DEVELOPMENTAL BIOLOGY 173, 408–419 (1996)
ARTICLE NO. 0036

In Vivo Regulation of the Early Embryonic Cell Cycle in *Xenopus*

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We report here the first extensive *in vivo* study of cell cycle regulation in the *Xenopus* embryo. Cyclin A1, B1, B2, and E1 levels, Cdc2 and Cdk2 kinase activity, and Cdc25C phosphorylation states were monitored during early *Xenopus* embryonic cell cycles. Cyclin B1 and B2 protein levels were high in the unfertilized egg, declined upon fertilization, and reaccumulated to the same level during the first cell cycle, a pattern repeated during each of the following 11 divisions. Cyclin A1 showed a similar pattern, except that its level was lower in the egg than in the cell cycles after fertilization. Cyclin B1/Cdc2 kinase activity oscillated, peaking before each cleavage, and Cdc25C alternated between a highly phosphorylated and a less phosphorylated form that correlated with high and low cyclin B1/Cdc2 kinase activity, respectively. Unlike the mitotic cyclins, the level of cyclin E1 did not oscillate during embryogenesis, although its associated Cdk2 kinase activity cycled twice for each oscillation of cyclin B1/Cdc2 activity, consistent with a role for cyclin E1 in both S-phase and mitosis. Although the length of the first embryonic cycle is regulated by both the level of cyclin B and the phosphorylation state of Cdc2, cyclin accumulation alone was rate-limiting for later cycles, since overexpression of a mitotic cyclin after the first cycle caused cell cycle acceleration. The activity of Cdc2 closely paralleled the accumulation of cyclin B2, but cell cycle acceleration caused by cyclin B overexpression was not associated with elevation of Cdc2 activity to higher than metaphase levels. Tyrosine phosphorylation of Cdc2, absent during cycles 2–12, reappeared at the midblastula transition coincident with the disappearance of cyclin E1. Cyclin A1 disappeared later, at the beginning of gastrulation. Our results suggest that the timing of the cell cycle in the *Xenopus* embryo evolves from regulation by accumulation of mitotic cyclins to mechanisms involving periodic G_1 cyclin expression and inhibitory tyrosine phosphorylation of Cdc2. © 1996 Academic Press, Inc.

INTRODUCTION

During embryogenesis a fertilized egg is divided by progressive divisions into a multicellular organism with distinct tissue types and organization. These embryonic cell divisions are precisely timed. In the *Xenopus* embryo the first cell cycle following fertilization is 90 min. Subsequent cleavages are rapid and synchronous, oscillating between DNA synthesis and mitosis every 25–30 min until cycle 13, at which point the cell cycle lengthens and cleavages become asynchronous (Graham and Morgan, 1966). This change in cell cleavage synchrony has been termed the midblastula transition (MBT) and is characterized by activation of zygotic transcription and increased cell motility (Newport and Kirschner, 1982). The regulation of mitotic timing in the early cell cycle in *Xenopus* embryos has not been biochemically characterized *in vivo,* as the majority of previous work on the embryonic cell cycle has been done in marine invertebrate embryos and in *Xenopus* egg extracts.

Both the accumulation of cyclins and the phosphorylation state of Cdc2 are thought to be important in determining the timing of entry into mitosis (Evans *et al.,* 1983; Hunt *et al.,* 1992; Murray and Kirschner, 1989; Solomon *et al.,* 1990). Cyclins are characterized by their periodic accumulation and destruction (Minshull *et al.,* 1989) and fall into two general classes, G_2/M cyclins (A and B) and G_1 cyclins (C, D, and E) (Pines, 1993; Sherr, 1993, 1994). In active MPF, Cdc2 is bound to cyclins B1 and B2 (Gautier *et al.,* 1990) and is phosphorylated on thr161 by the Cdc2 activating kinase, CAK (Solomon *et al.,* 1992). In some cell cycles phosphorylation of Cdc2 on tyr15 by the Wee1 kinase and on thr14 by a distinct protein kinase inactivates MPF (McGowan and Russell, 1993; Parker *et al.,* 1992; Parker and Piwnica-Worms, 1992). In these cycles the protein-tyrosine phosphatase Cdc25 initiates mitosis by dephosphorylating Cdc2 on tyr15 and probably on thr14, thus activating MPF

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(Dunphy and Kumagai, 1991; Gautier *et al.*, 1991; Lee *et al.*, 1992; Millar *et al.*, 1991; Strausfeld *et al.*, 1991). Cyclin A also forms active kinase complexes with Cdc2. These complexes have a G_2/M function in mammalian somatic cell cycles (Pagano *et al.*, 1992) and may also participate in S/M checkpoint control in *Xenopus* embryonic cycles (Walker and Maller, 1991).

In mammalian cells, A- and E-type cyclins associate with Cdk2, a cyclin-dependent kinase discovered in *Xenopus* but most thoroughly characterized in somatic tissue culture cells (Pines, 1993; Sherr, 1993). Cyclin E/Cdk2 complexes have been implicated in the regulation of the G_1/S transition (Duronio and O'Farrell, 1994; Koff *et al.*, 1993; Ohtsubo and Roberts, 1993; Resnitzky *et al.*, 1994), probably via phosphorylation of a retinoblastoma-related protein, p107 (Lees *et al.*, 1992; Nevins, 1992). They also are active in meiotic and embryonic cell cycles in *Xenopus* (Rempel *et al.*, 1995). Cyclin D1/Cdk4, which has been implicated as a regulator of retinoblastoma protein phosphorylation in G_1 (Quelle *et al.*, 1993; Resnitzky *et al.*, 1994), is undetectable in *Xenopus* eggs or embryos prior to the MBT (Dr. T. Kishimoto, Tokyo Institute of Technology, Tokyo and Drs. M. J. Cockerill and T. Hunt, Imperial Cancer Research Fund, South Mimms, UK, personal communication).

In contrast to cell cycle regulation based on periodic cyclin accumulation and differential phosphorylation, in the *Drosophila* embryo Cdc2/cyclin A and B complexes show little change in abundance, phosphorylation, or activity during the first 7 cycles. These cycles do not appear to require oscillations of cyclins or any known cell cycle regulators. In later cycles (cycles 8–13) there is a progressive increase in cyclin degradation at each mitosis and a corresponding oscillation of Cdc2 kinase activity (Edgar and O'Farrell, 1989, 1990; Edgar *et al.*, 1994). After cycle 13, cellularization occurs, maternal cyclin E and string (the *Drosophila* homolog of Cdc25) are destroyed, and zygotic Cdc25 transcripts control cycles 14–16 (Edgar and O'Farrell, 1989, 1990). Zygotic cyclin E is expressed in cycles 14–16 in a manner suggesting action as a regulator of the G_1/S transition (Duronio and O'Farrell, 1994; Knoblich *et al.*, 1994; Richardson *et al.*, 1993).

