REPORT

Hormonal stimulation of starfish oocytes induces partial degradation of the 3′ termini of *cyclin B* mRNAs with oligo(U) tails, followed by poly(A) elongation

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ABSTRACT

In yeast, plant, and mammalian somatic cells, short poly(A) tails on mRNAs are subject to uridylation, which mediates mRNA decay. Although mRNA uridylation has never been reported in animal oocytes, maternal mRNAs with short poly(A) tails are believed to be translationally repressed. In this study, we found that 96% of *cyclin B* mRNAs with short poly(A) tails were uridylated in starfish oocytes. Hormonal stimulation induced poly(A) elongation of *cyclin B* mRNA, and 62% of long adenine repeats did not contain uridine residues. To determine whether uridylated short poly(A) tails destabilize *cyclin B* mRNA, we developed a method for producing RNAs with the strict 3′ terminal sequences of *cyclin B*, with or without oligo(U) tails. When we injected these synthetic RNAs into starfish oocytes prior to hormonal stimulation, we found that uridylated RNAs were as stable as nonuridylated RNAs. Following hormonal stimulation, the 3′ termini of short poly(A) tails of synthesized RNAs containing oligo(U) tails were trimmed, and their poly(A) tails were subsequently elongated. These results indicate that uridylation of short poly(A) tails in *cyclin B* mRNA of starfish oocytes does not mediate mRNA decay; instead, hormonal stimulation induces partial degradation of uridylated short poly(A) tails in the 3′–5′ direction, followed by poly(A) elongation. Oligo(U) tails may be involved in translational inactivation of mRNAs.

Keywords: mRNA uridylation; poly(A); polyadenylation; oocyte; starfish; oligo(U)

INTRODUCTION

Generally, mRNAs with long poly(A) tails are stable and translationally activated, and shortening of the poly(A) tail decreases mRNA stability (Wilusz and Wilusz 2008; Norbury 2013). Recent work revealed that short poly(A) tails of mRNAs are uridylated in yeast (Rissland et al. 2007; Rissland and Norbury 2009), plant (Sement et al. 2013), and mammalian cells (Chang et al. 2014; Lim et al. 2014). The noncoded uridines decrease the stability of the mRNAs; specifically, the Lsm1–7 complex mediates degradation of 3′ uridylated mRNAs by recruiting decapping factors to 5′ caps (Rissland and Norbury 2009; Norbury 2013). Dis3l2, which interacts with oligo-uridines, is the 3′–5′ exonuclease responsible for the decay of these mRNAs (Malecki et al. 2013). In addition, oligo(U) tails of RNAs such as miRNAs, miRNA-directed cleavage products, and poly(A)$^-$ *histone* mRNA play a role in RNA decay, which is executed by RNA degradation factors including the Lsm1–7 complex, XRN1, the exosome, and Dis3L2 (Song and Kiledjian 2007; Mullen and Marzluff 2008; Chang et al. 2013; Hoefig et al. 2013; Malecki et al. 2013; Ustianenko et al. 2013; Lee et al. 2014; Slevin et al. 2014). Moreover, uridylation of the 3′ end of a polyadenylated luciferase reporter RNA represses translation of the RNA in *Xenopus* oocytes (Lapointe and Wickens 2013).

Although the poly(A) tails of stored mRNAs in animal oocytes are short, mRNAs such as *cyclin B* are believed to be stable as maternal mRNAs. In *Xenopus* oocytes, the poly(A) length of *cyclin B1* mRNAs in oocytes is dynamically controlled by uridine-rich cytoplasmic polyadenylation elements (CPE) in their 3′ UTRs and the CPE binding protein (CPEB), which allows binding of other proteins, including poly(A) polymerase (Gld2) and deadenylating enzyme (PARN) (Fox et al. 1989; Fox and Wickens 1990; Hake and Richter 1994; Copeland and Wormington 2001; Kwak et al. 2004). PARN is more active than Gld2, resulting in the shortening of poly(A) tails in immature oocytes (Kim and Richter 2006). After hormonal stimulation, CPEB is phosphorylated, causing the release of PARN from the RNP complex,

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followed by elongation of poly(A) tails by Gld2 (Paris et al. 1991; Mendez et al. 2000; Kim and Richter 2006; Richter 2007; Radford et al. 2008). Similar elongation of poly(A) tails of mRNAs has been reported in oocytes of mouse, fish, *Drosophila*, and *Spisula* (Sheets et al. 1994; Minshall et al. 1999; Tay et al. 2000; Benoit et al. 2008; Yasuda et al. 2010).

In starfish oocytes, the short poly(A) tails of *cyclin B* mRNA are elongated upon meiotic reinitiation (Hara et al. 2009) induced by a hormonal stimulation of 1-methyladenine (1-MA) (Kanatani et al. 1969). The 1-MA receptor is coupled to a heterotrimeric G protein (Tadenuma et al. 1991, 1992; Chiba et al. 1992), and Gβγ dissociated from Gα activates PI3-kinase (Chiba et al. 1993; Jaffe et al. 1993), PDK1 (Hiraoka et al. 2004), Akt (Okumura et al. 2002), cdc25 phosphatase (Okumura et al. 1996), and cdc2/cyclin B (Kishimoto 2015). Translational activation of *cyclin B* mRNA is required for meiotic division.

