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Early Development of Mouse Embryos Null Mutant for the Cyclin A2 Gene Occurs in the Absence of Maternally Derived Cyclin A2 Gene Products

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Progression through the mammalian cell cycle is regulated by the sequential activation and inactivation of the cyclin-dependent kinases. In adult cells, cyclin A2-dependent kinases are required for entry into S and M phases, completion of S phase, and centrosome duplication. However, mouse embryos lacking the cyclin A2 gene nonetheless complete preimplantation development, but die soon after implantation. In this report, we investigated whether a contribution of maternal cyclin A2 mRNA and protein to early embryonic cell cycles might explain these conflicting observations. Our data show that a maternal stock of cyclin A2 mRNA is present in the oocyte and persists after fertilization until the second mitotic cell cycle, when it is degraded to undetectable levels coincident with transcriptional activation of the zygotic genome. A portion of maternally derived cyclin A2 protein is stable during the first mitosis and persists in the cytoplasm, but is completely degraded at the second mitosis. The ability of cyclin A2-null mutants to develop normally from the four-cell to the postimplantation stage in the absence of detectable cyclin A2 gene product indicates therefore that cyclin A2 is dispensable for cellular progression during the preimplantation nongrowth period of mouse embryo development.

Key Words: **cell cycle; mouse oocyte; mitosis; transcription; protein degradation.**

INTRODUCTION

The developmental process in mammals and other animal species begins during oogenesis with the accumulation of both maternal RNA and protein as the oocyte grows and matures. In murine oocytes, mRNA accumulates linearly with respect to oocyte volume and thereafter remains constant (Paynton, 1988; Roller, 1989). The destruction of some maternal mRNA starts during oocyte maturation when the oocyte progresses from the germinal vesicle stage, arrested in the prophase of meiosis I, to an arrest in metaphase of meiosis II. A decrease of more than 50% in some mRNAs occurs (Bachvarova, 1985; Paynton, 1988), whereas the amount of other mRNAs remains unchanged (Moore, 1996). These more stable maternal mRNAs are presumably required for cell cycle progression and early development.

In many species, transcription is effectively silent during the period encompassing meiotic maturation and early development immediately following fertilization (Bolton, 1984; Wasserman, 1988). Thus, the earliest stages of em-

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bryogenesis are regulated by maternally inherited components stored within the oocyte. As development proceeds and maternally inherited components decay, the process of early embryogenesis becomes increasingly dependent on the expression of genetic information derived from the zygotic genome. In the mouse, embryo development can occur solely on maternal mRNA transcripts and proteins until the two-cell stage during the G2 phase of which the zygotic genome expression occurs on a large scale (Flach, 1982). At the same time a loss of about 70% of maternal polyadenylated mRNA (Clegg and Piko, 1983) and 40% of the total maternal RNA occurs. However, 30% of the total maternal RNA persists until the early blastocyst (Bachvarova and De Leon, 1980).

The cell "cleavage" divisions give rise to the blastocyst in the absence of growth. Cleavage divisions can be very rapid in invertebrates and amphibia and have provided experimental systems used to identify regulators of the cell cycle. The first to be identified were A- and B-type cyclins, which accumulate during interphase and are degraded at mitosis (Evans, 1983; Swenson, 1986). Two A-type cyclins have been characterized in vertebrates. In the mouse, cyclin A1 is expressed during meiosis in both male and female germ cells (Sweeney, 1996), but is essential only in male cells (Liu, 1998). In contrast, cyclin A2 is needed at two critical points in the mitotic cell cycle of somatic adult cells, at the G1- to S-phase transition and at the entry into mitosis (Girard, 1991; Pagano, 1992; Zindy, 1992). In mammalian somatic cells, cyclin A2 is degraded at each mitotic division and must be synthesized *de novo* during the following cycle. Cyclin A2 also plays a role during G2 to prevent rereplication of the genome. Mutations in *Drosophila* cyclin A lead to an arrest in G2 and endoreduplication of DNA in the absence of mitosis (Lehner and O'Farrell, 1990; Sauer, 1995).

