Developmental Biology **234,** 425–440 (2001)
doi:10.1006/dbio.2001.0260, available online at http://www.idealibrary.com on IDEAL®

Cyclin E and its Associated cdk Activity Do Not Cycle during Early Embryogenesis of the Sea Urchin

Jan L. Sumerel,*,† Jennifer C. Moore,*,† Bradley J. Schnackenberg,*,‡ Jason A. Nichols,*,§ Julie C. Canman,§ Gary M. Wessel,¶ and William F. Marzluff*,†,‡,§,[1]

**Program in Molecular Biology and Biotechnology, †Department of Biochemistry and Biophysics, ‡Lineberger Comprehensive Cancer Center, and §Department of Biology, University of North Carolina, Chapel Hill, North Carolina 27599; and ¶Department of Molecular Cell Biology and Biochemistry, Brown University, Providence, Rhode Island 02912*

Female sea urchins store their gametes as haploid eggs. The zygote enters S-phase 1 h after fertilization, initiating a series of cell cycles that lack gap phases. We have cloned cyclin E from the sea urchin *Strongylocentrotus purpuratus.* Cyclin E is synthesized during oogenesis, is present in the germinal vesicle, and is released into the egg cytoplasm at oocyte maturation. Cyclin E synthesis is activated at fertilization, although there is no increase in cyclin E protein levels due to continuous turnover of the protein. Cyclin E protein levels decline in morula embryos, while cyclin E mRNA levels remain high. After the blastula stage, cyclin E mRNA and protein levels are very low, and cyclin E expression is predominant only in cells that are actively dividing. These include cells in the left coelomic pouch, which forms the adult rudiment in the embryo. The cyclin E present in the egg is complexed with a protein kinase. Activity of the cyclin E/cdk2 changes little during the initial cell cycles. In particular, cyclin E-cdk2 levels remain high during both S-phase and mitosis. Our results suggest that progression through the early embryonic cell cycles in the sea urchin does not require fluctuations in cyclin E kinase activity.

Key Words: **sea urchin; cyclin; embryogenesis.**

INTRODUCTION

A cell's decision to divide or remain quiescent is governed by a complex system of growth-stimulating and growth-retarding signals. The best-characterized control points are the G_1-S transition point (START) that commits a cell to initiate DNA replication and the G_2-M transition point that commits a cell to enter and complete mitosis. It is the sequential formation, activation, and inactivation of cyclin/cdk complexes that together regulate the progression through these boundaries. The mitotic cyclins A and B were initially discovered in sea urchins as rapidly synthesized proteins that were degraded at mitosis during the first cell cycle (Evans *et al.*, 1983).

In early cleavage cycles of rapidly dividing animal embryos, gap phases are often missing, and M phase and S phase alternate. In these cycles, mitotic regulation is largely accomplished by oscillating levels of cyclins A and B (Hartley *et al.*, 1996). Even though a large amount of information has accumulated about the regulation of entry into and exit from mitosis in embryonic cells and the entry into S-phase of somatic cells, there is little information on the interactions of the G_1 cyclins with the cell cycle machinery during early embryogenesis.

In somatic cells, the decision to proliferate or remain quiescent is primarily made during G_1, and in mammalian cells the G_1-S transition requires the activation of cyclins D and E, which complex with cdk4/6 and cdk2, respectively (Sherr, 1994). In vertebrates, these G_1 cyclins are the essential rate-limiting molecules that determine whether a cell

[1] To whom correspondence should be addressed at: Program in Molecular Biology and Biotechnology, CB# 7100 University of North Carolina, Chapel Hill, NC 27599. Fax: (919) 966-6821. E-mail: marzluff@med.unc.edu.

0012-1606/01 $35.00

enters a proliferative state (Sherr, 1994). Mitogens activate the synthesis of cyclin E, and it associates with cdk2 (Koff *et al.*, 1992; Dulic *et al.*, 1992). While there are presumably multiple substrates for the G_1 cyclin/cdk complexes, their best-characterized function is to phosphorylate the pRb family of pocket proteins (Kato *et al.*, 1993; Hinds *et al.*, 1992). One consequence of phosphorylating pRb family members is their dissociation from E2F-containing complexes at promoters of genes required for the entry into S-phase, thereby allowing transcriptional activation (Dynlacht *et al.*, 1994; Beijersbergen *et al.*, 1995). Cyclin E has also been shown to play a critical role in the centrosome cycle in Xenopus embryos (Hinchcliffe *et al.*, 1999; Lacey *et al.*, 1999), and cyclin E has been implicated in the repeated centrosome duplication in sea urchin embryos blocked in S-phase (Hinchcliffe *et al.*, 1998).

In contrast to the mitotic cycle of somatic cells, the meiotic cycle of gametes has two successive M-phases without an intervening S-phase. These meiotic events require activation of the cyclin B-cdc2 kinase (maturation-promoting factor, MPF; Maller, 1990). In Xenopus oocytes, cdc2 is constitutively expressed, and translation of maternal cyclin B mRNA and degradation of cyclin B protein are both required for entry and exit of meiosis (King *et al.*, 1996). In most animals, oocytes are arrested at prophase of meiosis I, and prior to ovulation, they resume meiosis. Fertilization then occurs either before or after the completion of meiosis II followed by the initiation of early cleavage cycles. Since sea urchin female gametes are arrested as haploid eggs, the initial events after fertilization drive the sea urchin zygote into the first S phase, followed by a series of cell cycles that have alternating S and M phases. Unlike most other animals, the cell cycle events in the sea urchin required for oocyte maturation (meiosis) occur separately from those involved in regulation of the initial zygotic cell cycles, thereby making it an ideal system for studying the regulation of the early cleavage cell cycles.

The initial step(s) of embryogenesis involves the activation of a quiescent cell (an oocyte or egg) and the initiation of a program of gene expression that results in embryonic development. Although it is thought that critical maternal cell-cycle regulators present in an egg must somehow be kept inactive to maintain cell cycle arrest before fertilization (Edgar and Lehner, 1996), there is little or no evidence as to how this restraint is accomplished biochemically. Upon fertilization, the zygote enters into a series of rapid mitotic cell cycles followed by cell divisions with gap phases and cell differentiation. Therefore, molecular control of initial cleavage cell cycles must be different from the control of the subsequent cell cycles that have gap phases. We report here that cyclin E-cdk2 is constitutively active in sea urchin (*S. purpuratus*) eggs and early embryos, suggesting that regulation of entry into S phase must depend on events other than activation of cyclin E.

MATERIALS AND METHODS

Culturing Sea Urchin Embryos

Adult sea urchins were injected with 0.55 M KCl to shed their gametes. Eggs were shed into sea water, settled, and resuspended in fresh sea water. The jelly coat was removed by repeated passage through a Nitex mesh screen (150 microns). Sperm were collected separately and kept at 4°C. If embryos were harvested before hatching, 0.1% para-amino benzoic acid (PABA) was added to the dejellied eggs in order to soften fertilization membranes. Fertilization was checked by light microscopy and was routinely greater than 98%. Embryos were raised at a concentration of 5 g/L in sea water containing 0.1% Ampicillin (Sigma, St. Louis, MO) with constant aeration at 15°C and then harvested at different times during development. For embryos raised past the mesenchyme blastula stage, the embryos were collected after 18–24 h and resuspended in fresh sea water at a concentration of about 2.5 g of embryos per liter.

Preparation of Egg and Embryo Extracts

Embryos were harvested by settling and washed three times in 0.55 M KCl, 2 mM EDTA, and 2 mM EGTA. Eggs and embryos were resuspended in two volumes of Swelling buffer [0.22 M Sucrose, 0.1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2 mM EDTA, and 2 mM EGTA] and pelleted by centrifugation by allowing the centrifuge (Sorvall RC5B) to reach 5000 rpm and then switching it off. The volume of the pellet was measured, and the eggs were resuspended in an equal volume of lysis buffer containing 0.15 M NaCl, 0.5% NP-40, 50 mM Tris, pH 7.6, 1 mM DTT, 1 mM PMSF, and 1 mM sodium orthovanadate (Phelps and Xiong, 1997), and lysed by homogenization using 10–12 strokes in a Dounce homogenizer (tight pestle). Lysis was checked by light microscopy, and lysates were immediately frozen in a dry ice–ethanol bath and stored in liquid nitrogen. Because there is an increase in pH at fertilization, some extracts were prepared as above except that the Lysis Buffer was adjusted to pH 6.8 with HCl.

For experiments where the samples were assayed only by Western blotting, the embryos or eggs were washed once with 0.55 M KCl, followed by two washes with 0.5 M NaCl. The embryos were suspended in 2 volumes of 0.5 M NaCl, and 3 volumes of 10% SDS-5 mM EDTA were rapidly added. Typically, 5 ml of culture containing 0.05 ml of packed eggs or embryos were used and 0.25 ml of extract with a protein concentration of 10–20 mg/ml was obtained. If less SDS or a higher concentration of embryos was used, there was significant proteolysis of cyclin E (and other proteins).

Preparation of Radiolabeled Lysates from Embryos

In order to measure the synthesis of proteins, eggs or embryos were cultured in sea water with 1.0 mCi of ^{35}S-methionine-cysteine mix (NEN Translabel, Boston, MA) per 2.5 ml of embryos (in 2 liters of artificial sea water) for indicated times, and lysates were prepared as described above by using the NP-40 lysis buffer.

Design and Synthesis of Probes for Cloning Sea Urchin Cyclin E

Two 380-nt regions that encoded the conserved cyclin boxes from human cyclin E (Lew *et al.*, 1991) and Drosophila cyclin E

(Richardson *et al.*, 1993) were PCR-amplified in the presence of [α-^{32}P]dCTP. Briefly, primers were made to the 5′ and 3′ ends of the cyclin box from both cyclin E cDNAs. The 5′ and 3′ primers for amplification of the human and Drosophila cDNA clones were: (5′)-GGATTATTGCACCATCCAGA-(5′ (human) and (5′)-AAAGATTTGCTGGGGATACT (3′, human) and (5′)-GGAGG-ACGGTCTGCGCCAGT (5′, *Drosophila*) and (5′)-TTGCCTGC-CAATCTGGAGA (3′, *Drosophila*). The PCR product was labeled by carrying out the PCR in the presence of 50 μCi of [α-^{32}P]dCTP (specific activity 3000 Ci/mMol) and 2 nM dCTP in a 50-μl reaction. Routinely greater than 75% of the radiolabeled dCTP was incorporated into DNA. The DNA was quantified by staining with ethidium bromide after agarose gel electrophoresis and Cerenkov counting, and equal amounts of the human and *Drosophila* probes were mixed and used in the screen to clone sea urchin cyclin E.

