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Patterns of expression of cyclins A, B1, D, E and cdk 2 in preimplantation mouse embryos

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Summary

Cell cycle regulatory proteins have been characterized in somatic cells and exhibit phase-specific expression patterns. Changes in expression of these regulatory proteins have not been clearly characterized in early preimplantation mouse embryos. This study utilized indirect immunofluorescence to determine the expression pattern of G1/S phase cyclins D and E; S, G2/M phase cyclins A and B1, and cdk 2 during the first three cell cycles of mouse embryo development. Cyclin D demonstrated low expression throughout the first cell cycle but had a somatic-like pattern of expression in cycles 2 and 3 with peak expression at G1 declining through S phase to a low during G2. Cyclin E was present at peak levels in G1 declining through S to a low in G2 during both the first and third cell cycles, but remained at moderate levels during the entire second cell cycle. Cyclin A was maintained at moderate levels throughout the first two cell cycles but showed a somatic-like pattern with a low level in G1 increasing during S phase with peak levels during G2 of the third cell cycle. Cyclin B consistently demonstrated a pattern opposite to a somatic G2/M cyclin, with peak levels in G1 declining through S phase to a low in G2 during each of the three cell cycles examined. Cdk 2 was present at consistent levels during G1 and S phases of all three cell cycles declining slightly in G2.

Keywords: Cyclin-dependent kinase, Cyclins, Embryo, Mouse, Preimplantation

Introduction

Cell division is a highly controlled process which is regulated by many important endogenous factors. Two groups of proteins, cyclins and cyclin-dependent kinases (cdks), are key regulators in this reproductive process. The interaction between these two groups of proteins is necessary for progression through each phase of the cell cycle, i.e. G1, S and G2, in interphase and mitosis (Sherr, 1993). Cyclins are specialized activating proteins, which bind and activate cdks. Appropriately named, cyclins undergo a cycle of synthesis and degradation at specific times (Minshull *et al*., 1989). Once cdks are activated by cyclins, they are able to phosphorylate and further activate or inactivate target proteins as necessary for cell division activity. However, cdks are inactivated once cyclins are degraded. Absence or alterations of either protein result in an arrest of the cell cycle and, at times, cell death.

The duration of the cell cycle varies from one cell type to another, although for a given somatic cell type the cell cycle is consistent. In the first three cell cycles of mouse preimplantation development, however, division from one cycle to the next is quite variable. The first cell cycle is completed in 18–20 h, including 6–8 h in G1, 6 h in S and 6 h in G2 (Krishna & Generosa, 1977). During the second cell cycle, each cell spends approximately 1.3 h in G1, 6.1 h in S and 15.4 h in G2 (Sawicki *et al*., 1978). This cycle is completed in 22.8 h but can last up to 30 h. The third cell cycle is the shortest and only takes 9.5 h: 1.6 hours in G1, 7.4 h in S and 0.5 h in G2 (Sawicki *et al*., 1978).

In somatic cells, yeast, and some embryos such as clam, sea urchin and *Xenopus*, cyclin–cdk interactions have been studied extensively. Cyclins have been identified as phase-specific. The two major checkpoints in the cell cycle are at the completion of G1 before entry into S phase and at the end of G2 during entry

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into mitosis (Alberts *et al*., 1994). Cyclins specific for regulating these two checkpoints are referred to as G1/S cyclins and G2/M (mitotic) cyclins.

G1 cyclins D and E have been identified in mammalian cells such as human fibroblasts, rat fibroblasts, human tumour cells and a variety of epithelial cell lines (Sun *et al*., 1996). Three D-type cyclins have been characterized, which assemble into functional enzyme complexes with either cdk 4 or cdk 6 (Sherr, 1994). In human fibroblast cell lines, cyclin D1 appears early in G1, whereas cyclins D2 and D3 are expressed much later in G1 phase or early S phase respectively (Won *et al*., 1992; Pestell *et al*., 1999). The expression of all D-type cyclins in cultured cells can be regulated by specific growth factors such as colony-stimulating factor 1, platelet-derived growth factor and insulin-like growth factor (Furlanetto *et al*., 1994).

Cyclin E activates cdk 2 at the end of G1. Cyclin-E-dependent kinase activity is at its highest level in cultured cells at the G1/S transition. Once the cell has entered S phase, the two molecules dissociate and cyclin E is degraded (Sherr, 1994). Eliminating the activities of either protein complex during G1 by microinjecting antibodies against cyclin D1 or cdk 2 (partner of cyclin E) into normal fibroblasts prevents the cell from progressing to S phase (Baldin *et al*., 1993).

Two mammalian cyclins (A and B) have been identified as G2/M regulators. In a variety of mammalian cell lines, cyclin B associates solely with Cdc2 late in G2 phase for entry into M phase (reviewed in Sherr, 1993). The combination of B-type cyclins and Cdc2 (cdk 1) is known as maturation (or M phase) promoting factor (MPF), which is vital for cells to undergo mitosis. Two B-type cyclins, B1 and B2, have been identified in a variety of mouse tissue types and HeLa cells (Chapman & Wolgemuth, 1992; Pines & Hunter, 1989). Both proteins interact with Cdc2, although their subcellular locations are different. In HeLa cells, cyclin B1 enters the nucleus late in G2 to associate with microtubules, whereas cyclin B2 is often found associated with intracellular membranes, particularly the Golgi apparatus (Pines & Hunter, 1991; Jackson *et al*., 1995). Even though B-type cyclins are localized in different areas of the cell, some redundancy has been seen in their actions. Brandeis *et al*. (1998) created transgenic mice that were lacking either cyclin B1 or cyclin B2. Cyclin B1 proved to be an essential gene as no homozygous B1-null pups were born. However, mice lacking cyclin B2 developed normally without any obvious abnormalities. In fact, cyclin-B2-deficient mice expressed an increased level of cyclin B1 in both membranes and cytoplasm of the testes (Brandeis *et al*., 1998). It appears that cyclin B1 is the essential gene that is able to compensate for the loss of cyclin B2, activating the necessary Cdc2 molecules at specific locations in the cell.

Table 1 Embryo collection times relative to cell cycle stage

Cell cycle	Stage	Embryo collection time (hours post-fertilization)[a]
First	G1	3
First	S	12
First	G2	18
Second	G1	21
Second	S	27
Second	G2	32
Third	G1	44
Third	S	47
Third	G2	48

[a] Fertilization was assumed to occur at 12:00 midnight.

Cyclin A functions at two separate times during the cell cycle and has also been associated with the onset of DNA replication during S phase in mammalian fibroblasts (Girard *et al*., 1991). Similar to cyclin E, it complexes with cdk 2 during S phase. However, during G2, cyclin A has been shown to activate cdk 2 or Cdc2, while cyclin B will only activate Cdc2 for completion of the cell cycle (Sherr, 1993; Zindy *et al*., 1992). In HeLa cells, cyclin A will accumulate and be destroyed before cyclin B during G2 (Zindy *et al*., 1992).

