase/control mRNA injected, no progesterone; (C) luciferase/cyclin B1 mRNA injected, progesterone added; (D) luciferase/control mRNA injected, progesterone added.

oocyte) and luciferase activity was assayed (Fig. 3B). At this low dose, the mRNA carrying the wild-type 3′UTR sequence (WT) yielded little luciferase activity. Variant 1, mutated in the first 16 nt of the cyclin B1 reporter 3′UTR, was also repressed. However, Variant 2, carrying mutations in the central 22 nt of the sequence, and Variant 3, in which the last 20 nt had been replaced, were both derepressed in the oocyte: Variant 2 by sevenfold and Variant 3 by threefold. The increases in luciferase activity observed with Variants 2 and 3 appear to be due to disruption of low-dose repression since all mRNAs yielded similar luciferase activities when injected at a high dose (data not shown).

The sequences altered in Variants 2 and 3 contain multiple U-rich sequences that can stimulate cytoplasmic polyadenylation (CPEs; Fig. 3A; Stebbins-Boaz *et al.*, 1996; M. Sheets and M. Wickens, unpublished results). To test whether the CPEs were required for translational repression, we constructed Variant 4, in which all uridines in a tract of four or more consecutive nucleotides were changed to guanosines. At a low dose, Variant 4 was translated 10-fold more efficiently than the wild-type reporter mRNA, demonstrating both that the U-rich tracts are required for repression and that the repressive effect of U-rich sequences in the second and third blocks is additive. At a high dose, Variant 4 displayed the same efficiency as the unmodified 3′UTR sequence, demonstrating that the sequence alterations affect low-dose repression and not basal translational activity (data not shown). Similarly, the five mRNAs tested