Poly(A) tail length in oocytes is usually experimentally determined by poly(A) test assay (PAT assay), which takes advantage of oligo(dT) annealing to poly(A) tail (Sallés and Strickland 1995). Likewise, the most common method for cDNA synthesis is also based on oligo(dT) annealing. However, if the 3′ terminus of an mRNA contains oligo(U, G, or C) tails, oligo(dT) cannot anneal, resulting in synthesis of a cDNA that does not contain an exact copy of the original mRNA sequence at the 3′ terminus. Therefore, the actual 3′ terminal sequences of *cyclin B* mRNAs of oocytes remain unknown in many animals. In this study, we applied adaptor ligation to 3′ termini of mRNAs (Wada et al. 2012) in starfish oocytes to determine whether short poly(A) tails of *cyclin B* mRNAs are modified.

RESULTS AND DISCUSSION

Uridylated *cyclin B* mRNA in starfish oocytes

We ligated 23-nucleotide (nt) adaptors to the 3′ ends of total RNA from starfish oocytes, and performed RT-PCR using a 3′ adaptor primer and a *cyclin B*-specific primer (sfcycB Fwd primer1; see Materials and Methods) designed to hybridize ∼230 nt upstream of the polyadenylation site. Agarose gel electrophoresis of RT-PCR products from oocytes not subjected to hormonal stimulation revealed a single ∼290-bp band (Fig. 1A, −), suggesting that the tail length of *cyclin B* mRNA was 10–20 nt. The 320–380-bp mobility-shifted band observed in stimulated oocytes with hormone 1-MA (Fig. 1A, +) was consistent with the elongated poly(A) tail length measured by Hara et al. (2009) using the PAT assay.

To determine whether 3′ termini of poly(A) tails of starfish *cyclin B* mRNAs are modified, we cloned the RT-PCR product of *cyclin B* (Fig. 1A) and sequenced its 3′ terminal region. To our surprise, 96% ($n = 45$) of *cyclin B* mRNAs had a 1–8 nt oligo(U) tail (median, 2 nt) downstream from a short (4–18 nt) poly(A) tail (median, 12 nt) (Fig. 1B, [−] 1-MA treatment; 1C; 1D). The most frequent number of uridines was two (Fig. 1C). When we treated oocytes with 1-MA, we could not detect uridine residues in long poly(A) sequences of *cyclin B* mRNAs in 62% ($n = 55$) of all reads (Fig. 1B, [+] 1-MA treatment; 1D). The number of clones with uridine residues in the poly(A) regions was significantly reduced following 1-MA treatment ($P < 0.01$, χ^2 test). In some clones, the last several nucleotides immediately preceding the poly (A) tails ("AAAUGGAAAU," Fig. 1B, [−] 1-MA treatment) were trimmed into shorter sequences after hormonal stimulation (Fig. 1B, [+] 1-MA treatment), suggesting that exonuclease digestion in the 3′–5′ direction may occur prior to polyadenylation. Some long poly(A) tails contained oligo (U) segments (Fig. 1B, [+] 1-MA treatment: 38% [$n = 55$] of all reads), suggesting that mRNAs with oligo(U) tails can serve as substrates for poly(A) polymerase, and that U tails are not removed as a prerequisite for polyadenylation. In addition, to detect trimming activity in the 3′ region of *cyclin B* mRNA, we performed 3′ adaptor (23-mer) ligation of mRNA obtained from oocytes before and after hormonal stimulation. We subjected the resultant ligated RNA to RT-PCR using sfcycB short Fwd primer (22-mer), which hybridizes from 21 to 43 nt upstream of the polyadenylation site and the adaptor primer, followed by high-resolution electrophoresis (Fig. 1E). In oocytes without hormonal stimulation, we detected PCR products in a range of sizes (75–87 bp) corresponding to the variation in the length of poly(A) *cyclin B* mRNAs with oligo(U) tails (Fig. 1B, [−] 1-MA treatment). Immediately after GVBD, both shorter and longer *cyclin B* mRNAs appeared, corresponding to trimmed and poly(A)-elongated *cyclin B* mRNAs, respectively. The population of trimmed *cyclin B* mRNAs increased at 30–40 min and decreased at 50 min, whereas the population of elongated *cyclin B* mRNAs increased gradually over time. These results suggest that 3′ trimming and polyadenylation occurred simultaneously after GVBD, and that 3′ trimmed *cyclin B* mRNAs were substrates for polyadenylation. Thus, the majority of *cyclin B* mRNAs are first trimmed at the 3′ end and subsequently polyadenylated (Fig. 1B,E). In addition, some long poly(A) tails contain oligo(U) segments (Fig. 1B [+] 1-MA), indicating that some mRNAs with oligo(U) tails can also serve as substrates for poly(A) polymerase. These results suggest that removal of U tails is not a strict prerequisite for polyadenylation.