Cloning Sea Urchin Cyclin E

BB4 cells were infected with 1.2 million bacteriophage from a *S. purpuratus* sea urchin ovarian library in λZAP (Stratagene, La Jolla, CA) that had been prepared by the random-priming of sea urchin ovary poly(A)$^+$ mRNA (Laidlaw and Wessel, 1994). The phage were screened by hybridization in QuickHybe (Stratagene) containing 67 μg/ml denatured salmon sperm DNA (Gibco BRL, Gaithersburg, MD; 1.5 ml/filter). The filters were prehybridized for 15 min at 55°C. The probes were denatured at 100°C for 5 min and 1 μl (600,000 cpm/μl) of each probe was added per ml of the QuickHybe mixture and the filters hybridized for an additional 2 h at 55°C. After hybridization, the filters were washed twice for 15 min each in 2× SSC and 0.1% SDS at 45°C, followed by one wash with 0.1× SSC and 0.1% SDS at 25°C. The filters were autoradiographed overnight. A weak signal was obtained from 12 plaques, and these plaques were isolated and rescreened. Identical hybridization conditions were used in the secondary and tertiary screens, and a single positive phage was isolated. The purified phage contained a 2.1-kb *Eco*RI fragment that encoded the sea urchin cyclin E. Subclones were constructed, and both strands were sequenced.

Isolation of Total RNA from Sea Urchin Embryos

Total RNA was extracted from both eggs and embryos by using phenol extraction. Eggs and embryos were washed in 0.55 M KCl twice and resuspended in 5–10 volumes Swelling Buffer and pelleted by centrifugation. After resuspension, the eggs or embryos were added to an equal volume of a solution containing 2% SDS and 10 mM EDTA, pH 8.0. After mixing well, 1/10 volume of 3 M NaOAC, pH 5, was added. This solution was mixed with an equal volume of 4:1.5 phenol/chloroform solution and extracted at room temperature for 15 min. Additional phenol-chloroform extractions were done, and the nucleic acids were precipitated with three volumes of ethanol. The precipitated RNA was collected by centrifugation, dissolved at a concentration of about 0.5 mg/ml in a solution containing 0.1% SDS and 1 mM EDTA, extracted one more time with phenol-chloroform, and then ethanol precipitated again. For later stage embryos (when there is a large amount of chromosomal DNA), the initial extraction was performed at 55°C for 10 min to remove chromosomal DNA.

Northern Blot Analysis

A total of 5.0 μg of cyclin E DNA in pGEM-5Zf was digested with 20 units of *Xba*I, and 5.0 μg of cyclin A DNA in pCR2.1 was digested with 20 units of *Xho*I and *Nco*I for 1 h at 37°C. The resulting 666-bp *Xba*I fragment from cyclin E and the 1000-bp *Xho*I/*Nco*I fragment from cyclin A were separated on a 1% agarose gel and extracted by using a Qiaquik gel extraction kit (Qiagen). The probes were prepared by using a Random Primed DNA labeling kit (Boehringer Mannheim) with 50 μCi of [α-^{32}P]dCTP and purified with a ProbeQuant G-50 Micro Column (Amersham Pharmacia Biotech Piscataway, NJ). Approximately 60% of the label was incorporated into the probes. Total RNA samples (7.5 μg/lane) were dissolved in RNA Denaturation Buffer containing 20 mM MOPS, pH 7.0, 8 mM NaOAc, 1 mM EDTA, pH 8.0, 6% formaldehyde, and 50% formamide, heated to 65°C for 10 mins, and separated on an agarose gel containing 6% formaldehyde in 20 mM Mops, pH 7.0, 8 mM NaOAc, 1 mM EDTA, pH 8.0. The RNA was transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech), and the membrane was hybridized with 20 ng of cyclin E or cyclin A probe at 60°C for 4 h in QuickHybe (Stratagene), containing 0.1 mg/ml salmon sperm DNA. After hybridization, the filter was washed twice with 2× SSC/0.1% SDS at 60°C for 10 min, twice with 0.2× SSC/0.1% SDS for 10 min at 56°C, twice with 0.1× SSC/0.1% SDS for 10 mins at 56°C, and autoradiographed.

Preparation of Polyclonal Antibodies Against a C-Terminal Peptide of Sea Urchin Cyclin E

A synthetic peptide corresponding to the C-terminal amino acid sequence (with a cysteine residue added for coupling purposes) was synthesized in the UNC Protein Chemistry Facility, coupled to KLH, and injected into rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, Pa). The antibody was affinity purified from the rabbit serum by using a peptide-coupled sulfolink column (Pierce, Rockford, IL).

In Situ RNA Hybridization

A digoxygenin RNA probe was produced by isolating a DNA fragment corresponding to 610 nucleotides of the sea urchin cyclin E open reading frame (corresponding to nucleotides 608–1218 of *SpcycE*). *In situ* whole-mount RNA hybridizations were performed as described (Ransick *et al.*, 1993; Laidlaw and Wessel, 1994).

Immunofluoresence

Sea urchin ovaries, eggs, and embryos at different developmental stages were fixed in artificial sea water containing 4% paraformaldehyde. After fixation, they were washed twice in artificial sea water containing 0.05% Tween 20 (ASWt). The affinity purified α-cyclin E (0.25 mg/ml) was added at a concentration of 1:50 in ASWt. Samples were incubated at 25°C for 1 h followed by two washes in ASWt. Donkey α-rabbit serum conjugated to CY3 (Molecular Probes) was added at a concentration of 1:200 diluted in ASWt and incubated at room temperature for 1 h. After two additional washes in ASWt, ovaries, eggs, and embryos were visualized by fluorescent microscopy. Metamorph imaging software (Metamorph, Universal Imaging Corporation) was used for data collection and analysis.

For confocal microscopy, the eggs or early embryos were settled onto poly-L-lysine-coated coverslips, fixed in −20°C 90% MeOH/50 mM EGTA, pH 6.0 (Harris, 1986), rehydrated in PBS, and blocked with 5% milk in PBS containing 0.1% Tween 20 (PBST). Samples were incubated with anti-cyclin E antibodies overnight at 4°C, washed in PBST, incubated with rhodamine-conjugated sec-

ondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA), washed with PBST, and mounted in elvanol (Palazzo and Vogel, 1999). Images were collected by using a Zeiss LSM 410 laser scanning confocal system in conjunction with a Zeiss Axiovert 100 microscope equipped with a 40×/1.3NA objective lens.

Western Blotting

The protein concentration of extracts from sea urchin eggs and embryos was determined by Bradford analysis or BCA (Pierce Chemical). Total protein (10 μg) from each stage was resolved on a 12.5% SDS-polyacrylamide gel. Western blots were performed as described (Jenkins and Xiong, 1995).

Immunoprecipitation

Equal amounts of protein (typically 50 μg) from ^{35}S-methionine-labeled lysates were precleared in the presence of 10 μl Protein A agarose at 4°C for 1 h. After preclearing, samples were transferred to a new microfuge tube and immunoprecipitated with 1 μl α-cyclin E (approximately 200 μg/ml) at 4°C for 2 h. Following incubation with α-cyclin E, proteins that bound to the antibody were recovered by the addition of protein A agarose, and the samples were rocked at 4°C for 1 h. The beads were washed in Lysis Buffer four times and then boiled in sample buffer and resolved on a 12.5% SDS-polyacrylamide gel. The gel was fixed in Enhance (NEN, Boston, MA), and radiolabeled bands were detected by autoradiography.

Assay of Cyclin E-Dependent Kinase Activity

Immunoprecipitations were performed as described above by using extracts from unlabeled embryos. After isolating the antibody complexes with 10 μl of Protein A agarose, the beads were washed in Lysis Buffer four times. After washing, 25 μl of Kinase Assay Buffer (Phelps and Xiong, 1997) was added to the beads. Then, 4 μg of either calf thymus histone H1 (Sigma) or the C-terminus of human pRb conjugated to GST was added in the presence of 1 μCi[γ-^{32}P]ATP (specific activity 3000 Ci/mMol; NEN). Because the antibody was produced against a C-terminal peptide, the specificity of the reaction was determined by the addition of antigenic peptide to the antibody for 15 min at room temperature before carrying out the immunoprecipitation. In order to test the ability of a cdk inhibitor to block the reaction, the cyclin E immunoprecipitates were isolated on Protein A agarose and incubated for 10 min at 25°C in the presence of either p27-GST or p16-GST before performing the kinase assay.

RESULTS

To begin to understand the role of the G_1 cyclins and cdk complexes in the early events in embryogenesis, we have studied the expression and activity of cyclin E in sea urchin oocytes, eggs, and early embryos.

Identification and Characterization of Sea Urchin Cyclin E

Phage (1.2 million) from an *S. purpuratus* sea urchin ovarian library in bacteriophage λ were screened with a mixture of two probes: the cyclin boxes from human and *Drosophila* cyclin E. A single positive phage was obtained that contained a 2.1-kb insert that encoded the full-length cyclin E protein (Fig. 1). The plasmid SpcycE encoded a protein of 423 amino acids (predicted molecular weight 46.2 kDa), and contained 414 nt and 455 nt of 5′ and 3′ untranslated region (UTR), respectively. Cloning of sea urchin cyclin E from the species *Hemicentrotus pulcherrimus* has also been reported recently (Kurokawa *et al.*, 1997), and the nucleotide sequence of our clone is identical to that cDNA (as are several other genes from *H. pulcherrimus* and *S. purpuratus*), raising the likelihood that these two sea urchins are the same species.