The expression of cell cycle regulators has been well established in somatic cells, but has not been completely characterized in mammalian embryonic systems. The goal of this research was to examine the pattern of protein expression of five cell cycle regulators – cyclin A, cyclin B1, cyclin D, cyclin E and cdk 2 – during G1, S and G2 of the first three cell cycles of preimplantation mouse embryo development using indirect immunofluorescent staining techniques. The combined *in vivo* expression data for these cell cycle regulatory proteins supports unique roles especially for cyclins B1 and A in cell division during early mouse development.

Materials and methods

Embryo isolation

Female NSA mice (colony 217, Harlan Sprague Dawley, Indianapolis, IN) were injected intraperitoneally with 10 IU of pregnant mare serum gonadotropin (PMSG, Calbiochem, La Jolla, CA) followed 48 h later with 5 IU of human chorionic gonadotropin (hCG; Sigma Chemical). Superovulated female NSA mice were mated overnight with B6SJLF1/J males (Jackson Labs, Bar Harbor, ME).

Embryos (1-cell, 2-cell or 4-cell) were flushed from oviducts at varying stages of the cell cycle (G1, S or G2; see Table 1) with warmed Hank's balanced salt solution containing 0.4% bovine serum albumin

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(HBSS + BSA). If necessary, hyaluronidase (0.1% in 10% polyvinylpyrrolidone in phosphate-buffered saline (PBS)) was used to remove cumulus cells.

Fertilization was assumed to have occurred at 12:00 midnight, approximately 12 h after hCG injection. Flushed embryos were considered to be in G1 phase of the first cell cycle when a vaginal sperm plug was observed in the mother prior to dissection and sperm were present in the flushing drop after isolation. Fertilization was confirmed in some experiments by Hoechst 33258 staining. Embryos in S phase and G2 phase of the first cell cycle were confirmed by the presence of the female and male pronuclei. Embryos were analysed at G1 of the second cell cycle when, upon flushing an oviduct, some 1-cell and mostly 2-cell embryos were present indicating a transition between the first and second cell cycle. G1 of the third cell cycle was assumed when, upon flushing an oviduct, some 2-cell but mostly 3- and 4-cell embryos appeared, indicating a progression from the second to third cell cycle. Embryos were judged to be in S phase and G2 phase of the second and third cell cycles by timing based on published reports (Sawicki *et al*., 1978) and by the presence of entirely 2-cell embryos or 4-cell embryos in the flushing drop at the appropriate time (Table 1).

Embryo fixation and processing

Zonae pellucidae were removed by a brief treatment with acid Tyrode's solution or 0.5% pronase. Zona-free embryos were washed three times in HBSS + BSA and transferred into freshly prepared 4% paraformaldehyde (Sigma Chemical) in PBS. Fixation and permeabilization steps were modified from procedures described by Aoki *et al*. (1997), Simerly & Schatten (1993) and Stein *et al*. (1997). After 8–10 h in fixative at 4 °C, embryos were permeabilized in 0.05% Triton X-100 (Sigma Chemical) in calcium- and BSA-free CZB medium (Chatot *et al*., 1989) for 2 min (Aoki *et al*., 1997). After a brief recovery period in calcium- and BSA-free CZB, a second permeabilization step of 0.2% Triton X-100 was performed for 4 min. The embryos were washed three times in calcium- and BSA-free CZB medium and stained immediately. After fixation, some 1-cell embryos were stained for 30 min in Hoechst 33258 (Sigma Chemical.) to confirm fertilization.

Antibody staining

Permeabilized embryos were blocked in HBSS with 1% BSA for 1 h. Rabbit polyclonal primary antibodies against cyclin A, cyclin B1, cyclin D, cyclin E (Rockland, Gilbertsville, PA) or cdk 2 (Upstate Biotechnology, Lake Placid, NY) were diluted 1:500 in blocking solution (HBSS + 1% BSA). Embryos of various stages were incubated in each of the primary antibodies for 1 h at room temperature. After treatment, embryos were washed two times in dilute blocking solution (HBSS + 0.1%BSA) at 37 °C for 5 min. Embryos were stained in a 1:250 dilution of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit secondary antibody (Sigma Immunochemicals) for 1 h at room temperature. Embryos were washed three times in dilute blocking solution and were attached to coverslips coated with poly-L-lysine (2 mg/ml in dH_2O; Sigma Chemical). Coverslips were mounted on microscope slides in glycerol preservative solution (Glycerol-PBS 9:1 mixture containing 100 mg/ml of the antifade reagent 1,4-diazobicyclo[2,2,2,] octane (DABCO); Sigma Chemical) and sealed with clear nail polish. Embryos were analysed within 1 month of mounting onto a microscope slide to avoid fading of the fluorescence levels.

Controls

Negative controls in this experiment included embryos exposed only to the FITC-conjugated secondary antibody and embryos incubated with preimmune rabbit serum (Sigma Chemical.) in place of primary antibody followed by secondary antibody exposure. Controls with secondary antibody alone were included in every experimental group, whereas rabbit serum controls were performed twice on 1-cell embryos. Positive controls for a strong fluorescence signal included 1- and 2-cell embryos exposed to anti-cellular actin (Sigma Chemical) as the primary antibody followed by FITC-conjugated secondary antibody. This provided an intense staining model and was considered maximum fluorescence.

Data analysis

Cell cycle protein staining was examined under a Zeiss fluorescence microscope equipped with a fluorescein filter. Embryos were scored for fluorescence emitted on a scale of 1 (weak) to 4 (strong). Negative controls scored between 0 and 1, whereas positive controls scored between 3.5 and 4. Embryos stained for cell cycle regulatory proteins were scored on the basis of this scale. At each collection time, nine to 19 embryos which had been isolated from five or six different mice during two or three different experiments were scored and an average score was calculated for each antibody. The standard error of the mean (SEM) was calculated for all data points. Representative photographs were taken for each collection time using a Nikon 35 mm camera attached to the Zeiss fluorescence microscope. Pictures were taken under ×400 magnification at an exposure time of 15 s for all stages. Selected pictures with unique staining patterns were photographed under ×1000 magnification with immersion oil at an exposure time of 15 s.