Previously we have shown that murine embryos lacking the cyclin A2 gene develop to the late blastocyst stage, implant in the uterine wall, but fail to develop past 5.5 days postcoitum (pc) (Murphy, 1997). In this study, we have examined the stability of maternally inherited cyclin A2 mRNA to determine whether it might be essential to support these cell divisions in the murine embryo. We also assessed the levels and cellular distribution of the cyclin A2 protein. The results show that maternal stocks of both cyclin A2 mRNA and protein are degraded to undetectable levels during the two- to four-cell transition. Therefore, cyclin A2 is not required for the subsequent cell division cycles until 5.5 days pc. We also show that cyclin A2 is not needed for DNA replication in the early mouse embryo.

MATERIALS AND METHODS

Collection and Culture of Oocytes and Embryos

Wild-type F1 mice (C57B/6 × CBA) were obtained from INRA (Jouy-en-Josas, France). Mice heterozygous (+/−) or homozygous wild type (+/+) for the cyclin A2 gene (Balb/c × 129 Sv/J mixed genetic background) were genotyped by PCR using primers specific for the *lacZ* reporter gene, which replaces the cyclin A2 transcribed region in the mutant allele (Murphy, 1997). Mice harboring the cyclin A2 wild-type Balb/c allele were selected by restriction digest of tail DNA samples followed by Southern blotting and used for all experiments.

Immature oocytes displaying a germinal vesicle (GV) were obtained from ovaries removed from 5- to 6-week-old female mice and transferred to prewarmed (37°C) M2 medium supplemented with 4 mg/mL bovine serum albumin (M2+BSA; Whittingham, 1971), with or without 50 μg/mL dibutyryl cyclic AMP (dbcAMP; Sigma), which prevents germinal vesicle breakdown (GVBD).

Ovulated oocytes arrested in metaphase II (MII) were recovered from the fallopian tubes of female mice superovulated by intraperitoneal injections of 10 iu of pregnant mare's serum gonadotrophin (Intervet) and human chorionic gonadotrophin (Intervet) 48 h apart. To obtain embryos, females were paired overnight with males. Embryos at the one-cell to morula stages were either flushed from the oviducts at the stage to be examined or derived by culture *in vitro* of earlier stages in T6 medium supplemented with BSA under oil (BDH Chemicals, UK) in 5% CO_2 at 37°C.

Embryo bisection was carried out according to Tarkowski and Rossant (1976) modified by Kubiak (1993). After bisection, 300 nucleate or anucleate approximately equal-sized halves of one-cell embryos or 600 equal-sized halves of two-cell embryo blastomeres were washed in M2 medium containing 4 mg/mL polyvinylpyrrolidone (M2+PVP) and collected for immunoblotting.

Blastocysts 3.5 days pc were flushed from the uterine horns and either collected immediately for analysis or cultured *in vitro* on gelatin-coated glass coverslips in supplemented DMEM (Hogan, 1994; Hendrey, 1995).

Staining for β-Galactosidase Activity

Expression of the *lacZ* reporter gene was assessed in oocytes and embryos fixed in 0.1 M phosphate buffer (pH 7.3) containing 0.2% glutaraldehyde, 5 mM EGTA, and 2 mM $MgCl_2$ for 10 min at room temperature and then permeabilized by three rinses in phosphate buffer with 2 mM $MgCl_2$, 0.1% sodium deoxycholate, 0.2% NP-40. After extensive washing in phosphate buffer, β-galactosidase activity was detected using the chromogenic substrate X-gal (Vernet, 1993).

α-Amanitin, Cycloheximide, and Nocodazole Treatments

Fertilized eggs and embryos were cultured in the presence or absence of 11 μg/mL α-amanitin to inhibit specifically and irreversibly RNA polymerase II in mouse embryos (Levey and Brinster, 1978). At the end of the incubation period and careful rinsing in M2+PVP, individual oocytes and embryos were collected in a 2-μL volume of diethyl pyrocarbonate-treated double distilled sterile water and frozen immediately at −80°C for RT-PCR analysis.

One-cell or two-cell stage embryos, recovered in G2 phase of the cell cycle, were cultured in the presence or absence of 400 μM cycloheximide, known to inhibit more than 95% of protein synthesis (Howlett, 1986). At the end of the incubation period, groups of divided and nondivided embryos were collected separately, rinsed in M2+PVP, and lysed in sample buffer (Laëmmli, 1970) for immunoblot analysis.