The protein encoded by the cDNAs was identified as cyclin E on the basis of the highly conserved cyclin box, which was very similar to both the mammalian and Drosophila cyclin sequences. A comparison of the cyclin E sequences from different phyla is shown in Fig. 1. In addition to the similarity in the cyclin box of cyclin E among the different species, the sequence around threonine-380 (in mammalian cyclin E), which is responsible for regulation of the stability of cyclin E in mammalian cells (Kelly *et al.*, 1998; Clurman *et al.*, 1996), is also conserved in the sea urchin cyclin E (denoted by a ★ in Fig. 1).

The 5′ untranslated region of the cyclin E mRNA is unusually long and contains a number of potential small open reading frames in all three possible reading frames. An in-frame stop codon is present 176 nt upstream of the ATG designated as the initiator methionine, confirming that the initiator methionine has been properly identified (not shown). Long 5′ UTRs with open reading frames are characteristic of many translationally regulated mRNAs, particularly those involved in cell growth (Geballe and Morris, 1994). Rescreening of the library with the cyclin E cDNA resulted in isolation of multiple independent clones of the same cDNA. None of these extended further 5′ than the initially isolated clone.

A polyclonal antibody was raised against a C-terminal peptide containing the last 15 amino acids of cyclin E and affinity purified. To test the ability of the antibody to specifically recognize cyclin E, we labeled sea urchin embryos with ^{35}S-methionine and cysteine for 2 h after fertilization. The lysates were immunoprecipitated with α-cyclin E and analyzed by SDS-polyacrylamide gel electrophoresis. The precipitation was also performed in the presence of the peptide used as the antigen. A single major labeled polypeptide of 46 kDa, the same molecular weight as the *in vitro* translation product (not shown), was precipitated with the α-cyclin E (Fig. 2A, lane 2). There is also a 36-kDa band which is a proteolytic product of cyclin E (see below). Precipitation of both of these proteins was competed by the peptide antigen (Fig. 2A, lanes 3). Neither of these proteins was precipitated by the protein A beads in the absence of antibody (Fig. 2A, lane 4), although there was a nonspecific band at about 43 kDa present in all samples.

The antibody also detected a single polypeptide of about 46 kDa by Western blotting in extracts prepared from 8-cell

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H. sapiens          ------------------PRERRERDAKERDTMKEDGGAEFSARSRKRKANVTVFLQDPDEEMAKIDRTARDQCGSQ-PWDNN-AVCAD
M. musculus         ------------------P---RERDSTDHSNMKEEGGSDLSVRSRKRKANVAVFLQDPDEEIAKIDKTVKSEDSSQ-PWDDNSA-CVD
X. laevis           ----------------------PVISNPAVEKSTKDEGTASCSVRSRKRKADVAIFLQDPDETLDSLEMTKKKQYQDRGPWSNE-MTCKS
D. melanogaster     DSPDSPPSPDRGSKQTPVVVRYAAEQVVTSTVVTQKTEDDDLLDDSCEDYSYDEDDEDDVEEEDDDVEIYSSTISPASSGCSQQQAVNG
S. purpuratus       ---------------SRRSGRLQSRQDNQPLSECISDENNLPMCTRKRKTREQDTTGVSKAEEVQRRRQQFTIENRWVPISE--SSSIE

H. sapiens          PCSLIP---TPDKEDDDRV----YPNSTC-KPRIIAPSRGSPLPVLSWANREEVWKIMLNKE--KTYLRDQHFLEQHPLLQPKMRAILL
M. musculus         PCSFIP---TPNKEEDNELE---YPRTAF-QPRKIRPPRASPLPVLNWGNREEVWRIMLNKE--KTYLRDEHFLQRHPLLQARMRAVLL
X. laevis           PHKLIP---TPEKEEHEPNP---TNYSHF-ASLRFSPVSVSPLPRLGWANQDDVWRNMLNKD--RIYLRDKNFFQKHPQLQPNMRAILL
D. melanogaster     ERTPGLP--KHQEQIHHPVSDLMINMRTPMSPAVENGLRQCPLPALAWANAADVWRLMCHRDEQDSRLRSISMLEQHPGLQPRMRAILL
S. purpuratus       TSLLVPMQTKEPSTPSEELMDTANWVTFRNLFPAHVSDRASPVPLLHWDDLPEVWTIMTRKE--ALCPRKHDCLKSHPSLGERMRAILL

H. sapiens          DWLMEVCEVYKLHRETFYLAQDFFDRYMATQENVVKTLLQLIGISSLFIAAKLEEIYPPKLHQFAYVTDGACSGDEILTMELMIMKALK
M. musculus         DWLMEVCEVYKLHRETFYLAQDFFDRYMASQQNIIKTLLQLIGISALFIASKLEEIYPPKLHQFAYVTDGACSGDEILTMELMMMKALK
X. laevis           DWLMEVCEVYKLHRETFYLAQDFFDRFMATQKNVIKSRLQLIGITSLFIAAKLEEIYPPKLHQFSFITDGACTEDEITRMELIIMKDLG
D. melanogaster     DWLIEVCEVYKLHRETFYLAVDYLDRYLHVAHKVQKTHLQLIGITCLFVAAKVEEIYPPKIGEFAYVTDGACTERDILNHEKILLQALD
S. purpuratus       DWLIEVCEVYRLHRESFYLAADFVDRYLAAKENVPKTKLQLIGITSLFVAAKLEEIYPPKLHEFAYVTDGACTDDQILDQELIMLMTLN

H. sapiens          WRLSPLTIVSWLNVYMQVA----------------YLNDLHEVLLPQYPQQIFIQIAELLDLCVLDVDCLEFPYGILAASALYHFSSSE
M. musculus         WRLSPLTIVSWLNVYVQVA----------------YVNDTGEVLMPQYPQQVFVQIAELLDLCVLDVGCFRFPYGVLAASALYHFSSLE
X. laevis           WCLSPMTIVSWFNVFLQVA----------------YIRELQQFLRPQFPQEIYIQIVQLLDLCVLDICCLEYPYGVLAASAMYHFSCPE
D. melanogaster     WDISPITITGWLGVYMQLNVNNRTPASFSQIGRQKSAEADDAFIYPQFSGFEFVQTSQLLDLCTLDVGMANYSYSVLAAAAISHTFSRE
S. purpuratus       WDLTPITVNTWLNAFMQICNAE------------EIAHRKTNFHFPSYSSTEFVQVAQLLDVCTLDIGSMDFDYSILAASALYHVTNEE

H. sapiens          L-MQKVSGYQWCDIENCVKWMVPFAMVIRETGSSKLKHFR----------------GVADEDAHNIQTHRDSLDLLDK------ARAKK
M. musculus         L-MQKVSGYQWCDIEKCVKWMVPFAMVIREMGSSKLKHFR----------------GVPMEDSHNIQTHTNSLDLLDK------AQAKK
X. laevis           L-VEKVSGFKVTELQGCIKWLVPFAMAIKEGGKSKLNFFK----------------GVDIEDAHNIQTHSGCLELMEK------VYINQ
D. melanogaster     M-ALRCSGLDWQVIQPCARWMEPFFRVISQKA-PYLQLNEQNEQVSNKFGLGLICPNIVTDDSHIIQTHTTTMDMYDEVLMAQDAAHAM
S. purpuratus       V-TLSVTGLKWDDIAACVQWMSTFAMTIREVGVAQLKNFK----------------NIYAGDAHNIQTHCSSLELLDK------SHEKQ

                                    ★
H. sapiens          AMLSEQNRASP-LPSGLLTPPQSGKK--QSSGPEMA
M. musculus         AILSEQNRISP-PPSVVLTPPPSSKKAEQRAGDRMTKPAIASKDSGVEAAAWSSLCCLGGQEPLQMLCSMERCISSGAAGVCKMPWMEV
X. laevis           ALLEEQNRTSP-IPTGVLTPPQSNKKQKSDRAD
D. melanogaster     RSRIQASPATALPPESLLTPPASSHKPDEYLGDEGDETGARSGISSTTTCCNTAASNKVGKSSSNNSVTSCSSRSNP
S. purpuratus       RLLREASCYSPVQVPGVLTPPQSDKKTKKGVL
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FIG. 1. Sea urchin cyclin E. The alignment of human, mouse, *Xenopus*, sea urchin, and *Drosophila* cyclin E sequences is shown. The shaded amino acids are identical in all these species. The cyclin box is underlined. The sea urchin sequence is 60% identical to human cyclin E1 and 64% identical to *Drosophila* cyclin E in the cyclin box. The peptide used as the antigen is underlined. The ★ indicates the conserved threonine that is phosphorylated to target cyclin E for degradation.

embryos (Fig. 2B, lane 1), and the reaction was blocked by competition with the antigenic peptide (Fig. 2B, lane 2). Thus, this antisera specifically recognizes sea urchin cyclin E and both immunoprecipitates and detects the cyclin E polypeptide by Western blotting.

Cyclin E Is Expressed at High Levels in Eggs and Early Embryos

To determine the expression pattern of cyclin E during development, we prepared extracts from embryos at various times after fertilization, resolved the equal amounts of total protein by SDS-gel electrophoresis, and probed Western blots with the cyclin E antibody. Surprisingly a large amount of cyclin E protein was detected in the unfertilized eggs (Fig. 3A, lane 1), which have completed meiosis and are quiescent. There was as much cyclin E in the egg as in any other stage of development. The egg contained more cyclin E protein than the cleaving embryo (4–8 h), when cell number increases from 8 cells to over 100 cells. The levels of cyclin E decreased about 2-fold between 2 and 4 h of development (2–8 cell embryo; Fig. 3A, lanes 4 and 5), but there was a larger drop (an additional 5-fold) between 10.5 and 12.5 h of development (at the very early blastula stage). Thus, the early blastula (12.5 h) embryo contains about 5–10% the amount of cyclin E present in the unfertilized egg. In later stages (after 24 h), cyclin E protein was barely detectable by Western blotting of whole embryo extracts (not shown).