Poly(A) elongation is controlled by 3′ UTR sequence of *cyclin B* mRNAs

Because synthesized RNAs containing AAUAAA and U-rich sequences are polyadenylated in *Xenopus* oocytes (Fox et al. 1989), we investigated whether the 3′ UTR of *cyclin B* mRNA containing such sequences (Supplemental Fig. S1) was sufficient for poly(A) elongation. Using T7 RNA polymerase and blunt-end DNA templates, we obtained synthesized RNA with unexpected complementary sequences

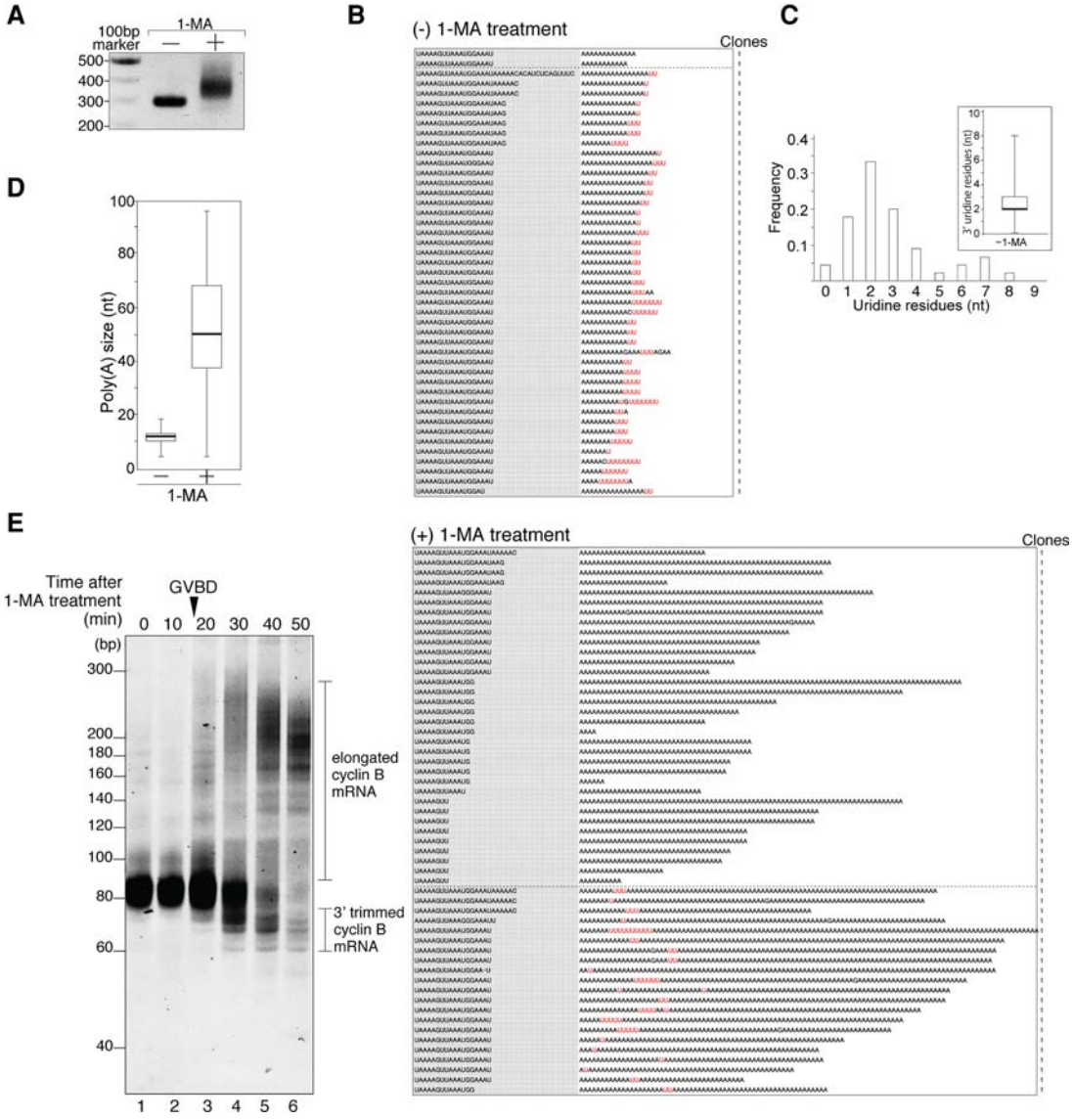

FIGURE 1. Uridylated short poly(A) tail of *cyclin B* mRNA in starfish oocytes and nonuridylated long poly(A) tail of *cyclin B* mRNA in oocytes stimulated with the hormone 1-MA. (*A*) Elongation of the 3′ region of mRNA after hormonal stimulation. Adaptor-ligated *cyclin B* mRNAs from oocytes treated with (+) or without (−) 1-MA were RT-PCR amplified. The products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. (*B*) Sequencing results of the 3′ terminal region of *cyclin B* mRNA from oocytes treated with [(+) 1-MA treatment] or without 1-MA [(−) 1-MA treatment]. Gray shaded sequences show the 3′ terminal portion of the 3′ UTR, and the following sequences are the tail regions of *cyclin B* mRNA. The number of clones obtained is indicated. (*C*) Histogram of the number of uridine residues at the 3′ ends of *cyclin B* mRNAs in starfish oocytes not subjected to hormonal stimulation. *Inset* shows box-plot analysis of oligo(U) tail length. The 25th and 75th percentiles are shown by box edges, the median value is indicated by the thick line, and whiskers show the maximum and minimum values. (*D*) Box plot analysis of poly(A) tail length of *cyclin B* mRNAs in oocytes. Median of poly(A) size increased from 12 to 50 nt after hormonal stimulation. (*E*) Trimming activity at the 3′ region of *cyclin B* mRNA after hormonal stimulation. GVBD occurred 18 min after 1-MA treatment. At the indicated time after hormonal stimulation, total RNA was purified from oocytes, and adaptor-ligated *cyclin B* mRNAs were RT-PCR amplified using 3′ adaptor Rev primer 1 and sfcycB short Fwd primer. The PCR products were subjected to high-resolution acrylamide gel electrophoresis (15%) and visualized by SYBR-Green I staining.

(Supplemental Fig. S2A), whereas polyadenylation of the microinjected RNAs occurred after hormonal stimulation (Supplemental Fig. S2B,C). When we used a DNA template with 5′-protruding termini, we obtained RNAs with extra C and U residues (Supplemental Table S1; Supplemental Fig. S3A-No. 1; Milligan et al. 1987; Krupp 1988). To decrease the number of extra bases, we increased the ATP concentration in the transcription reaction (Supplemental Table S1; Fig. S3A-No. 4) and purified the RNA designated cycB-oligo(A) (Supplemental Fig. S4-[3]). To produce cycB-oligo(A) with an oligo(U) tail (Supplemental Fig. S4-[4]), we used poly(U) polymerase and a fluorescently labeled