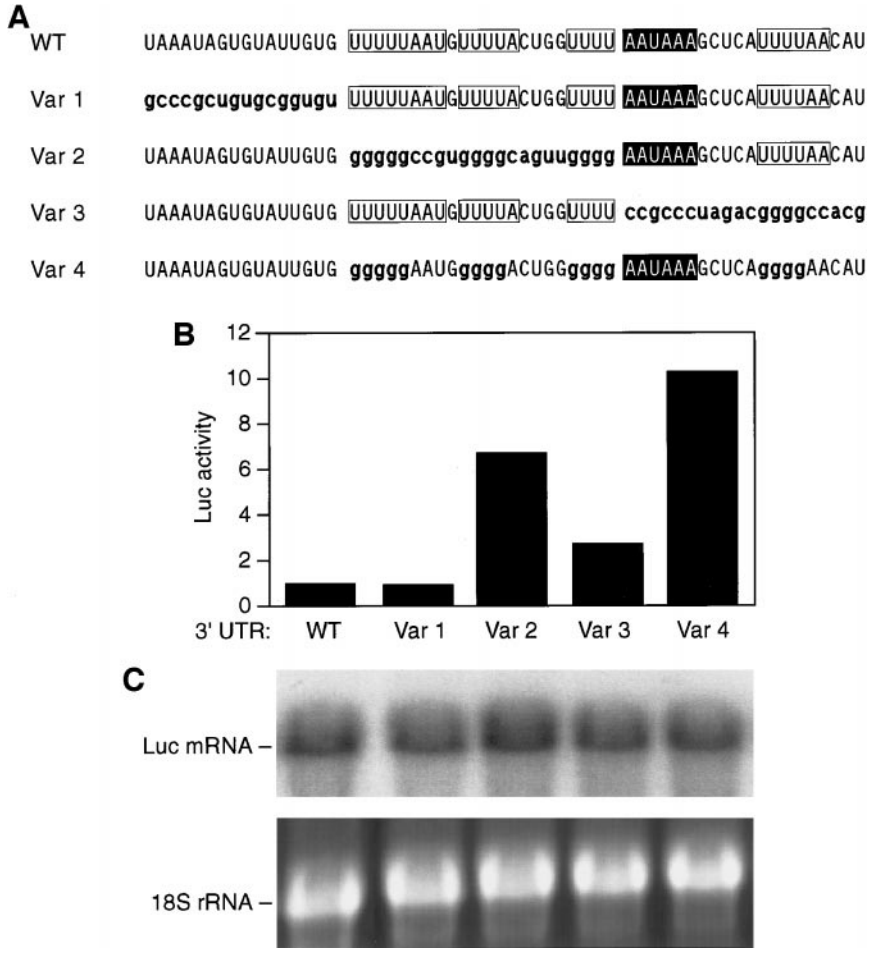

FIG. 3. CPEs are required for translational repression. Oocytes were injected with 0.1 fmol/cell radiolabeled luciferase reporter mRNA. Oocytes were collected for luciferase assays and RNA analysis following 4 h of incubation. The experiment was repeated three times, and a representative experiment is shown. (A) Sequences of the 3′UTRs of the reporter mRNAs. Polyadenylation signals (AAUAAA) are enclosed in a black box; putative cytoplasmic polyadenylation elements are in white boxes; nucleotides that have been changed from the wild-type sequence are shown in lowercase letters. The wild-type sequence of the cyclin B1 3′UTR in the luciferase/cyclin B1 reporter mRNA used throughout this paper is shown at the top (WT). Sequences of the 3′UTRs in the mutant luciferase/cyclin B1 reporters are shown below (Var 1–4). (B) Histogram of luciferase activities produced from the constant amount of mRNA injected, normalized to the luciferase activity directed by luciferase/cyclin B1 (wild type) mRNA. (C) PhosphorImager scan of denaturing agarose gel, showing the reporter mRNAs following isolation from oocytes. Ethidium-stained 18S ribosomal RNA is shown in the bottom.

were equally stable and were present at the same concentrations in the oocyte (Fig. 3C), thus the differences observed (Fig. 3B) reflect changes in relative translational activity. We conclude that the sequences required for repression overlap with CPEs.

Other mRNAs Containing CPEs Are Not Translationally Repressed

To determine whether the presence of a CPE is sufficient to cause translational repression, several other reporter mRNAs carrying 3′UTRs that promote cytoplasmic polyadenylation were constructed. In particular, we prepared luciferase mRNAs with the 3′UTRs of *X. laevis* cyclin A1, cyclin B2, and c-*mos* (Fig. 4A). Previous work established that each of these mRNAs supports cytoplasmic polyadenylation and identified the U-rich sequences as being responsible for this activation (Sheets *et al.*, 1994; Fox *et al.*, 1989; McGrew *et al.*, 1989). In addition, we analyzed both the ribosomal protein L1 3′UTR, which lacks a CPE, and a variant of the L1 3′UTR (L1+CPE), into which UUUUUUAU (a canonical CPE) had been inserted. The L1+CPE 3′UTR receives poly(A) during maturation, while the wt L1 3′UTR does not (Verrotti *et al.*, 1996). Each mRNA was injected into resting oocytes at a low dose (0.1 fmol per cell), and luciferase activity was determined.