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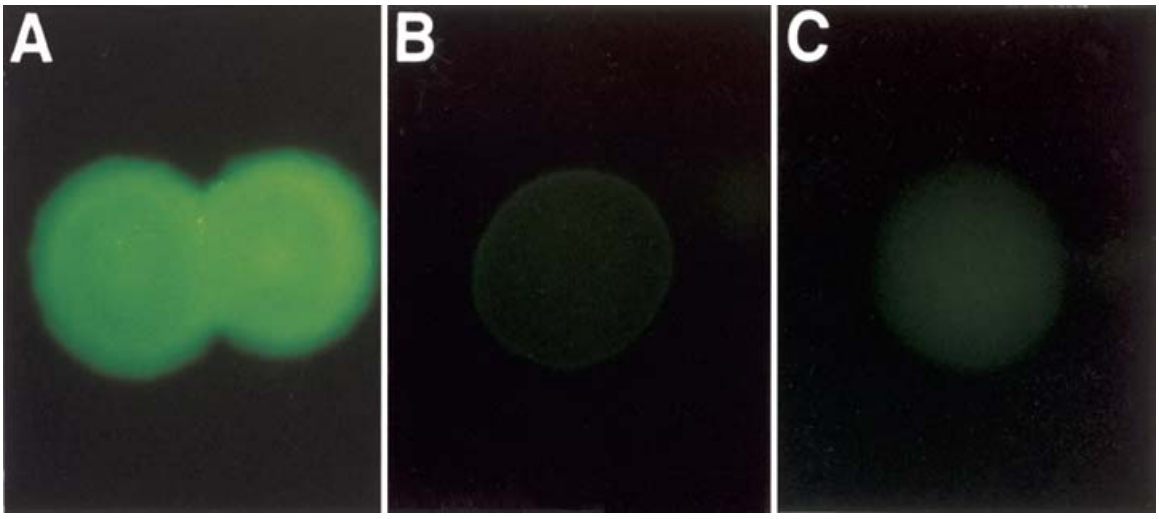


Figure 1 Controls for immunofluorescence in 1-cell and 2-cell preimplantation mouse embryos. Two-cell mouse embryos were fixed, blocked and stained with anti-actin (*A*). One-cell mouse embryos were fixed, blocked and stained with goat anti-rabbit IgG secondary antibody alone (*B*) or with normal rabbit serum followed by goat anti-rabbit IgG secondary antibody (*C*). (*A*), (*B*) and (*C*) were taken at a magnification of ×400 under a fluorescence microscope with an exposure time of 15 s.

Results

Preimplantation mouse embryos were collected at various stages of the first three cell cycles (Table 1) and stained with antibodies against cyclins A, B1, D, E or cdk 2. Embryos were scored for relative levels of fluorescence using a numerical scoring system with a scale of 0 to 4. An average fluorescence score (AFS) was determined for each cell cycle phase for each individual antibody.

Controls

Three sets of controls were used in this study. In order to obtain a maximum and minimum fluorescence value, positive and negative controls were used. To assess maximum fluorescence, embryos were stained with anti-actin (cellular). It is known that cellular actin is ubiquitously expressed during development at high levels. Therefore, a strong signal was seen and these embryos were given a score of 4 ($n = 11$, AFS 3.5–4) (Fig. 1*A*). This represented the maximum fluorescence reference value when other embryos were evaluated after cell cycle protein staining. Two sets of negative controls were used to evaluate any background fluorescence. Embryos were exposed to secondary antibody alone. Regardless of cell cycle stage, little to no fluorescence was detected with this set of controls (Fig. 1*B*). Embryos comparable to this group were scored in the 0 to 1 category. The other negative control consisted of embryos exposed to preimmune rabbit serum and then secondary antibody to assess the presence of natural factors in rabbit serum that non-specifically bind to the embryo causing background fluorescence. Again, minimal fluorescence was emitted in this set of controls ($n = 15$, AFS 0.5–1.5; Fig. 1*C*). Embryos showing a similar staining pattern were given a score of 0.5–1.5. If embryos stained with cell cycle proteins exhibited a fluorescence pattern comparable to either of the negative control groups, it was considered background fluorescence.

Immunofluorescence patterns for G1/S cyclins E and D and cdk 2

During the first cell cycle, cyclin E was present at high levels in G1 with an AFS of 3.6 ± 0.18 (Fig. 2*A*). Expression declined sequentially in S and G2/M with AFSs of 2.5 ± 0.14 and 1.7 ± 0.12 respectively (Fig. 2*B*, *C*). During G1 and S of the first cell cycle, cyclin E was localized in both the cytoplasm and the pronuclei of the embryo. Cyclin E was expressed at moderate levels throughout the second cell cycle with AFSs of 2.5 ± 0.12, 2.5 ± 0.20 and 2.2 ± 0.11 respectively in G1, S and G2/M (Fig. 2*D*, *E*, *F*). During the third cell cycle, cyclin E returned to the typical somatic cell pattern of expression, being high in G1 (AFS $= 2.9 \pm 0.20$) and declining in S and G2/M (AFS $= 1.6 \pm 0.17$ and 1.6 ± 0.14 respectively) (Fig. 2*G*, *H*, *I*; Fig. 3*A*).

Embryos stained for cyclin D exhibited background levels of fluorescence throughout the first cell cycle with AFSs of 0.4 ± 0.15 in G1, 1.1 ± 0.27 in S and 0.1 ± 0.07 in G2, but had a somatic-like pattern of expression in cycles 2 and 3 with peak expression at G1 declining through S phase to a low during G2. AFSs for the second cell cycle were 3.1 ± 0.17 in G1, 0.7 ± 0.45 in