Total RNA was prepared from the same stages of development as the protein samples, and the amount of cyclin E mRNA was determined by Northern analysis. A single cyclin E mRNA, about 5.5 kb in length, was detected. The level of cyclin E mRNA was high in eggs and remained high

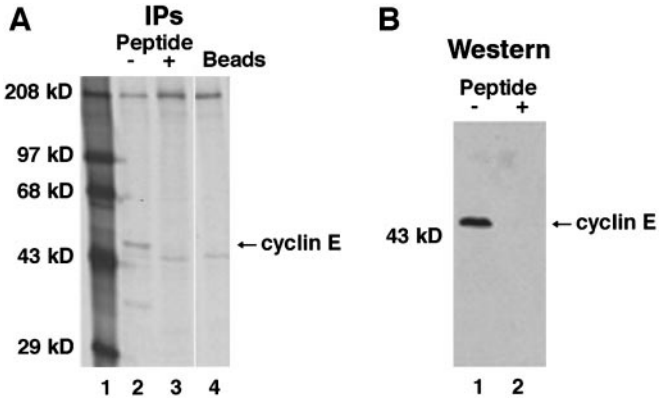

FIG. 2. Characterization of sea urchin cyclin E protein. (A) Newly synthesized cyclin E can be immunoprecipitated from embryos. Immediately after fertilization, embryos were incubated in sea water with ^{35}S-methionine-cysteine, and radiolabeled extracts were prepared at 100 min after fertilization. Both the 46-kDa intact cyclin E and the 32-kDa proteolytic product are precipitated by the antibody and competed by the peptide. Lane 1, Molecular weight markers. Lane 2, 100 μg of the extract was immunoprecipitated with α-cyclin E. Lane 3, α-cyclin E was preincubated in the presence of 0.5 μg of competing peptide. Lane 4, The extract was treated with protein A beads without added antibody. (B) Western blot of 8-cell embryonic extracts probed with α-cyclin E. Protein (10 μg) from 8-cell (4-h) embryos was resolved by SDS–PAGE and transferred to nitrocellulose. The filter was cut in half and one lane blotted with α-cyclin E (lane 1). The other lane was blotted with α-cyclin E that had been preincubated with 1 μg of the peptide antigen (lane 2).

throughout the first 10–12 h of development (early blastula; Fig. 3B, lanes 3–8, top panel). Cyclin E mRNA levels then declined slowly (Fig. 3B, lanes 9 and 10, top panel), decreasing about 5-fold between 10 and 23 h (mesenchyme blastula) of development.

The mitotic cyclins A and B are very abundant mRNAs in early embryos (Minshull *et al.*, 1989; Pines and Hunt, 1987). To compare the levels of the mitotic cyclin mRNAs with cyclin E mRNA, we analyzed the same RNA samples by Northern blotting using similar sized probes of identical specific activity. More than 10 times as much cyclin A mRNA than cyclin E mRNA was present in eggs and early embryos (Fig. 3B, lanes 1 and 2). The level of cyclin A mRNA was also high in unfertilized eggs, and declined much more sharply after the blastula stage than the levels of cyclin E mRNA (Fig. 3B, lanes 3–10, bottom panel).

The level of cyclin E mRNA and cyclin E protein did not change in parallel, but the cyclin E protein levels declined much more rapidly than the cyclin E mRNA levels. In particular, cyclin E protein levels dropped about 10-fold between 2 and 10 h of development, a time when cyclin E mRNA levels were constant. In addition, cyclin E protein was less than 5% of the level in the egg by 15.5 h (Fig. 3A, lane 10), and even lower in 23-h embryos (not shown), a time when there is still significant amounts of cyclin E mRNA (Fig. 3B, lane 10). Cyclin E mRNA levels decline slowly later in development (not shown), although the protein is barely detectable by Western blotting (not shown). Therefore, a major component of regulation of cyclin E protein levels during this time must be translational or posttranslational.

Synthesis of Cyclin E in Early Embryogenesis

Major changes occur in the rates of translation of preformed maternal mRNAs in animal cell embryos, as a result of selective recruitment of mRNAs to polyribosomes (Winkler *et al.*, 1985; Nelson and Winkler, 1987). In sea urchins, the overall rate of protein synthesis rises more than 50-fold during the first 4 h of development (Winkler *et al.*, 1985). Among the proteins synthesized by the embryo immediately after fertilization are the mitotic cyclins A and B, and these proteins are not being synthesized at a significant rate in the unfertilized egg (Evans *et al.*, 1983).

For experiments examining cyclin E synthesis and activity during the first two cell cycles, we used highly synchronous populations of embryos. After fertilization sea urchin embryos proceed synchronously through the first cell cycle, and the events of the first cell cycle have been very well documented (Whitaker and Patel, 1990). To ensure that we were studying synchronous populations of embryos, we used eggs from a single female (up to 10–20 ml of eggs could be obtained) and fertilized and cultured them in dilute suspensions (0.5–1 ml eggs/100 ml). The eggs were fertilized (>98%) in less than 2 min after addition of sperm. Over 90% of the embryos cleaved to the 2-cell stage within a 5-min window, indicating that they progressed through the first cell cycle synchronously. In some experiments, the cultures were monitored by polarization microscopy, and we observed that nuclear envelope breakdown and mitosis and cytokinesis occurred synchronously in the culture.

To determine whether cyclin E was synthesized immediately after fertilization, we preloaded eggs for 4 h with ^{35}S-methionine and then fertilized the eggs and prepared extracts from eggs and embryos 40, 80, and 110 min postfertilization. Inclusion of the label resulted in a slightly slower first cell cycle, with cytokinesis occurring at about 120 min in this experiment. Sea urchin eggs import amino acids poorly, and the 4-h incubation of the eggs with labeled methionine allows equilibration of the amino acid pool as well as labeling of the proteins being synthesized by the eggs. Following fertilization, the labeled methionine that was imported during the 4-h preincubation is used for protein synthesis, and incorporation of radiolabel into total protein was linear for at least 3 h (not shown). The cyclin E protein was then precipitated with the anti-cyclin E antibody and the proteins resolved by gel electrophoresis. Two labeled proteins were observed, a major band at 46 kDa (full-length cyclin E) and a less intense band at 36 kDa, which is a proteolytic fragment of cyclin E. Note that the precipitation of both the 36-kDa polypeptide and full-length

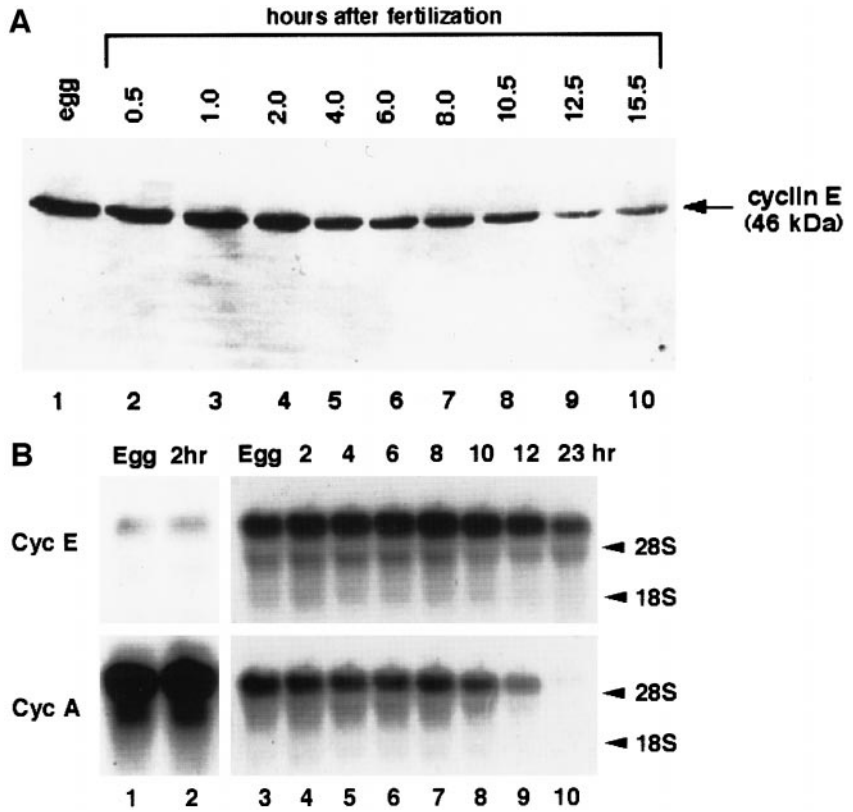

FIG. 3. Expression of cyclin E during development. (A) Extracts were prepared from eggs and embryos at different times after fertilization by using the SDS extraction procedure. Equal amounts of protein (as judged both by protein assay and staining of the gels) were resolved by SDS–PAGE. Proteins were transferred to nitrocellulose and Western blotted with α-cyclin E. Lane 1, Unfertilized eggs. Lanes 2–10, Extracts prepared at the indicated times after fertilization. Embryos were at the 2-cell stage at 2 h, 8-cell stage at 4 h, and in the early blastula stage at 12.5 h. (B) Equal amounts of RNA from eggs and 2-h embryos were resolved by gel electrophoresis. The filter was probed with cyclin E (lanes 1 and 2, top) or cyclin A (lanes 1 and 2, bottom) probes of equal specific activities. The filters were washed and exposed to the same piece of X-ray film to assess the relative amounts of cyclin E and cyclin A mRNAs. In lanes 3–10, duplicate total cell RNA samples from eggs and embryos from the indicated time of development were resolved by gel electrophoresis and probed with cyclin E (top) or cyclin A (bottom) probes. The cyclin E blot was exposed for 20 times longer than the cyclin A blot.

cyclin E was competed by the antigenic peptide (Fig. 2A). Lysis of eggs in lysis buffer results in a cleavage of cyclin E yielding a 36-kDa fragment detected by Western blotting (not shown). This cleavage is a result of the activity of proteases that are released from the egg at fertilization (Vacquier *et al.*, 1972), and is present at low levels in some, but not all, embryo extracts. The 36-kDa band is not cdk2, which migrates at 29 kDa, and is not synthesized at a significant rate in eggs or early embryos (J.A.N., unpublished results).