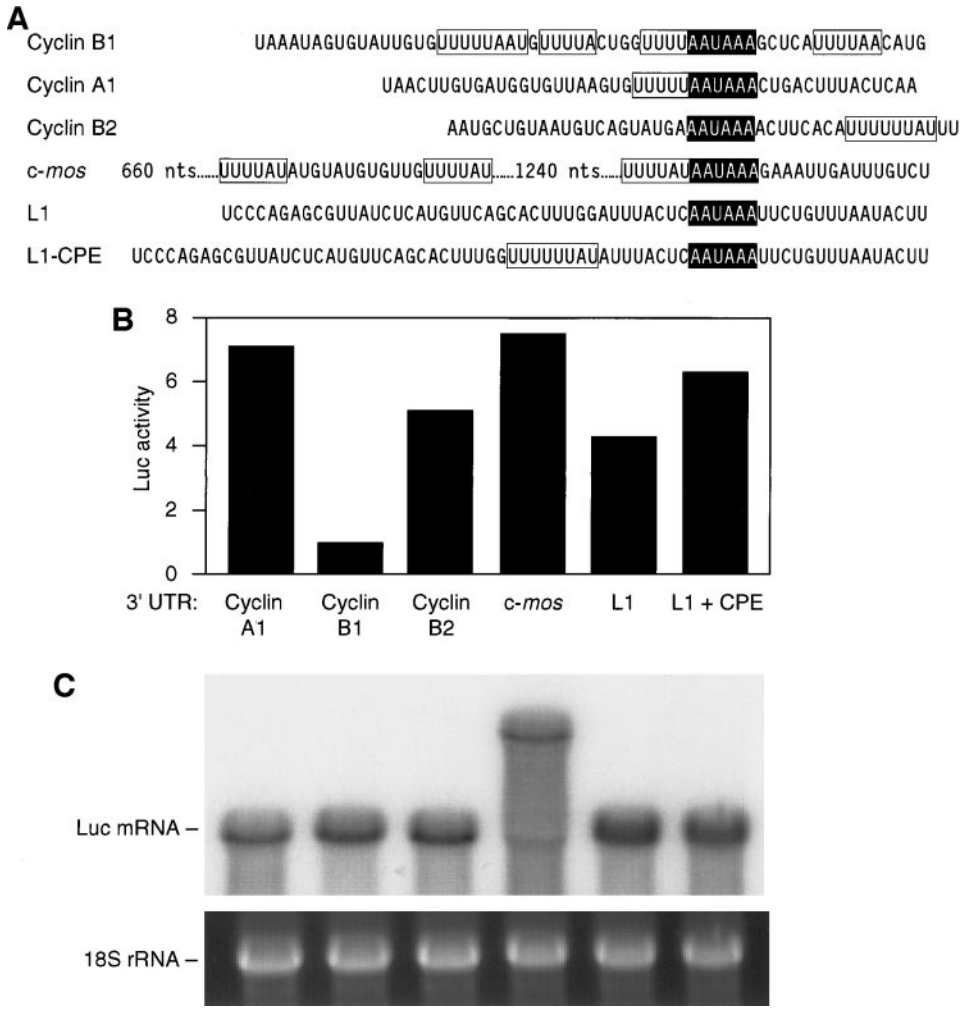

FIG. 4. Other mRNAs containing CPEs are not translationally repressed. Oocytes were injected with 0.1 fmol/cell radiolabeled luciferase reporter mRNA. Oocytes were collected for luciferase assays and RNA analysis following 4 h of incubation. The experiment was repeated twice, and a representative experiment is shown. (A) Sequences of the 3′UTRs of the reporter mRNAs are shown. The polyadenylation signal (AAUAAA) is enclosed in a black box; putative cytoplasmic polyadenylation elements are in white boxes. (B) Histogram of luciferase activities produced from the constant low (0.1 fmol) amount of mRNA injected, normalized to the luciferase activity directed by luciferase/cyclin B1 (wild type) mRNA. (C) PhosphorImager scan of a denaturing agarose gel, showing the reporter mRNAs following isolation from oocytes. Ethidium-stained 18S ribosomal RNA, providing a control for RNA loading, is shown at the bottom.

At low doses the luciferase/cyclin B1 reporter mRNA was repressed (Fig. 4B). No other mRNA was comparably repressed. Rather, the other mRNAs yielded luciferase activities 4.5- to 7.5-fold higher than that of the cyclin B1 chimera (Fig. 4B). Translation of the mRNA carrying the L1+CPE 3′UTR was actually higher than that of the same mRNA lacking the CPE (Fig. 4B). The translational efficiency of only the luciferase/cyclin B1 mRNA was altered at high doses (10 fmol per cell; data not shown). Differences were not due to differential stability of the transcripts (Fig. 4C). We conclude that the mere presence of a CPE is insufficient to cause translational repression. Instead, some facet of the specific sequence within the cyclin B1 3′UTR, that overlaps with or encompasses its CPEs, is responsible for translational repression.

Derepression of Cyclin B1 Occurs after GVBD

To systematically evaluate when repression directed by the cyclin B1 3′UTR was relieved, we injected the luciferase/cyclin B1 reporter mRNA into oocytes at low concentrations, induced meiotic maturation, and collected oocytes at various times throughout meiotic maturation. Translation increased substantially only after GVBD occurred (Fig. 5). Similarly, cytoplasmic polyadenylation of cyclin B1 mRNA occurs at or shortly after GVBD (Ballan-

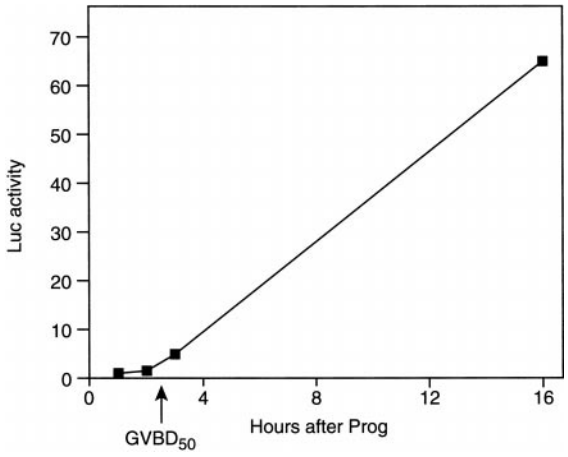

FIG. 5. Derepression of cyclin B1 occurs after GVBD. Oocytes were injected with 0.1 fmol/cell luciferase/cyclin B1 mRNA. All oocytes were then treated with progesterone to induce meiotic maturation. Oocytes were collected and frozen at 1, 2, 3, and 16 h after progesterone application. In this experiment, $GVBD_{50}$ occurred at 2.5 h. At 2 h all oocytes were still immature, and at 3 h all oocytes had undergone GVBD. Luciferase activity is shown relative to that in the 1-h time point sample. This experiment was repeated twice, and a representative experiment is shown.

tyne *et al.*, 1997). These data are consistent with models in which relief of low-dose repression requires GVBD and/or poly(A) addition.