Information about *in vivo* regulation of the early embryonic cell cycle is lacking for *Xenopus* or any other vertebrate, and it is important to evaluate whether vertebrate embryonic cell cycles are regulated by periodic accumulation and degradation of regulatory cyclins and tyrosine phosphorylation of Cdc2 or whether they resemble the earliest cycles of *Drosophila.* Work in *Xenopus* cell-free extracts has shown that addition of exogenous cyclin B mRNA is sufficient to restore cycling of extracts arrested by depletion of endogenous cyclins (Murray and Kirschner, 1989). Previous work *in vivo* has demonstrated that at least the first two cell cycles following fertilization in *Xenopus* are characterized by oscillating MPF activity (Gerhart *et al.*, 1984; Wasserman and Smith, 1978). These results were obtained before molecular characterization of MPF. In this paper, we analyze the regulation of embryonic cell cycles in the *Xenopus* embryo in molecular terms. The data indicate that there are divergent mechanisms of cell cycle regulation in *Xenopus* before and after the midblastula transition that are distinct from those in *Drosophila.*

MATERIALS AND METHODS

Embryos and mRNA microinjections. Embryos were obtained by *in vitro* fertilization, dejellied in 2% cysteine (pH 7.8), cultured in 0.1× MMR, and staged according to Nieuwkoop and Faber (1967). For time courses, embryos were collected either every 5 or every 15 min beginning 15 min postfertilization, frozen on dry ice, and stored at −80°C. Unfertilized *Xenopus* eggs or embryos were homogenized in 5–10 vol of extraction buffer (EB) and microcentrifuged for 5 min to remove insoluble and yolk proteins, and the supernatants were used for immunoprecipitations, *in vitro* kinase assays, and immunoblots. EB is composed of 20 m*M* Hepes (pH 7.5), 80 m*M* β-glycerophosphate, 20 m*M* EGTA, 15 m*M* $MgCl_2$, 1 m*M* dithiothreitol, 50 m*M* NaF, 1 m*M* sodium vanadate, 3 μg/ml leupeptin, 0.5 m*M* phenylmethylsulfonyl fluoride, 0.2 m*M* ammonium molybdate, 30 m*M* *p*-nitrophenyl phosphate, and 1 μM microcystin.

The *Xenopus* cyclin B2 cDNA clone in pGEM-1 was kindly provided by Dr. T. Hunt (ICRF, South Mimms, UK). The histidine-tagged cyclin B2 construct was generated by cloning the entire open reading frame extending from the *Hpa*II site just 5′ of the initiating ATG to a polylinker *Hpa*II site 3′ of the B2 stop codon into pRSETB (Invitrogen). The tagged cyclin B2 protein has a predicted size 4 kDa larger than endogenous cyclin B2. The mRNA for microinjection was generated by *in vitro* transcription of the cyclin B2 pRSET plasmid using T7 polymerase (mMessage mMachine, Ambion) and quantitated by both spectrophotometry and agarose gel electrophoresis. Actin mRNA was generated identically using a pTRI-β-actin plasmid. Dejellied embryos were manually injected with 5 ng of either actin or cyclin B2 *in vitro* transcribed mRNA. In preliminary experiments, different amounts of cyclin B2 mRNA were injected into both 1- and 2-cell embryos. It was found that concentrations 10-fold lower (0.5 ng) than that used in the experiments shown did not perturb the cell cycle, whereas concentrations 10-fold higher (50 ng) caused cleavage arrest. We routinely injected 5.0 ng and even at this concentration a proportion of the embryos arrested with high histone H1 kinase activity, presumably caused by overexpression of exogenous cyclin B2 beyond the level capable of being degraded by the cyclin degradation system (Roy *et al.*, 1991; Fig. 7C). Microinjections were always performed within a 10- to 20-min window at the 1-cell stage (between 50 and 70 min postfertilization). Embryos were maintained in a solution of 1× MMR containing 2% Ficoll after microinjection.

Antibodies and immunoblot analysis. Antisera were raised in sheep against full-length *Xenopus* cyclins A1, B1, and B2, as described in Gautier *et al.* (1990) and used without further purification. Cyclin E1 antiserum was raised in a goat using full-length recombinant *Xenopus* cyclin E1 and affinity-purified as described (Rempel *et al.*, 1995). Antiserum was raised in rabbits against a synthetic peptide of the C-terminus of *Xenopus* Cdk2 and affinity-purified on a Cdk2 peptide column as previously described (Gabrielli *et al.*, 1992b; Rempel *et al.*, 1995). Antiserum to Cdc2 was generated in rabbits against a synthetic peptide encoding the C-terminus of *Xenopus* Cdc2 and affinity-purified using a Cdc2 peptide column. Antibodies to the bacterially expressed C-terminal half of *Xenopus* Cdc25C were raised in rabbits (Izumi *et al.*, 1992) and affinity-purified for immunodetection using nitrocellulose blots (Olmsted, 1981) as described below. Recombinant Cdc25C

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was fractionated on 10% SDS–polyacrylamide gels and transferred to nitrocellulose. Blots were stained with 0.2% Ponceau red in 3% trichloroacetic acid and the band of Cdc25C was excised. The nitrocellulose strips containing Cdc25C were neutralized in PBS, blocked in blotto (10% nonfat dry milk in PBS) for 2 hr, and incubated with 10 ml crude serum mixed with 10 ml PBS overnight at 4°C. The strips were washed with PBS-0.05% Tween 20 and the protein-bound antiserum was eluted with 0.1 *M* glycine (pH 2.5). The eluate was neutralized by adding one-tenth vol of 1 *M* Tris–HCl (pH 8.0) and diluted with 4 vol of PBS containing 1% BSA and filter sterilized. This blot-purified antibody solution could be reused several times. Antiphosphotyrosine antibodies were the kind gift of Dr. Wilfried Merlevede (Katholieke Universiteit te Leuven, Belgium).

For immunoblot analysis, extracts were subjected to electrophoresis on SDS–polyacrylamide gels and transferred to supported nitrocellulose membranes using a semidry blotting apparatus (LKB). Membranes were blocked with blotto and incubated for 1 hr at room temperature with primary antibody diluted in blotto for the cyclins, Cdc2 and Cdk2, for affinity-purified Cdc25C antibody, or for phosphotyrosine antibody in PBS-1% BSA. Membranes were then incubated in the appropriate second antibody conjugated to either alkaline phosphatase or peroxidase (Jackson Immunochemicals) and developed with an alkaline phosphatase color reagent (Bio-Rad) or by enhanced chemiluminescence (Amersham).

Immunoprecipitations. Immunoprecipitation of cyclin B1- and cyclin E1-associated kinase activity was performed essentially as described previously (Gabrielli *et al.,* 1992a). Briefly, egg or embryo extracts (10 embryos in 100 μl EB prepared as described above under Embryos and mRNA Microinjections) were diluted in EB to a final volume of 300 μl and precleared with protein G–Sepharose. Precleared samples were separated into two equal aliquots and incubated with either affinity-purified cyclin E1 antiserum or cyclin B1 antiserum for 2 hr on ice. Twenty-five microliters of 50% protein G–sepharose was then added and samples were mixed for 1 hr, washed with low and high salt buffers (20 m*M* Tris–HCl, pH 7.4, 5 m*M* EDTA, 0.1% Triton X-100, and either 100 m*M* or 1 *M* NaCl, respectively), and finally washed with kinase assay buffer (20 m*M* Hepes, pH 7.5, 15 m*M* $MgCl_2$, 5 m*M* EGTA, 1 m*M* DTT). Immunoprecipitates were incubated in 25 μl of kinase assay buffer containing 0.2 mg/ml BSA, 1.0 mg/ml histone H1, and 200 μM [γ-^{32}P]ATP (2 cpm/fmol). Samples were incubated at 25°C for 20 min and the reaction was stopped by the addition of 25 μl of 2× sample buffer. Samples were boiled for 3 min, electrophoresed on 12.5% Laemmli polyacrylamide gels, and analyzed for histone H1 phosphorylation by scintillation counting of the excised H1 band.