Incorporation of label into cyclin E was readily detectable within 40 min after fertilization (Fig. 4A, lane 3), and the amount of labeled protein reached a steady-state by 80-110 min (Fig. 4A, lanes 4 and 5), although the amount of total labeled protein was still increasing at a linear rate at this time (not shown). In this experiment, the first cell division was between 100 and 105 min. Both mitotic (80 min) and cells immediately after cytokinesis (110 min) contained similar amounts of newly synthesized cyclin E. Since incorporation of label into total protein continued at a linear rate for more than 2 h and incorporation of label into cyclin E reached a constant level in about 80 min, cyclin E must have a relatively short half-life during the first cell cycle. When the total embryo protein was analyzed and the newly synthesized proteins were detected by autoradiography, a protein comigrating with cyclin E was not visible (not shown). This is consistent with the much lower levels of cyclin E mRNA in the egg than cyclin A mRNA (Fig. 3B).

To determine how rapidly we could see an increase in cyclin E synthesis after fertilization, we prelabeled eggs for 4 h and then fertilized the embryos and harvested them 10 min after fertilization. There is a small amount of cyclin E synthesized in the unfertilized egg, all of which is in the 36-kDa proteolytic fragment (Fig. 4A, lane 6). We detected a clear increase in the amount of cyclin E (46-kDa band plus 36-kDa band), analyzed by immunoprecipitation, synthe-

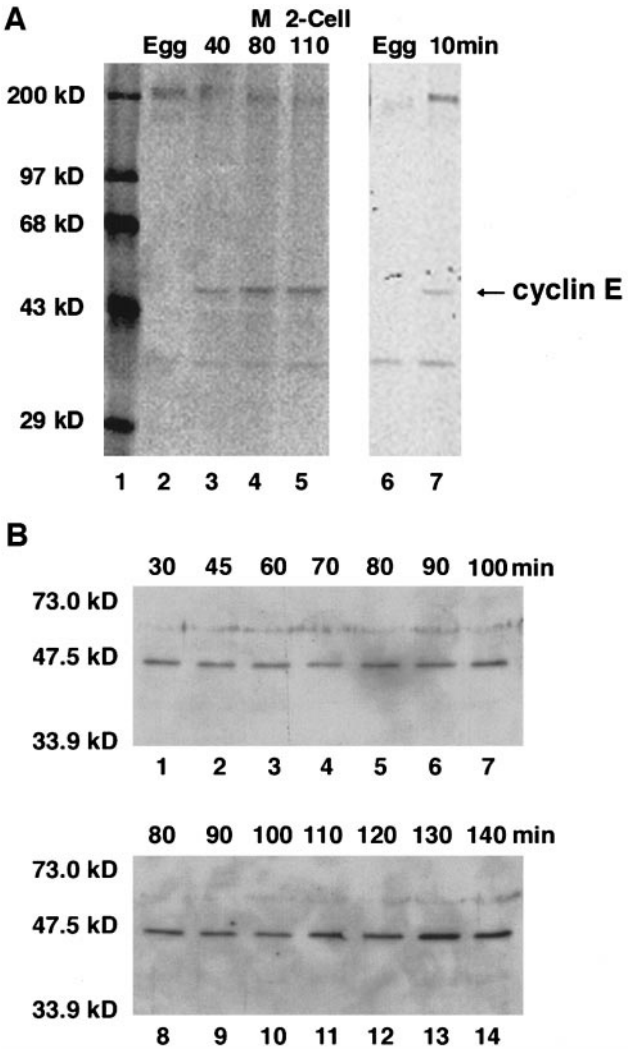

FIG. 4. Synthesis of cyclin E in early embryos. (A) Eggs were preloaded with ^{35}S-methionine-cysteine for 4 h, and then the eggs were fertilized. Extracts were prepared from eggs and from embryos 40, 80, and 110 min after fertilization. Each extract (100 μg) was immunoprecipitated with α-cyclin E. Lane 1, Molecular weight markers. Lane 2, Egg. Lane 3, 40 min after fertilization. Lane 4, 80 min (in mitosis) after fertilization. Lane 5, 110 min (just completed cytokinesis) after fertilization. Lanes 6 and 7 show a separate experiment in which extracts were prepared from the radiolabeled eggs and embryos 10 min after fertilization. The band at about 36 kDa is a proteolytic fragment of cyclin E. (B) Extracts of *S. purpuratus* embryos from a single female were prepared by suspension in SDS lysis buffer. Equal amounts (10 μg) of protein were resolved by SDS-gel electrophoresis and cyclin E detected by Western blotting using the anti-cyclin E antibody. The samples were resolved on two SDS-polyacrylamide mini-gels. To account for differences in transfer, the 80-, 90-, and 100-min time points were loaded on both gels, allowing us to standardize the relative amounts of cyclin E as detected by Western blot. The time in minutes after fertilization is shown above each lane. More than 90% of the embryos completed cytokinesis between 120 and 130 min in this experiment. The position of the molecular weight markers is indicated.

sized in the first 10 min after fertilization (Fig. 4A, lane 7) compared to the amount of label incorporated in cyclin E by eggs in a 4-h incubation (Fig. 4A, lane 6). These results demonstrate that cyclin E protein is synthesized in sea urchin eggs, and that translation of cyclin E mRNA is increased immediately after fertilization.

The rapid increase in the rate of synthesis of cyclin E after fertilization did not result in an increase in the absolute amount of cyclin E protein (Fig. 3A, lanes 1 and 2). We analyzed the amount of cyclin E by Western blotting at 10-min intervals during the first cell cycle in a highly synchronous population of embryos derived from a single female (Fig. 4B). There was no change in the amount of cyclin E protein during the first and second cell cycles. In this experiment, the embryos cleaved to give two cell embryos between 120 and 130 min. In particular, there was no change in cyclin E levels as the embryos progressed either through S-phase (Fig. 4B, lanes 2–4) or mitosis (Fig. 4B, lanes 5–12). These results suggest that cyclin E must be continually turning over after fertilization, since cyclin E is being synthesized, but the steady-state amount of cyclin E is not increasing. It is likely that there is an increase in both the rate of synthesis and degradation of cyclin E after fertilization. Consistent with this conclusion is the recent report of an increase in proteasome activity after activation of echinoderm eggs (Chiba *et al.*, 1999).

Kinase Activity Associated with Cyclin E

In somatic cells, the major function of cyclin E is to bind to cdk2 and phosphorylate critical substrates, resulting in progression of cells into S-phase. We determined the level of cyclin E kinase activity in eggs and embryos by immunoprecipitation of cyclin E followed by use of either histone H1 or the pocket fragment of the retinoblastoma protein, pRb, as a substrate. We readily detected kinase activity using either histone H1 or pRb as a substrate in immunoprecipitates of egg and embryo extracts with anti-cyclin E (Figs. 5A and 5B). The activity was completely blocked by including excess antigenic peptide in the immunoprecipitation (not shown, see Fig. 6A). This assay provides a quantitative estimate of the amount of cyclin E activity in a particular extract since immunoprecipitation of increasing amounts of extract resulted in recovery of increasing amounts of kinase activity (not shown).

Sea urchin embryogenesis initiates with a rapid period of cell division lasting until the early blastula stage. During this time, there is an exponentially increasing rate of DNA synthesis on a per embryo basis. We measured the activity of cyclin E kinase activity at various times after fertilization. We observed the highest levels of cyclin E-associated kinase in eggs and early embryos and the level of cyclin E-associated kinase decreased as development proceeded. Activity was reduced about 3-fold in the 6-h (32–64 cell) embryo (Fig. 5A, lane 4) compared to the 2-cell embryo (Fig. 5A, lane 3). There is a further drop in activity during the morula stage to a level about 10% of that in the egg (7.5 h;

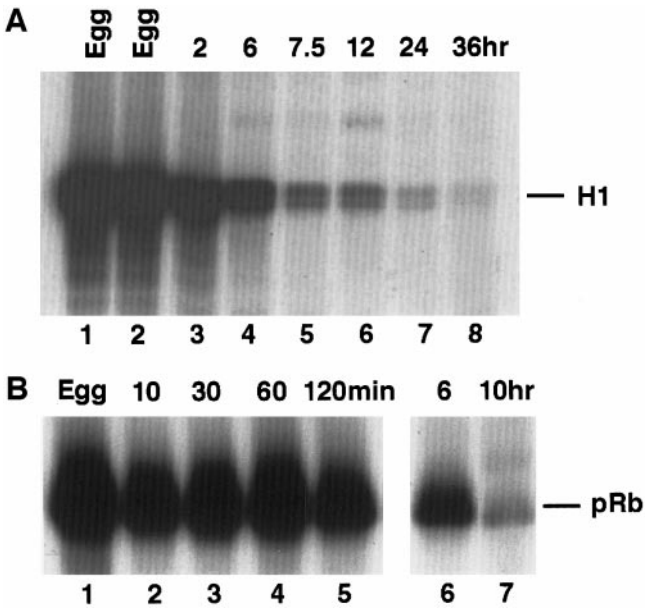

FIG. 5. Cyclin E-associated kinase activity during development. (A) Two independent extracts from eggs (lanes 1 and 2) and embryos were prepared at the indicated times after fertilization. Protein (100 μg) was immunoprecipitated with α-cyclin E. Immunoprecipitated proteins were washed with Lysis buffer and assayed for kinase activity by using histone H1 as a substrate. The reaction was resolved by SDS–PAGE and phosphorylated proteins detected by autoradiography. Lane 3, 2-h (2-cell) embryonic extract. Lane 4, 6-h (30–60 cell) embryonic extract. Lane 5, 7.5-h (morula) extract. Lane 6, 12-h (early blastula) extract. Lane 7, 24-h (mesenchyme blastula) extract. Lane 8, 36-h (early gastrula) extract. (B) Extracts from eggs and embryos were prepared at different time points through the first cell cycle. Protein (200 μg) was immunoprecipitated with α-cyclin E. Immunoprecipitated proteins were washed with Lysis buffer and assayed for kinase activity using pRb as a substrate. The reaction was resolved by SDS–PAGE and phosphorylated proteins detected by autoradiography. Lane 1, Egg extract. Lane 2, 10 min after fertilization. Lane 3, 30 min after fertilization. Lane 4, 60 min after fertilization. Lane 5, 120 min (late mitosis) after fertilization. Lane 6, 6-h (30–60 cells). Lane 7, 10-h (early blastula).