RNA (42 nt) as a marker substrate to determine the number of uridines by electrophoresis (Supplemental Fig. S3B,C). As expected, we could produce cycB-oligo(A) with one to four uridines (median, 2 nt), which we named cycB-oligo (A) + U (Supplemental Fig. S3D,E).

To determine whether the 3′ termini of synthetic *cyclin B* mRNAs would be polyadenylated, we microinjected cycB-oligo(A) + U into oocytes. After the oocytes were stimulated with or without 1-MA, we recovered total RNA and performed adaptor ligation. To amplify the injected RNA, we performed RT-PCR using primers against the exogenous sequence (Supplemental Fig. S4, exogenous sequence tag) and the adaptor sequence. We detected a single band from oocytes not subjected to hormonal stimulation and a shifted band from stimulated oocytes (Fig. 2A, left and middle lanes), but no band from oocytes that did not receive cycB-oligo(A) + U (Fig. 2A, right lane). Therefore, in vitro synthesized cycB-oligo(A) + U was polyadenylated like endogenous *cyclin B* mRNA after hormonal stimulation. Next, we cloned and sequenced these RT-PCR products in Figure 2A to check the sequences of the 3′ regions of injected cycB-oligo(A) + U with or without stimulation by 1-MA. In stimulated oocytes, the median length of the poly(A) tail increased to 62 nt, and most reads did not contain uridine residues in the long adenine repeat (Fig. 2B, [+] 1-MA; Fig. 2C, +1-MA). In contrast, uridine residues were stable in unstimulated oocytes, and the median length of oligo(A) was 13 nt (Fig. 2B, [−] 1-MA; Fig. 2C, −1-MA), almost identical to that of the uninjected control (Fig. 2B, Control; Fig. 2C, Control). These results indicate that endogenous polyadenylation of *cyclin B* mRNAs could be recapitulated using in vitro synthesized cycB-oligo (A) + U. On the basis of these findings, we conclude that the 3′ UTR alone is sufficient for polyadenylation of *cyclin B* mRNA during oocyte maturation.

mRNA stabilization and decay in oocytes

Several groups have proposed that uridylation at the 3′ ends of mRNAs induces mRNA decay (Rissland et al. 2007; Rissland and Norbury 2009; Sement et al. 2013; Chang et al. 2014; Lim et al. 2014). If the oligo(U) tail of *cyclin B* mRNAs in starfish oocytes in the absence of hormonal stimulation is also involved in mRNA degradation, *cyclin B* mRNA with oligo(U) should be turned over more rapidly than the mRNA without oligo(U). Alternatively, given that many maternal mRNAs in oocytes are stored stably in the cytoplasm (Barckmann and Simonelig 2013), uridylated *cyclin B* mRNA may be stable in oocytes. To determine which hypothesis is correct, it is necessary to measure the stability of uridylated *cyclin B* mRNA in oocytes in the absence of hormonal stimulation.

