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Cyclin D and cdk4 Are Required for Normal Development beyond the Blastula Stage in Sea Urchin Embryos

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***cdk4* mRNA and protein are constitutively expressed in sea urchin eggs and throughout embryonic development. In contrast, cyclin D mRNA is barely detectable in eggs and early embryos, when the cell cycles consist of alternating S and M phases. Cyclin D mRNA increases dramatically in embryos at the early blastula stage and remains at a constant level throughout embryogenesis. An increase in cdk4 kinase activity occurs concomitantly with the increase in cyclin D mRNA. Ectopic expression of cyclin D mRNA in eggs arrests development before the 16-cell stage and causes eventual embryonic death, suggesting that activation of cyclin D/cdk4 in cleavage cell cycles is lethal to the embryo. In contrast, blocking cyclin D or cdk4 expression with morpholino antisense oligonucleotides results in normal development of early gastrula-stage embryos but abnormal, asymmetric larvae. These results suggest that in sea urchins, cyclin D and cdk4 are required for normal development and perhaps the patterning of the developing embryo, but may not be directly involved in regulating entry into the cell cycle.**

Entry of somatic cells into the cell cycle is stringently controlled in response to both growth-stimulating and growth-inhibitory signals. One result of this regulation is to ensure that cells do not commit to replicating their DNA and dividing unless the cell has enough nutrients to complete the process. In mammalian cells, cyclin-dependent protein kinases (cdk's) are the major proteins regulating the cell cycle (42). In order for cdk's to become active, they must bind their cyclin partner and be phosphorylated by a cdk-activating kinase (42). Each cyclin contains a region called the cyclin box, which is involved in the binding of specific cdk's (30). The best-defined points of the cell cycle that are controlled by cyclins and cdk's are the G_1/S and G_2/M transitions.

Mammalian cells have two G_1 cyclins: cyclin D, which binds cdk4 and -6, and cyclin E, which binds cdk2. Cyclin D plays a major role in the transition of a cell from a resting to a growing state. Resting mammalian cells have very small amounts of cyclin D/cdk4 kinase activity, because of both very small amounts of cyclin D protein and the presence of specific p16/ink4 cdk inhibitors, which bind to free cdk4 and -6 and inhibit their kinase activity (48, 60). When somatic mammalian cells are stimulated to reenter the cell cycle, cyclin D mRNA levels increase rapidly and remain elevated as long as mitogens are present (36). This expression results in the formation of an active kinase complex composed of cyclin D/cdk4. The major substrate for cyclin D/cdk4 is pRb, and phosphorylation of pRb results in dissociation of the E2F complex from Rb (7, 17, 29, 56). The free E2F proteins in turn stimulate transcription of genes required for DNA synthesis (20, 56).

In cycling cells, cyclin D is constitutively synthesized but continuously turned over as a result of phosphorylation of a conserved threonine by glycogen synthase kinase-3β, followed by targeting to the proteasome (5). cdk4 is maintained at a constant level through the cell cycle in all mammalian cells examined (37, 38, 40). In contrast, cyclin E/cdk2 activity cycles in mammalian somatic cells and an increase in cyclin E/cdk2 activity are necessary to commit a cell to enter S phase (31). Cyclin E/cdk2 also phosphorylates pRb as well as other molecules directly involved in chromosome replication (49).

In addition to control of the cell cycle through regulation of the level of cyclin proteins, there are also two families of cdk inhibitors that are important in cell cycle regulation (50). The p21 family members bind to the cyclin/cdk complexes, while the p16/ink4 family members bind directly to cdk4 and cdk6. Homologues of p21 family members are present in all metazoans and are important in regulation of the cell cycle in embryonic development in *Drosophila melanogaster* (4, 33) and *Caenorhabditis elegans* (24), while the p16 family has only been identified in mammals thus far.

The role of the G_1 cyclins in the early embryo is less well understood. Many organisms, including sea urchins, go through a period of rapid cell division after fertilization, in which the cell cycles consist of alternating S phases and mitoses, with no gap phases. These cell cycles are controlled in part by the oscillation of cyclins A and B (16, 22). Two additional cell cycle events occur during embryogenesis that are unique to development. The first is the precise timing of the introduction of gap phases, and the second is the complex mechanism that ends maternal control of the embryo and initiates zygotic control. Additionally, the embryo must correctly signal the patterning and differentiation of cell lineages within the embryo to form a viable larva.

An unusual characteristic of sea urchins is that their female

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gametes are stored as haploid eggs. The zygote enters S phase directly after fertilization rather than having to first complete meiosis. A large amount of cyclin E is complexed with the cdk2 present in the unfertilized egg, and cyclin E/cdk2 levels do not change during the initial cell cycles (52). Thus, unlike somatic cell cycles, early cell cycles do not require degradation of cyclin E for completion. Although some of the mechanisms that control the switching of the cell cycles from S/M cycles to cycles with gap phases have been elucidated in *D. melanogaster* cells (13, 15), very little is known about this mechanism in vertebrates or other invertebrates. Recent data from *D. melanogaster* and *C. elegans* suggest that cyclin d/cdk4 complexes may have some alternative functions other than cell cycle control in these invertebrates. Specifically, these data show that cyclin D and cdk4 appear to be more involved in the regulation of cell growth and size than in cell cycle progression.

Here we report that in sea urchin embryos, cyclin D mRNA expression is increased only at the end of cleavage, while cdk4 mRNA is constitutively expressed. The increase in cyclin D mRNA is accompanied by an increase in cdk4 kinase activity. Premature expression of cyclin D is lethal to the early embryo, while blocking cdk4 or cyclin D expression results in abnormal development at the gastrula stage as a result of inappropriate patterning of the embryo rather than an obvious cell cycle defect. These results demonstrate that cyclinD/cdk4 is essential for early embryogenesis in the sea urchin, with the primary regulatory signal being zygotic expression of cyclin D mRNA.

MATERIALS AND METHODS

Culturing sea urchin embryos. Sea urchin gametes (*Strongylocentrotus purpuratus* and *Lytechinus variegatus*) were obtained by injecting the urchins with 0.55 M KCl. Eggs were collected in seawater at 16°C, and sperm was collected and stored dry at 4°C. Eggs were dejellied by passage through 75-μm Nitex (Sefar America, Depew, N.Y.) and packed by centrifugation at 2,000 × *g* for 5 min. The eggs were resuspended in seawater at a concentration of 0.5 ml of packed embryos per 100 ml of seawater with 0.1% ampicillin (Sigma, St. Louis, Mo.) with constant aeration. If the embryos were harvested before hatching, 1 mM *para*-aminobenzoic acid was added to the dejellied eggs before fertilization to inhibit cross-linking of the fertilization envelope. Embryos that were grown past the mesenchyme blastula stage were collected at 18 to 24 h and resuspended at a concentration of less than 2.5 ml of embryos per liter of seawater for continued growth. Fertilization was checked by light microscopy and was greater than 95%.

Embryo extracts were prepared essentially as described previously (52). Embryos were washed twice with 0.55 M KCl. The embryo pellet was resuspended in 8 volumes of 0.22 M sucrose–10 mM Tris (pH 7.6)–1 mM EGTA–1 mM EDTA–1 mM dithiothreitol. The embryos were pelleted again and resuspended in 3 volumes of lysis buffer (50 mM Tris [pH 7.6], 0.15 M NaCl, 0.5% NP-40, 1 mM dithiothreitol, 1 mM $NaVO_4$, 1 mM phenylmethylsulfonyl fluoride). The embryos were homogenized with a Dounce homogenizer (tight pestle) and checked by light microscopy to ensure complete cell lysis.

For analysis of proteins by Western blotting, some extracts were prepared by resuspending the 0.55 M KCl-washed embryos in 1 volume of 0.5 M NaCl, followed by addition of 2 volumes of a 10% sodium dodecyl sulfate (SDS)–5 mM EDTA solution. The embryos were then pipetted up and down several times to immediately lyse the cells and inactivate proteases and stored at −20°C.

Cloning sea urchin cdk4 and cdk2. A fragment of *cdk4* cDNA was isolated from DNA prepared from a sea urchin ovary cDNA library by degenerate PCR (Fig. 1A). The primers used were forward (amino acids HRDLKPQN), 5′-CA Y(A/C)GNGAYYTNAARCCN(G/C)ARAA and reverse (amino acids WYRAPE), 5′-YTCNGGNGCNC(G/T)RTACCA. The amplified fragments were cloned, and we obtained 75 clones containing 110-nucleotide fragments encoding a portion of sea urchin cdk's. Three of the 75 clones sequenced encoded 33 amino acids of the sea urchin homologue of CDK4, based on the presence of the QMALT sequence that is unique to vertebrate CDK4 and CDK6. The fragment encoding a portion of the sea urchin cdk4 was labeled by PCR in the presence of [α-^{32}P]dCTP and used as a probe to screen an *S. purpuratus* ovary library (32); 1.2 million phages were screened, and two clones were obtained, one of which encoded the full-length cdk4 protein (accession no. AY044637). The coding region of the *S. purpuratus cdk4* was amplified and used to screen an *L. variegatus* cDNA library prepared from 4-h (morula stage) embryos (12). Several full-length clones encoding *L. variegatus* cdk4 were obtained and sequenced (accession number AY044638).

We cloned *S. purpuratus cdk2* from the *S. purpuratus* ovary library with the *cdk2* from *Sphaerechinus granularis* (41) as a probe.