Cytoplasmic Polyadenylation Is Required for Derepression

To test whether cytoplasmic polyadenylation is required to relieve repression by the cyclin B1 3′UTR, we injected a luciferase/cyclin B1 reporter mRNA containing a point mutation in AAUAAA (AAgAAA). This mutation prevents cytoplasmic polyadenylation (Fox *et al.*, 1989). If relief from repression were independent of polyadenylation, then an mRNA carrying this mutation would still show increased translational activity during maturation.

In resting oocytes, as expected, the mutant reporter mRNA was translationally repressed, yielding a level of luciferase comparable to that of an mRNA carrying a wild-type cyclin B1 3′UTR (Fig. 6A, Mut vs WT, −progesterone). Additionally, a dose–response curve obtained using this mRNA demonstrated low-dose repression (data not shown). These data demonstrate that AAUAAA is not required for repression. Upon induction of meiotic maturation, translation of the mutant reporter mRNA did not change significantly, indicating that repression was not relieved (Fig. 6A, Mut, +progesterone). In contrast, the activity of the wild-type reporter was stimulated more than 60-fold (Fig. 6A, WT, +progesterone). Both mRNAs were stable throughout meiotic maturation (Fig. 6B), indicating that the difference in luciferase expression was due to a difference in translation and not mRNA stability.

Polyadenylation Confers Resistance to Translational Repression

To determine the effect of a preexisting poly(A) tail on translational repression, we constructed a luciferase/cyclin B1 reporter mRNA with a poly(A) tail of 65 adenosines following its 3′UTR. This mRNA was injected into oocytes at three different concentrations. For comparison, a nonadenylated luciferase/cyclin B1 reporter mRNA was injected into oocytes at equal concentrations. Consistent with the known stimulatory effects of poly(A) on translation (reviewed in Richter, 1996; Gray and Wickens, 1998), the polyadenylated reporter mRNA was translated more efficiently than the nonadenylated mRNA, as assessed by luciferase activity. Importantly, the polyadenylated mRNA was translated with comparable efficiency (luciferase activity per fmol of RNA injected) from 0.01 to 10 fmol of mRNA injected (Fig. 7). Thus the stimulatory effects of the poly(A) tail were not significantly abrogated by repression.

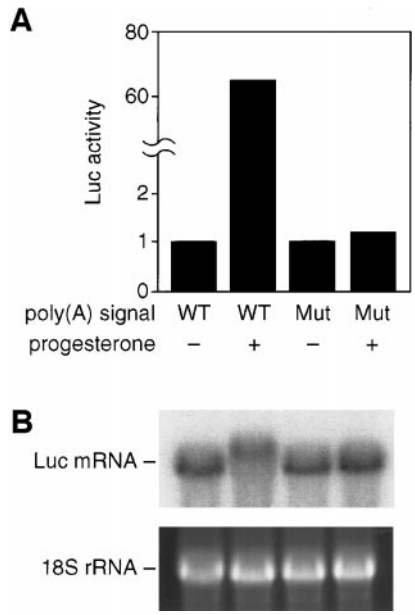

FIG. 6. Cytoplasmic polyadenylation is required for derepression. Oocytes were injected with 0.1 fmol/cell radiolabeled luciferase reporter mRNA. Half of each batch of oocytes was treated with progesterone to induce meiotic maturation. At the end of maturation oocytes were collected for either luciferase assays or RNA analysis. The two reporter mRNAs differ only in one nucleotide, the third nucleotide of the hexanucleotide polyadenylation signal. "WT" has AAUAAA whereas "Mut" has AAgAAA. The experiment was repeated three times, and a representative experiment is shown. (A) Histogram of luciferase activities produced from the constant amount of mRNA injected, normalized to the luciferase activity directed by luciferase/cyclin B1 mRNA. (B) PhosphorImager scan of a denaturing agarose gel, showing the reporter mRNAs following isolation from oocytes. Ethidium-stained 18S ribosomal RNA, providing a control for RNA loading, is shown at the bottom.

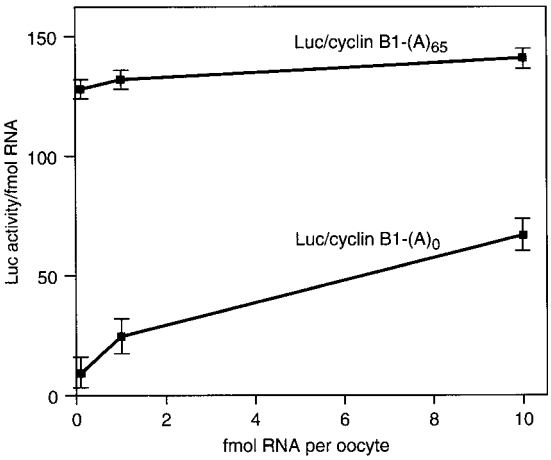

FIG. 7. Polyadenylation confers resistance to translational repression. Oocytes were injected with three different concentrations of either luciferase/cyclin B1-$(A)_0$ reporter mRNA (bottom trace) or luciferase/cyclin B1-$(A)_{65}$ reporter mRNA (top trace). The three concentrations were 0.1, 1.0, and 10.0 fmol/cell. Following incubation for 4 h oocytes were collected for luciferase assays. The experiment was repeated twice, and the results obtained were averaged. The average translational efficiency (luciferase activity/fmol RNA) as a function of amount of RNA injected is displayed.