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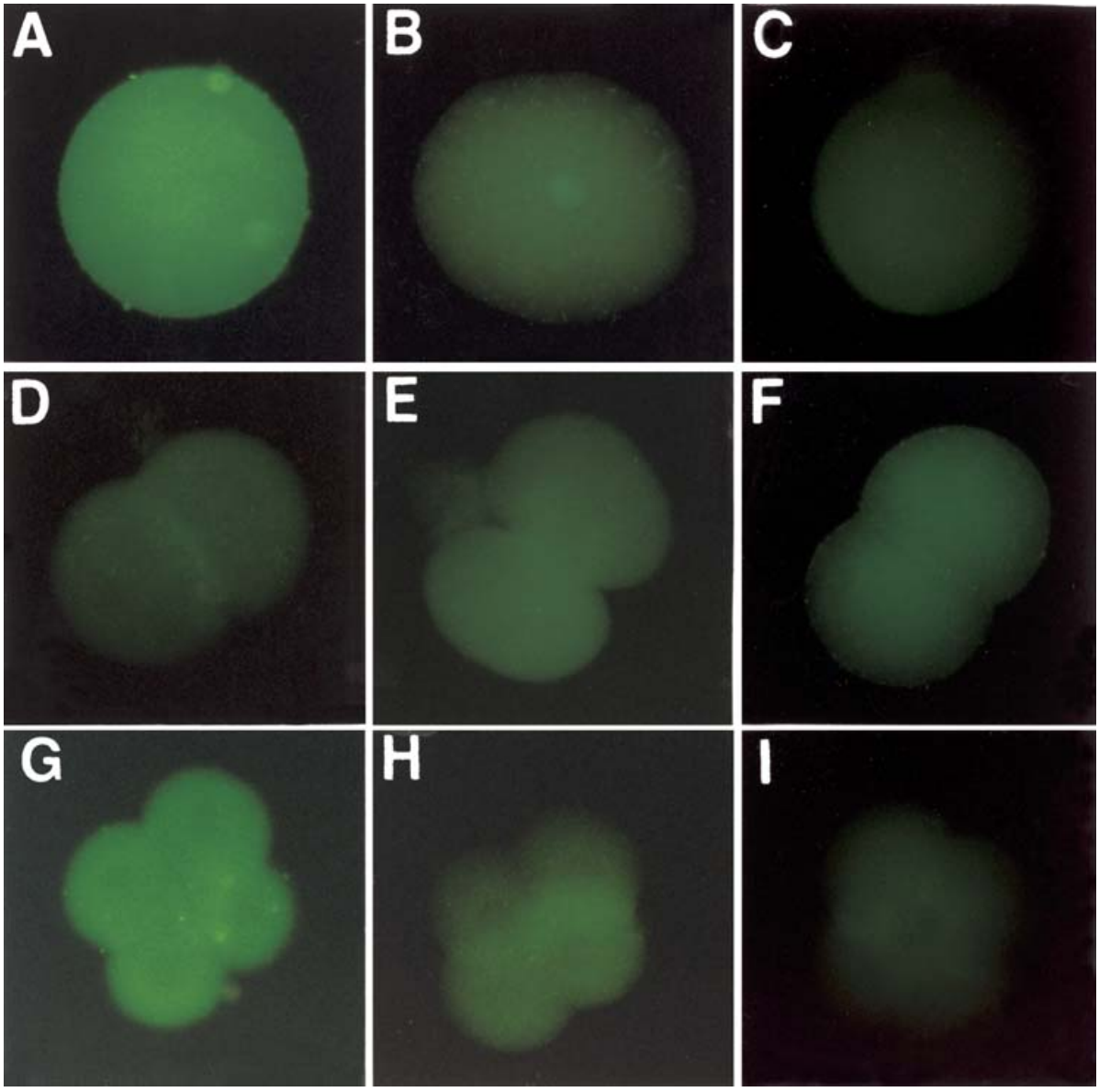


Figure 2 Preimplantation mouse embryos stained with antibodies against cyclin E during the first three cell cycles of development. One-cell embryos were fixed at G1, S and G2 of the first three cell cycles and stained with antibodies against cyclin E. (*A*), (*B*) and (*C*) represent embryos during G1, S and G2 of the first cell cycle; (*D*), (*E*) and (*F*) are photographs of embryos during G1, S and G2 of the second cell cycle; while (*G*), (*H*) and (*I*) are embryos isolated at G1, S and G2 of the third cell cycle. All photographs were taken with an exposure time of 15 s and a magnification of ×400.

S and 1.4 ± 0.28 in G2, and for the third cell cycle were 2.6 ± 0.19 in G1, 1.1 ± 0.17 in S and 2.8 ± 0.16 in G2 (Fig. 3*B*).

Cyclin-dependent kinase 2 (Cdk 2) was present at moderately high levels in G1 (2.7 ± 0.17, 2.7 ± 0.17 and 2.7 ± 0.14) and S phase (3.0 ± 0.16, 3.0 ± 0.19 and $2.9 \pm .018$) for each of the three cell cycles respectively. Immunofluorescence staining declined somewhat to AFSs of 1.8 ± 0.20, 1.6 ± 0.13 and 1.9 ± 0.15 during G2 of cell cycles 1, 2 and 3 respectively (Fig. 3*E*).

Immunofluorescence patterns for S/G2/M cyclins A and B1

Cyclin A was present at very low levels in the first cell cycle, with an AFS of 1.6 ± 0.26 in G1, rising to 2.2 ± 0.13 and 2.0 ± 0.13 in S and G2/M respectively, as would be expected of a G2/M cyclin (Fig. 4*A*, *B*, *C*). During the second cell cycle, cyclin A was detected at AFSs of 2.0 ± 0.18, 2.3 ± 0.11 and 2.0 ± 0.25 in G1, S and G2/M (Fig. 4*D*, *E*, *F*). Cyclin A levels dropped precipitously to background levels in G1 of the third cell cycle (AFS

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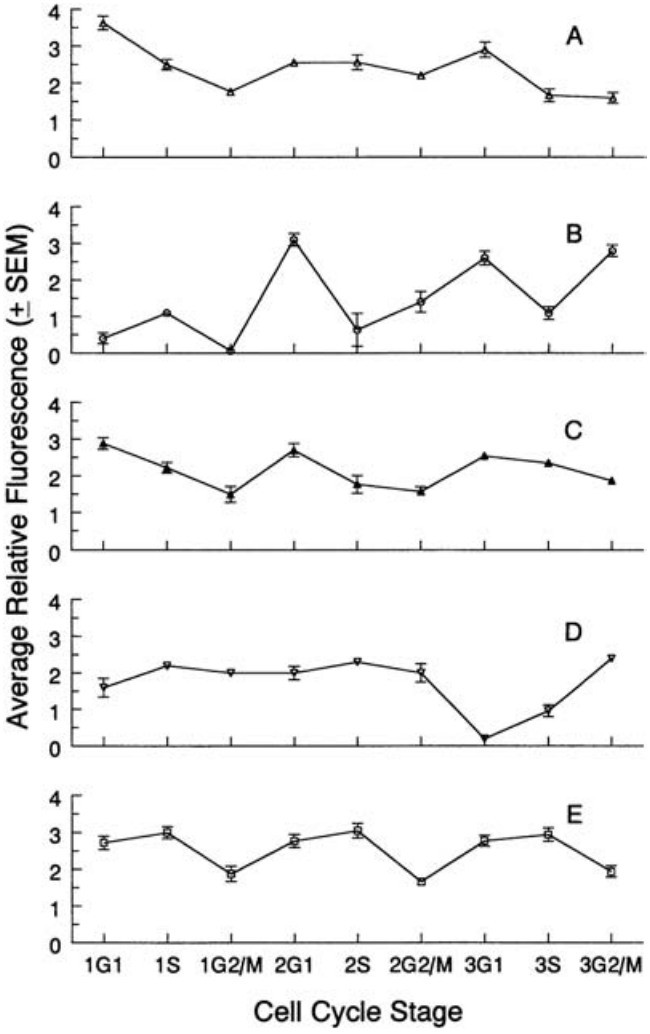


Figure 3 Cyclin A, B1, D, E and cdk 2 expression during the first three cell cycles of mouse development. The *y*-axis represents an average fluorescence score per each group of embryos (*N* range = 9–19) Scoring: no fluorescence = 0, background fluorescence = 1, minimal fluorescence = 2, moderate fluorescence = 3, and maximum fluorescence = 4. The *x*-axis represents each phase of the cell cycle for the first three cell divisions (1G1 = G1 of the first cell cycle, 1S = S phase of the first cell cycle, 1G2 = G2 of the first cell cycle, 2G1 = G1 of the second cell cycle, 2S = S phase of the second cell cycle, 2G2 = G2 of the second cell cycle, 3G1 = G1 of the third cell cycle, 3S = S phase of the third cell cycle, and 3G2 = G2 of the third cell cycle). Error bars represent the standard error of the mean. Graphs represent: (*A*) cyclin E; (*B*) cyclin D; (*C*) cyclin B1; (*D*) cyclin A; and (*E*) cdk 2.