Fig. 5A, lane 5), despite the fact that, during this time, there is at least a 50-fold higher rate of DNA synthesis/embryo than during the first cell cycle. Activity declined to barely detectable levels by the mesenchyme blastula stage (24 h of development; Fig. 5A, lane 7) and the early gastrula stage (Fig. 5A, lane 8). Figure 5B shows a separate experiment using pRb as a substrate, again showing that the cyclin E-associated kinase activity initially declines after the first 2 h (Fig. 5B, lanes 6 and 7), and continues to decline throughout cleavage. The activity of cyclin E-associated kinase qualitatively parallels the level of cyclin E protein (Fig. 3A). The one exception is the large decrease in cyclin E kinase activity after 7.5 h (Fig. 5A, lane 5; Fig. 5B, lane 7), before there is a similar decrease in cyclin E protein which doesn't occur until early blastula stage, after 10 h of development (Fig. 3A, lanes 7–9). The reason for this discrepancy is not known, but it is possible that cyclin E-associated kinase activity has been reduced at this time as the result of the appearance of a cdk inhibitor or an inhibitory modification of cdk2.

Cyclin E Is Active in Sea Urchin Eggs

Surprisingly, we detected the highest levels of cyclin E-associated kinase activity in extracts of sea urchin eggs, as assayed by phosphorylation of both histone H1 and pRb. Similar results were found in six different batches of egg and embryo extracts, and, in each case, the cyclin E-associated kinase activity in eggs was as high or higher than the activity in 2-cell embryos, even though the eggs are quiescent and are not capable of DNA replication. At these early stages, the activity of cyclin E-associated kinase does not change during the first cell cycle and, importantly, is present during "G_1" (Fig. 5B, lanes 2 and 3), S-phase (Fig. 5B, lane 4), and mitosis (Fig. 5B, lane 5, and unpublished results).

The finding that there were equally high levels of cyclin E-associated kinase activity in unfertilized eggs and early embryos was surprising, since (1) the egg is not replicating DNA, (2) it has already completed meiosis, and (3) eggs are stored for weeks in a quiescent state in the ovary prior to spawning. Therefore, we performed several control experiments to ascertain that we were specifically measuring cyclin E-associated kinase activity, both in the egg and in the embryo samples. Parallel immunoprecipitations were performed from the egg extract in the presence of increasing amounts of the peptide used as an antigen. The activity of these immunoprecipitates was assayed by using both histone H1 and pRb as substrates. Precipitation of the kinase activity toward both substrates in the egg extracts was completely prevented by preincubation of the antibody with the synthetic peptide (Fig. 6A, lanes 2 and 6).

A characteristic of the cyclin E-associated kinases is that they are inhibited by the p21 family of cyclin-dependent kinase inhibitors (Sherr and Roberts, 1995), and we took advantage of this specific inhibition as a further demonstration of cyclin E kinase activity (Sherr and Roberts, 1999). The cyclin E-associated kinase was immunoprecipitated from the egg extract with the anti-cyclin E antibody, and then the kinase activity assayed in the presence of GST-p27, a p21 family member, or GST alone. p27 specifically blocked the kinase activity (Fig. 6B, lane 4), while the GST protein had no effect (Fig. 6B, lane 3). The p16 cdk inhibitor, which is specific for the cdk4/6-associated kinase activity (Guan *et al.*, 1994), also had no effect on the cyclin E-associated kinase activity (Fig. 6B, lane 5). We conclude that our activity assay specifically measures cyclin E-dependent kinase activity in extracts from sea urchin eggs.

One of the critical changes which occurs at fertilization is an increase in the pH of the sea urchin egg, and this increase

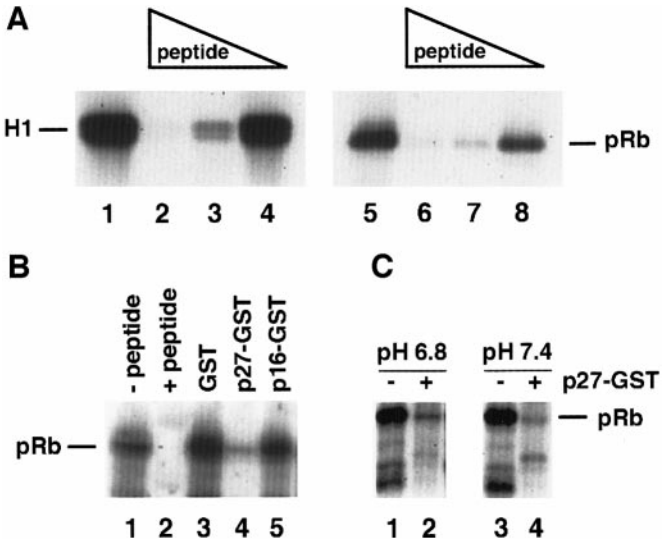

FIG. 6. Characterization of cyclin E kinase activity in eggs. (A) α-Cyclin E antibody was incubated in the absence (lanes 1 and 5) or presence (lanes 2–4, 6–8) of varying amounts of the peptide antigen. After incubation, 100 μg of egg extract was added to each sample followed by immunoprecipitation with protein A beads. Immunoprecipitated proteins were washed with Lysis buffer and assayed for kinase activity by using histone H1 (lanes 1–4) or pRb (lanes 5–8) as a substrate. The proteins were resolved by SDS–PAGE and phosphorylated proteins detected by autoradiography. Lanes 2 and 6, 1 μg antigenic peptide. Lanes 3 and 7, 0.01 μg antigenic peptide. Lanes 4 and 8, 0.001 μg antigenic peptide. (B) Equal amounts of egg extract (100 μg protein) were immunoprecipitated with α-cyclin E antibody. The immunoprecipitates were analyzed for kinase activity by using pRb as a substrate. In lane 2, the antibody was incubated with 1 μg of peptide antigen. In lanes 1 and 2, no proteins were added to the immunoprecipitates prior to the kinase assay. In lanes 3–5, equal amounts of GST, p27-GST, or p16-GST, respectively, were added prior to the kinase assay. The phosphorylated proteins were resolved by gel electrophoresis and detected by autoradiography. (C) Egg lysates were prepared by using lysis buffers at pH 6.8 and pH 7.4. Equal amounts (100 μg of protein) of extracts were immunoprecipitated with α-cyclin E. The immunoprecipitated proteins were washed with Lysis buffer and assayed for kinase activity at the appropriate pH using pRb as a substrate in the presence of GST (lanes 1 and 3) or p27-GST (lanes 2 and 4). The phosphorylated proteins were resolved by gel electrophoresis and detected by autoradiography.

in pH is sufficient to initiate the increase in protein synthesis (Grainger *et al.*, 1979; Winkler *et al.*, 1980; Winkler and Steinhardt, 1981) and DNA replication in eggs (Mazia and Ruby, 1974; Mazia, 1974). Since the extracts we had assayed were all prepared at pH 7.4, it was possible that we had activated latent cyclin E kinase activity by increasing the pH of the extract compared with that of the unfertilized egg. We prepared extracts at either pH 7.4 or pH 6.8 from the same batch of eggs and assayed the cyclin E-associated kinase activity in the immunoprecipitates. The pH in the assay buffer was the same as in the corresponding extract. Identical activities were found in both extracts (Fig. 6C, lanes 1 and 3), and the activity was inhibited by the cdk inhibitor p27 (Fig. 6D, lanes 2 and 4). Thus, we conclude that the cyclin E-dependent kinase in eggs exists in a form which is catalytically active.

Localization of Cyclin E in Eggs and Early Embryos

To determine the expression characteristics of cyclin E, we used both *in situ* hybridization to detect the cyclin E mRNA and immunofluorescence to detect the cyclin E protein. Using *in situ* hybridization, we observed high levels of cyclin E mRNA in both immature and mature oocytes, while there was no detectable cyclin E mRNA in the surrounding ovarian tissue (Fig. 7A). Cyclin E mRNA is present in oocytes throughout oogenesis, and is uniformly distributed in the cytoplasm of oocytes and eggs (Fig. 7B). Cyclin E mRNA remained at similar levels in all cells through development to the early blastula stage (Fig. 7C). In later embryos, cyclin E mRNA levels were significantly lower (Fig. 7B) and all detectable signal was present in select cells and tissues. In mesenchyme blastula embryos, cyclin E mRNA accumulated selectively in the vegetal plate, as well as in a small patch of cells at the animal pole (Fig. 7D). During gastrulation, cyclin E mRNA was detectable in both invaginating gut precursor cells as well as in single cells within the ectoderm (Figs. 7B and 7E). These latter cells appeared to be pigment cells, based on their morphology, presence in the ectoderm, and spacing, although, following the fixation and hybridization protocol used in these experiments, the pigment is extracted, so it is not possible to definitively identify them. In larvae, the ciliated band and regions of the gut, tissues known to be still undergoing cell division, continue to accumulate detectable levels of cyclin E mRNA (Fig. 7F). In larvae cultured for several days, significant cyclin E mRNA was also detected in the left but not the right coelomic pouch (Fig. 7G). The embryo develops coelomic pouches following gastrulation, one on either side of the foregut, just below the mouth. Both coelomic pouches develop and expand into elaborate mesodermal tissues during the larval stage. The left coelomic pouch, however, contains the cells destined to form the adult rudiment, where, at metamorphosis, the immature adult everts and the remainder of the larval tissues histolyze. The assymetry in cyclin E levels between the two pouches is not coincident with the onset of coelomic pouch development, since late gastrula/prism embryos (Figs. 7B and 7E) have apparent pouches, but no significant cyclin E mRNA accumulation in the pouches. This suggests that expression of cyclin E and the proliferation of the pouch derivatives is a result of mitogenic induction, and not remaining from cells which were continuously cycling and had maintained high cyclin E mRNA levels.