Cytoplasmic Polyadenylation Is Required for the Translational Activation of Endogenous B1 mRNA

The previous experiments, using reporter mRNAs, suggest that translational activation of cyclin B1 mRNA is dependent upon cytoplasmic polyadenylation. To determine whether the activation of endogenous cyclin B1 mRNA during meiotic maturation was polyadenylation-dependent, we inhibited polyadenylation using the influenza virus protein, NS1. NS1 interacts with the 30-kDa subunit of cleavage and polyadenylation specificity factor (CPSF; Nemeroff *et al.*, 1998). Overexpression of NS1 in the oocyte prevents cytoplasmic polyadenylation (Dickson *et al.*, 1999) and so provides a specific reagent with which to block polyadenylation *in vivo*.

mRNAs encoding NS1 and a mutant form of NS1 ($NS1^{mut}$), which does not interact with CPSF (Qian *et al.*, 1994), were prepared by *in vitro* transcription and injected into oocytes. After overnight incubation to permit protein production, meiotic maturation was induced in both uninjected and NS1-expressing cells. To assess the effect of NS1 on polyadenylation of endogenous cyclin B1 mRNA, total RNA was isolated and the length of cyclin B1 mRNA analyzed by Northern blotting. Cyclin B1 mRNA normally receives approximately 200 As during meiotic maturation, resulting in a detectable change in its electrophoretic mobility (Fig. 8A, lane 2 vs lane 1). The addition of a long poly(A) tail is prevented by expression of NS1 (Fig. 8A, lane 4 vs lane 3), but not by the mutant form of NS1 that does not interact with CPSF (Fig. 8A, lane 5 vs lane 6).

The effect of NS1 on the translation of cyclin B1 mRNA was assessed by sedimentation analysis of polyribosomes. In matured oocytes lacking NS1 protein, a fraction of cyclin B1 mRNA is associated with polyribosomes and distributed through the faster sedimenting region of the gradient (Fig. 8B(1); the positions of 80S subunits and polyribosomes are indicated in the absorbance trace at the bottom). Only a minority of cyclin B1 mRNA associates with polysomes after maturation, as observed with other endogenous mRNAs (e.g., Tafuri and Wolffe, 1993). Expression of NS1 protein abrogates the apparent recruitment of the mRNA onto large polyribosomes (Fig. 8B(2)).

A series of control experiments was performed to test the specificity of both the sedimentation assay and the inhibitory effect of NS1. In these studies, the gradients were separated into fewer fractions (Fig. 8C). The data demonstrate that the faster sedimentation of cyclin B1 mRNA requires maturation, as it was observed only after progesterone treatment (Fig. 8C(1) vs 8C(2)). EDTA treatment of extracts of oocytes that had undergone maturation abolished the rapid sedimentation, consistent with dissociation of polysomes by this agent (data not shown). Importantly, while expression of NS1 protein prevented cyclin B1 mRNA from loading onto large polyribosomes during maturation (Fig. 8C(3) vs 8C(2)), expression of $NS1^{mut}$ did not (Fig. 8C(4) vs 8C(2)). We infer that full translational recruitment of cyclin B1 mRNA during meiotic maturation is dependent upon addition of poly(A).

In principle, inhibition of cyclin B1 translation by NS1 could be indirect. For example, translation of another mRNA that is NS1-sensitive might be required to recruit cyclin B1 mRNA. However, several lines of evidence suggest that the NS1 effect on cyclin B1 is direct. First, reporter mRNA experiments suggest cytoplasmic polyadenylation is required for derepression (Figs. 6 and 7). Second, cleavage of the poly(A) tail from endogenous cyclin B1 mRNA, using oligonucleotides directed at the 3′UTR, prevents translational recruitment of cyclin B1 mRNA (N.K.G. and M.W., unpublished).

DISCUSSION

MPF activity is tightly regulated to control the meiotic cell cycle (reviewed in Lohka, 1998). It is therefore not surprising that cyclin B1 protein, a component of MPF, is stringently controlled at multiple levels. The importance of repressing cyclin B1 activity in resting oocytes is illustrated by the observation that injection of high concentrations of cyclin B1 mRNA induces precocious meiotic maturation (Freeman *et al.*, 1991; Pines and Hunt, 1987; Westendorf *et al.*, 1989).