Immunoprecipitation of total cyclin B/Cdc2 H1 kinase activity was performed essentially as described above except that an equal mixture of cyclin B1 and B2 antisera were used to precipitate kinase activity from five embryos in a final volume of 150 μl. Immunoprecipitation of Cdc2 was also performed as described above except that 20 embryos per timepoint were immunoprecipitated in a volume of 200 μl. After the kinase wash 5× sample buffer containing 10 m*M* *N*-ethylmaleimide instead of β-mercaptoethanol was added to the immunoprecipitate, and samples were processed for immunoblot analysis of cyclin B2 as described in the above section.

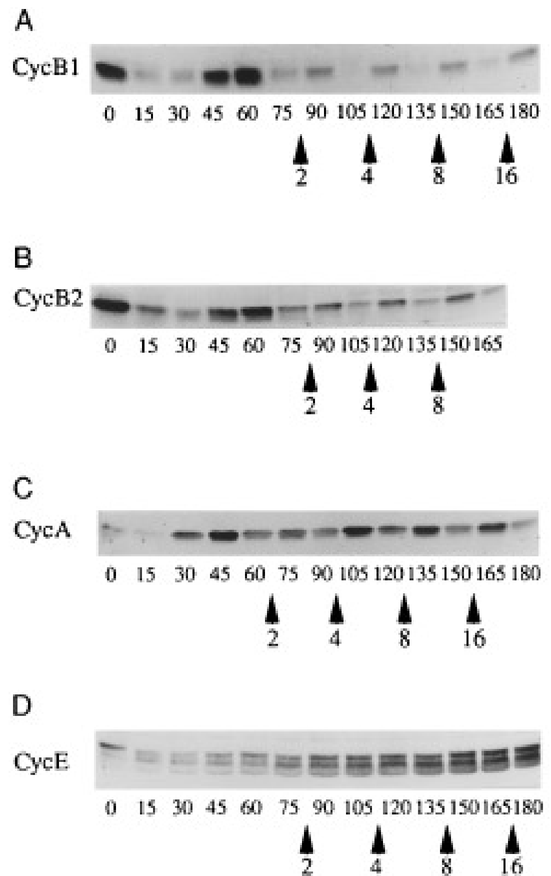

FIG. 1. Cyclin protein levels oscillate during embryonic cell cycles. Immunoblots of embryos collected every 15 min after fertilization are shown. Time postfertilization is denoted in min (numbers directly underneath blots). The cell number is shown under the arrowheads. Blots were probed with antibodies to cyclin B1 (A), cyclin B2 (B), cyclin A1 (C), and cyclin E1 (D). The equivalent of one embryo was loaded per lane. This experiment was repeated several times with similar results.

RESULTS

Cyclins B1, B2, and A1 Oscillate during Early Embryonic Cell Cycles

Previous studies in *Xenopus* reported the oscillation of MPF activity immediately after fertilization (Gerhart *et al.,* 1984; Wasserman and Smith, 1978). To characterize these oscillations of MPF activity in the early *Xenopus* embryo in biochemical and molecular terms, we first examined the types and abundance of the cyclins bound to Cdc2 or Cdk2 by immunoblot analysis of developing embryos. Figure 1A shows that the amount of cyclin B1 was high in the unfertilized egg (Time 0), which is arrested in metaphase of meiosis II, but had dropped to a low level by 15 min after fertilization due to degradation (Glotzer *et al.,* 1991) (also see Fig. 2B). Cyclin B1 then gradually reaccumulated to the same level as that at meiosis II, peaking at 60 min postfertilization, prior to the first cleavage, and dropping again during cytokinesis. In subsequent cycles the level of cyclin B1 dropped and reaccumulated with a 30-min periodicity, coincident with the 30-min division cycle; this oscillation in the level of cyclin B1 could be seen through at least 8 hr postfertilization (Figs. 2B and 3B). Cyclin B2 had a pattern

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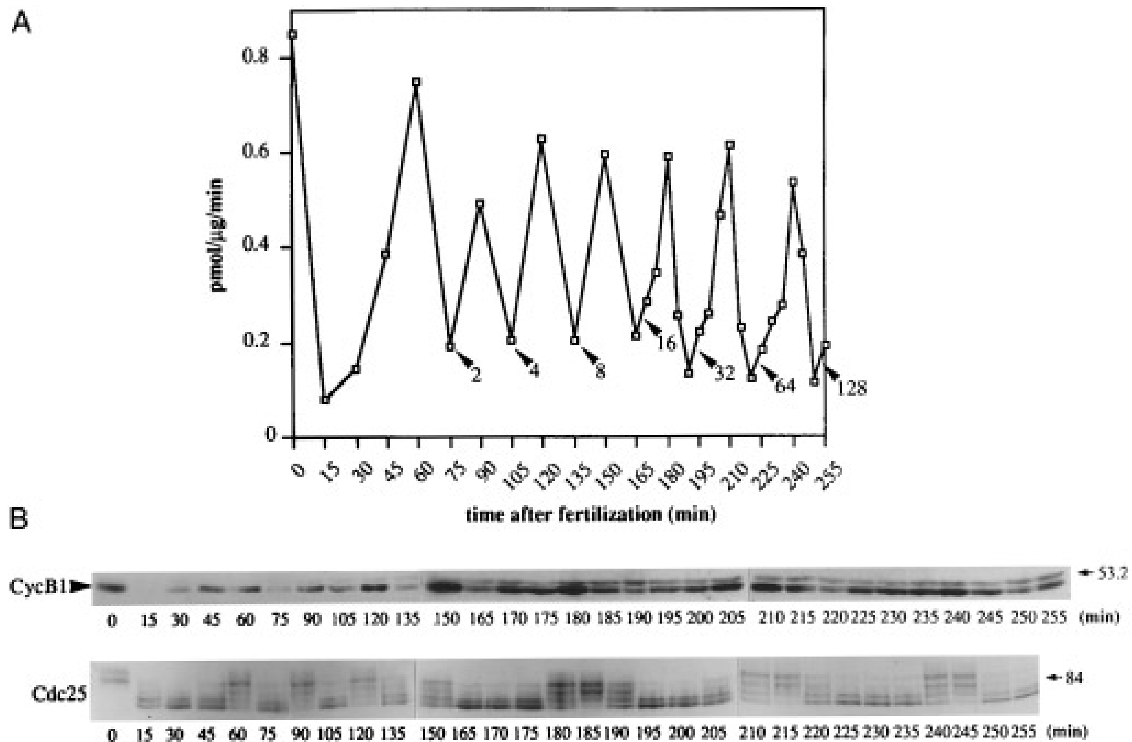

FIG. 2. Cyclin B1/Cdc2 kinase activity oscillates in parallel with cyclin accumulation and degradation and with the phosphorylation state of Cdc25C. Histone H1 kinase activity immunoprecipitated from an embryo timecourse with antibodies against cyclin B1 is shown in (A); the accompanying immunoblots probed with antibodies to cyclin B1 (CycB1) and Cdc25C (Cdc25) are shown in (B). Timepoints were taken every 15 min for the first three cycles, and then every 5 min. Each point in (A) represents the kinase activity from five immunoprecipitated embryos. Cytokinesis is shown by the arrowheads and cell number is indicated. The numbers in (B) indicate time after fertilization in min. The equivalent of one embryo was loaded per lane. The arrowhead denotes the cyclin B1 doublet; the nonspecific species above cyclin B1 is always present after the first few cell cycles. Immunoreactivity of this protein was not blocked when antibody was preincubated with cyclin B1 before blotting and its level did not oscillate during the cell cycle. Arrows to the right indicate the positions of molecular weight markers in kilodaltons. This figure shows data from a single experiment that was repeated three times with similar results.