Cloning sea urchin cyclin D. The open reading frame of *S. purpuratus* cdk4 was cloned into the yeast two-hybrid vector pGBT8 and used as bait to screen a 20-h sea urchin cDNA library in the vector pGAD10 (a gift from Bob and Lynn Angerer). Since yeast strains expressing sea urchin cdk4 did not transform efficiently, a mating yeast two-hybrid screen was performed. The bait and the prey plasmids were transformed into two complementary *Saccharomyces cerevisiae* mating strains, JP69-4A and JP69-4α (a gift from Niranjan Pandey and Tom Maniatis). The complementary strains were mated in 1% yeast extract–2% peptone–2% dextrose and then plated on synthetic dropout medium lacking LWH and containing 5 mM 3-aminotriazole to select for the most stringent interactions. Five positive colonies were obtained and sequenced from 10^7 colonies screened. Four of the five colonies represented portions of the same cDNA and encoded the homologue of the vertebrate cyclin D's (Fig. 1B).

The fragment obtained from the yeast two-hybrid screen was radiolabeled with [α-^{32}P]dCTP by random primer labeling and used to screen the *S. purpuratus* phage library as described above. From this screen, three positive colonies were obtained and sequenced (accession number AF318615). These cDNAs overlapped, and the final sequence of sea urchin cyclin D is shown in Fig. 1B. The 4-h *L. variegatus* library was also screened to obtain full-length cyclin D from that species.

In vitro translation. The open reading frames for cdk4 and cyclin D were cloned into the pGEM5 vector, and 1 μg of the purified DNA template was used in a 50-μl coupled transcription and translation kit (TNT kit; Promega, Madison, Wis.). The reaction mixture contained 25 μl of the TNT rabbit reticulocyte lysate, 40 μM amino acid mixture without methionine, and 5 μCi of [^{35}S]methionine-cysteine (Translabel; New England Nuclear, Boston, Mass.). The reaction mixture was incubated at 30°C for 2 h, and the resulting protein was resolved on an SDS–15% polyacrylamide gel, fixed in En^{3}Hance (New England Nuclear), dried, and autoradiographed.

Total RNA isolation. Total RNA was extracted from both eggs and embryos by phenol extraction, essentially as previously described (27). Eggs and embryos were washed in 0.55 M KCl twice and resuspended in 5 to 10 volumes of 0.25 M sucrose–1 mM EDTA 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 sodium acetate (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 performed, and the nucleic acids were precipitated with 3 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 blots. To make probes for Northern blots, the 1,100-bp *Nco*I fragment from *cdk4* was purified by preparative gel electrophoresis with a Qiaquik gel extraction kit (Qiagen, Valencia, Calif.), and a 1,600-bp PCR fragment of the cyclin D coding region was amplified. Then 100 ng of the DNA was labeled with 50 μCi of [α-^{32}P]dCTP with a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Ind.) and purified by elution through a ProbeQuant G-50 microcolumn (Amersham Pharmacia Biotech, Piscataway, N.J.). Samples containing equal amounts of total RNA (10 μg/lane) were dissolved in 20 mM MOPS (morpholinepropanesulfonic acid, pH 7.0), 8 mM sodium acetate, 1 mM EDTA (pH 8.0), 6% formaldehyde, and 50% formamide, heated to 65°C for 10 min, and separated on an agarose gel containing 6% formaldehyde in 20 mM MOPS (pH 7.0)–8 mM sodium acetate–1 mM EDTA (pH 8.0). The RNA was transferred to a Hybond nylon membrane (Amersham Pharmacia Biotech, Piscataway, N.J.), and the membrane was hybridized with the cdk4 or cyclin D probe at 60°C for 4 h in QuickHybe (Stratagene, La Jolla, Calif.), containing 0.1 mg of salmon sperm DNA per ml. After hybridization, the filter was washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at 58°C for 15 min and once with 0.1× SSC–0.1% SDS at 58°C for 30 min and autoradiographed.

Antibodies. Peptides corresponding to the C-terminal 15 amino acids of *S. purpuratus* cyclin D (CVDEVEIITMPSGLS), cdk4 (CDSSQSQDVTPTNKR),

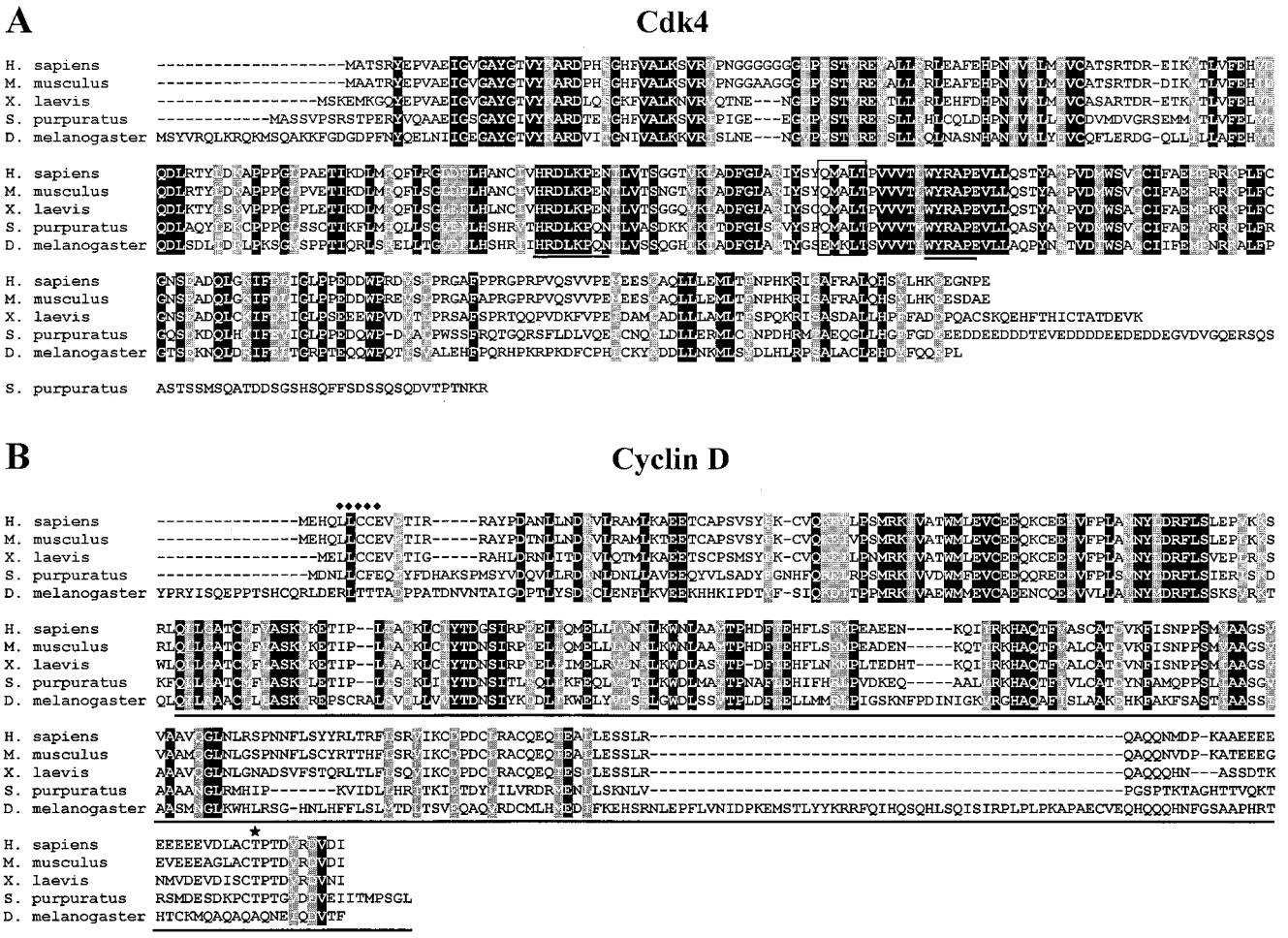

FIG. 1. Sea urchin cdk4 and cyclin D. The protein sequences of sea urchin *S. purpuratus* cdk4 (panel A) and cyclin D (panel B) are compared to the sequences of cyclin D1 from vertebrates (*Homo sapiens*, *Mus musculus*, and *Xenopus laevis*) and cyclin D from *D. melanogaster*. In panel A, the amino acid sequences used to construct primers for PCR to identify the *cdk* genes are underlined. The QMALT sequence characteristic of the vertebrate *cdk4*/*cdk6* genes is boxed. In panel B, the asterisk indicates the threonine identified as the phosphorylation site that determines cyclin D half-life (6). The diamonds indicate the Rb binding motif. Dark shading indicates identical residues, and light shading indicates similar residues.

and cdk2 (CPYFKDVKMVPPPRL) were synthesized at the University of North Carolina Protein Chemistry Facility, coupled to keyhole limpet hemocyanin and used to generate polyclonal antibodies in rabbits (Pocono Rabbit Farm and Laboratory, Canadensis, Pa.). The resulting antibodies were purified with the antigenic peptide cross-linked to a Sulfolink column (Pierce Chemical, Rockford, Ill.). Two milliliters of serum was incubated with the column, and after being washed extensively with phosphate-buffered saline, the antibodies were eluted with 0.1 M glycine-HCl (pH 2.5) and immediately neutralized with 1 M Tris-HCl (pH 9.5). The antibodies recognized the proteins expressed as glutathione *S*-transferase (GST) fusions in *Escherichia coli* or in baculovirus by Western blotting, although the anti-cdk4 was more than 10-fold more sensitive than the anti-cyclin D and precipitated in vitro-synthesized protein labeled with [^{35}S]methionine.

Western blotting. The protein concentration of extracts from sea urchin eggs and embryos was determined by Bradford or bicinchoninic acid analysis (Pierce Chemical). Equal amounts of total protein (typically 20 μg) from all stages were resolved on an SDS–12.5% polyacrylamide gel. Western blots were performed as described before (28, 52).