One-cell or two-cell stage embryos, recovered in G2 phase of the cell cycle, were cultured in the presence of 10 μM nocodazole for a

maximum of 3 h until they had arrested in metaphase. Embryos were then either collected or cultured for a further period without the drug until the onset of cytokinesis.

mRNA Detection Using RT-PCR

Single oocytes and embryos were analyzed for the presence of cyclin A2, cdc25, or β-tubulin mRNAs using RT-PCR. Reverse transcription was carried out in a final volume of 20 μL of reaction mix containing 10 mM Tris–HCl (pH 8.0), 50 mM KCl, 5 mM $MgCl_2$, 62.5 μM dNTP (Gibco BRL), 100 ng random primers (Pdn6; Pharmacia Biotech), 200 U reverse transcriptase (MMLV; Gibco BRL), and 20 U RNase inhibitor. Samples were kept frozen until addition of the reaction mix and incubation at 37°C for 1 h. The total contents of each RT reaction tube were then added to PCR tubes prepared with 30 μL of standard reaction mix under paraffin oil (Sigma) and mixed by pipetting. Using 38 cycles of amplification and an annealing temperature of 58°C, cyclin A2 primers generate a diagnostic 352-bp fragment, β-tubulin primers a 550-bp fragment, and cdc25 primers a 463-bp fragment. The primers used were RTA2U, 5′-TCAGGAAGACCAAGAGAATG-3′, and RTA2L, 5′-GGATAGTCAAGAGGTG TCAG-3′, which are specific to the 5′ end of the murine cyclin A2 coding sequence spanning nucleotides 181 to 200 and 514 to 533 of the cDNA, respectively (Sweeney, 1996); 1830, 5′-CCTTCTTGTTCGGTACCTACA-3′, and 1829, 5′-GTATTCATGATACGGTCAGGA-3′, located at the 3′ end of the coding region of the murine β-tubulin gene spanning nucleotides 18 to 38 and 555 to 575, respectively; and cdc25.S, 5′-CAAGTTCAGCAACATCGTGG-3′, and cdc25.AS, 5′-CTGGTCTTGCAGCCTGCTACA-3′, located at the 5′ end of the coding sequence of the human cdc25 gene, spanning nucleotides 1236 to 1255 and 1678 to 1698, respectively (Yokoi, 1993). Positive and negative control samples were added to the analysis, during both the RT reaction (reaction mix with 1 μg of mouse intestine RNA for the positive and reaction mix alone for the negative control) and the PCR step (mouse intestine cDNA for the positive and water for the negative controls).

Amplified DNA produced by RT-PCR was run out on 2% agarose gels in 1× TBE and transferred to Hybond N+ membranes (Amersham, UK) in 0.4 N NaOH overnight at room temperature. Membranes were rinsed for 1 min in 2× SSC buffer and prehybridized in 10 mL QuikHyb solution (Stratagene) at 65°C for 30 min. Labeling of radioactive probes was performed using [α-^{32}P]dCTP (110 TBq/mmol; Amersham, UK) and the kit Megaprime (Amersham, UK) according to the manufacturer's recommendations. The DNA fragments used as probes were derived from RT-PCR-amplified products starting with RNA extracted from mouse intestine. Hybridization was carried out in QuikHyb with 1 million counts per milliliter of labeled probe at 65°C for 2 h. The membranes were washed to a stringency of 0.1× SSC, 0.1% SDS at 65°C. Autoradiography was carried out at −80°C with intensifying screens.

Antiserum Anti-cyclin A2

The antigen used comprised the first 148 residues of mouse cyclin A2 expressed in a recombinant form in *Escherichia coli.* The first injection was made in Freund's complete adjuvant into the popliteal lymph node of a rabbit. Subsequent injections were subcutaneous using Freund's incomplete adjuvant. The antiserum used was that giving the highest titer on Western blotting against murine thymus lysates and was used for the immunofluorescence analyses.

Detection of Cyclin A2 by Immunoblotting

Groups of oocytes and embryos were collected in sample buffer (Laëmmli, 1970) and heated to 100°C for 5 min. The proteins were separated by SDS–PAGE (10% gels) and electrically transferred to nitrocellulose membranes (Amersham Hybond-C or Schleicher and Schuell; pore size 0.45 μm). Following transfer and blocking for 2 h in 3% milk in TBS (10 mM Tris–HCl, pH 7.5, 140 mM NaCl), containing 0.1% Tween 20 (TBS/Tween), membranes were incubated overnight at 4°C with a polyclonal anti-cyclin A2 antibody diluted 1/2000 in blocking solution. The membranes were then incubated in the second-layer antibody (anti-rabbit immunoglobulin; Amersham) conjugated to horseradish peroxidase diluted 1/2000 in blocking solution for 2 h at room temperature. After three rinses in TBS/Tween, the cyclin A2 protein signal was revealed using the SuperSignal substrate Western blotting system (Pierce). All experiments were repeated at least three times.