of expression identical to that of cyclin B1 (Fig. 1B). Cyclin B2 is less abundant and more difficult to detect in early embryos than cyclin B1, thus changes in its levels could be seen only upon overexposure of the immunoblots. Unlike the B cyclins, the level of cyclin A1 was low in the unfertilized egg (Fig. 1C), decreased at fertilization, and increased above the meiosis II level shortly thereafter, peaking just before the first cytokinesis, which began at 75 min postfertilization in this timecourse. Cyclin A1 then oscillated every 30 min with each cell cycle, as did both of the B cyclins. In contrast to the A and B cyclins, cyclin E1 did not oscillate with the cell cycle (Fig. 1D). Instead, the amount of cyclin E1 increased two- to threefold during the first cell cycle and additional forms with retarded electrophoretic mobility appeared. These forms of cyclin E1 differ in phosphorylation state and reflect cyclin E/Cdk2 complex activity (Rempel *et al.,* 1995). In later cycles, the amount of cyclin E1 stabilized and there was no change in amount of protein through 6 hr postfertilization (Fig. 5).

Cdc2 Kinase Activity and the Cdc25C Phosphorylation State Correlate with Accumulation of Mitotic Cyclins

In *Xenopus,* tyrosine phosphorylation of a 34-kDa band presumed to be Cdc2 occurs in the first cell cycle and not in cycles 2–12 (Ferrell *et al.,* 1991). Since dephosphorylation of tyr15 activates Cdc2, one could predict that Cdc2 kinase activity might be maintained at a high level during cycles 2–12, as reported for the first 7 cycles in *Drosophila* embryos (Edgar *et al.,* 1994). On the other hand, the fluctuation in mitotic cyclins suggests the possibility of an accompanying fluctuation in Cdc2 kinase activity. To address these conflicting predictions, cyclin B1/Cdc2 activity was determined during early cell cycles by immunoprecipitating cyclin B1 and assaying the associated histone H1 kinase activity. Figure 2A shows the pattern of cyclin B1/Cdc2 activity during an embryo timecourse in which activity was measured every 15 min for cycles 1–3 and every 5 min for cycles 4–6. The kinase activity

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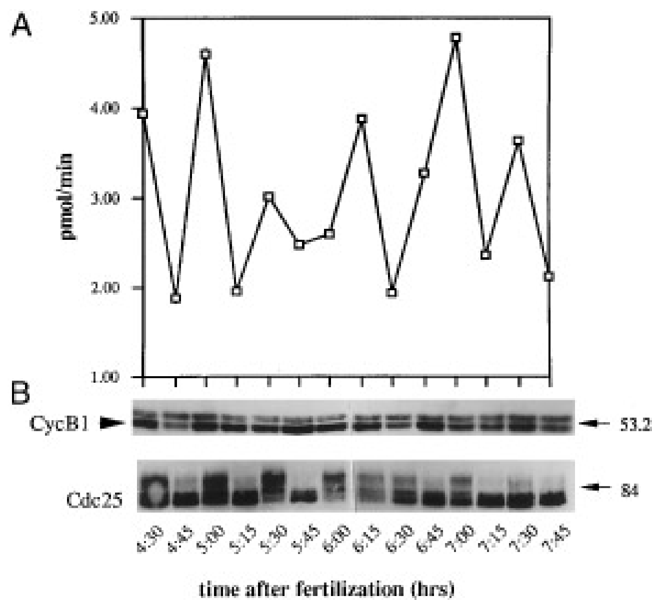

FIG. 3. Cyclin B1/Cdc2 kinase activity, the level of cyclin B1, and Cdc25C phosphorylation oscillate through the midblastula transition. (A) Histone H1 kinase activity immunoprecipitated from later timepoints of the same embryo timecourse as shown in Fig. 2. (B) The corresponding immunoblots probed with antibodies specific for cyclin B1 and Cdc25C, respectively. Samples were collected every 15 min for the time period shown. As in Fig. 2, each point in (A) represents the kinase activity from five immunoprecipitated embryos. In (B), the equivalent of one embryo was loaded per lane. Arrowhead denotes cyclin B1 doublet; the doublet above cyclin B1 is always present and does not oscillate. Arrows to the right indicate the positions of molecular weight markers in kilodaltons. This experiment was repeated three times with similar results.

oscillated with every division, paralleling the cyclin accumulation cycle (compare Figs. 2A and 2B). Kinase activity was high in the unfertilized egg, plummeted within 15 min after fertilization, coincident with the decrease in cyclin B1 protein, and increased as cyclin levels increased, peaking at 60 min postfertilization, prior to the first cleavage and coincident with the maximum cyclin B1 level. With each cycle, the rise and fall of kinase activity paralleled the increase and decrease of B cyclins, with the maximum kinase activity occurring at the peak of cyclin accumulation (peaks at 60, 90, 120, 150, 180, 210, and 240 min). This pattern of alternating high and low kinase activity occurred until at least 8 hr postfertilization (Fig. 3A). Analysis of timepoints taken every 5 min showed that the rise in kinase activity and accumulation of cyclin B1 to the maximum level occurred over approximately 20 min, while both reached a low 10 min later (Fig. 2B, 165–255 min, 16- to 64-cell embryos).

In view of the periodic pattern of cyclin B1/Cdc2 kinase activity with each cell cycle, we examined the phosphorylation state of the Cdc25C tyrosine phosphatase, which is known to activate Cdc2 kinase by dephosphorylation of tyr15 and probably of thr14 (Dunphy and Kumagai, 1991; Gautier *et al.,* 1991; Lee *et al.,* 1992; Millar *et al.,* 1991; Strausfeld *et al.,* 1991). Cdc25C phosphatase activity is regulated by phosphorylation, being low in interphase and high in M-phase, with reduced electrophoretic mobility upon activation (Izumi and Maller, 1995; Izumi *et al.,* 1992; Kumagai and Dunphy, 1992). Therefore, immunoblots were used to monitor the phosphorylation state of Cdc25C. Figure 2B (bottom) shows an immunoblot of Cdc25C during the first six cycles of *Xenopus* development. In the unfertilized egg, Cdc25C was present in the more highly phosphorylated active form, and upon fertilization its mobility changed to that of the more quickly migrating inactive protein. The changes in the phosphorylation state of Cdc25C paralleled the fluctuation in cyclin B1 and cyclin B1/Cdc2 kinase activity, with phosphorylation occurring when kinase activity was high and cyclin B1 was at its peak (Figs. 2 and 3). The phosphorylation state of Cdc25C continued to cycle until at least 8 hr postfertilization (Fig. 3B).