Immunoprecipitations. Samples (10 μl) from in vitro translations were cleared in the presence of 10 μl of protein A-agarose at 4°C for 1 h. After clearing, samples were transferred to a new microcentrifuge tube and immunoprecipitated with 1 μl of antibody (approximately 200 μg/ml) at 4°C for 2 h. Following immunoprecipitation, the antibodies were incubated with protein A-agarose at 4°C for 1 h. The antibody complex was collected by centrifugation, washed in lysis buffer four times, boiled in sample buffer, and resolved on an SDS–12.5% polyacrylamide gel. The gel was fixed in En^{3}Hance (New England Nuclear), and radiolabeled bands were detected by autoradiography.

Protein kinase assays. Immunoprecipitations were done as previously described with extracts from sea urchin embryos at the indicated times after fertilization (52) and antibodies to either cyclin E or cdk4. After isolation of 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 (19) was added to the beads. Then 4 μg of the C terminus of human pRb conjugated to GST was added in the presence of 1 μCi of [γ-^{32}P]ATP (specific activity, 3,000 Ci/mmol; New England Nuclear). 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 situ hybridization. A digoxigenin RNA probe was prepared by in vitro transcription of cyclin D in the presence of digoxigenin-11-UTP (Boehringer Mannheim, Indianapolis, Ind.). Sea urchin eggs and embryos were fixed, washed, rehydrated, and treated with proteinase K as described previously (46). The fixed embryos were hybridized, washed, and visualized as described previously (52).

mRNA microinjections. The sea urchin cyclin D and *cdk4* open reading frames were cloned into the pXFRM expression vector (55) behind an SP6 promoter and in vitro transcribed with the Message Machine transcription kit from Ambion (Austin, Tex.). *L. variegatus* eggs were collected and dejellied as described above and plated in seawater on plates that had been coated with protamine

sulfate. The eggs were fertilized and injected with 0.2 to 1.0 pg of RNA within 5 min after fertilization (35). Development of the embryos was monitored by light microscopy.

In vitro translation reaction mixtures containing morpholino oligonucleotides. Morpholino oligonucleotides complementary to the 5′ untranslated region (UTR) (25 nucleotides before the start codon) of cyclin D and *cdk4* were synthesized by Gene-Tools Inc. (Corvallis, Oreg.). The *cdk4* morpholino was ATGCCATCCTCTTCTCTGGTCCCTT, and the cyclin D morpholino was ATCGTCCCACAAGTTCGGTTTGCGG. A total of 10 pmol of either the cdk4, cyclin D, or control morpholino was incubated with in vitro-transcribed RNA at 37°C for 15 min. The RNA morpholino mixture was then in vitro translated in the presence of [^{35}S]methionine (TNT kit; Promega, Madison, Wis.), and the products were analyzed by gel electrophoresis and detected by autoradiography.

Morpholino oligonucleotide injection. With the cdk4 and cyclin D morpholinos described above, *L. variegatus* embryos were injected less than 5 min after fertilization with 1 to 2 pl of a morpholino mixture that contained 2.5×10^{-16} mol ($\approx 1.5 \times 10^{8}$ molecules) of morpholino, 20% glycerol, and 10 pg of dextran-tetramethyl rhodamine (molecular weight, 10,000; Molecular Probes, Eugene, Oreg.). Development was monitored by light microscopy.

Staining nuclei to determine total cell number. Injected embryos were fixed in 90% methanol–50 mM EGTA (pH 6.0) at −20°C (21), washed two times for 5 min in 2× SSC, and treated with 100 μg of RNase A per ml in 2× SSC for 20 min at 37°C. After three rinses with 2× SSC, the embryos were incubated in 5 μM Syto11 (Molecular Probes, Eugene, Oreg.) at room temperature for 5 min. The embryos were then washed three times for 10 min each with 2× SSC. Embryos were then flattened by a coverslip and mounted in Elvanol (44). Images were collected with a Zeiss LSM 410 laser scanning confocal system in conjunction with a Zeiss Axiovert 100 microscope equipped with a ×40/1.3NA objective lens.

RESULTS

Identification of cdk4. We amplified a conserved region of the cyclin-dependent kinases with degenerate primers to the underlined amino acids in Fig. 1A and obtained clones that contained 110 nucleotides of the sea urchin homologue of the various cdk's. cdk4 was identified based on the fact that the QMALT sequence (boxed in Fig. 1A), unique to vertebrate cdk4 and cdk6, was present in this fragment, and we used the fragment to isolate the *S. purpuratus cdk4* cDNA. This cDNA encoded a protein with a predicted molecular mass of 42 kDa (372 amino acids) (Fig. 1A). We arbitrarily designated this clone cdk4, because it is equally similar to mammalian cdk4 and cdk6. We do not know if there is a second member of this class of cdk's in sea urchins.

Sea urchin cdk4 is longer than any other known cdk and contains a glutamic/aspartic acid-rich region near the C terminus. We also cloned cdk4 from the sea urchin *L. variegatus* with the *S. purpuratus cdk4* as a probe. The *L. variegatus* cdk4 is also a 372-amino-acid protein and contains the C-terminal acidic region (not shown). It is 94% identical with the *S. purpuratus* cdk4. Hence, the unusual size of cdk4 is likely a general characteristic of the sea urchin cdk4, since the extended C-terminal acidic region is not found in any other known cdk. The longest cDNA clone that we obtained also contained a 5′ UTR of 604 nucleotides. Since the mRNA for cdk4 is at least 8 kb (see below), the 3′ UTR must be over 5 kb. There was only limited similarity between the 5′ UTRs of the cdk4 cDNAs from the two sea urchin species.

Sea urchin cdk4 interacts with mammalian cyclin D and p16. A characteristic of cdk4 is that it binds tightly to cyclin D and the ink4 class of cdk inhibitors. To determine whether the putative sea urchin cdk4 sequence has the properties of cdk4, we tested its ability to interact with human cyclin D and/or the cdk inhibitor p16 in directed yeast two-hybrid assays. Sea urchin cdk4 was subcloned into the pGBT8 yeast two-hybrid vector and transformed into the yeast strain HF7c. Yeast cells expressing *S. purpuratus* cdk4 were then transformed with human cyclin D1, human cyclin D2, or human p16 and cloned as fusion proteins to the Gal4 activation domain in pGAD10. Yeast cells containing both plasmids were streaked onto selective medium and transferred to a nitrocellulose membrane to test for the transcription of the *lacZ* reporter gene. As shown in Fig. 2, sea urchin cdk4 is able to tightly bind human cyclins D1 (Fig. 2, panel 6) and D2 (Fig. 2, panel 5) and human p16 (Fig. 2, panel 4), and thus we conclude that the sequence encodes a functional cdk4. As shown in Fig. 2 (panel 2), yeast cells expressing cdk4 fused to the Gal4 DNA binding domain (DBD) in the absence of an interacting protein fused to the Gal4 activation domain are unable to grow on medium lacking histidine.

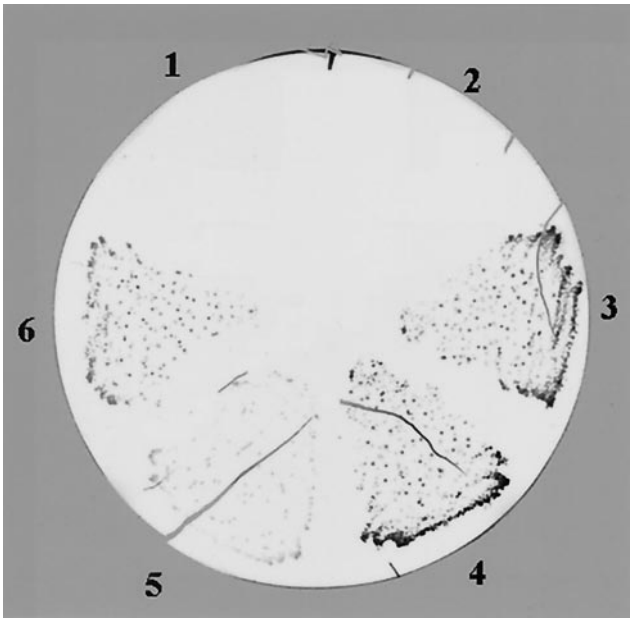

FIG. 2. Sea urchin cdk4 interacts with human cyclin D and p16, The *S. purpuratus cdk4* open reading frame was fused to the Gal4 DNA binding domain and transformed into yeast cells expressing the Gal4 activation domain (sector 2) or the Gal4 activation domain fused to human p16 (sector 4), human cyclin D2 (sector 5), or human cyclin D1 (sector 6). Sector 1 is the HF7c yeast strain with no vectors, and sector 3 is yeast cells expressing the human *cdk4* open reading frame fused to the Gal4 DNA binding domain transformed with human cyclin D1. The yeast cells were grown on plates lacking leucine and tryptophan and then streaked onto plates lacking leucine, tryptophan, and histidine. The colonies were transferred to nitrocellulose and then assayed for β-galactosidase activity.