Our results suggest that full translational control of cyclin B1 mRNA is achieved through two separate but related mechanisms: translational repression and cytoplasmic polyadenylation. We draw the following main conclu-

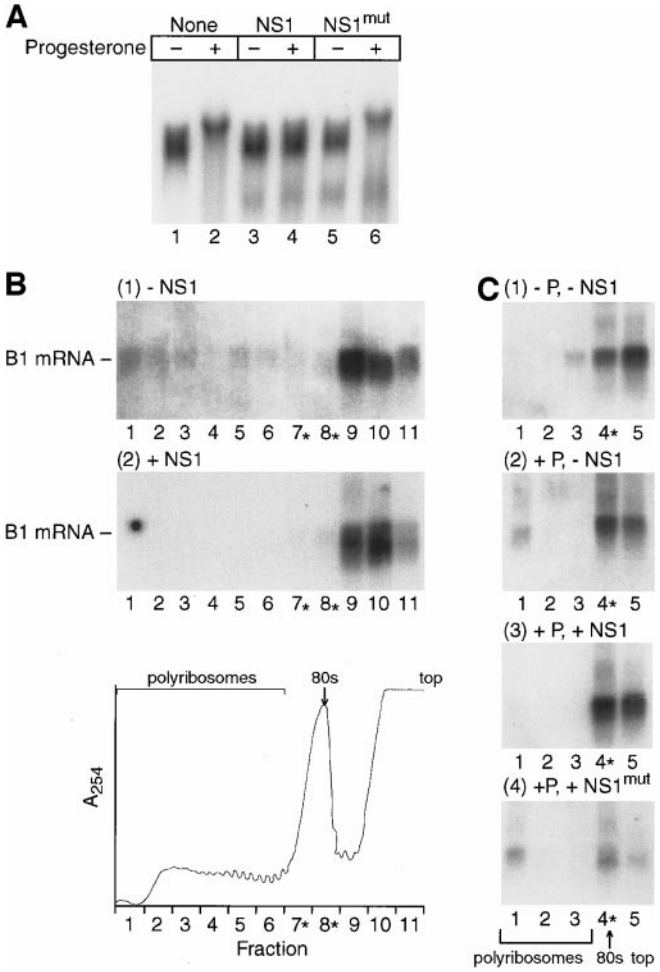

FIG. 8. Cytoplasmic polyadenylation of endogenous B1 mRNA is required for translational stimulation. Oocytes were injected with NS1 mRNAs and incubated overnight to allow protein production. Uninjected and NS1-expressing cells were treated with progesterone to induce meiotic maturation. At the end of meiotic maturation oocytes were collected. (A) Total RNA was isolated from uninjected cells (lanes 1 and 2), cells injected with NS1 mRNA (lanes 3 and 4), and cells injected with NS1^{mut} mRNA (lanes 5 and 6). Cells were either treated with progesterone (lanes 2, 4, and 6) or left untreated (lanes 1, 3, and 5). RNAs were separated on a 0.8% agarose gel and transferred to membrane. Cyclin B1 mRNA was visualized by Northern blotting using a cDNA probe against the cyclin B1 message. A decrease in the mobility of the cyclin B1 mRNA was indicative of cytoplasmic poly(A) addition. (B) Oocytes were injected with mRNA encoding NS1 or were not injected. (1) Not injected; (2) injected with NS1 mRNA. After addition of progesterone and maturation, extracts were prepared and subjected to sucrose gradient density centrifugation. RNA was extracted from the fractions and analyzed by Northern blotting, as above. The A_{254} profile is shown at the bottom of (B); sedimentation was from right ("top") to left. As indicated in the bottom panel of (B), polyribosomes were distributed in fractions 1–6, the monosomal ("80S") peak was located in fractions 7 and 8 (indicated with an asterisk), and the untranslated mRNPs were located in fractions 9–11. (C) Oocytes were injected with mRNAs encoding NS1 or NS1^{mut} or were not injected, as indicated above each blot. Progesterone was then added or omitted, as indicated. (1) No progesterone added, no mRNA injected; (2) progesterone added, no mRNA injected; (3) progesterone added, NS1 mRNA injected; (4) progesterone added, NS1^{mut} mRNA injected. The remainder of the analysis was performed as described in (B), except that fewer fractions were collected. As indicated, polyribosomes were in fractions 1–3, the monosomal peak was located in fraction 4 (indicated with an asterisk), and the untranslated mRNPs were located in fraction 5.

sions. Translational repression of cyclin B1 mRNA in the oocyte is due to specific sequences that overlap with the CPE. Relief of repression during meiotic maturation is mediated by the cyclin B1 3′UTR and requires polyadenylation. The presence of a long poly(A) tail enhances translation of cyclin B1 mRNA in an oocyte, demonstrating that repression does not significantly interfere with the stimulatory effect of poly(A). In one simple hypothesis, a *trans*-acting repressor binds to the repressive sequences in resting oocytes and is overridden by a polyadenylation-dependent mechanism during maturation.