To determine whether the 3′ oligo(U) tail of *cyclin B* mRNA plays a role in mRNA decay, we microinjected uniformly radiolabeled cycB-oligo(A) with or without cold U-tail [cycB-oligo(A) + U or cycB-oligo(A)] into oocytes, and analyzed the amount of injected RNA remaining after 0 or

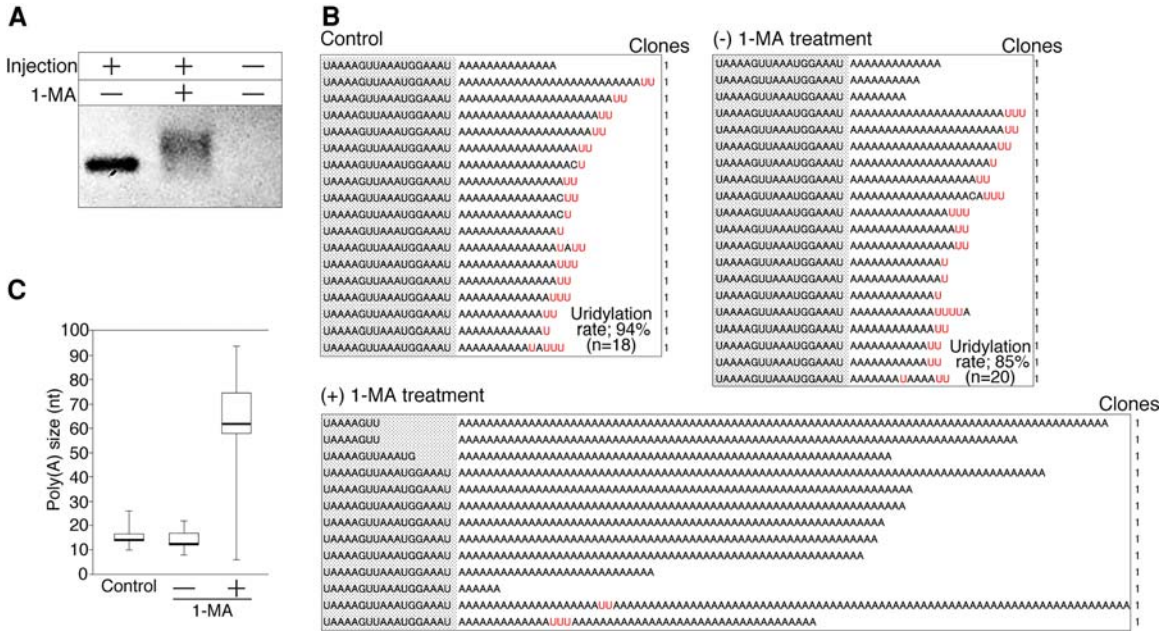

FIGURE 2. The 3′ UTR of *cyclin B* mRNAs is sufficient to recapitulate endogenous poly(A) elongation. (*A*) Band shift of cycB-oligo(A) + U in injected oocytes after hormonal stimulation (*left* and *middle* lanes), and the control RT-PCR product from oocytes not subjected to RNA injection (*right* lane). (*B*) Sequencing results of the 3′ terminal region of cycB-oligo(A) + U before injection (Control), after injection without hormonal stimulation ([−] 1-MA treatment), and after injection with hormonal stimulation ([+] 1-MA treatment). (*C*) Quantitation of poly(A) tail lengths. Layout and labeling are the same as in *B*.