Cyclin/Cdk2 Changes during Embryogenesis

Due to synthesis in meiosis II, cyclin E1 complexed to Cdk2 was already present in the unfertilized egg (Fig. 1D; Rempel *et al.,* 1995) but unlike the B cyclins, cyclin E1 remained stable during early embryogenesis (Fig. 1D). Cdk2 is bound to cyclin E1 but not to cyclin A1 at this stage of development (Rempel *et al.,* 1995). Although the level of cyclin E1 protein did not oscillate during *Xenopus* embryogenesis, cyclin E1/Cdk2 histone H1 kinase activity did cycle (Fig. 4) with a pattern different from that of the mitotic cyclins. There was a two- to threefold change in cyclin E1/Cdk2 activity twice during each cell cycle, with minimum activity never reaching zero (Fig. 4). In order to observe this pattern of cyclin E1/Cdk2 activity consistently, it was necessary to analyze embryos every 5 min beginning 15 min postfertilization. The first peak of cyclin E1/Cdk2 activity occurred in S-phase (when cyclin B/Cdc2 activity is low), while the second occurred slightly before the peak of cyclin B1/Cdc2 activity (mitosis) (Fig. 4). In each successive cell cycle this pattern was repeated. Even with analysis at 5-min intervals, a dampening was seen in the oscillation of both cyclin E1/Cdk2 and cyclin B1/Cdc2 kinase activities, beginning at about 120 min (the third cell cycle in this experiment). This is probably due to the slight metachrony of cell cycles in different regions of the embryo (vegetal blastomeres divide more slowly than animal blastomeres) and asynchrony in the embryo population as a whole. It is more obvious with cyclin E1/Cdk2 kinase activity, as the net difference between high and low Cdk2 activity is less than that of high and low Cdc2/cyclin B activity (Gabrielli *et al.,* 1992b).

The MBT in *Xenopus* is marked by changes in the embryonic cell cycle, with the cycle increasing in length and cells becoming asynchronous as the cycle expands (Graham and Morgan, 1966; Newport and Kirschner, 1984). Cycles 13 and 14 last approximately 41 and 90 min, respectively (Newport

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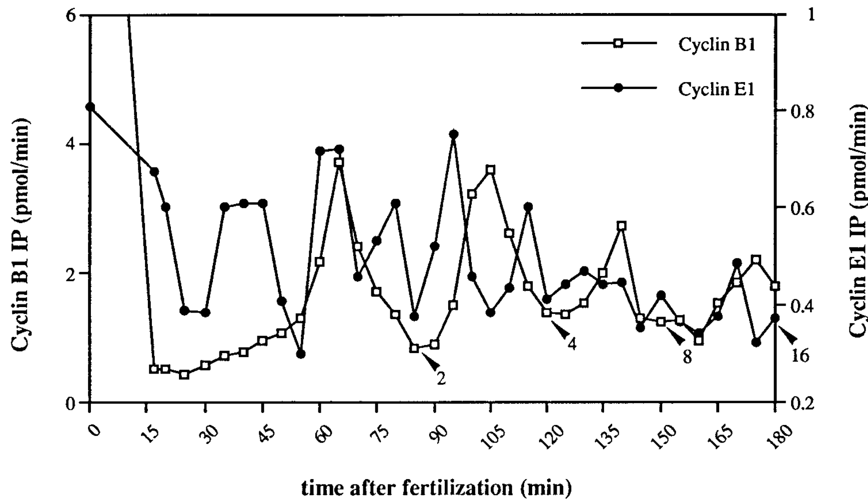

FIG. 4. Cyclin E1/Cdk2 kinase activity oscillates twice for each oscillation in cyclin B1-associated kinase activity. Histone H1 kinase activity immunoprecipitated from an embryo timecourse with antibodies to either cyclin B1 or cyclin E1. Samples were taken every 5 min beginning 15 min postfertilization. Each point represents the activity immunoprecipitated from five embryos. Cyclin B1-associated kinase activity for the egg sample (Time 0) is off scale. This experiment was repeated once with similar results.

and Dasso, 1989). The majority of zygotic transcription also begins during this time, with embryos no longer solely dependent on maternal transcripts (Newport and Kirschner, 1984). We were interested in whether there were changes in the levels and/or activity of cell cycle regulators during this transition. Accordingly, we examined cyclins, Cdc2, and Cdk2 in embryos beginning 5 hr (midblastula) through 11 hr (midgastrula) postfertilization. We previously showed that protein levels of both cyclins E1 and A1 decreased sometime between Stage 8 and Stage 10.5 (Rempel *et al.,* 1995). As seen in Fig. 5, during a more detailed timecourse the level of cyclin B1 showed an increase after 7 hr postfertilization (Fig. 5, top, and Fig. 3). In contrast, the level of cyclin A1, initially fairly constant, dropped to a nearly undetectable level beyond 9 hr postfertilization, corresponding to the beginning of gastrulation (Fig. 5, middle). Cyclin E1 declined even earlier, between 6 and 7 hr postfertilization, coincident with the onset of the MBT (Fig. 5, bottom). The increase in cyclin B1 and the decrease in cyclin E1 paralleled the appearance of a more slowly migrating band on Cdc2 immunoblots (Fig. 6, top). Such a shift has been previously shown in egg extracts to result from phosphorylation of tyr15 in Cdc2 (Parker *et al.,* 1991; Solomon *et al.,* 1990). The appearance of this band in the embryo was also due to tyrosine phosphorylation of Cdc2, as shown by the antiphosphotyrosine blot in Fig. 6 (middle), and also by immunoprecipitation of Cdc2 followed by immunoblotting with phosphotyrosine antibodies (data not shown). The level of Cdk2 remained fairly constant (Fig. 6, bottom), but we observed that a shift up to its inactive (thr160 dephosphorylated) form corresponds temporally with the decrease in cyclin E1 (data not shown).

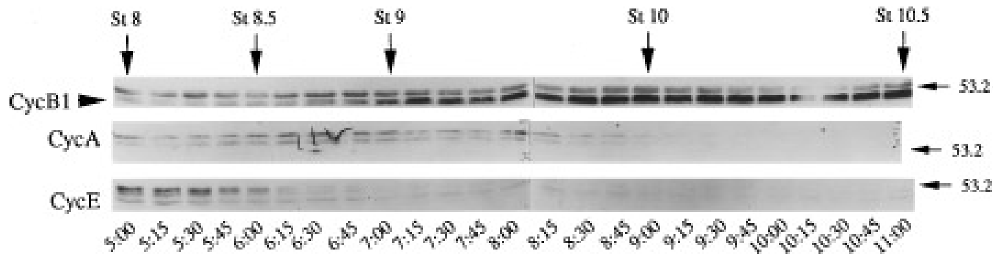

FIG. 5. Cyclin A1 and cyclin E1 levels decrease after the MBT. Immunoblots of embryos collected every 15 min between 5 and 11 hr postfertilization are shown. Time after fertilization is denoted below the blots in hr and min. Blots were probed with antibodies to cyclin B1 (CycB1), cyclin A1 (CycA), and cyclin E1 (CycE). The arrowhead to the left denotes the cyclin B1 doublet, arrows to the right indicate the position of molecular weight markers in kilodaltons, and top arrows indicate the stage of development. One embryo equivalent was loaded per lane. This experiment was repeated twice with similar results.