The antibodies to cyclin E also allowed us to study the cyclin E protein accumulation by immunofluorescence.

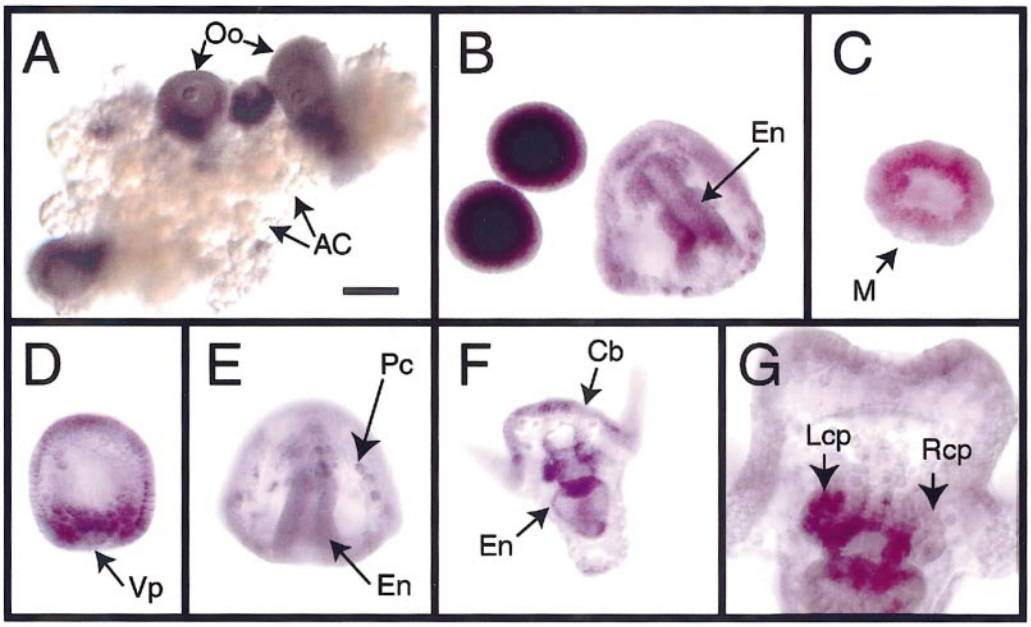

FIG. 7. Detection of cyclin E mRNA by *in situ* hybridization. Cyclin E mRNA was localized in situ following hybridization to digoxygenin probes as described in Materials and Methods. The embryos were hybridized to the same batch of probe and developed for similar times. (A) An explant of ovary tissue showing both immature (small) and full-grown oocytes. Note accessory cells (AC) are unlabeled. (B) Eggs and a gastrula embryo hybridized on the same cover slip. Overall, the gastrula embryo contains significantly less cyclin E mRNA, although some tissues are enriched, especially the endoderm (En). (C) Early blastula showing micromere (m) derivatives. (D) Mesenchyme blastula showing enriched cyclin E mRNA in the vegetal plate (Vp). (E) Gastrula embryo focused on the surface to reveal the pigment cells (Pc) within the ectoderm. (F) Pluteus larva showing low overall cyclin E signal. The tissues with detectable cyclin E mRNA include the ciliary band (Cb), endoderm (En) and shown best in a higher magnification of a different embryo (G) label in the left coelomic pouch. Note that the right coelomic pouch is not detectably labeled, nor are pigment cells at this stage. Bar in (A) = 50 μm.

The mature oocytes contained large amounts of cyclin E, and it was largely, if not exclusively, restricted to the germinal vesicle (Fig. 8A), as judged by both whole-mount immunofluorescence and confocal microscopy. After germinal vesicle breakdown, cyclin E protein was uniformly distributed throughout the egg cytoplasm, and by confocal microscopy was not found concentrated in any particular region of the cytoplasm, and there was only a slight concentration of cyclin E in the pronucleus (Fig. 8A). In the one-cell zygote, cyclin E is concentrated in the nucleus after pronuclear fusion, and, in two-cell, four-cell, and 16-cell embryos, the cyclin E protein was localized in the nucleus of interphase cells (Fig. 8B). Strikingly, cyclin E was present in similar concentrations in the nuclei of all cells, including the micromeres, of the 16-cell embryo (Fig. 8B). The micromeres are the first cells to exit the cell cycle, and their progression through the next cell cycle will be delayed relative to the rest of the blastomeres. In later stages of embryogenesis, there was labeling of a subset of cell nuclei using the antibody to cyclin E (Fig. 8C). In blastula embryos, there was labeling of a subset of cell nuclei, with labeling clearly concentrated in the vegetal plate (Fig. 8C). In the gastrula and early pluteus embryo, there was also labeling of a small subset of cell nuclei, including some of the pigment cells that were also labeled by *in situ* hybridization.

DISCUSSION

During oogenesis, many of the macromolecules that are necessary for early embryogenesis are packaged in the developing egg. At fertilization, the quiescent egg undergoes a number of changes that result in the initiation of DNA replication and entry into the cleavage cell cycles. These unusual rapid cell cycles continue during the initial stages of embryogenesis and are subsequently replaced by cell cycles that are more similar to the somatic cell cycles in larva and adults. However, many of the molecules involved in regulating the later somatic cell cycles are likely also involved in regulating the early cell cycles that lack gap phases.

The cyclin/cdk complexes play a critical role in cell cycle regulation. While the role of the mitotic cyclins A and B are well understood during oogenesis and embryogenesis, it is less clear how the initiation of DNA replication (entry into S-phase) is regulated during embryogenesis. Cyclin E has been directly implicated in controlling initiation of DNA

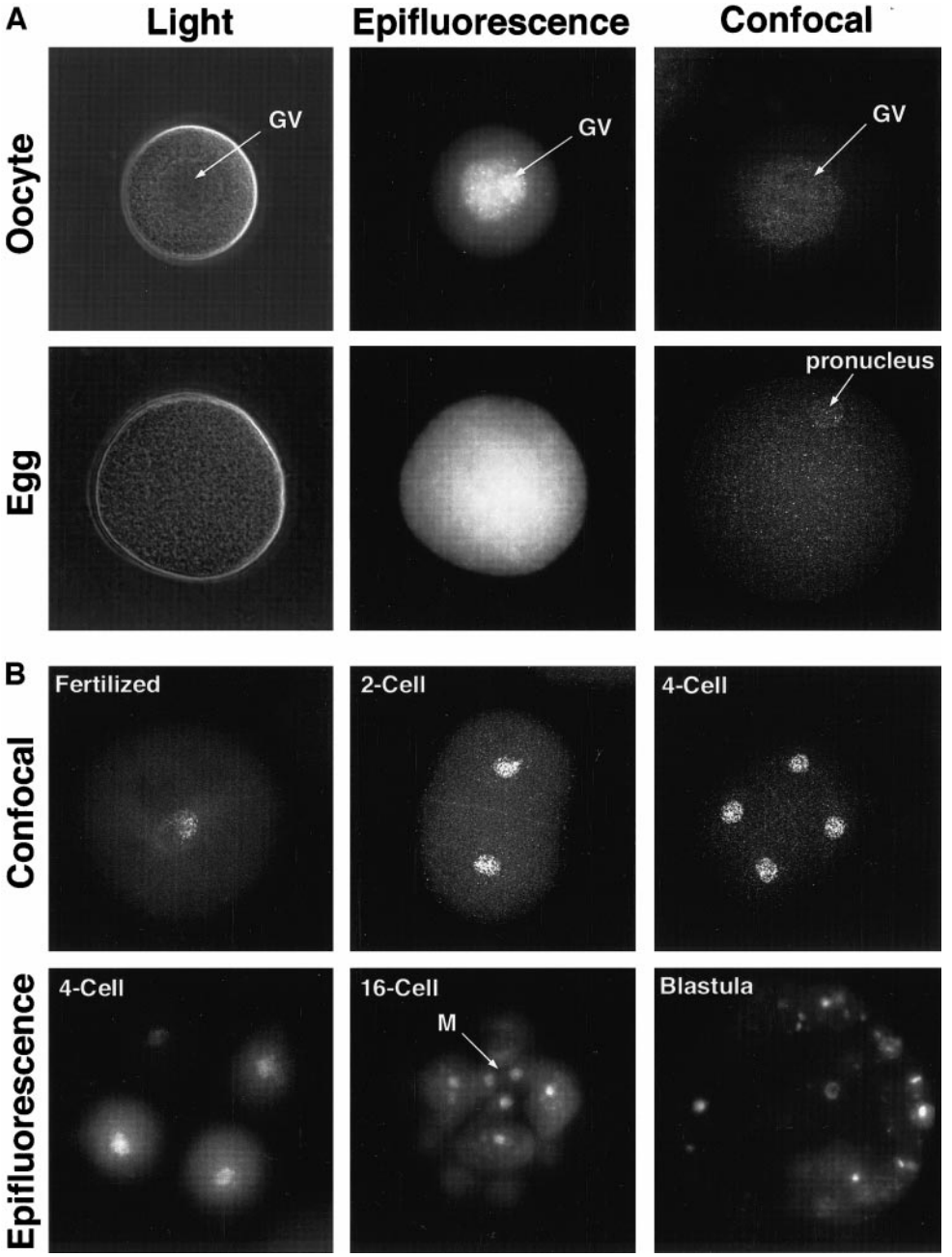

FIG. 8. Immunolocalization of cyclin E during sea urchin development. Oocytes, eggs, and embryos were fixed in paraformaldehyde and immunostained with α-cyclin E and a CY3-conjugated secondary antibody. For confocal microscopy, the samples were fixed in methanol and immunostained with α-cyclin E and rhodamine-conjugated secondary antibody. (A) Oocyte and egg. Phase contrast microscopy (light), epifluorescence, and confocal microscopic images are shown. Note the intense staining in the germinal vesicle (GV) and the lack of staining in the pronucleus of the unfertilized egg. (B) One-cell zygote after pronuclear fusion (fertilized); 2-cell embryo; 4-cell embryo; 16-cell embryo; and mesenchyme blastula embryo. Both epifluorescence and confocal images of 4-cell embryos are shown. (C) Mesenchyme blastula (24-h), gastrula (48-h), and pluteus (72-h) embryo. Phase contrast picture on the left (light), epifluorescence on the right. The vegetal plate (Vp) is labeled in the mesenchyme blastula (out of focal plane) as well as some individual nuclei (which were visible in the phase contrast image of the same embryo (not shown). This is the same embryo shown in (B). Among the cells labeled in the pluteus embryo are some of the pigment cells (Pc).