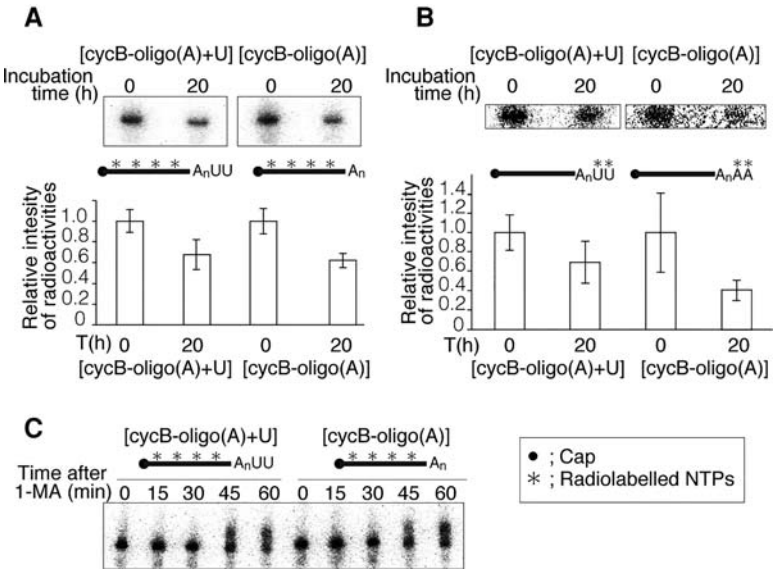

FIGURE 3. Uridylated and nonuridylated RNAs were similarly stable in starfish oocytes. Asterisks in the schematics denote locations of radiolabels. (*A*) Similar stability of uniformly labeled uridylated and nonuridylated RNAs in oocytes without hormonal stimulation. *Upper* panel shows representative autoradiography results. The intensity of each band was calculated using ImageQuant. *Lower* graphs show the remaining amounts of injected RNA after 20 h incubation. Error bars represent standard deviation from more than three experiments. (*B*) An experiment similar to the one shown in *A* was performed using 3′-end radiolabeled cycB-oligo(A) + U or cycB-oligo(A). *Upper* panel shows representative autoradiography results, and *lower* graphs show the remaining amounts of injected RNA after 20 h incubation. Error bars represent standard deviation ($n = 3$). (*C*) Similar band shifts of uniformly labeled uridylated and nonuridylated RNAs in oocytes after hormonal stimulation. Bands for both cycB-oligo(A) + U and cycB-oligo(A) were shifted up after 45–60 min.

20 h of incubation. After 20 h, the levels of both injected cycB-oligo(A) + U and cycB-oligo(A) were reduced (Fig. 3A, upper panel), and the remaining amount of radioactivity did not differ significantly between the two RNAs (Fig. 3A, lower panel). To determine the stability of 3′ termini of uridylated and nonuridylated RNAs, we synthesized cold cycB-oligo(A) with radiolabeled terminal oligo(U) or oligo (A) using hot UTP or ATP, and then performed an experiment similar to the one shown in Figure 3A. Again, the remaining radioactivity did not differ significantly between 3′ uridine and adenine residues after 20 h incubation (Fig. 3B). These results confirm that 3′ uridine modification does not induce mRNA decay in oocytes prior to hormonal stimulation.

Finally, to determine whether uridylated mRNA is degraded after hormonal stimulation, we stimulated oocytes injected with uniformly radiolabeled cycB-oligo(A) with or without cold oligo(U) tails with 1-MA, and then analyzed the injected RNAs by denaturing acrylamide gel electrophoresis and autoradiography (Fig. 3C). Similar band shifts were observed in both cycB-oligo(A) + U and cycB-oligo(A) (Fig. 3C), indicating that polyadenylation per se does not require the 3′ uridine modification, and the oligo(U) tail does not induce remarkable mRNA decay during meiotic progression following hormonal stimulation (Fig. 3C).

In this study, we found that *cyclin B* mRNAs with oligo(U) tails were stored in starfish oocytes. After hormonal stimulation, *cyclin B* mRNAs could be trimmed in the 3′–5′ direction, and poly(A) elongation occurred. To our knowledge, this is the first report showing that uridylated mRNAs are stored in oocytes and that they can be activated translationally.

In somatic cells, factors involved in degradation of uridylated mRNAs localize to P-bodies (Sheth and Parker 2003; Decker and Parker 2012; Malecki et al. 2013); in some cases, stored mRNAs within P-bodies can reenter polysomes for translation (Brengues et al. 2005; Anderson and Kedersha 2006; Bhattacharyya et al. 2006). If storage of mRNAs in P-bodies is regulated by uridylation, deuridylation should be required for reentry of the stored mRNAs into polysomes, suggesting that uridylation is involved in translational inactivation. Indeed, uridylated poly(A)$^+$ reporter luciferase RNA is translationally inactive in *Xenopus* oocytes (Lapointe and Wickens 2013). Therefore, modification of the 3′ oligo(U) tail in starfish oocytes may translationally inactivate certain mRNAs prior to hormonal stimulation.

MATERIALS AND METHODS

Animals and oocytes

Starfish (*Asterina pectinifera*) were collected on the Pacific coast of Honshu, Japan, and kept in laboratory aquaria supplied with circulating seawater. Immature oocytes were treated with cold calcium-free seawater (SW) to remove follicle cells, and then incubated in normal SW containing 1 μM 1-MA to induce germinal vesicle breakdown (GVBD). Usually, GVBD was induced 20–30 min after 1-MA treatment. All experiments were performed at 20°C unless otherwise stated.

Microinjection of in vitro synthesized RNA into immature oocytes

For rapid microinjection of synthesized RNA, oocytes were held between two coverslips separated by a double layer of Scotch tape, and then released after injection by peeling off the tape (Chiba et al. 1992). Microinjections were performed using a constricting pipet filled with in vitro synthesized RNA (80 ng/μL); the injection volume was 10–30 pL per oocyte. Injected oocytes were incubated for the indicated periods, and 100 oocytes were used for each experiment.

Extraction of RNA from oocytes

Total RNA from oocytes was extracted using the TRI reagent (Molecular Research Center, Inc.). In some experiments, 1 µg yeast tRNA was used to coprecipitate RNA. In oocytes stimulated with 1-MA, total RNA was extracted 30 min after GVBD.