Functional cloning of sea urchin cyclin D. With the sea urchin cdk4 as bait, 10 million clones were screened for proteins that interacted with cdk4 with a yeast two-hybrid cDNA library from 20-h *S. purpuratus* embryos. To increase the efficiency of the screen, a cDNA library that had been transformed into the JP69-4A yeast strain was used. The JP69-4α yeast strain was transformed with *gal4*(DBD)-*cdk4*, and the library was screened by mating the two yeast strains. Four positive clones were identified, each of which contained fragments of sea urchin cyclin D, based on their homology to cyclin

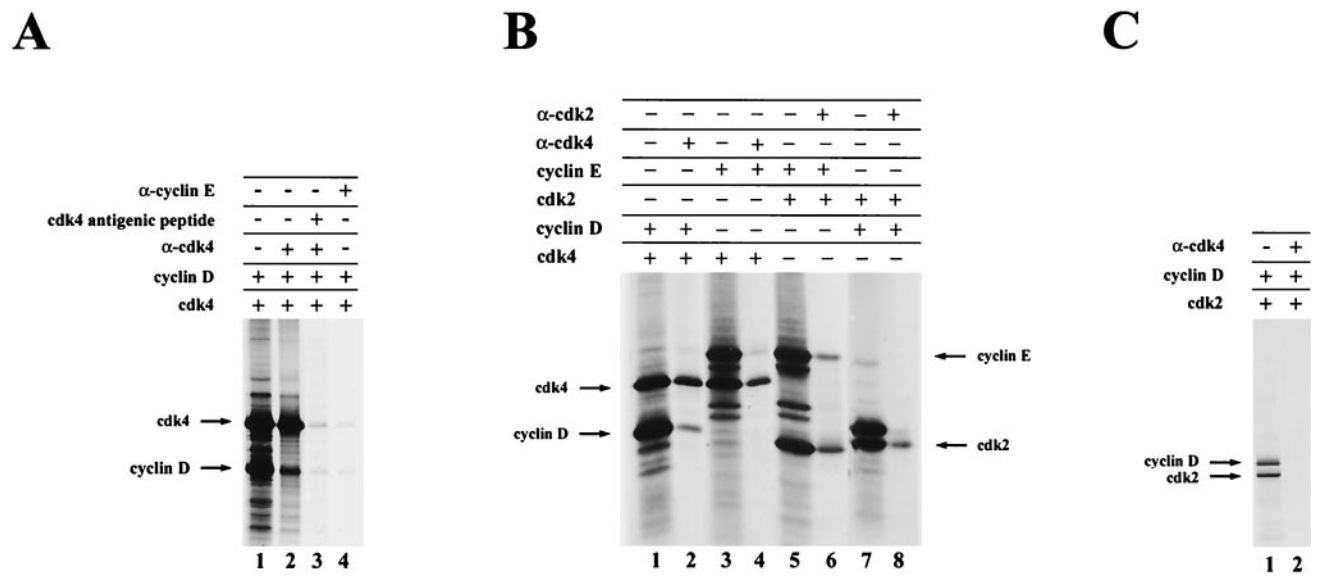

FIG. 3. Sea urchin cyclin D and cdk4 form a complex in reticulocyte lysates. (A) Synthetic mRNAs encoding *S. purpuratus* cyclin D and cdk4 were mixed and translated in a rabbit reticulocyte lysate containing [^{35}S]methionine. Equal amounts of lysate were analyzed directly (lane 1) or precipitated with the anti-cdk4 antibody in the absence (lane 2) or presence (lane 3) of competing antigenic peptide or the antibody to the C terminus of cyclin E (lane 4). (B) mRNAs encoding *S. purpuratus* cdk4 and cyclin D (lanes 1 and 2), cdk4 and cyclin E (lanes 3 and 4), cyclin E and cdk2 (lanes 5 and 6), or cyclin D and cdk2 (lanes 7 and 8) were cotranslated in a rabbit reticulocyte lysate system containing [^{35}S]methionine. The resulting proteins were analyzed directly (lanes 1, 3, 5, and 7) or after precipitation with α-cdk4 (lanes 2 and 4) or α-cdk2 (lanes 6 and 8). The cyclin E and cdk2 were analyzed directly or after precipitation with α-cyclin E (lane 4). (C) mRNAs encoding *S. purpuratus* cdk2 and cyclin D were cotranslated in the presence of [^{35}S]methionine (lane 1). The resulting proteins were immunoprecipitated with α-cdk4 (lane 2).

D from other organisms. Clones encoding the complete cyclin D coding region were subsequently obtained from both *S. purpuratus* and *L. variegatus* by screening phage cDNA libraries.

Sea urchin cyclin D is a 303-amino-acid protein which is most similar to the vertebrate proteins in the cyclin box. Figure 1B shows the sequence similarity between cyclin D from various organisms. In addition to the cyclin box, two key regions are conserved among the cyclin D proteins in sea urchins and vertebrates. These are the phosphorylation site, CTPT, which is responsible for determining the half-life of cyclin D in mammalian cells, and the LXCXE motif, which is responsible for binding to the Rb family of proteins (Fig. 1B, asterisk and diamond) (6, 7, 29). Since cyclin D mRNA is about 6.0 kb long, as analyzed by Northern blots (see Fig. 4B), there must be a long 3′ UTR, as there is in human cyclin D1 (59). The largest clone that we obtained had 1,800 nucleotides of 3′ UTR and no polyadenylation signal. The longest 5′ UTR that we obtained was 90 nucleotides. The cyclin D from the sea urchin *L. variegatus* was 85% identical to the *S. purpuratus* cyclin D, differing in 45 amino acids from the *S. purpuratus* cyclin D, most of which were conservative changes.

Sea urchin cyclin D forms a complex with sea urchin cdk4. A synthetic peptide representing the C terminus of cdk4 was used to raise a rabbit polyclonal antibody. In order to demonstrate that the cdk4 and cyclin D cloned here from sea urchins are capable of forming a complex, these proteins were synthesized together by in vitro translation, and the reaction mixture was immunoprecipitated with α-cdk4 and α-cyclin D. The cdk4 antibody successfully coimmunoprecipitated cdk4 and cyclin D (Fig. 3A, lane 2), and the precipitation of the two proteins was blocked by preincubation with the antigenic peptide (Fig. 3A, lane 3). The α-cdk4 did not precipitate cyclin D in the absence of coexpression of cdk4 (Fig. 3C, lane 2). We coexpressed cdk4 and sea urchin cyclin E in the reticulocyte lysate and tested whether cdk4 could bind cyclin E as assayed by coimmunoprecipitation with α-cdk4. Only background levels of cyclin E were precipitated with the α-cdk4 (Fig. 3B, lane 4). As an additional control, we used α-cyclin E, which did not immunoprecipitate either cyclin D or cdk4 (Fig. 3A, lane 4), although it does precipitate cyclin E efficiently (52).

We also cloned *S. purpuratus* cdk2 and prepared an antibody against the C-terminal peptide. We coexpressed cyclin E and cdk2 in the reticulocyte lysate (Fig. 3B, lane 5) and also coexpressed cyclin D and cdk2 (Fig. 3B, lane 7) and tested whether α-cdk2 could precipitate a cyclin/cdk2 complex. The α-cdk2 precipitated both cdk2 and cyclin E (Fig. 3B, lane 6), but did not coprecipitate cyclin D (Fig. 3B, lane 8).

We prepared several antibodies with peptides or recombinant cyclin D, but we only obtained low-affinity antibodies, which precipitated only small amounts of cyclin D from in vitro translation reaction mixtures and did not detect cyclin D on Western blots of embryos. The antibody against the C terminus of cyclin D did coimmunoprecipitate cyclin D and cdk4, but with much lower efficiency than the α-cdk4 antibody (data not shown). Thus, as in mammalian cells, cdk4 formed a complex with sea urchin cyclin D but not cyclin E, and cdk2 formed a complex with cyclin E but not cyclin D.

Expression of cdk4 and cyclin D mRNA during development. We used Northern blots to determine the amounts of *cdk4* and cyclin D mRNAs present during sea urchin development. Equal amounts of total RNA (as determined by levels of

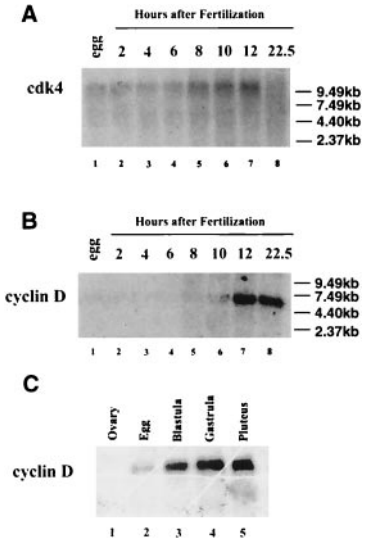

FIG. 4. Expression of *cdk4* and cyclin D mRNA during development. Total RNA was prepared from *S. purpuratus* eggs and embryos at the indicated times after fertilization and culturing at 15°C. Equal amounts of total RNA were resolved by Northern blotting, transferred to nitrocellulose, and probed with random primed labeled *cdk4* cDNA (A) or cyclin D cDNA (B and C). Eggs are shown in lane 1, and embryos at the indicated times of development are shown in lanes 2 to 8 of panel B and lanes 2 to 5 of panel C. The 12-h embryos in panel B were at early blastula stage.