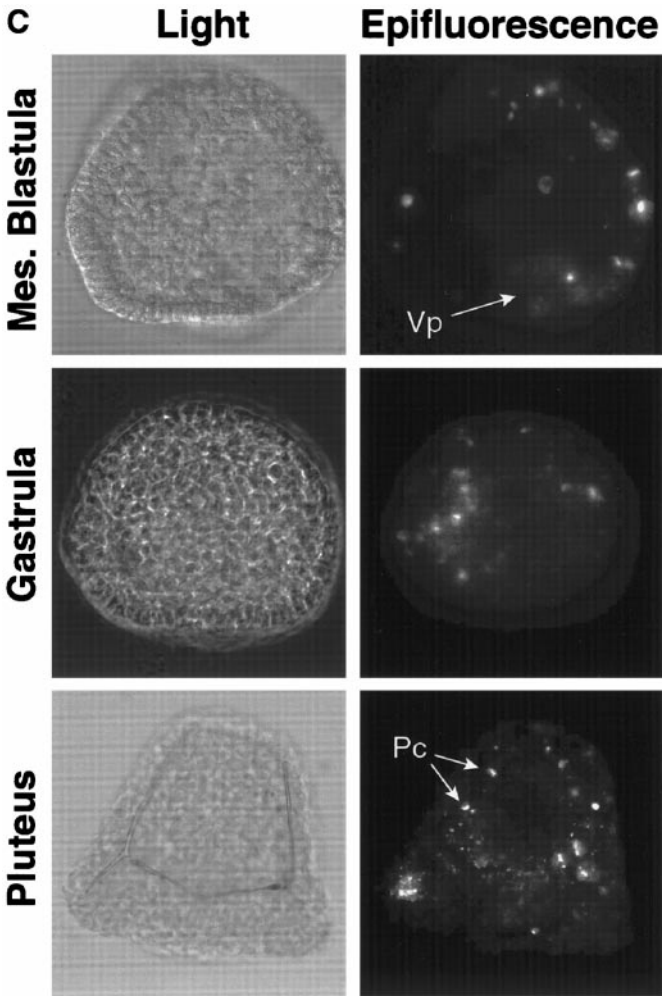

FIG. 8—*Continued*

replication in Xenopus embryos (Strausfeld *et al.,* 1996; Jackson *et al.,* 1995). Cyclin E has also been implicated in controlling the transition from G_1 to S-phase in somatic cells (Ohtsubo *et al.,* 1995; Knoblich *et al.,* 1994; Koff *et al.,* 1992; Duronio and O'Farrell, 1995; Chevalier *et al.,* 1996). In "normal" cell cycles, cyclin E/cdk2 has low kinase activity at the beginning of the gap phase before DNA synthesis (G_1), but rises right before entry into S-phase as a result of both activation of cyclin E expression as well as degradation of inhibitors of cyclinE/cdk2, particularly p27 (Vlach *et al.,* 1997; Coats *et al.,* 1996; Pagano *et al.,* 1995; Polyak *et al.,* 1994). The activity of cyclin E-cdk2 is then reduced during S-phase, and cyclin E-cdk2 does not become active again until the next cell cycle. In the mitotic cell cycles, activation of cyclin E-cdk2 is a major event committing the cell to replicate its DNA. The degradation of cyclin E (or the inactivation of cyclin E-cdk2) is thought to be necessary for cells to complete S-phase and reenter the next cell cycle.

In frog embryos, cyclin E is essential for initiation of DNA replication, since depletion of cyclin E from the Xenopus cell-free system blocks initiation of DNA replication (Strausfeld *et al.,* 1996; Jackson *et al.,* 1995). Cyclin E is also postulated to play a role in meiosis, since significant amounts of cyclin E are synthesized after treatment of stage VI oocytes with progesterone prior to fertilization (Rempel *et al.,* 1995). Other workers claim that the increase in cyclin E-cdk2 at this time has no role in meiosis (Furuno *et al.,* 1997). An additional role for cyclin E is in the replication of the centrosome, and cyclin E is associated with the centrosome in frog embryos (Hinchcliffe *et al.,* 1999; Lacey *et al.,* 1999).

In Drosophila, cyclin E protein is present continuously throughout the initial syncytial cell cycles, as is cdk2. These proteins are found both in the nucleus of interphase cells and in the cytoplasm during mitosis (Sauer *et al.,* 1995). Cyclin E-cdk2 kinase is constitutively active throughout these cell cycles, which are characterized by a lack of a G_1-phase (Sauer *et al.,* 1995). In contrast, later in embryogenesis when endoreduplication cycles occur, cyclin E-cdk2 activity is cyclic, decreasing in the gap phase and then increasing shortly before entry into the subsequent S-phase (Sauer *et al.,* 1995).

Some workers have reported an oscillation of cyclin E-cdk2 activity in frog embryos during the initial cell cycles (Rempel *et al.,* 1995; Hartley *et al.,* 1996), while others have reported that the cyclin E-cdk2 kinase activity is constant during the initial cell cycles *in vitro* (Fang and Newport, 1991; Hua *et al.,* 1997). The levels of cyclin E-cdk2 proteins do not vary during the cleavage cell cycles (Greaves *et al.,* 1989; Fang and Newport, 1991; Howe and Newport, 1996). Newport and coworkers have postulated that the high levels of cyclin E-cdk2 present in nuclei of early embryos inhibits re-replication of DNA prior to the completion of mitosis. At mitosis, the cyclin E-cdk2 is greatly diluted (effectively inactivated) allowing the cell to prepare for the subsequent reinitiation of DNA replication which occurs after formation of the nuclei and import of cyclin E into nuclei (Howe and Newport, 1996). According to this model, supported by *in vitro* experiments in frog egg extracts, there is effectively little difference in the role of cyclin E in the early embryonic cycles and the "normal" somatic cell cycles.

Role of Cyclin E in Sea Urchin Eggs?

We have clearly shown the presence of both cyclin E protein and cyclin E-dependent kinase activity in otherwise quiescent sea urchin eggs, and we see little change in the kinase activity as the embryo progresses through the first cell cycle. Thus, while cyclin E-cdk2 activity may be necessary for the initiation of DNA replication, the activation of cyclin E-cdk2 is not one of the signals which is produced after fertilization resulting in DNA replication. Since protein synthesis is not necessary for entry into or completion of the first S-phase (Hinchcliffe *et al.,* 1998), the

signaling pathway initiated by fertilization (and the increase in pH in the sea urchin embryo), must result in activation of key components for replication by posttranslational mechanisms. Either these mechanisms normally act downstream of cyclin E, affect the phosphatase activity that would alter the balance of modification on cyclin E-cdk2 substrates, or affect a parallel pathway to cyclin E that is necessary for initiation of the first cell cycle. Alternatively, the concentration of cyclin E in the nucleus after pronuclear fusion (Fig. 8B) could trigger the initiation of DNA replication. Recent findings suggest, however, that cyclin E associates with pronuclei well before pronuclear fusion and initiation of DNA replication (B.J.S. and W.F.M., unpublished results), suggesting that there must be additional limiting steps other than concentration of cyclin E-cdk2 activity necessary for initiation of DNA replication.

It is possible that cyclin E-cdk2 also has a role in maintenance of the unfertilized egg in a state that is poised to undergo DNA replication. Alternatively, since cyclin E-cdk2 is not concentrated in the egg pronucleus, there may be cytoplasmic substrates for this complex, either in the egg or after fertilization.

Cyclin E Doesn't "Cycle" during the Initial Cell Cycles

The initial cell cycles in sea urchin embryogenesis don't have gap phases, and cyclin E protein levels and kinase activity are constant through the first cell cycle. In particular, cyclin E kinase activity is high during mitosis and immediately after cytokinesis. These results provide a serious paradox for using cyclin E kinase activity as a paradigm for regulation of the entry into and out of S phase. Clearly, embryonic cells are committed to S phase in spite of the continual cyclin E kinase activity, and they also go through mitosis in the presence of high G_1 cyclin activity. In early embryogenesis in the sea urchin, DNA replication is actually initiated during anaphase as vesicles form around portions of the condensed chromosomes allowing some replication for the next cell cycle to occur during telophase (Ito *et al.*, 1981).

It is not known when the first gap phases are introduced during sea urchin embryogenesis. There is a drop in total cyclin E protein levels between 2 and 4 h (8 cells) and a further drop as embryos enter the blastula stage (between 10 and 12 h, Fig. 3A). However, the levels of cyclin E mRNA are constant throughout this time, suggesting that the levels of cyclin E protein must be translationally or post-translationally controlled as cells enter the blastula stage. By immunofluorescence, only a subset of cells in the blastula embryo contain high concentrations of cyclin E protein, while cyclin E mRNA is present in most if not all cells. These results are consistent with the possibility that the cells in the blastula embryo contain gap phases and that the somatic pattern of cyclin E expression has been established in these cells. These possibilities are currently under investigation.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants GM 58921 (to W.F.M.) and HD28152 and HD01170 (to G.W.) and a grant from National Science Foundation IBN-9816683 (to G.W.). B.J.S. was supported by National Cancer Institute Training Grant T32CA09156 and postdoctoral fellowship F32GM20151. J.C. was supported by a NIH predoctoral training grant (T32GM08581). We thank Yue Xiong for a gift of the GST-p27, p16, and pRb expression plasmids and Yue Xiong and Bob Duronio for many helpful suggestions. We thank Dr. Z. F. Wang for assistance in the library screening.

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Received for publication June 1, 2000
Revised March 7, 2001
Accepted March 7, 2001
Published online May 1, 2001