Detection of Cyclin A2 by Immunofluorescence

Samples were processed for immunofluorescence staining as described (Sweeney, 1996; Murphy, 1997), with the following modifications that were essential to abolish background staining. Oocytes and embryos were collected in M2+BSA and the zonae were removed by brief treatment with acid Tyrode's solution (Nicholson, 1975). All samples were fixed immediately first in 4% paraformaldehyde in PBS containing 1 mg/mL polyvinyl alcohol for 30 min and then in the same solution with the addition of 0.3% Triton X-100 for a further 30 min. The solutions used for rinsing and dilution of the antibodies (anti-cyclin A2 antiserum and fluorescein-conjugated $F(ab)'_2$ fragments of anti-rabbit IgG; Boehringer) contained 0.1% Tween 20. Samples were also stained with propidium iodide (5 μg/mL for 10 min; Sigma) and mounted on glass slides in Vectashield mounting medium (Vector Laboratories) to prevent bleaching. All steps of the procedure were carried out at room temperature.

The cellular localization of the cyclin A2 protein was assessed by Confocal Laser Scanning Microscopy using a Leica confocal imaging system (TCS4D; Leica Lasertechnik GmbH Germany). Images were collected using an oil immersion lens (63×, NA I.4 plan Apochromat). For fluorescein and propidium iodide excitation, an argon krypton laser operating at 488 nm and 568 lines, respectively, was used. The pinhole of the confocal system was closed to allow a minimum field depth. Optical sections were collected at 2-μm intervals, corresponding to the increment between two adjacent sections in sequential mode (FITC first and propidium iodide second). Line averaging mode was used to integrate the signal collected over eight lines in order to reduce noise with a scan time of 2 s per frame and raster size of 512 × 512 pixels. Micrographs were processed with Adobe PhotoShop 6.0 and images were assembled and printed directly from the computer on a dye sublimation printer (Colorease Kodak).

BrdU Incorporation in Cultured Blastocysts and Early Postimplantation Stage Embryos

Blastocysts cultured for 3 or 4 days *in vitro* were labeled with 10 μM 5-bromo-2′-deoxyuridine (BrdU) for 50 min prior to fixation in 70% ethanol in 50 mM glycine–HCl buffer (pH 2.0) for 20 min at −20°C. BrdU-positive cells were detected using a monoclonal antibody against BrdU and an alkaline phosphatase-conjugated secondary antibody (BrdU Labeling and Detection Kit II; Boehringer

Mannheim), according to the manufacturer's instructions, and examined under a light microscope.

Pregnant mice (6.5 days pc) received a single intraperitoneal injection of 400 μL BrdU (10 mg/mL in PBS) 3 h before sacrifice. Intact deciduae were fixed overnight in 70% ethanol at 4°C and embedded in paraffin. Sagittal sections 5 μm thick were rehydrated and treated with 2 N HCl for 45 min, washed twice in PBS containing 0.1% Tween (PBT) for 10 min, and then incubated in proteinase K (20 μg/mL in PBS) for 15 min. After three washes with PBT, 10 min each, embryo sections were blocked in goat serum (1% in PBT; Sigma) for 1 h. BrdU incorporation was detected using an FITC-conjugated anti-BrdU monoclonal antibody (dilution 1/20 in PBT + 1% goat serum; Caltag) and incubated overnight at 4°C. Finally, after two washes in PBT, the sections were mounted in Vectashield and analyzed under a fluorescence microscope. All steps were carried out at room temperature unless stated otherwise.

RESULTS

The Cyclin A2 Gene Is Transcribed Actively in GV-Stage Oocytes and Two-Cell Stage Embryos Onward

We first wished to determine in detail the transcriptional activity of the cyclin A2 gene during early mouse embryo development. We used a strain of mice heterozygous for cyclin A2 (Murphy, 1997), in which the *lacZ* reporter gene is under the control of the cyclin A2 promoter in the cyclin A2-null allele (Table 1 and Fig. 1).