0.2 ± 0.13),_ rising slowly in S to an AFS of 1.0 ± 0.16 and then accumulating in G2/M to an AFS of 2.4 ± 0.13 (Fig. 4*G*, *H*, *I*; Fig. 3*D*). Staining at all stages was general to the cytoplasm and did not appear to localize to the nucleus.

Cyclin B1 was not expressed as a G2/M cyclin, as was expected. During the first cell cycle, cyclin B1 was present at moderate to high levels during G1 of the cell cycle with an AFS of 2.8 ± 0.15 (Fig. 5*A*). This level declined steadily in S and G2/M to 2.2 ± 0.14 and 1.5 ± 0.21 respectively (Fig. 5*B*, *C*). This pattern repeated itself during the second and third cell cycles, with AFSs in G1, S and G2/M of 2.7 ± 0.17, 1.7 ± 0.23 and 1.5 ± 0.21 (second cell cycle)(Fig. 5*D*, *E*, *F*) and 2.5 ± 0.13, 2.3 ± 0.12 and 1.5 ± 0.13 (third cell cycle) (Fig. 5*G*, *H*, *I*; Fig. 3*C*). Staining was frequently punctate and appeared to be associated with small vesicles in the cytoplasm. Staining did not localize to the nucleus.

Localization of unique staining patterns

Some embryos emitting a strong signal had unique staining patterns throughout the cytoplasmic cortex of the embryo. For example, G1 embryos from the first cell cycle stained with anti-cyclin E exhibited an intense fluorescence with a punctate pattern (Fig. 6*A*). This speckled appearance was also seen in G1 1-cell embryos stained for cyclin B1 and S phase 1-cell embryos stained for cyclin E. In other cases, a more diffuse but still punctate pattern was observed around larger circular areas. Four-cell embryos in S phase of the third cell cycle stained for cdk 2 appear to have a localized stain around an unknown cellular element along with a speckled fluorescence (Fig. 6*B*). The staining in these embryos spanned the entire periphery as well as cytoplasmic and nuclear areas.

Discussion

The results of this study describe the protein expression patterns of G1 cyclins D and E, S/G2/M cyclins A and B1, and the cyclin-dependent kinase cdk 2 in preimplantation mouse embryos. For cyclins D and E, with slight variation, expression peaked at G1 of each of the first three cell cycles. This is in agreement with cyclin D and E expression in most somatic cells.

Cyclin D protein was not present during the first cell cycle of preimplantation mouse embryo development. This is similar to the pattern of expression observed for cyclin D in sea urchin embryos, where neither the mRNA nor the protein was expressed until the mid-blastula transition (Moore *et al*., 2002). Moore *et al*. (1996) characterized the expression of mRNA for cyclins D1, D2 and D3 during the first two cell cycles of preimplantation mouse embryo development. While cyclin D2 mRNA was present in high abundance during the early first cell cycle declining to very low levels after 8 h post-insemination and cyclin D3 mRNA was present at fluctuating levels throughout both cell cycles 1 and 2, the expression of mRNA for cyclin D1 agrees with the pattern of protein staining observed in this study. Cyclin D1 RNA was at low

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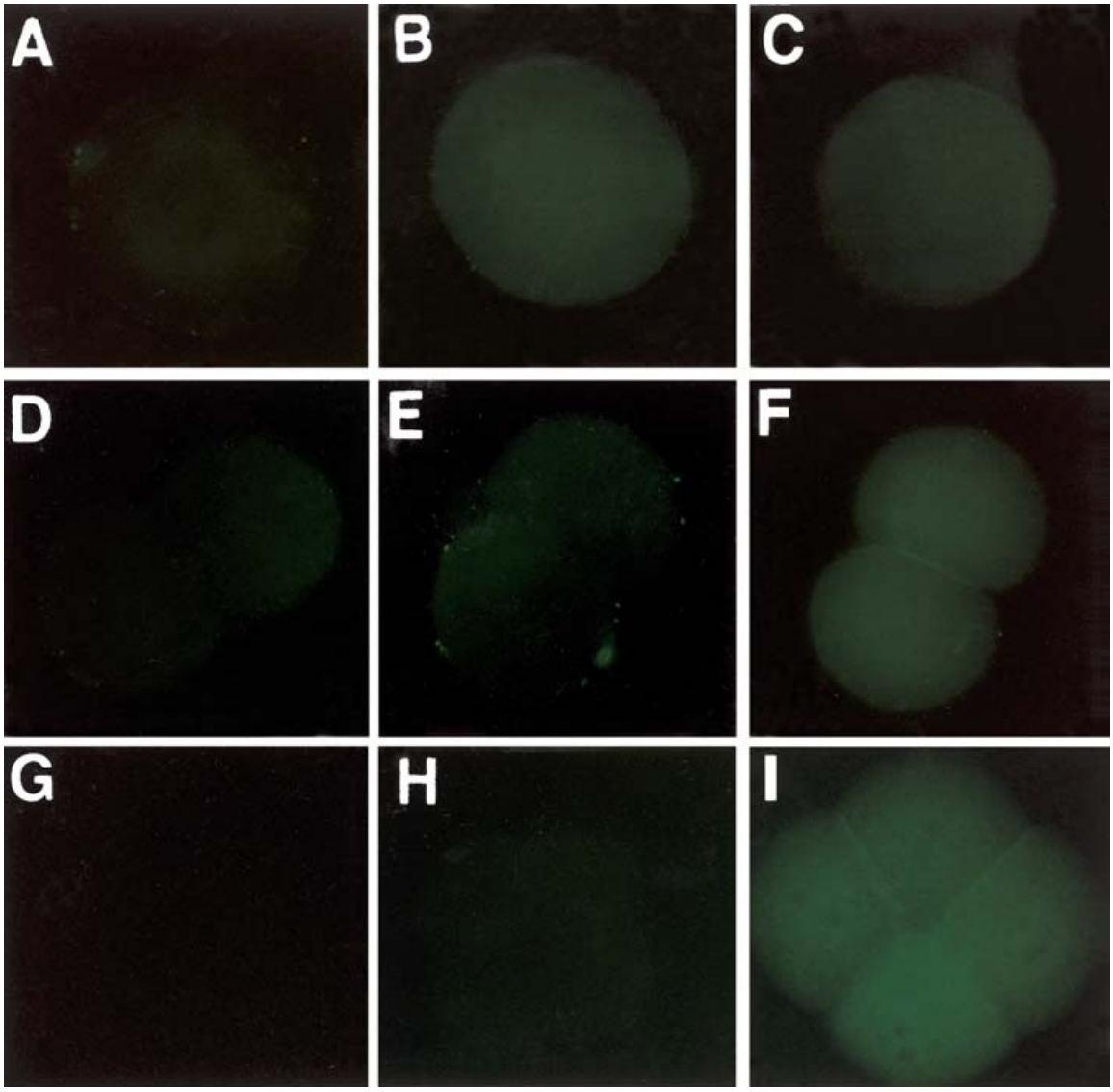