Electrophoresis

RNA was analyzed by a 10% polyacrylamide gel in TBE (89 mM Tris borate [pH 8.0], 89 mM boric acid, 2 mM EDTA) containing 7.5 M urea.

RT-PCR with 3′ adaptor ligation and tail sequence

Synthesis of cDNA with biotinylated 3′ adaptor ligation was performed using small RNA Cloning Kit (Takara Bio) as reported previously (Wada et al. 2012), followed by PCR using a gene-specific primer and a 3′ adaptor primer (primer sets are listed below). In some experiments, in order to increase the amount of DNA available for cloning, secondary nested PCR was performed using internal (nested) PCR primers (primer sets for the second amplification are listed below). PCR products were purified by agarose gel electrophoresis and extracted from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega). Purified PCR products were cloned into a pCR2.1-TOPO vector (Invitrogen), and the insert sequences were determined.

The following primer sets were used to target endogenous *cyclin B* mRNAs: for the first amplification, sfcycB Fwd primer 1 (5′-TTGTGCTAGATATCCGAGACC-3′) and 3′ adaptor Rev primer 1 (5′-GTCTCTAGCCTGCAGGA-3′); for the second amplification, sfcycB Fwd primer 2 (5′-TCCCACACAACTTCAGATGAAC-3′) and PCR-R&RT primer (5′-GTCTCTAGCCTGCAGGATCGATG-3′). In Figure 1E, PCR was performed using sfcycB short Fwd primer (5′-GCTTTTGTCTGTCAGTCTTTGT-3′) and 3′ adaptor Rev primer 1.

The following primer sets were used to target in vitro synthesized RNAs encoding the 3′ region of *cyclin B* mRNA: for the first amplification, exo tag Fwd primer 1 (5′-CCCCTCGAGAAAGATCTGC-3′) and 3′adaptor Rev primer 1; for the second amplification, exo tag Fwd primer2 (5′-GAAAGATCTGCAGCGTGCGTCA-3′) and 3′adaptor Rev primer 2 (5′-AGCCTGCAGGATCGATG-3′).

DNA cloning and plasmids for in vitro RNA synthesis

To create pcDNAsfcycB3′UTR1, the 3′ UTR of starfish *cyclin B* cDNA was amplified by RT-PCR using a sfcycB Fwd primer 1 and a (dT)12-anchor primer (5′-GCGAGCTCCGCGGCCGCG TTTTTTTTTTTT-3′) (Wada et al. 2012), which was TA-cloned into vector pCR2.1-TOPO (Invitrogen).

Plasmids encoding the T7 promoter, exogenous sequence tag (5′-AAAGATCTGCAGCGTGCGTCA-3′, 21 nt), *cyclin B* 3′ UTR of starfish oocyte (Supplemental Fig. S1), short poly(A) tail, and a FokI site (see also Supplemental Fig. S4-[1]) were generated by the following two-step procedure: (i) A DNA fragment encoding the exogenous sequence tag and the portion of the 3′ UTR of *cyclin B* (nucleotides 1946–2179, Supplemental Fig. S1) was PCR amplified from pcDNAsfcycB3′UTR1 using a forward primer (5′-GCTCGAG AAAGATCTGCAGCGTGCGTCATTGTGCTAGA TATCCGAGACC-3′; exogenous sequence tag underlined and XhoI site double underlined) and reverse primer (5′-CGAATTC GTATACAAAGACTGACAGACAAAAGC-3′; Bst1107I [SnaI] site underlined and EcoRI site *double*-underlined). The PCR product was cloned into the XhoI and EcoRI sites of pBluescript SK II (Stratagene), and the resultant plasmid was named pcDNA sfcycB3′UTR2. (ii) A synthetic double-stranded DNA (5′-CTGTC AGTCTTTGTAAATAAAAGTTAAATGGAAATAAAAAAAAAAAA CGCATGTTCCATCCTACGAATTCCTGCAG-3′/5′-CTGCAGGAA TTCGTAGGATGGAACATGCGTTTTTTTTTTTTTATTTCCATTTAA CTTTTATTTACAAAGACTGACAG) encoding the portion of the *cyclin B* 3′ UTR (nucleotides 2180–2199, Supplemental Fig. S1), a short poly(A) tail, and a FokI site were cloned into the SnaI site of pcDNAsfcycB3′UTR2 by the In-Fusion (Takara Bio) method, and the resultant plasmid was named pcDNAsfcycB3′UTRFokI.