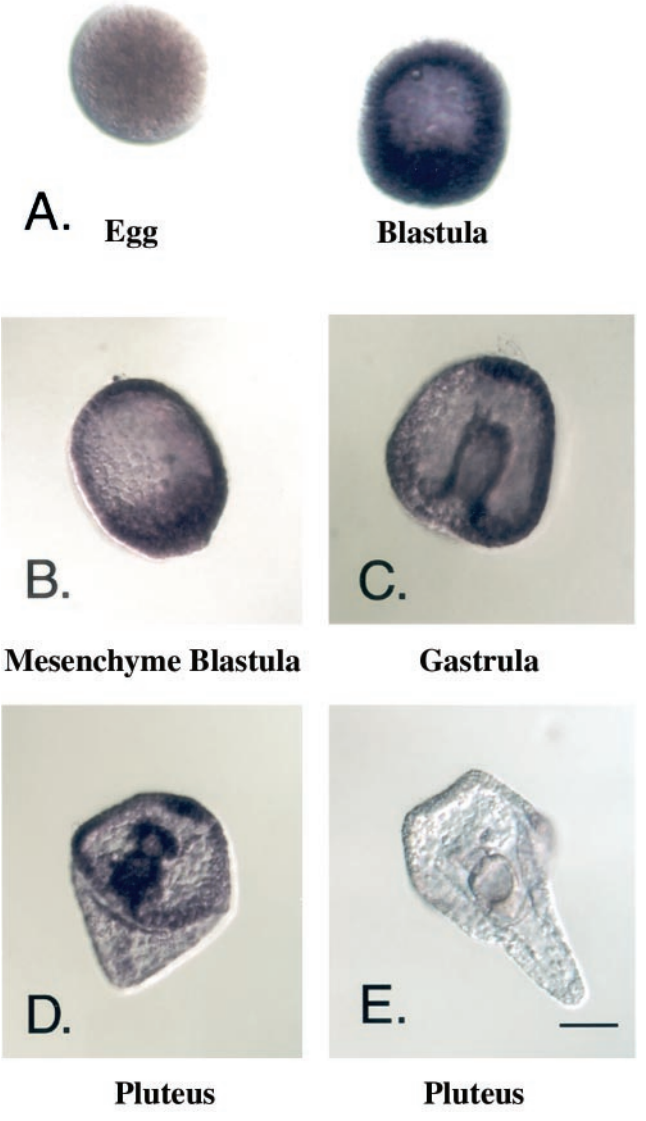

FIG. 5. Expression of cyclin D mRNA during embryogenesis by in situ hybridization. *L. variegatus* embryos at different stages were fixed and analyzed for cyclin D mRNA by in situ hybridization. Eggs (left) and early embryos (A) have no detectable signal until blastula stage (embryo at right), when the signal appears uniform throughout the embryo. At mesenchyme blastula (B), the mRNA is concentrated in the vegetal plate and the future oral ectoderm. This ectodermal regionalization persists through gastrulation (C) to the larval stage (D), and cyclin D mRNA remains concentrated in regions of the ciliary band. Endoderm labeling also increases during gastrulation to maximal levels in plutei. Panel E is a pluteus larva hybridized with a sense strand probe. The slight purple color in the egg is due to the pigment in the egg and not to hybridization.

rRNA) were resolved by agarose gel electrophoresis and analyzed with the coding region of cdk4 as a probe. *cdk4* mRNA is about 8 to 10 kb long and is present at constant low levels in the unfertilized egg and through the first 8 h of development (Fig. 4A, lanes 1 to 5). The levels of *cdk4* mRNA then increased slightly between 8 and 12 h (Fig. 4A, lanes 5 to 7) and decreased to levels similar to those in the first 8 h of development by 23 h (mesenchyme blastula; Fig. 4A, lane 8).

In contrast to *cdk4* mRNA, the levels of cyclin D mRNA changed dramatically during development. Cyclin D mRNA (6 kb) was present at low levels in the unfertilized egg, and the levels remained low during the first 8 h of development through the morula stage (Fig. 4B, lanes 1 to 6). There was a large increase (at least 10-fold) in cyclin D mRNA in early blastula embryos, by 12 h of development, and mRNA levels remained high throughout blastula (Fig. 4B, lanes 7 and 8) and persisted through the pluteus stage (Fig. 4C).

In addition to the Northern blots that were performed to quantitate levels of cyclin D message, in situ hybridizations were done to determine the localization of cyclin D mRNA in the developing embryos. In agreement with the Northern blots, there were not significant levels of cyclin D mRNA in sea urchin eggs compared to those in later-stage embryos (Fig. 5A). In agreement with the Northern blot, cyclin D mRNA levels were first detectable by in situ hybridization at the blastula stage (6 h in *L. variegatus*), and cyclin D mRNA was expressed in all cells at this stage. In mesenchyme blastula- and gastrula-stage embryos, cyclin D mRNA was concentrated in the vegetal plate and in the ectoderm on the oral side of the embryo (Fig. 5B and C). In pluteus embryos, cyclin D mRNA was concentrated in the gut and ciliary band (Fig. 5D).

cdk4 protein levels during development. To test whether our cdk4 antibody could detect small amounts of cdk4, we synthesized cdk4 in a coupled in vitro transcription and translation reaction. We readily detected the sea urchin cdk4 by Western blotting as a single band migrating at 41 kDa (Fig. 6A, lane 1), which was not present in the reticulocyte lysate (Fig. 6A, lane 2).

The α-cdk4 also detected a single 41-kDa protein in sea

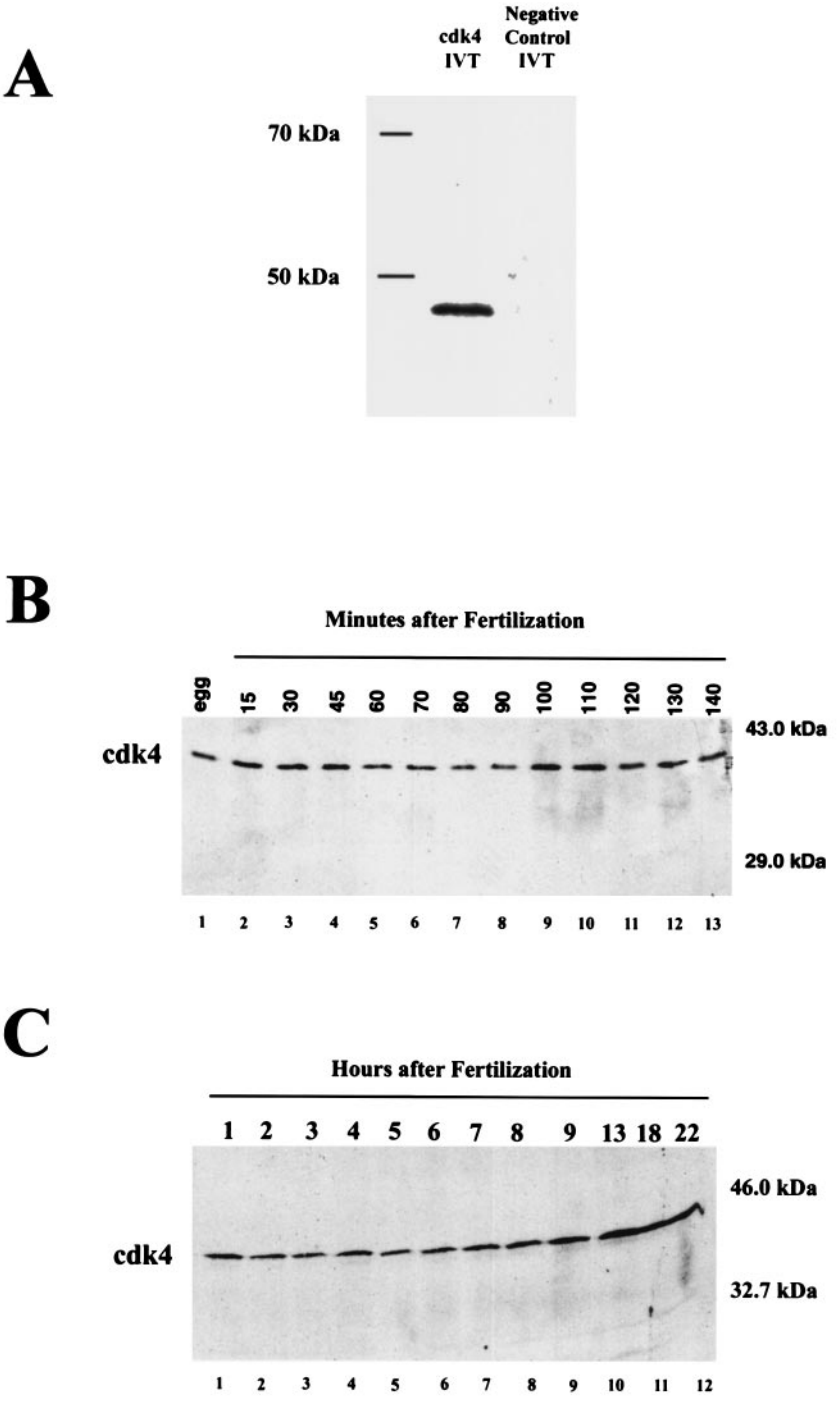

FIG. 6. Expression of cdk4 protein during development. (A) A coupled transcription-translation reaction in a reticulocyte lysate programmed with a plasmid expressing *cdk4* mRNA (lane 1) or a plasmid (lane 2) was analyzed by Western blotting with the anti-cdk4 antibody. IVT, in vitro translation. (B) Extracts were prepared from *S. purpuratus* eggs (lane 1) and embryos at various times of development. In panel B, embryos were harvested at the indicated time throughout the first two cell cycles. The embryos divided between 110 and 120 min. (C) Embryos were harvested at the indicated time after fertilization. Equal amounts of total protein were resolved by SDS gel electrophoresis, transferred to nitrocellulose, and analyzed by Western blotting with the anti-cdk4 antibody.

urchin embryo extracts by Western blotting (Fig. 6B and C). Equal amounts of protein from lysates prepared at different developmental stages were analyzed by Western blotting to determine the amount of cdk4 during development. Figure 6B shows a Western blot of cdk4 over the first cell cycle. The amount of cdk4 did not change during the first 2 h of development. Equal amounts of protein from extracts made every hour for the first 12 h of development were also analyzed for

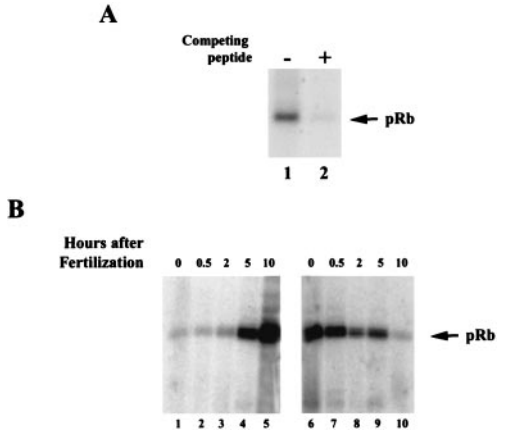

FIG. 7. Activity of cdk4 kinase during development. (A) Extracts were prepared from *S. purpuratus* embryos cultured at 18°C 10 h after fertilization (early blastula). The extracts were immunoprecipitated with α-cdk4 in the absence (lane 1) or presence (lane 2) of antigenic peptide. The precipitates were assayed for kinase activity with GST-pRb as a substrate (52). (B) Extracts were prepared from a single batch of embryos at the indicated times after fertilization and culturing at 18°C. Equal amounts of total protein were immunoprecipitated with α-cdk4 (lanes 1 to 5) or α-cyclin E (lanes 6 to 10), and the immunoprecipitates were assayed for kinase activity. The 10-h embryos were at early blastula (the same stage as the 12-h embryos in Fig. 4).

cdk4 with α-cdk4 (Fig. 6C). The cdk4 protein levels did not change significantly during the first 9 h of development and showed a slight increase between 9 and 13 h that correlated with the increase in *cdk4* mRNA.

cdk4 kinase activity. To determine whether the cdk4 was associated with protein kinase activity, we analyzed the ability of immunoprecipitates generated with the α-cdk4 antibody to phosphorylate a GST-Rb fusion protein with the same approach that we used previously to detect cyclin E-associated kinase activity (52). When extracts from 10-h embryos were analyzed, cdk4 kinase activity could be detected with a portion of human pRb fused to GST as a substrate (Fig. 7A, lane 1). Preincubation of α-cdk4 with its antigenic peptide blocked precipitation of kinase activity (Fig. 7A, lane 2). No activity was observed toward GST or after incubation of the beads in the extract without antibody (data not shown).