Figure 4 Preimplantation mouse embryos stained with antibodies against cyclin A during the first three cell cycles of development. One-cell embryos were fixed at G1, S and G2 of the first three cell cycles and stained with antibodies against cyclin A. (*A*), (*B*) and (*C*) represent embryos during G1, S and G2 of the first cell cycle; (*D*), (*E*) and (*F*) are photographs of embryos during G1, S and G2 of the second cell cycle; while (*G*), (*H*) and (*I*) are embryos isolated at G1, S and G2 of the third cell cycle. All photographs were taken with an exposure time of 15 s and a magnification of ×400.

levels until the end of the first cell cycle and then rose steadily during the second cell cycle. This supports the appearance of cyclin D1 protein in G1 of the second cell cycle and suggests that this protein is first made from the embryonic genome at the start of zygotic gene activation. Although the antibody used in this study was not identified as being targeted for a specific D-type cyclin, the current evidence suggests it is specific for cyclin D1.

Cyclin D deficiency can be overcome by the presence of sufficient cyclin E, based on cyclin E knockin experiments in the mouse. Geng *et al*. (1999) demonstrated that the knockin of cyclin E into the cyclin D gene location in mice can rescue the cyclin D1 deficiency phenotype. These data suggested that cyclin E is a downstream target of cyclin D and can replace cyclin D in the mouse. The presence of cyclin E protein during G1 of cell cycle 1 in mouse embryos may be sufficient to drive entry into S phase. Alternatively, cyclins D2 and/or D3, whose mRNAs are expressed during the first cell cycle, may be present to regulate the G1 phase of the first cell cycle. Cyclin D1 knockout mice were small with retinopathy and mammary acinar cell development deficiency, but nonetheless were viable and fertile (Fantl *et al*., 1995), supporting the ability of the other D-type cyclins to compensate for the absence of D1. Ciemerych *et al*. (2002) developed double-knockout

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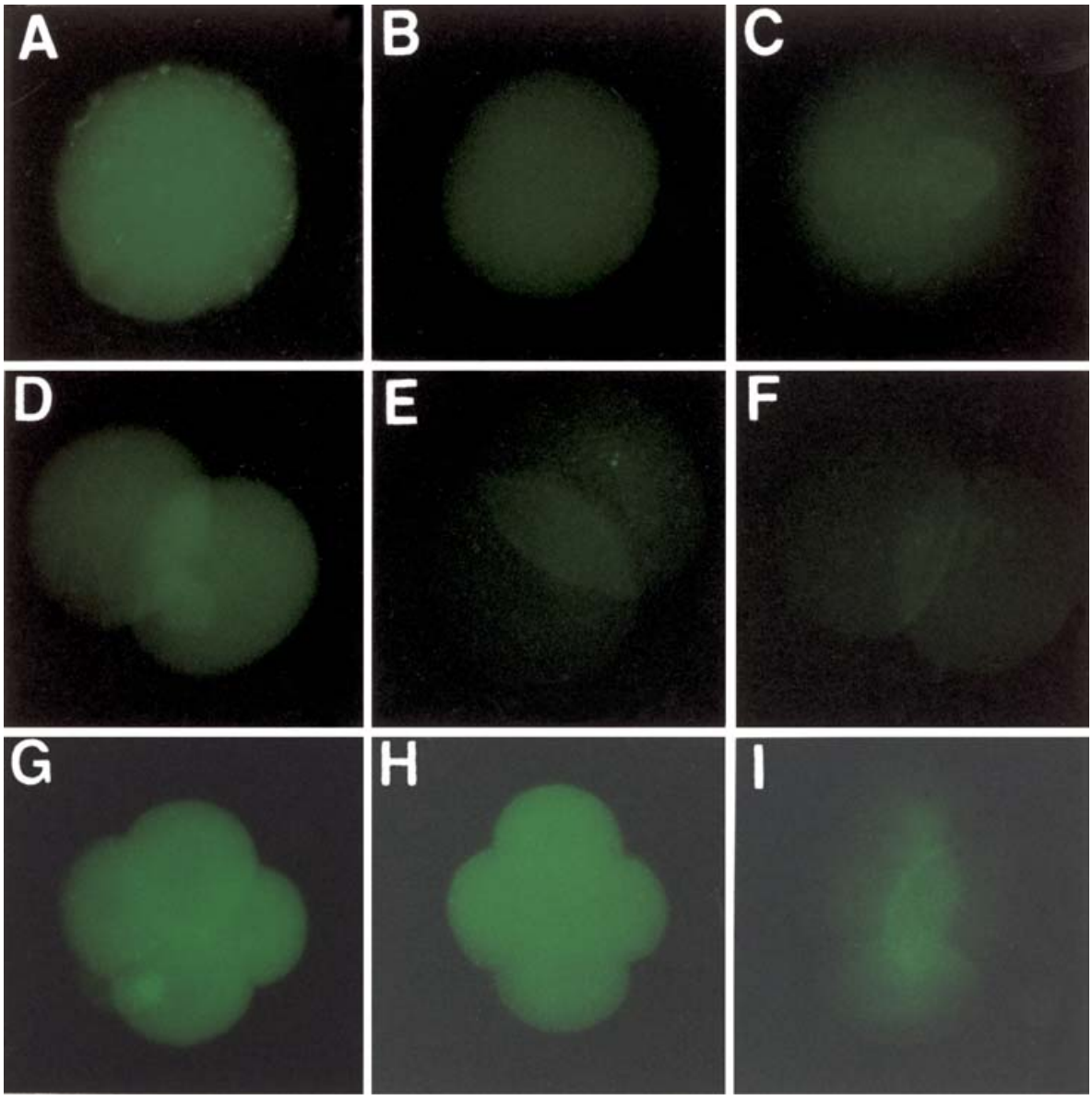