In vitro transcription

A DNA template for in vitro transcription of cycB-oligo(A) was PCR amplified from pcDNAsfcycB3′UTRFokI using a forward primer (5′-GCGTAATACGACTCACTATAGG-3′; T7 promoter sequence underlined) and a universal M13 reverse primer (5′-CAGGAAAC AGCTATGAC-3′). The PCR product was digested with FokI, and the objective fragment was purified by agarose gel electrophoresis using the Wizard SV Gel and PCR Clean-Up System, followed by phenol/chloroform extraction and ethanol precipitation, and the resultant DNA pellet was subsequently dissolved in sterile water. Using this DNA as a template, in vitro transcription was performed using the mMESSAGE mMACHINE T7 kit (Invitrogen) with the following modified NTP concentrations: 18.75 mM ATP, 3.75 mM CTP, 3.75 mM CTP, 0.75 mM GTP, and 3 mM cap analog unless otherwise indicated (see also text). Transcripts were purified by phenol/chloroform extraction and ethanol precipitation and were subsequently separated by electrophoresis on a 5% polyacrylamide gel in TBE (89 mM Tris [pH 8.0], 89 mM boric acid, 2 mM EDTA) containing 8 M urea. The gel was stained with SYBR Green I, and ~318-nt RNA fragments were cut out, crushed, and eluted from gel slices in four volumes of TE buffer (10 mM Tris [pH 8.0] 0.1 mM EDTA) at 37°C for 4–5 h. Eluted RNA was recovered by phenol/chloroform extraction and ethanol precipitation, and the resultant RNA pellet was dissolved in sterile water.

In vitro uridylation/adenylation at the 3′ ends of RNAs

To produce cycB-oligo(A) + U RNAs, the uridylation reaction shown in Supplemental Figure S3C was carried out in reaction mixture A [40 unit/mL of poly(U) polymerase (NEB: M0337S), 1.6 unit/µL RNase inhibitor (Promega:N2611), 50 mM NaCl, 10 mM Tris–HCl (pH 7.9), 10 mM $MgCl_2$, 1 mM DTT, 450 nM of a 42-nt synthetic RNA 5′-end labeled with Alexa Fluor 488 (5′-Alexa488UGUCAGUCUUUGUAAAUAAAAGUUAAAUGGAAAU AAAAAAAA-3′, marker substrate) and 50 nM cycB-oligo(A) RNA] with 1 mM UTP. When the uridylation reaction was performed without cycB-oligo(A) in Supplemental Figure S3B, 500 nM 42-nt synthetic RNA 5′-end labeled with Alexa Fluor 488 was used. The reaction mixture was incubated at 37°C for 60 sec or as indicated. The reaction was stopped by addition of EDTA (28 mM final concentration). Reaction products were purified by phenol/-chloroform extraction and ethanol precipitation and separated on

a 10% polyacrylamide gel in TBE containing 7.5 M urea. The gel was scanned on a Fuji-imager LAS 4000 mini with or without SYBR-Green I staining.

In vitro synthesis of radiolabeled RNA

To produce uniformly radiolabeled cycB-oligo(A) + U and cycB-oligo(A), hot cycB-oligo(A) was produced and purified by the in vitro transcription procedure with 111 KBq/μL [α-^{32}P]UTP in T7 polymerase reaction mixture, and the 3′ end of hot cycB-oligo(A) was uridylated or adenylated in reaction mixture A with 1 mM cold UTP or 1 mM cold ATP, respectively. Reaction mixtures containing UTP or ATP were incubated at 37°C for 60 sec or 25°C for 90 sec, respectively. The reaction was stopped by addition of EDTA (28 mM final concentration). The resultant uniformly radiolabeled cycB-oligo(A) + U and cycB-oligo(A) RNAs were purified using the RNA Clean-Up and Concentration Kit (Norgen Biotek Corporation).

To radiolabel the 3′ end of cycB-oligo(A) RNA with [α-^{32}P]UTP or [α-^{32}P]ATP in Figure 3B, 11.1 KBq/μL [α-^{32}P] UTP + 2.5 mM UTP or 11.1 KBq/μL [α-^{32}P]ATP + 2.5 mM ATP were added to reaction mixture A, respectively. Reaction mixtures containing [α-^{32}P] UTP or [α-^{32}P] ATP were incubated at 37°C for 60 sec or at 25°C for 90 sec, respectively, followed by addition of EDTA (28 mM final concentration) to stop the reaction. 3′ radiolabeled cycB-oligo(A) + U and cycB-oligo(A) were purified using the RNA Clean-Up and Concentration Kit (Norgen Biotek Corporation).

RNA stability assay

Radiolabeled cycB-oligo(A) + U or cyclinB-oligo(A) was injected into oocytes without hormonal stimulation, and total RNA was extracted before or after 20 h incubation (Fig. 3A,B). In addition, injected oocytes were treated with 1−MA and extracted every 15 min after hormonal stimulation (Fig. 3C). Purified RNAs were separated by electrophoresis on a 10% polyacrylamide gel in TBE containing 8 M urea, followed by autoradiography (Typhoon FLA 7000, GE Healthcare). The bands of labeled RNAs incubated in oocytes were quantitated using the Image Quant TL software (GE Healthcare).

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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Hormonal stimulation of starfish oocytes induces partial degradation of the 3′ termini of *cyclin B* mRNAs with oligo(U) tails, followed by poly(A) elongation

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