We then used this assay to determine the amount of cdk4 during the first 13 h of development. As a control, we also determined the amount of cyclin E-associated kinase activity in the same extracts. Equal amounts of total embryo extract were precipitated with either the cdk4 antibody (Fig. 7B, lanes 1 to 5) or the cyclin E antibody (Fig. 7B, lanes 6 to 10), and the immunoprecipitates were analyzed for protein kinase activity. As reported previously (52) the levels of cyclin E-associated protein kinase activity were high in sea urchin eggs and during the first cell cycle (Fig. 7B, lanes 6 and 7). The levels of cyclin E-associated kinase decreased dramatically by 10 h of development and remained low through 13 h (Fig. 7B, lanes 9 and 10.

The pattern of cdk4 kinase activity was very different. Only background levels of cdk4 kinase activity (the same as observed when the extracts were incubated with protein A beads without antibody) were observed in eggs (Fig. 7B, lane 1) and during the first 2 h of development (Fig. 7B, lanes 1 to 3). Cyclin D kinase activity was detected by 5 h after development, but the levels increased dramatically by 12 h of development, after the large increase in cyclin D mRNA levels (Fig. 4). Cyclin D mRNA was present in eggs and early embryos, since we isolated the cyclin D clones from two-hybrid libraries prepared at that stage, and low levels of mRNA were detected by Northern blotting (Fig. 4). The levels of cdk4-associated kinase activity clearly increased before the large increase in cyclin D mRNA levels. The kinase assay is very sensitive, so the small changes observed at 5 h might not have been reflected in the Northern blot. However, it is also possible that in addition to increased expression of cyclin D, there is activation of a small amount of cyclin D/cdk4 present in early embryos or a small increase in cyclin D as a result of posttranscriptional regulatory events. Since the cdk4 protein levels are constant during this time, as analyzed by Western blotting (Fig. 6), it is likely that this increase in cdk4 kinase activity is a result of the synthesis of cyclin D.

Ectopic expression of cyclin D in early embryos is lethal. Since there is abundant cdk4 protein in the unfertilized egg and probably very little cyclin D (based on the low levels of cyclin D mRNA and cdk4 kinase activity present), we tested the effect of precocious cyclin D expression by injecting synthetic polyadenylated cyclin D mRNA into *L. variegatus* zygotes within 5 min of fertilization. The premature expression of cyclin D induced in this manner resulted in the death of 95% of more than 200 injected embryos. The injected embryos arrested and died within 4 h of cyclin D expression, usually after only one or two cell divisions. The death of these embryos was characterized by extensive blebbing of the cells (Fig. 8). Injection of two negative control RNAs, a cyclin D mRNA that had a stop codon early in the cyclin D open reading frame and cdk4 mRNA, had no effect on development in more than 200 injected embryos. Since this same preparation of cdk4 mRNA was able to rescue the embryos depleted of cdk4 (see below), overexpression of cdk4 has no effect on embryonic development.

The effect of the cyclin D mRNA was directly related to the amount of cyclin D mRNA injected. Various amounts of cyclin D mRNA, between 0.2 and 1 pg/embryo, resulted in changing the time of developmental arrest from the 1-to-2-cell stage (1 pg) to the 16-cell stage (0.2 pg). In all cases, when more mRNA was injected, the embryos completed fewer divisions. The expression of cyclin D early in development, prior to the normal time of expression, was lethal, probably because of the inappropriate activation of cyclin D-cdk4 kinase activity, resulting in disruption of the cleavage cell cycles.

Antisense morpholino oligonucleotides block embryonic development. Antisense morpholino oligonucleotides are a recently developed and powerful tool to probe the role of gene activity during embryonic development (1). This technique utilizes long-lived and nontoxic oligonucleotides that sterically prevent translation of the annealed message (25, 53). The function of a gene can then be rescued by injection of a synthetic mRNA that is not complementary to the antisense morpholino oligonucleotide. We synthesized synthetic mRNAs that contained 45 nucleotides of the 5′ UTR from *L. variegatus* cdk4 and the cdk4 coding region from *S. purpuratus* that contained the human β-globin 5′ UTR. The resulting mRNAs

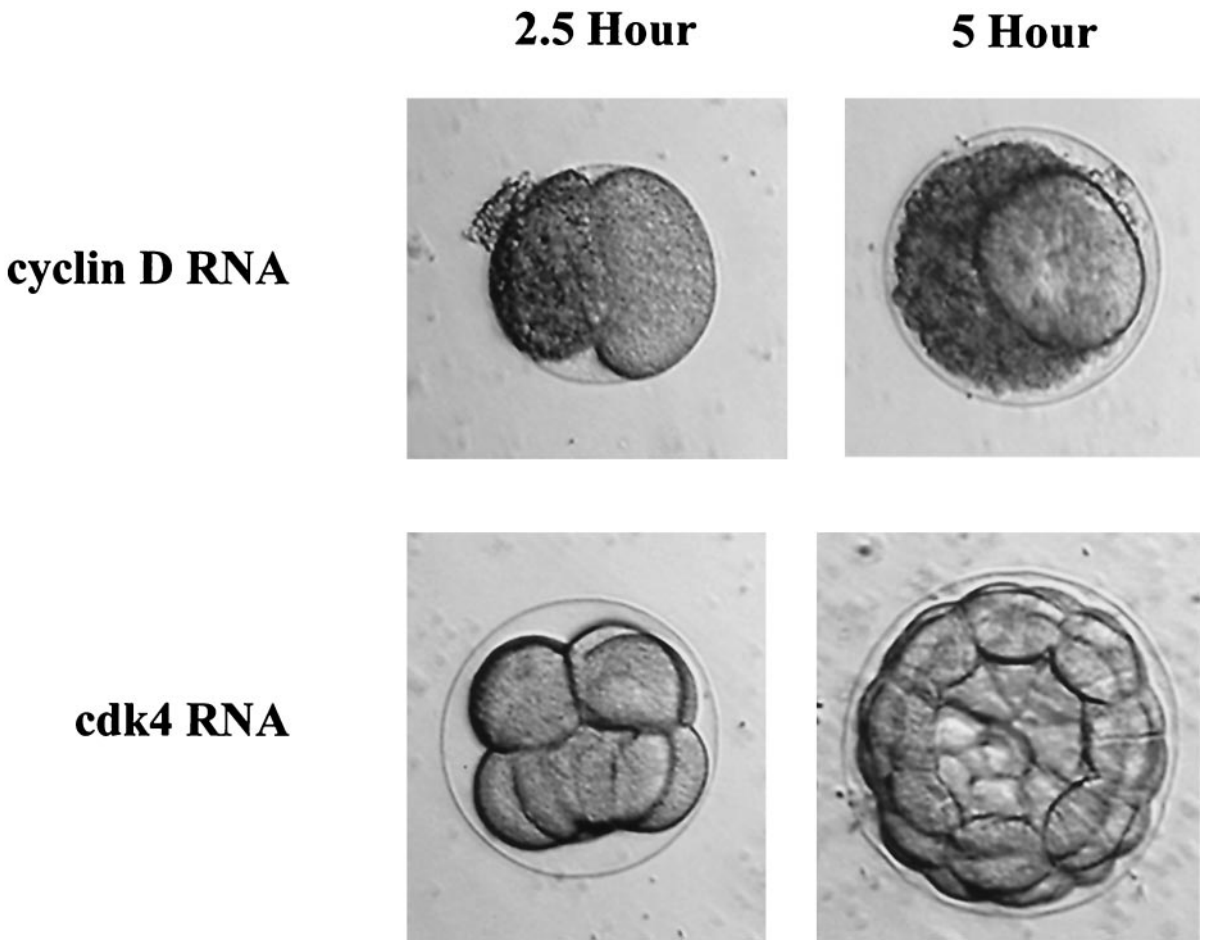

FIG. 8. Premature expression of cyclin D mRNA is lethal to sea urchin embryos. Fertilized *L. variegatus* sea urchin eggs were injected with cyclin D (top) or cdk4 (bottom) mRNA and photographed 2.5 and 5 h after fertilization.

were incubated with a 25-nucleotide antisense morpholino complementary to *L. variegatus cdk4* cDNA from positions −18 to +7 or a control nonspecific morpholino oligonucleotide and assayed in an in vitro translation reaction (Fig. 9A). Translation of *L. variegatus cdk4* mRNA was inhibited by the cdk4 antisense morpholino (Fig. 9A, lane 2), while translation of the *S. purpuratus cdk4* mRNA was not affected by the *L. variegatus* cdk4 morpholino (Fig. 9A, lane 4).