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Overexpression of Cyclin B2 Causes Premature Entry into Mitosis

The accumulation and degradation of B cyclins was directly correlated with the rise and fall of H1 kinase activity in each of the early cell cycles (Fig. 2), indirectly supporting the hypothesis that cyclin accumulation controls the timing of the early embryonic cell cycle (Murray and Kirschner, 1989). This hypothesis is based on studies in egg extracts, where cyclin synthesis alone is sufficient to drive the cell cycle. These studies also suggest a threshold requirement for cyclin B, whereby the timing of mitosis is controlled by cyclin B accumulation (Murray and Kirschner, 1989; Murray *et al.,* 1989). To date, this model has been based solely on extracts in which the only synthesized protein is cyclin; so it is not clear whether other proteins normally synthesized in embryos also affect cell cycle timing. In order to determine whether reaching a certain "threshold" of cyclin B can control the timing of embryonic cell cycles *in vivo,* we injected 1-cell embryos with cyclin B2 mRNA to allow overexpression of cyclin B2 protein and to potentially perturb cell cycle timing (Fig. 7). Embryos were injected during the first cycle after cortical rotation and before cytokinesis, either with cyclin B2 mRNA or with actin mRNA as a negative control. The timing of the microinjections was such that the first postfertilization cell cycle, which involves tyrosine phosphorylation of Cdc2, would be completed before substantial cyclin accumulation. Samples were taken every 5 min for the next two to three divisions, and cell cycle progress was monitored by immunoprecipitation of cyclin B/Cdc2 kinase activity. The synthesis of exogenous and endogenous cyclin B2 protein was analyzed on immunoblots. Exogenous cyclin B2 protein (open arrowhead) has a higher apparent molecular weight than endogenous cyclin B2 (solid arrowhead), since the mRNA injected encodes a histidine-tagged protein.

Figure 7A shows that total cyclin B/Cdc2 kinase activity immunoprecipitated from cyclin B2 mRNA-injected embryos rose earlier than that in actin mRNA-injected embryos and shows that these embryos entered mitosis before control embryos. In Fig. 7A, the second cell cycle after microinjection was accelerated (the third cleavage, from 4 to 8 cell), with peak activity in cyclin B2 mRNA-injected embryos occurring at 1:45 (1 hr 45 min postfertilization) and in control embryos at 2 hr postfertilization. Kinase activity was minimal in B2 mRNA-injected embryos when kinase activity in actin mRNA-injected control embryos was peaking. The fourth cycle was also accelerated in cyclin B2 mRNA-injected embryos, with Cdc2 kinase activity at mitotic levels, while actin mRNA-injected embryos exhibited interphase levels. This experiment was performed four times with similar results. Cytokinesis was also faster in embryos injected with cyclin B2 mRNA compared to that in those injected with actin mRNA. Microinjection of cyclin B1 mRNA resulted in the same cell cycle acceleration seen with cyclin B2 mRNA (data not shown). Microinjections into one blastomere at the 2-cell stage caused asynchrony between blastomeres, with the injected blastomere cleaving prior to the uninjected side or, at higher concentrations, undergoing cleavage arrest (data not shown).

In general, the accumulation and degradation of total cyclin B2 protein paralleled kinase activity (Fig. 7A, bottom). Exogenous cyclin B2 was synthesized and degraded in a cyclical manner similar to endogenous cyclin B2, except that the peak in accumulation of cyclin B2 protein in B2 mRNA-injected embryos preceded the peak in accumulation of cyclin B2 in actin mRNA-injected controls. Although the faster rate of accumulation of cyclin B2 was associated with earlier activation of Cdc2, supporting a threshold model for mitotic timing, the level of Cdc2 kinase activity at metaphase was the same despite elevated cyclin B levels. This may be due to activation of the cyclin B degradation pathway by metaphase levels of Cdc2 kinase activity (Luca *et al.,* 1991). To confirm that exogenous cyclin B2 formed complexes with Cdc2, Cdc2 was immunoprecipitated from cyclin B2 mRNA-injected embryos and blotted for cyclin B2. As shown in Fig. 7B, exogenous cyclin B2 was coimmunoprecipitated with Cdc2. The degradation of both exogenous and endogenous cyclin B2 in injected embryos confirms that synthesized cyclin B2 is associated with Cdc2, since Cdc2 binding is required for cyclin B2 degradation (Stewart *et al.,* 1994).

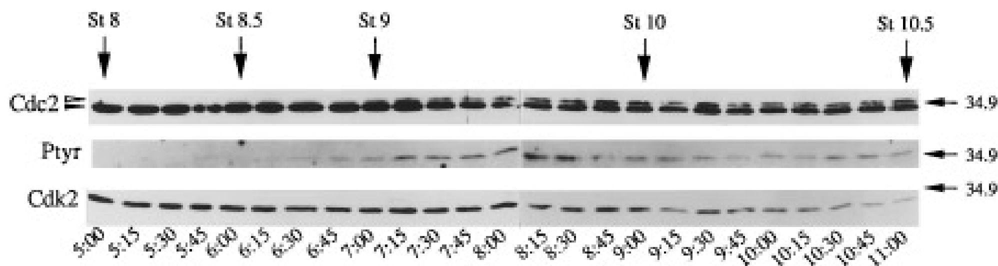

FIG. 6. Cdc2 becomes tyrosine phosphorylated during the MBT. Immunoblots of embryos collected every 15 min between 5 and 11 hr postfertilization. Samples are identical to those in Fig. 5. Time after fertilization is denoted in hr and min. Blots were probed with antibodies to Cdc2 (Cdc2), phosphotyrosine (ptyr), and Cdk2 (Cdk2). Open and solid arrowheads to the left indicate the two forms of Cdc2. Arrows to the right denote molecular weight markers in kilodaltons, and those on the top indicate the stage of development. One embryo equivalent was loaded per lane. This experiment was repeated twice with similar results.

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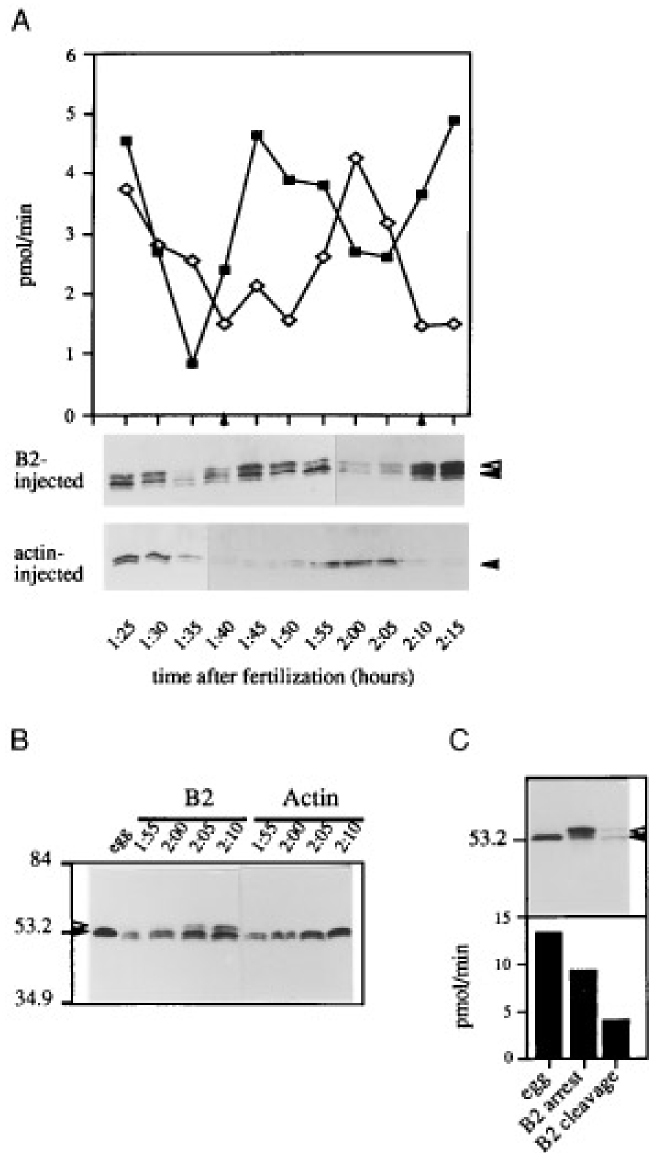