Translational Repression by the Cyclin B1 3′UTR: Not All CPEs Are Equal

Translational repression of cyclin B1 mRNA is mediated by sequences in the last 58 nt of the cyclin B1 3′UTR. To detect repression, it was critical that the amount of mRNA injected be comparable to that present in the cell: higher concentrations overwhelmed the repression mechanism. In contrast, these higher concentrations do not saturate the cytoplasmic polyadenylation machinery nor its positive effects on translation during maturation (e.g., Sheets *et al.*, 1994). Nevertheless, our results emphasize the importance of RNA concentration in attempting to reconstitute regulation *in vivo*.

The cyclin B1 sequences required for translational repression consist, at least in part, of several oligo(U) tracts. These same oligo(U) tracts promote cytoplasmic polyadenylation (Stebbins-Boaz *et al.*, 1996; M. Sheets and M.W., unpublished). Thus the elements required for repression overlap with those required for its activation. The first suggestion that the same control element in a 3′UTR could be both positive and negative arose in studies of mouse tPA mRNA, in which elements that cause poly(A) removal prior to maturation overlap with those that cause poly(A) addition once maturation has begun ("ACE" elements; Salles *et al.*, 1992). Our results are generally consistent with this study and recent work on cyclin B1 (de Moor and Richter, 1999) and other (Stutz *et al.*, 1998; Minshall *et al.*, 1999; Ralle *et al.*, 1999) mRNAs. In particular, our results demonstrate that a single, small region of the cyclin B1 3′UTR can both activate and repress translation. Additionally, our results demonstrate that multiple sequences within the cyclin B1 3′UTR, which contain CPEs, repress translation and act

independently. This follows from the finding that mutations upstream and downstream of the AAUAAA have additive effects on repression (Fig. 3). These findings are consistent with the fact that multiple copies of the cyclin B1 CPE motif repress more efficiently than does a single copy (de Moor and Richter, 1999).

Our data support a model in which CPEs contribute to and are required for repression, but are insufficient in and of themselves. This conclusion is based on the finding that several 3′UTRs that mediate polyadenylation, and thus contain demonstrable CPEs, fail to repress the mRNAs in which they reside. The data are consistent with two interpretations. First, the putative *trans*-acting repressor may not recognize the CPE per se, but sequences that overlap with that element. Alternatively, qualitative differences in the arrangement or context of the cyclin B1 CPEs might cause that 3′UTR to have a repressive character. Mutational analysis of the cyclin B1 CPEs suggests that CPEs are required for repression and that mutations in CPEs affect repression and polyadenylation comparably. This implies either competition for this site or utilization of a common RNA binding component (de Moor and Richter, 1999). Individual CPE sequences inserted into a neutral RNA backbone can function in repression, arguing that, at least in some contexts, CPEs not only are required but also are sufficient for repression (de Moor and Richter, 1999).

An RNA-binding protein of the RRM family, CPEB, binds to CPEs and is required for cytoplasmic polyadenylation (Hake and Richter, 1994, 1998; Stebbins-Boaz *et al.*, 1996). A CPEB homologue in clams appears to cause translational repression of CPE-containing mRNAs (Minshall *et al.*, 1999; Walker, 1999). To try to determine whether *X. laevis* CPEB was sufficient to cause translational repression, we overexpressed the protein in resting oocytes. We did not observe significant repression at higher amounts of reporter mRNA injected, as would be predicted if CPEB were solely responsible for this effect (data not shown). Consistent with this observation, the CPEs of c-*mos*, A1, and B2 mRNAs bind to CPEB (Stebbins-Boaz *et al.*, 1996) and promote polyadenylation, but do not repress translation (Fig. 7). It is therefore unclear whether mere binding of CPEB is sufficient to cause repression. Repression could be mediated by CPEB, but only when bound in the distinctive arrangement found in the cyclin B1 3′UTR. This could be related to the cyclin B1 3′UTR's ability to bind multiple, adjacent CPEB molecules and its particularly high polyadenylation activity (Sheets *et al.*, 1994; Hake *et al.*, 1998; Stebbins-Boaz *et al.*, 1998). Alternatively, repression may require other *trans*-acting factors, acting either alone or in combination with CPEB.

Recent studies of a *X. laevis* lamin mRNA identified two new proteins that interact with CPEs in its 3′UTR and may mediate its repression (Ralle *et al.*, 1999), supporting the idea that factors other than the canonical CPEB may be involved in cyclin B1 mRNA repression. Multiple CPEB homologs may exist with distinct activities. Indeed, the *C. elegans* and zebrafish genomes encode multiple CPEB homologs and, in *C. elegans*, these CPEBs have distinct biological functions (C. Luitjens and M.W., personal communication). It is also possible that the activity of a single form of CPEB varies, depending on the protein partners with which it interacts.