We performed a similar analysis for *L. variegatus* and *S. purpuratus* cyclin D mRNA. The translation of *L. variegatus* cyclin D mRNA samples containing 67 nucleotides of 5′ UTR was also inhibited by the cyclin D morpholino (complementary from −27 to −2) (Fig. 9B, lane 2), but incubation with the control morpholino had no effect on translation of either mRNA (Fig. 9B, lanes 1 and 3). *S. purpuratus* cyclin D mRNA that contained the human β-globin 5′ UTR was not affected by the *L. variegatus* cyclin D antisense morpholino (Fig. 9B, lane 4). We then used these antisense oligonucleotides and synthetic mRNAs to determine whether cdk4 or cyclin D is necessary for early development in sea urchins.

Inhibition of cyclin D expression blocks development after the blastula stage. To determine if cyclin D was necessary for normal development, the morpholino oligonucleotide complementary to the 5′ UTR of the *L. variegatus* cyclin D cDNA was injected into fertilized *L. variegatus* eggs. The injected embryos never completed normal gastrulation or developed into normal larvae, although many embryonic structures, including pigment cells and spicules, were formed (Fig. 10). Although spicules were formed, they did not develop into properly organized skeletons. Although embryos injected with cyclin D morpholino became mutant larvae, their development appeared normal through the mesenchyme blastula stage (about 8 to 10 h, since *L. variegatus* develops faster than *S. purpuratus*). More than 95% of the embryos injected with the cyclin D morpholino oligonucleotide developed abnormally, and 95% of embryos showed no abnormalities when injected with a control oligonucleotide. Thus, the activation of expression of cyclin D at the proper time is essential for the correct completion of gastrulation and the subsequent development into normal plutei. Injection of antisense morpholino oligonucleotides against other cell cycle regulators, cyclin E and cyclin A, resulted in arrested development at an early stage (before 32 cells), as predicted from the expression pattern of these cell cycle regulators (52).

cdk4 expression is also required for normal development. The delay in cyclin D expression until the blastula stage raises the question of whether cdk4 has an essential function prior to the expression of cyclin D. To test this possibility, a morpho-

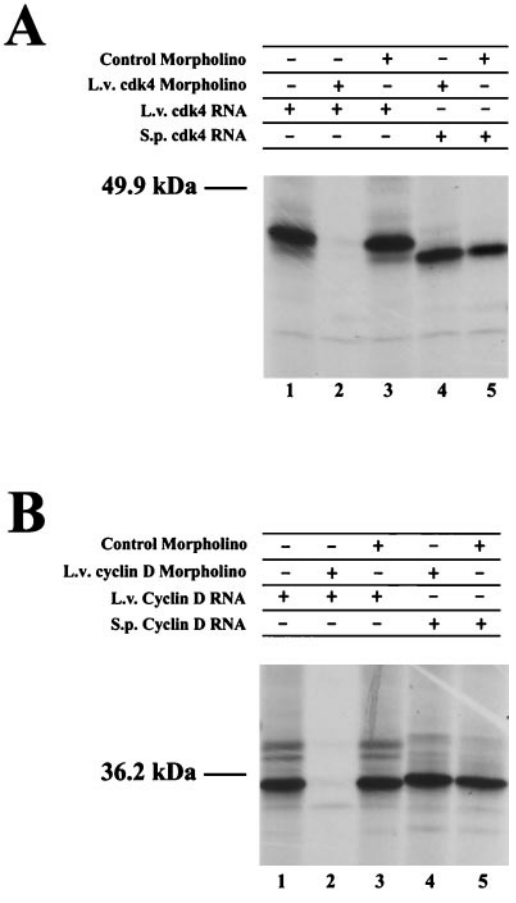

FIG. 9. Morpholino oligonucleotides inhibit in vitro translation of *cdk4* mRNA (A) and cyclin D mRNA (B). (A) Synthetic *L. variegatus* (L.v., lanes 1 to 3) or *S. purpuratus* (S.p., lanes 4 and 5) *cdk4* mRNAs were translated in a reticulocyte lysate. The *L. variegatus* cdk4 antisense morpholino oligonucleotide was included in lanes 2 and 4, and the control morpholino was included in lanes 3 and 5. (B) Synthetic *L. variegatus* (lanes 1 to 3) or *S. purpuratus* (lanes 4 and 5) cyclin D mRNAs were translated in a reticulocyte lysate. The *L. variegatus* cyclin D antisense morpholino oligonucleotide was included in lanes 2 and 4, and the control morpholino was included in lanes 3 and 5.

lino oligonucleotide complementary to the 5′ end of the *cdk4* mRNA was injected into one-cell *L. variegatus* embryos. Like embryos injected with the cyclin D morpholino, embryos injected with the cdk4 morpholino failed to develop into normal larvae. Importantly, the development of the embryos was not affected until after the blastula stage, even though cdk4 protein is present throughout development. Since cdk4 protein is present in the early embryo, any effect of the morpholino antisense oligonucleotide requires that the maternal store of cdk4 protein turn over. It is likely that by the blastula stage there has been turnover of the maternal cdk4 protein. Any requirement for cdk4 in the very early stages of development would not have been detected by this assay. The phenotypes of the embryos injected with the cyclin D and cdk4 morpholino oligonucleotides were identical; in each there was development of pigment cells and spicules, but a failure to properly complete gastrulation and develop normal larvae (Fig. 10). Embryos injected with the control morpholino developed normally.

To determine if the phenotypic alteration resulting from the antisense morpholino oligonucleotide was only due to inhibition of cdk4 activity, we coinjected the cdk antisense morpholino oligonucleotide together with a synthetic *cdk4* mRNA. This mRNA, which contained the β-globin 5′ and 3′ UTRs, encoded *S. purpuratus* cdk4 and did not contain the antisense oligonucleotide target sequence. Fertilized eggs from the same sea urchin were injected with either the antisense cdk4 morpholino, the oligonucleotide plus the synthetic mRNA, or the synthetic *cdk4* mRNA alone. The embryos coinjected with the antisense oligonucleotide and synthetic *cdk4* mRNA developed normally, while the embryos injected with the oligonucleotide were abnormal in the ways detailed above (Fig. 10). As shown before, the injection of *cdk4* mRNA alone had no effect on development.

From the rescue experiments described above, we conclude that the effects of the cdk4 oligonucleotide were due to their specific effect on cdk4 protein expression and not to a general toxicity or inhibition of other mRNAs. Since expression of the cyclin D protein early in development is lethal early in development, we were not able to rescue the effect of the cyclin D oligonucleotide by injection of cyclin D mRNA into fertilized eggs. The results of the microinjection experiments are summarized in Table 1.

Total cell number is not affected by inhibition of expression of cyclin D or cdk4. To determine if the developmental defects in the embryos injected with the cdk4 or cyclin D morpholino were accompanied by a change in the total number of cells, the nuclei of injected embryos were stained with Syto11, and the embryos were flattened with a coverslip, visualized by confocal microscopy, and counted. Embryos injected with a control morpholino, the cdk4 morpholino, or the cyclin D morpholino and collected at 24 h postfertilization all contained about 1,400 ± 200 nuclei (data not shown), showing that there were not large differences in cell numbers in the various embryos. These data suggest that inhibiting cyclin D and cdk4 expression did not result in large differences in the number of cell divisions, suggesting instead that it may play a direct role in the correct patterning of the embryo.

DISCUSSION

Although cyclins E, A, and B have been shown to play conserved roles in the cell cycle regulation of all metazoans, the role of cyclin D/cdk4 in nonmammalian species is less clear. In both *C. elegans* and *D. melanogaster*, genetic analysis indicates a role for the cyclin D/cdk4/pRb pathway that does not involve the response to mitogens and the passage of a cell from G_1 or G_0 to S phase. Instead, cyclin D/cdk4 plays a role in regulating cell size and growth in developing embryos. *D. melanogaster* flies that lack cdk4 (there is only a single homologue of cdk4 and -6 in both *D. melanogaster* and *C. elegans*) are viable but are smaller than normal flies and have cells that are smaller than normal, but show no apparent defects in cell cycle regulation (3, 39). The overexpression of cdk4/cyclin D complexes leads to larger than normal cells (3, 39). The degree of the enlarged-cell phenotype is proportional to the amount of cdk4/cyclin D expressed (3).