FIG. 7. Overexpression of cyclin B2 accelerates the cell cycle. (A) Profile of total cyclin B/Cdc2 kinase activity immunoprecipitated from embryos injected with either cyclin B2 or actin mRNAs and accompanying cyclin B2 immunoblots. On the graph, solid squares depict B2 mRNA-injected embryos; open diamonds depict actin mRNA-injected embryos. Each timepoint represents the activity from five embryos. Arrowheads on the x-axis indicate the approximate time of cytokinesis for B2 mRNA-injected embryos. Embryos were injected between 50 and 67 min postfertilization. Below the cyclin B2 immunoblots, time postfertilization is indicated in hr and min. The open arrowhead indicates exogenous cyclin B2, and the solid arrowhead indicates endogenous cyclin B2. The equivalent of one embryo was loaded per lane. This experiment was repeated twice with similar results. (B) Western blot of cyclin B2 associated with immunoprecipitated Cdc2 in embryos injected with either cyclin B2 or actin mRNAs. Time postfertilization is indicated on top in hr and min, molecular weight markers are in kilodaltons to the left. The open arrowhead indicates exogenous cyclin B2 and the solid arrowhead indicates endogenous cyclin B2. Microinjections were performed between 50 and 70 min postfertilization. (C) Cyclin B2 immunoblot and corresponding cyclin B-associated kinase activity of cleavage-arrested and dividing embryos injected with cyclin B2 mRNA. Embryos are from the same experiment as (A). Bars represent the activity immunoprecipitated from one unfertilized egg (egg) or a B2 mRNA-injected embryo which either arrested at the 2-cell stage (B2 arrest) or progressed to Stage 8 (B2 cleavage). One embryo was loaded per lane.

DISCUSSION

The results in this paper describe cell cycles in the early *Xenopus* embryo and attempt to determine the mechanism of cell cycle regulation *in vivo.* Cdc2 kinase activity oscillated in each cell cycle, as did the levels of mitotic cyclins. Accompanying these oscillations, the phosphorylation state of Cdc25C changed, such that it was present in its more highly phosphorylated active form when Cdc2 kinase activity was high and in its less phosphorylated inactive form when Cdc2 kinase activity was low. This is consistent with evidence implicating a positive feedback loop between Cdc2 and Cdc25C (Hoffmann *et al.,* 1993; Izumi and Maller, 1993, 1995; Strausfeld *et al.,* 1994a) and may not correspond to an actual need for Cdc25 during these cycles. It was suggested previously that Cdc2 is tyrosine phosphorylated during the first cell cycle in *Xenopus* embryogenesis and after the MBT but not in cycles 2–12 (Ferrell *et al.,* 1991). This conclusion was based on anti-phosphotyrosine blotting of a 34-kDa protein in embryos. In this paper we have shown that an electrophoretic shift and tyrosine phosphorylation of Cdc2 were absent until loss of cyclin E1 at the MBT (Fig. 6). Therefore, in cycles 2–12 it appears that Cdc25C activity would not be necessary for dephosphorylation of Cdc2.

A possible substrate for Cdc25C in cycles 2-12 in addition to Cdc2 could be the cyclin E1/Cdk2 complexes. Cyclin E1/Cdk2 complexes have been shown to be an *in vitro* substrate for Cdc25 (Gabrielli *et al.,* 1992a). Moreover, Cdk2 has been found to contain phosphotyrosine when immunoprecipitated from interphase extracts in which S-phase is arrested with aphidicolin (Gabrielli *et al.,* 1992a). In addition, Cdc25 is phosphorylated and activated by cyclin E1/Cdk2 complexes in both *Xenopus* egg extracts (Izumi and Maller, 1995) and human cell lines (Hoffmann *et al.,* 1994). It is notable that tyrosine phosphorylation of Cdc2 returns at the MBT coincident with the disappearance of cyclin E1 (Figs. 5 and 6). In view of these data, it is interesting to speculate that active cyclin E1/Cdk2 complexes could be acting on Cdc25C in a positive feedback loop. Since oscillations in the phosphorylation state of Cdc25C are not coincident with those of cyclin E1-associated kinase activity, the feedback mechanism would be more complex than a simple loop involving only cyclin E1/Cdk2 and Cdc25C.

Consistent with the hypothesis that cyclin accumulation times the cell cycle, oscillations in Cdc2 kinase activity paralleled the accumulation of cyclin B, with the maximum cyclin level coincident with the peak kinase activity (see

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Figs. 2 and 3). Further support for this is seen in the cyclin mRNA microinjection experiments (Fig. 7). Overexpression of cyclin B2 (or of cyclin B1) caused an earlier rise and fall of Cdc2 kinase activity, with the peak corresponding to mitosis. The combined expression of endogenous and exogenous protein paralleled the kinase activity, with cyclin B2 protein accumulating sooner and being degraded more rapidly in cyclin B2 mRNA-injected embryos. These results support the hypothesis that cyclin B accumulation controls the timing of the early embryonic cell cycle and the existence of a threshold of cyclin which must be reached before entry into mitosis. The threshold hypothesis was suggested following experiments which showed that the length of the cell cycle increased as decreasing amounts of cyclin mRNA were added to mRNA-dependent cell cycle extracts (Murray and Kirschner, 1989). Our experiments also show that a certain amount of cyclin must accumulate before mitotic entry, but the total accumulation at metaphase differed between B2-injected and actin-injected embryos. For example, the blots in Fig. 7A show that at peak kinase activity, there is severalfold more total cyclin B2 protein in cyclin B2 mRNA-injected embryos than in actin mRNA-injected embryos. The additional cyclin B2 in Fig. 7 did not result in a greater level of H1 kinase activity at metaphase, perhaps due to activation of cyclin B degradation. However, much higher concentrations of cyclin B did cause cleavage arrest and persistent elevation of Cdc2 kinase to levels above metaphase (Fig. 7C). The ability of high levels of B-type cyclins to cause cleavage arrest is similar to the metaphase arrest caused by overexpression of cyclin A in mitotic extracts (Roy *et al.*, 1991) or after expression of nondegradable forms of cyclin B (Murray *et al.*, 1989).