Figure 5 Preimplantation mouse embryos stained with antibodies against cyclin B1 during the first three cell cycles of development. One-cell embryos were fixed at G1, S and G2 of the first three cell cycles and stained with antibodies against cyclin B1. (*A*), (*B*) and (*C*) represent embryos during G1, S and G2 of the first cell cycle; (*D*), (*E*) and (*F*) are photographs of embryos during G1, S and G2 of the second cell cycle; while (*G*), (*H*) and (*I*) are embryos isolated at G1, S and G2 of the third cell cycle. All photographs were taken with an exposure time of 15 s and a magnification of ×400.

mice expressing only one of the three possible cyclin D proteins. Tissues in mice of all different possible genotypes ubiquitously expressed the single cyclin D available and developed well beyond preimplantation stages, suggesting that any of the D-type cyclins are sufficient to drive preimplantation development. Additional experiments are under way to characterize the expression patterns of cyclin D2 and D3 proteins in preimplantation mouse embryos.

Cyclin E and cdk 2 appeared to cycle in mouse embryos with a pattern similar to mammalian cells in culture, in that cyclin E complexes with cdk 2 during mid-G1 and remains active into early S phase (Rempel *et al.*, 1995). Cyclin E is thought to be rate-limiting during G1 phase of the cell cycle and regulates the initial stages of DNA replication (Jackson *et al.*, 1995). Results from the first and third cell cycles in mouse embryos further support this role for cyclin E. It was expressed at high to moderate levels during G1 and S but declined slightly during G2. During the second cell cycle, cyclin E levels remained at a consistent moderate level. It did not appear to cycle at all throughout the three phases of the second cell cycle, although there may be an indication of a slight decrease at G2. Cyclin E

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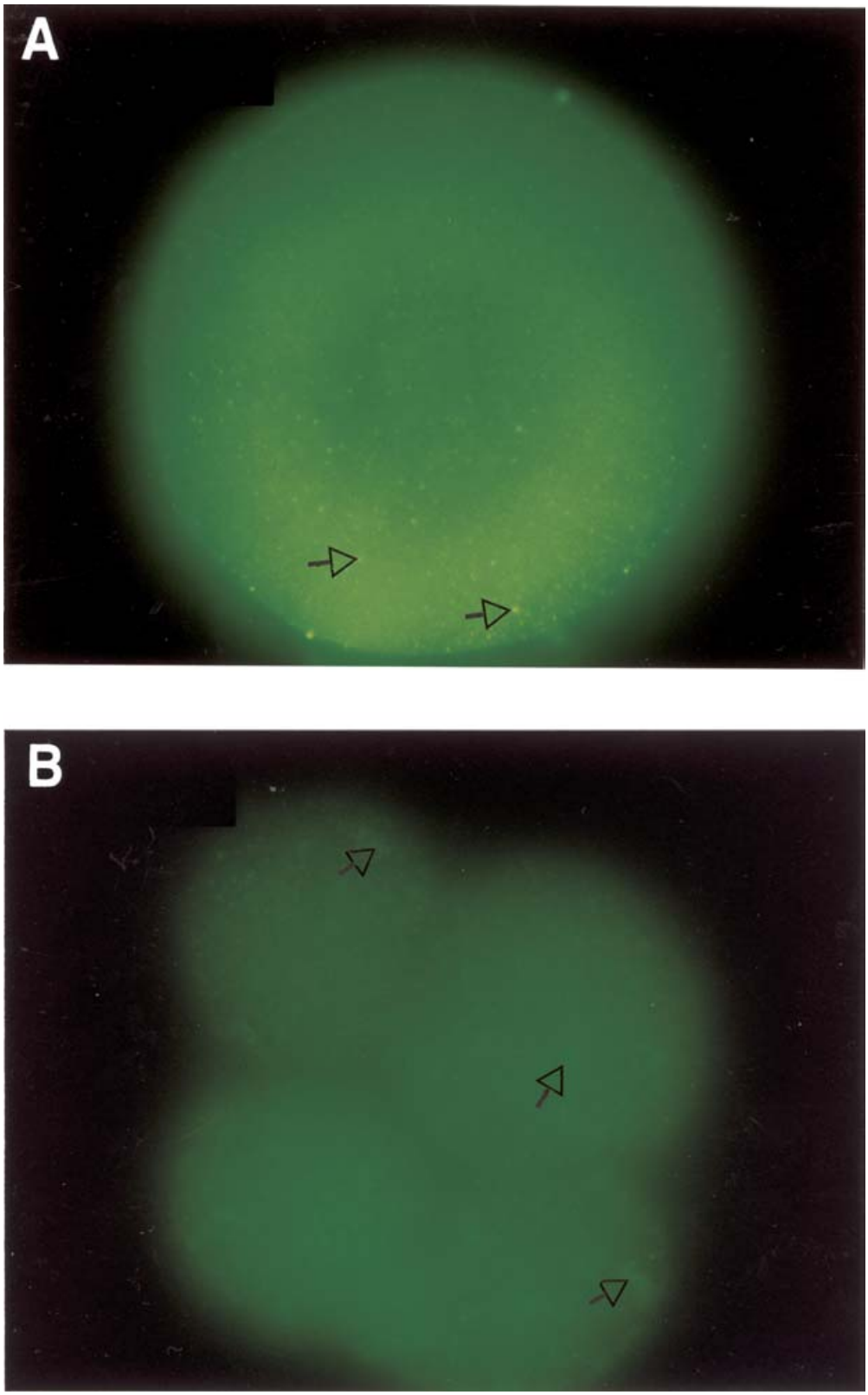


Figure 6 Preimplantation mouse embryos exhibiting unique staining patterns. One-cell and 4-cell mouse embryos were fixed, blocked and stained for cyclin E or cdk 2. (*A*) Represents a 1-cell embryo at G1 stained with anti-cyclin E. (*B*) Represents a 4-cell embryo during S phase stained with anti-cdk 2. Magnification for these photographs was ×1000 under oil immersion. Exposure times were consistent at 15 s. Arrows in (*A*) indicate punctate staining; arrows in (*B*) indicate stained cytoplasmic elements.

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in sea urchin and *Xenopus* does not cycle during the cleavage stages of development (Hartley *et al*., 1996; Sumerel *et al*., 2001). In *Xenopus*, a second peak in cyclin E/cdk 2 activity was detected just prior to M phase, suggesting a possible role for cyclin E in mitosis as well (Hartley *et al*., 1996), and the use of the cdk inhibitor p21 results in the build-up of inactive cyclin B/cdc2 and blocks progression into M phase (Guadagno & Newport, 1996). In *Drosophila* embryos, cyclin E maintains a role not only in G1 and S phase but also in mitosis (Knoblich *et al*., 1994) by stabilizing the M phase cyclins. The lack of a dramatic G2 decline in cyclin E in the mouse embryos may be indicative of a similar role.