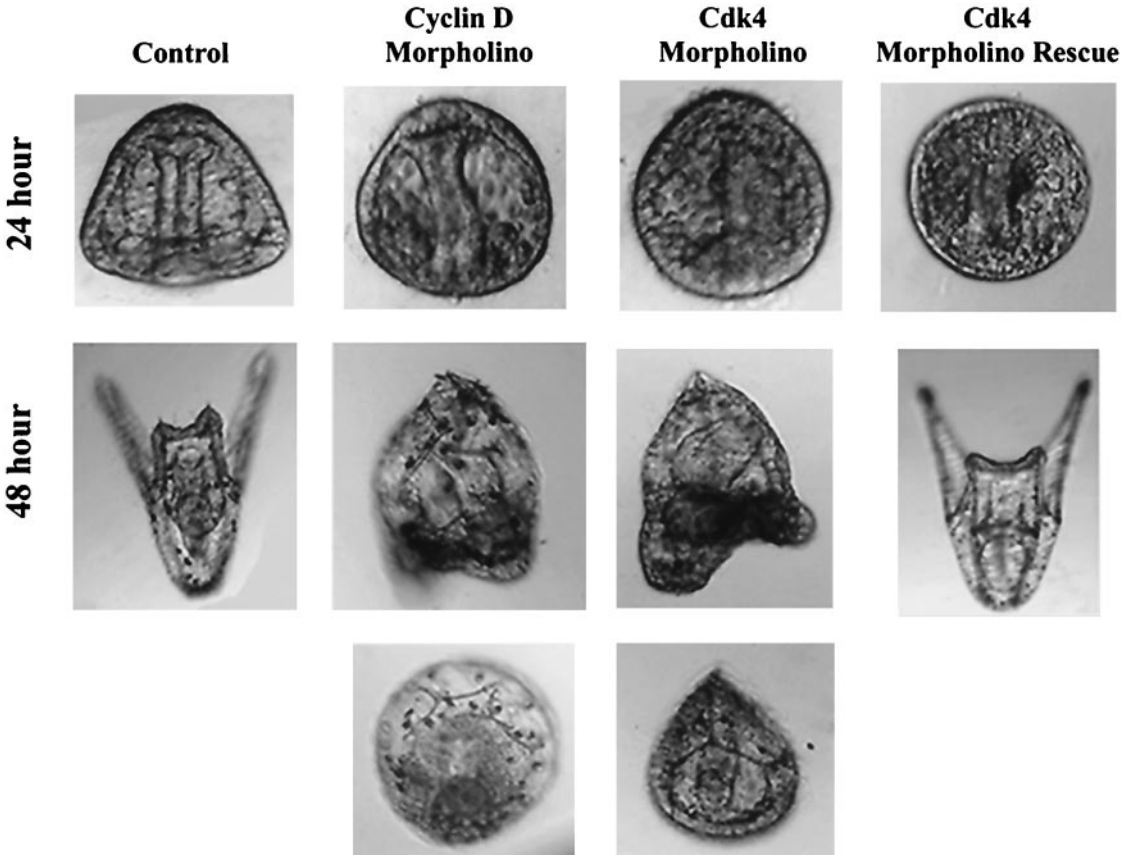

FIG. 10. Cyclin D and cdk4 are required for the normal development of sea urchin larvae. Fertilized *L. variegatus* sea urchin eggs were injected with a morpholino antisense control oligonucleotide, a morpholino antisense oligonucleotide to cyclin D, a morpholino antisense oligonucleotide to cdk4, or a mixture of the antisense oligonucleotide and a synthetic *S. purpuratus cdk4* mRNA. Embryos were photographed 24 and 48 h after fertilization. Two examples of the embryos injected with antisense cyclin D or antisense cdk4 are shown.

A similar situation is found in *C. elegans*, in which it has been shown that neither cdk4 nor cyclin D is required for early development (45). With RNA interference, Park and Krause showed that loss of cyclin D function results in normal hatching and development until the L2 stage (45). These animals, however, did have a defect in the germ line, preventing gonad elongation and resulting in the gonads' being arrested as a ball of cells (45). Loss of cdk4 function in *C. elegans* (through RNA interference or a mutation in cdk4 that disrupts the ATP binding domain) showed a phenotype similar to that for the loss of cyclin D, with the exception that the embryos that had lost cdk4 function were able to survive to later stages (45). These results are consistent with cyclin D/cdk4 functioning as a complex in *C. elegans* and suggest that it does not have an obligatory role in early development.

TABLE 1. Effect of injection of antisense morpholino oligonucleotides and synthetic mRNAs on development[a]

Injected nucleic acid	Phenotype (% of embryos)
Control morpholino	Normal (95 ± 5)
Cyclin D morpholino	Abnormal gastrulation (85 ± 10)
cdk4 morpholino	Abnormal gastrulation (85 ± 7)
cdk4 mRNA	Normal (95 ± 3)
Cyclin D mRNA	Arrest by 4-cell stage (75 ± 10); arrest by 16-cell stage (90 ± 10)
cdk4 morpholino + cdk4 mRNA	Normal (75 ± 14)

[a] One picoliter of solution containing either 250 μM morpholino oligonucleotide or 0.5 to 0.7 pg of synthetic polyadenylated and capped mRNA was injected into *L. variegatus* zygotes within 5 min after fertilization. The embryos were allowed to develop for 48 h, and the development of the embryos was monitored by light microscopy. Each oligonucleotide or mRNA was injected into at least five different sets of zygotes, between 200 and 600 embryos were scored for each treatment, and the percentage of embryos showing the indicated phenotype is given in parentheses, with the range of individual experiments. The abnormal gastrulation phenotype is that shown in Fig. 8B.

A major downstream target of cyclin D/cdk4 in mammalian cells is the pRb family of proteins, which play a critical role in regulation of transcription of genes involved in cell cycle regulation (56). However, it is not known whether cyclin D/cdk4 acts through pRb in invertebrates. The *D. melanogaster* pRb homologue RbF is not necessary for the introduction of G_1 phases during embryogenesis, but RbF is necessary for the maintenance of cells in the G_1 phase of the cell cycle (8). However, the E2F/DP proteins in *D. melanogaster*, major downstream targets of pRb, are clearly involved in cell cycle regulation in *D. melanogaster*, regulating transcription of many of the same genes that are E2F regulated in mammalian cells during the cell cycle (9–11, 47). *C. elegans* mutants carrying a mutation in the pRb homologue show inappropriate hyperactivation of the Ras pathway and do not have an obvious cell cycle phenotype (34). The same Ras pathway phenotype is

found in *C. elegans* with mutant E2F and DP, two proteins that are downstream targets of pRb, suggesting that in *C. elegans* this pathway is not primarily involved in cell cycle regulation (2, 43).

We have detailed here the cloning of *S. purpuratus* cdk4 and its partner cyclin D. Both of these proteins are homologous to their vertebrate counterparts. In addition to interacting with cyclin D, sea urchin cdk4 interacts with mammalian p16, a cdk4-specific inhibitor, even though members of this class of inhibitor have not been reported in any organisms except mammals. We have also shown that cdk4 and cyclin D interact only with each other and not with other cyclins and cdk's, such as cyclin E and cdk2. The time of induction of cyclin D during sea urchin development corresponds to the time of reduction in cyclin E (52) and may coincide with the change in cell cycle control from cycles that lack gap phases to cycles that include gap phases. Although cdk4 protein is present in eggs and during embryogenesis, cyclin D mRNA does not accumulate significantly until 10 h after fertilization, and cdk4 kinase activity is initiated only at 5 h postfertilization and increases as the levels of cyclin D mRNA increase. Thus, cdk4 is probably not active until the blastula stage. In support of this, knocking out *cdk4* with an antisense morpholino oligonucleotide did not affect development up to the blastula stage, but disrupted later development. The same phenotype was observed when expression of cyclin D was prevented, consistent with the possibility that the critical function of cdk4 is to form a complex with cyclin D.

In mammalian tissue culture cells, cyclin D has been shown to be the critical factor induced by mitogenic signals that is essential for the cells to reenter the cell cycle and progress to S phase (36, 58). A primary effect of cdk4 is phosphorylation of pRb, ultimately resulting in the activation of transcription of a number of genes required for entry into S phase (56). Early developing embryos likely do not need transcription and translation of the genes involved in entry into S phase, since these proteins and mRNAs are constitutively present. In addition, since cells progress immediately from mitosis into the next S phase, active cyclin D/cdk4 is not necessary to stimulate exit from G_1 phase. Since conventionally cycling cells need to make a decision after mitosis when to commit to enter the next S phase, cyclin D may play a key role in that decision. The signals that stop cells after mitosis likely are not produced in the rapidly cycling cells.

There are three cyclin D's in mammalian cells, in contrast to the single cyclin D in *C. elegans* and *D. melanogaster*, and these are likely to have overlapping functions. The phenotypes of cyclin D1 and cyclin D2 knockouts in mice were relatively mild (18, 51). Mice lacking cyclin D1 were smaller than their littermates and showed defects in their retinas and mammary glands, but there were no severe developmental defects (18). Similarly, mice lacking cyclin D2 were indistinguishable from their littermates, but mutant females were infertile due to ovarian failure (51). Cyclin D is first expressed in large amounts just prior to gastrulation in mouse embryos (57), similar to its delayed expression in embryogenesis in both sea urchins and frogs (54). The lack of severe phenotypes and embryonic lethality is likely a result of the ability of other members of the cyclin D family to compensate for the loss of cyclin D1 or cyclin D2.

Ectopic expression of cyclin D in cleavage embryos as a result of injecting cyclin D mRNA into sea urchin eggs disrupts the cleavage cell cycles, resulting in embryonic death, with a direct correlation to the amount of mRNA injected. A similar observation has been made in frog embryos; premature expression of cyclin D blocks cleavage of frog embryos (54). The effect of premature expression of cyclin D could be due to storage of many of the components (except for cyclin D) necessary for the switch in cell cycle regulation, from S/M cycles to cycles with gap phases, in the egg and early embryo. This switch may involve the synthesis of cyclin D as well as changes in other cell cycle regulators, such as a reduction in cyclin E (23, 26, 52). A precedent for this type of regulation has been seen in *D. melanogaster* embryos, in which cyclins A and B are titrated away during the continuous S/M cycles, allowing the embryo to develop an interphase lag and activating degradation of the *D. melanogaster* cdc25 homologue (14).

The mutant embryos that develop as a result of cyclin D or cdk4 loss of function do not differ significantly from control embryos in total cell number. This suggests that even in later stages of sea urchin embryogenesis, cyclin D/cdk4 kinase activity is not directly involved in entry into DNA synthesis from G_1. Like the role of cdk4/cyclin D in *D. melanogaster*, in sea urchins cdk4/cyclin D may be more intimately involved in cell growth and the patterning of embryonic cells. This is supported by the demonstration that loss of cyclin D or cdk4 function only affects some cell lineages in the developing embryo. Some lineages found in larvae, such as pigment cells, are clearly present.

Thus, while the cyclins A, B, and E have had conserved functions throughout metazoan evolution, the biological role of cyclin D appears to have changed. Unlike *C. elegans* and *D. melanogaster*, sea urchins have a clear requirement for cyclin D/cdk4 to complete early embryonic development, but it does not appear that this results from a disruption of a function in cell cycle regulation. One possibility is that this effect is due to relatively subtle perturbation of the cell cycle or the incorrect signaling of differentiation in specific lineages, and this will be the subject of future investigations.

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