Role of Polyadenylation in the Translational Activation of Cyclin B1 mRNA

During meiotic maturation cyclin B1 is released from a translationally inactive state. Polyadenylation of injected mRNAs bearing the cyclin B1 3′UTR mRNA increases their translational activity (Ballantyne *et al.*, 1997; de Moor and Richter, 1997; this paper). Our data demonstrate that polyadenylation, and not maturation per se, leads to derepression. We suggest that full translational activation of endogenous cyclin B1 mRNA is an additive effect of cytoplasmic polyadenylation and derepression.

Cytoplasmic polyadenylation circumvents repression. The addition of a poly(A) tail during maturation apparently eliminates low-dose repression; additionally, it elevates translation more than 50-fold (Fig. 2). Endogenous cyclin B1 mRNA, prior to maturation, carries a 30-nt poly(A) tail that is extended by some 200 nt during maturation (Sheets *et al.*, 1994). *In vivo*, recruitment of endogenous cyclin B1 mRNA onto polyribosomes is specifically prevented by inhibiting cytoplasmic polyadenylation or by removing its 3′UTR (Fig. 8; data not shown). Similarly, a poly(A) tail of 30 adenosines does not overcome repression of an injected mRNA in oocytes (de Moor and Richter, 1999), while an mRNA carrying a 65-nt poly(A) tail was translated efficiently (Fig. 7). Our findings therefore suggest that a poly(A) tail of at least 65 nt must be added to endogenous cyclin B1 mRNA to relieve repression. In contrast, polyadenylation is not required for derepression of mouse tPA mRNA as its short preexisting poly(A) tail is sufficient for its translational activation (Stutz *et al.*, 1998). Our results suggest that the repressor can only block translation of cyclin B1 mRNA containing a short poly(A) tail. Consequently, cytoplasmic polyadenylation leads to derepression of cyclin B1 mRNA.

Models for the Translational Activation of Cyclin B1 mRNA

Several models for the regulation of cyclin B1 mRNA can be considered. In one, maturation causes both relief of repression (e.g., inactivates the repressor) and activation of cytoplasmic polyadenylation. These events occur simultaneously, but are independent. In a second model, meiotic maturation causes relief of repression, which is a prerequisite for the acquisition of the polyadenylation machinery to the mRNA. In a third model, meiotic maturation promotes polyadenylation of the mRNA, which in turn causes relief of repression. Both the second and the third models suggest competition for overlapping 3′UTR binding sites; polyadenylation could displace or modify a repressor or removal of the repressor could allow the polyadenylation machinery to access the mRNA.

Several lines of evidence support the third model. First, poly(A) function is not significantly prevented by the presence of repressing elements in oocytes (Fig. 7). Second, repression is maintained on mRNAs that are not polyadenylated after maturation (Fig. 6). This suggests that polyadenylation, and not another maturation-specific event, is required. Finally, polyadenylation is necessary to recruit cyclin B1 mRNA onto polysomes (Fig. 8 and data not shown).

One simple molecular incarnation of the third model follows. Prior to meiotic maturation the mRNA has a short poly(A) tail and is repressed by the binding of a repressor to its 3′UTR. This repressor may include a polyadenylation-inactive form of CPEB, on its own or bound to additional repressor proteins. The cytoplasmic polyadenylation machinery is not active at this stage (Dworkin and Dworkin, 1985; Fox *et al.*, 1989). During meiotic maturation, the polyadenylation apparatus is activated, enabling polyadenylation factors to bind to the RNA, in part through AAUAAA. The relevant polyadenylation factors may be a cytoplasmic form of CPSF (Dickson *et al.*, 1999), a polyadenylation-competent form CPEB (reviewed in Richter, 1996), or a combination of the two. The mRNA is then polyadenylated and efficiently translated. Either the binding of the polyadenylation machinery or the enhanced translational activity that results displaces or modifies the repressor allowing maximal translation. Support for a dual role for CPEB in repression and activation has emerged recently: p82, a clam CPEB homologue, acts as both a translational repressor in the oocyte and a polyadenylation factor during maturation (Minshall *et al.*, 1999). The mechanisms by which repression is maintained and relieved are key issues for future research.

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Note added in proof. Vaillant, M., *et al.* (1999) have shown that inhibition of MPF activation, which blocks polyadenylation of cyclin B1 mRNA (Ballantyne *et al.*, 1997), does not prevent the increase in cyclin B1 translation upon progesterone addition. Thus, the impact of cytoplasmic polyadenylation on translation of injected and endogenous mRNAs may differ.

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