In the second cell cycle, a steady signal may also be the result of a variety of changes in gene expression regulation occurring in mouse embryos at this time. During the second cell cycle, mouse embryos undergo embryonic gene activation (Wang & Latham, 1997). Therefore, a steady level of cyclin E might be present during the entire second cell cycle as protein is made from newly transcribed zygotic cyclin E mRNA. Once the switch to embryonic control is made, the levels appear to cycle again. Cyclin E mRNA levels remain constant through the entire first cell cycle and most of the second cell cycle, but increase 2- to 3-fold during G2 of the second cell cycle (Moore *et al*., 1996). This sharp mRNA increase during the second G2 could account for the increase in immunofluorescence observed in the current study during G1 of the third cell cycle.

Cyclin E was present in both the pronuclei and the cytoplasm during G1 and S of the first cell cycle. Moore *et al*. (1999) have suggested that cyclin E enters the nucleus by binding to the α subunit of the importin heterodimer through a basic nuclear localization sequence.

Cdk 2 protein appeared relatively stable throughout the cell cycle; however, immunofluorescence appeared to be slightly stronger in G1, declining somewhat during S and G2 in the preimplantation mouse embryo. Although cdks are considered to be more stable than cyclins, the polyclonal antibody used could be recognizing some epitopes exposed only when cdk 2 is complexed with a G1 cyclin. The observed decline may be indicative of a release of this complex.

Cyclin A protein levels were consistent during the first two cell cycles, demonstrating only a slight rise in S and G2. This correlates with the constant levels of cyclin A2 mRNA detected during the first 2 days of mouse development by Moore *et al*. (1996). In the third cell cycle, the pattern of cyclin A protein levels appeared typical of somatic cells, with low levels present during G1 rising in S and building to a peak in G2. It is possible that some of the cyclin A present during the first two cell cycles is from stored maternal protein in addition to being made from stored maternal mRNA. The dramatic decline in cyclin A levels at the beginning of the third cell cycle is consistent with the degradation of any stored protein and mRNA and the beginning of embryonic gene transcription in S/G2 of the third cell cycle (Winston *et al*., 2000). Cyclin A2 protein has been shown to accumulate as early as 6 h into the first cell cycle in the mouse while cyclin A1 was expressed primarily in eggs and early 1-cell embryos (Sweeney *et al*., 1996; Fuchimoto *et al*., 2001). This differential expression between eggs and embryos occurs although the mRNA for both A-type cyclins is present in the preimplantation mouse embryo and the mouse oocyte. The presence of both mRNAs could explain the ability of cyclin-A2-deficient mice to develop to early postimplantation stages (Murphy *et al*., 1997).

In somatic cells cyclin B1 has been described as a mitotic cyclin, accumulating late in the cell cycle (G2) and activating cyclin-dependent kinases (Cdc2) necessary for cell division (Nurse, 1994; Moore *et al*., 1999). After cell division, the cyclin B1–Cdc2 complex is inactivated by inhibitory phosphorylation of a Cdc2 subunit (Thr-14 and Tyr-15) as a result of the WEE1 kinase and cyclin B1 is degraded (reviewed in Morgan, 1995). The data presented here suggest that cyclin B1 may be expressed differently in early mouse embryos. With the antibody utilized, cyclin B1 was not expressed as a G2/M cyclin. Its pattern of expression was that of a G1 cyclin, peaking at G1 and declining during S and G2. The function of cyclin B1 in embryonic cells could be different from that in somatic cells. Alternatively, the antibody used in this study could be recognizing another B1-like cyclin that is expressed in G1 in the embryo. The data presented here conflict with data reported by Ohashi *et al*. (2001) who demonstrated by immunoblotting that cyclin B1 protein is present in S and G2 phases at the 2-cell stage of mouse embryo development. These two studies differ in the strain of mouse used and also in the antibody used. Either of these conditions could account for the observed differences. Studies are under way to compare the two antibodies used, to reconcile these differences.

Cyclin B1 remaining from oocyte maturation could be an explanation for the unusual cyclin B1 expression during G1 of the first cell cycle. Cyclin B1 has been shown to accumulate up to the metaphase II arrest during oocyte maturation and to remain active in the egg until fertilization (Taieb *et al*., 1997). It is possible that some of the mouse embryos fixed for this experiment at 3 h of development (G1) were still unfertilized or just entering G1. Hoechst 33258 staining of embryos isolated at comparable times showed approximately 70–80% fertilization as indicated by fluorescence of both the metaphase plate of the egg and the chromatin of the sperm cell (data not shown). However, this would not explain the consistent cyclin B1 peak also observed during G1 of the second and third cell cycles.

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It is apparent from the current results that there is a specific trend for cyclin B1 or cyclin-B-like signalling during the first three cell cycles. A cyclin B-like protein may interact with a different cdk molecule and play a role earlier in cell cycle regulation in preimplantation mouse embryos than in somatic cells. Moore *et al*. (2003) have demonstrated in *Xenopus* extracts that cyclin B1/cdc2 can promote S phase if provided with a nuclear localization signal and appropriate activation. Finally, it is possible that cyclin B1 is not necessary for early preimplantation development. Cyclin B1 might play a much different role early in mouse development as opposed to later in development.

Unique subcellular staining patterns were shown in some of the phases of the cell cycle. A punctate staining pattern was seen in the cytoplasm of 1-cell G1 embryos stained with antibodies against cyclins B1 and E, 1-cell S phase embryos stained with anti-cyclin E, and 4-cell S phase embryos stained with anti-cdk 2. Four-cell embryos exhibited a different punctate staining pattern compared with 1-cell embryos. During S phase of the third cell cycle, cdk 2 emitted a signal that appeared to localize around oval intracellular components that are much larger than the punctate pattern observed at the 1-cell stage. Studies with immunogold electron microscopy are planned to help elucidate some of these subcellular structures.

Cyclin expression in the preimplantation mouse embryo is in some cases consistent with patterns seen in somatic cells but in others reflects the unique properties of cleavage-stage embryos in terms of maternal storage of RNA and protein and of embryonic gene activation. These unique expression patterns for cyclins in early mouse development compared with typical somatic cell patterns are consistent with differences recently described by Sumerel *et al*. (2001) for the sea urchin, in which cyclin E and its associated cdk did not cycle during early embryogenesis. This suggests that embryos may not always follow typical somatic cell rules for cyclin and associated cdk activities.

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