Developmental Regulation of Cyclins A1 and E1

Unlike the B cyclins and cyclin A1, the level of cyclin E1 did not oscillate during embryonic cell cycles, but surprisingly, cyclin E1/Cdk2 kinase activity oscillated twice for every cycle of cyclin B1-associated activity. The two- to threefold difference between high and low kinase activity is similar to that seen previously for cyclin E/Cdk2 complexes in *Xenopus* (Gabrielli *et al.*, 1992b; Rempel *et al.*, 1995). This biphasic pattern of kinase activity suggests a role for cyclin E1 in both S phase and mitosis. The mechanism responsible for the oscillation in cyclin E/Cdk2 activity is not clear at present. Figures 1 and 5 in this paper and Rempel *et al.* (1995) show that it is not due to changes in the level of cyclin E or tyrosine phosphorylation of Cdk2. Other data show that there is no electrophoretic shift of cyclin E during the oscillations, although the rapidity of the cell cycle could obscure such a shift. A novel cyclin-dependent kinase inhibitor with high affinity for cyclin E/Cdk2 has been identified recently in *Xenopus* (Su *et al.*, 1995), but whether it is involved in regulating the activity of cyclin E/Cdk2 in the embryo is not yet clear. It is of interest that there is an M-phase peak in activity of cyclin E/Cdk2, particularly since this complex has defined roles only for the initiation of DNA synthesis in *Xenopus* egg extracts (Gabrielli *et al.*, 1992b; Rempel *et al.*, 1995; Strausfeld *et al.*, 1994b; Su *et al.*, 1995). However, in *Drosophila* there is genetic and biochemical evidence for a role for cyclin E during both S- and M-phase. *Drosophila* embryos in which cyclin E has been deleted fail to progress through S-phase once maternal stores have been depleted (Knoblich *et al.*, 1994) as expected for a DNA synthesis function, but cyclin E also plays a mitotic role by influencing the stability of mitotic cyclins. When cyclin E is ectopically expressed in *Drosophila* during the final mitosis (cycle 16), cyclins A and B accumulate again, resulting in an additional cell cycle (Knoblich *et al.*, 1994). The addition of recombinant cyclin E1 to *Xenopus* egg extracts slows the decline in cyclin B/Cdc2 activity associated with the release from M-phase arrest, but not by competing for destruction machinery since *Xenopus* cyclin E1 does not have a destruction box (R.E.R. and J.M., unpublished results). This supports the evidence that Cdk2/cyclin E may be a component of cytostatic factor, the activity responsible for metaphase arrest in unfertilized eggs (Gabrielli *et al.*, 1993; Rempel *et al.*, 1995). Thus, one role for cyclin E1 in mitosis may be to regulate mitotic cyclin stability in the rapid embryonic divisions.

The level of cyclin E1 remains constant until midblastula stages, declining only at the MBT, between 6 and 7 hr postfertilization (Fig. 5), concomitant with tyrosine phosphorylation of Cdc2. The disappearance of cyclin A1 following the decline in cyclin E1 further supports a role for cyclin E1 in stabilizing mitotic cyclins. In contrast, cyclin B1 is readily detectable through 11 hr (Fig. 5) and further (at least until Stage 19/20, R.E.R., unpublished observations). It is likely that the increase in cyclin B1 is due to zygotic transcription, as our cyclin B1 antibody does not appear to be specific for maternal B1. Based on analogies with *Drosophila,* cyclin E1 is probably a maternal cyclin E that is replaced at the MBT with a zygotic form (Richardson *et al.*, 1993). The disappearance of cyclin A1 corresponds to the increased expression of a second *Xenopus* cyclin A, cyclin A2 (Howe *et al.*, 1995). The disappearance of both cyclin E1 and A1 coupled to the additional regulation of Cdc2 and Cdk2 by tyrosine phosphorylation corresponds temporally with lengthening of the cell cycle and activation of zygotic transcription. In agreement with Rempel *et al.* (1995) and this paper, Howe *et al.* (1995) have also recently shown that cyclin A1 declines around gastrulation and that Cdc2, Cdk2, and cyclin B2 levels remain constant.

Xenopus and Drosophila: A Comparison of Developmental Systems

Unlike early *Xenopus* cycles, in *Drosophila* only divisions 10–13 are timed by cyclin accumulation (Edgar *et al.*, 1994). Cyclin accumulation does not control the timing of early (1–9) or late (14–16) cycles, and there are no fluctuations in cyclin levels or Cdc2 kinase activity in the first 7 cell cycles in *Drosophila.* The transition from maternal to zygotic control occurs after cycle 13 in *Drosophila,* at the time of cellularization and a gradual lengthening of the cell cycle. Maternal string is degraded after cycle 13 and mitoses

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14–16 are controlled by zygotic transcription of the *string* gene (Edgar and O'Farrell, 1990). Thus, the mechanisms regulating early *Xenopus* cell cycles are similar to those operating during the cyclin-limited *Drosophila* cycles, as both rely on cyclin accumulation and not on inhibitory tyrosine phosphorylation of Cdc2. Tyrosine phosphorylation of Cdc2 occurs again in *Xenopus* cell cycles past the MBT, suggesting that Cdc25 is again important in the regulation of these cycles, as in the *string*-limited cycles of *Drosophila. Drosophila* cycles lacking oscillations in cyclins and Cdc2 kinase activity differ from those of *Xenopus* in that they occur in a syncitial cytoplasm. Edgar and coworkers (1994) suggest that these syncitial cycles may be driven by degradation of cyclin B associated with the mitotic apparatus. This hypothesis predicts that more cyclin would be progressively degraded at each mitosis. In *Xenopus,* however, the early mitoses are accompanied by cytokinesis and complete degradation of cyclin B still occurs in anuclear *Xenopus* egg extracts (R.S.H. and R.E.R., unpublished observations), suggesting that cyclin degradation is independent of the mitotic apparatus in vertebrate embryos.

Our data show that the transition from a rapid and simple early embryonic cell cycle to an extended somatic cell-like cycle that includes G_1- and G_2-phases is accompanied by changes in cell cycle regulators. The disappearance of cyclin E1 and A1 occurs during and after the MBT when the cell cycle is lengthening due to an extension of S-phase (Frederick and Andrews, 1994; Newport and Dasso, 1989). We presume that expression of zygotic forms of cyclins is tightly and differentially regulated during ensuing gastrulation and differentiation. An important question concerns whether lengthening of the cell cycle results directly from the loss of cyclin E1 and A1 or whether the lengthening of the cycle directly or indirectly causes the loss of cyclin E1 and A1. A related question is whether the changes seen in cell cycle regulators would occur without progression through the cell cycle or transcription. There is evidence for this in the case of *Drosophila,* where cyclin E expression and downregulation occur normally even in the absence of cell division (Knoblich *et al.,* 1994). On the other hand, it has been suggested that the short length of the early mitotic cycles in *Drosophila* limits gene activity so that only small transcripts can be translated (Rothe *et al.,* 1992). Thus the lengthening of the cycle at the initiation of transcription would allow synthesis of an expanded range of proteins. Previous studies in *Xenopus* also suggest that stopping progression through the cell cycle can stimulate transcription and cell motility (Kimelman *et al.,* 1987). It is likely that in *Xenopus,* as in *Drosophila,* the changes which occur in the cell cycle at the initiation of transcription are a result of maternal programming. Further study is necessary to understand the relationship between developmental transitions in cell cycle control and the onset of differentiation and morphogenesis.

ACKNOWLEDGMENTS

We thank Andrea Lewellyn for invaluable assistance with the embryo timecourses and microinjections, Olivier Haccard for useful discussion and help with microinjections and the manuscript, Dr. Wilfried Merlevede for antiphosphotyrosine antibodies, and Dr. Tim Hunt for the cyclin B2 cDNA clone. This work was supported by the Howard Hughes Medical Institute. R.S.H. and R.E.R. are Associates and J.L.M. is an Investigator of the Howard Hughes Medical Institute.

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Received for publication September 7, 1995
Accepted November 15, 1995

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