β-Galactosidase activity was detected in all germinal vesicle stage oocytes (Table 1, "GV + dbcAMP" and Fig. 1B), disappeared during germinal vesicle breakdown (Table 1, "GV − dbcAMP"), and was never observed in mature MII oocytes recovered from mice heterozygous for cyclin A2 (+/−) nor in wild-type oocytes at any stage of oocyte development (Table 1, Fig. 1A). We were also unable to detect β-galactosidase activity in one-cell or recently divided two-cell embryos derived from crosses, including at least one heterozygous parent (Murphy, 1997, and Table 1). The absence of activity also indicates that β-galactosidase is rapidly turned over in the early mouse embryo and is therefore a reliable reporter of the up- and downregulation of the adjacent promoter. β-Galactosidase staining reappeared in late two-cell embryos, in the proportions expected according to the parental genotypes (Table 1). Thus, cyclin A2 promoter is silenced in mouse oocytes from the resumption of meiotic maturation until activation of the zygotic genome at the late two-cell stage.

To establish the normal pattern of cyclin A2 gene transcription and distribution of the transcripts after implantation, the presence of β-galactosidase activity was assessed *in vivo* in embryos between days 3.5 and 9.5 pc obtained from different crosses between mice wild-type (+/+) and mice heterozygous (+/−) for cyclin A2 (Table 1 and Figs. 1E–1H) and in embryos 6.5 days pc developed *in vitro* (Table 1 and Fig. 1D). The data show that the cyclin A2 gene is actively transcribed in the blastocyst, as shown by the blue staining of all the nuclei in both trophectoderm and inner cell mass cells (Fig. 1C). Transcription continued in both cell types in blastocyst outgrowths cultured for 3 days (Fig. 1D). However, staining of *in vivo* embryos at 6.5 and 7.5 days pc (Figs. 1E and 1F, respectively) revealed that cyclin A2 gene transcription is restricted to the embryonic zone during gastrulation. Most of the embryonic cells were stained until the somites appeared, at which time β-galactosidase staining was particularly prominent in the neural folds (Fig. 1G). Later in development, the percentage of positively stained cells and the level of β-galactosidase activity were considerably reduced, indicating that the overall transcriptional activity of the cyclin A2 gene was repressed (9.5 days pc, Fig. 1H).

In summary, the cyclin A2 gene activity is repressed after implantation, initially during gastrulation in the extraembryonic zone, and later within the embryo.

TABLE 1

β-Galactosidase Activity in Mouse Oocytes and Embryos

Stage	♀ +/+ × ♂ +/−	♀ +/− × ♂ +/−
Oocytes GV + dbcAMP	0/198 (0%)	122/122 (100%)
Oocytes GV − dbcAMP	0/23 (0%)	8/12 (67%)
Oocytes MII	0/40 (0%)	0/14 (0%)
Late 1-cell embryos	0/39 (0%)	ND
Early 2-cell embryos	0/29 (0%)	ND
Late 2-cell embryos	25/45 (55%)*	16/20 (80%)*
Blastocysts	42/81 (52%)*	12/18 (67%)*
Implanted embryos 6.5 dpc	13/22 (59%)*	12/18 (67%)
Implanted embryos 7.5 dpc	3/11 (27%)[a]	2/4 (50%)[a]
Implanted embryos 8.5 dpc	16/30 (53%)*	11/14 (79%)
Implanted embryos 9.5 dpc	9/14 (64%)*	12/16 (75%)

Note. Wild-type (cyclin A2 +/+) or heterozygous (cyclin A2 +/−) females were crossed with heterozygous males. The table gives the percentage of oocytes and embryos stained by the chromogenic substrate X-gal at various stages of development. GV, germinal-vesicle-stage oocytes recovered in the presence or absence of dibutyryl cAMP (+ and − dbcAMP, respectively). MII, mature oocytes arrested in metaphase of meiosis II. Preimplantation stage embryos, prior to (1- and early 2-cell) and after (late 2-cell and blastocysts) the major transcriptional activation of the zygotic genome. Postimplantation-stage embryos developed *in vivo* from 6.5 to 9.5 days pc. Two or more litters were analyzed in each group, unless otherwise stated. ND, not determined.

[a] Only one litter analyzed.

* Not significantly different from the expected proportions of 50% (♀ +/+) and 75% (♀ +/−) X-gal-stained embryos using a χ^2 analysis ($P < 0.05$).

Detection of the Maternal Cyclin A2 mRNA Stock in Single Oocytes and Embryos

To explore how cyclin A2-null mutants complete preimplantation development, we examined the presence and stability of the maternal stock of cyclin A2 mRNA in

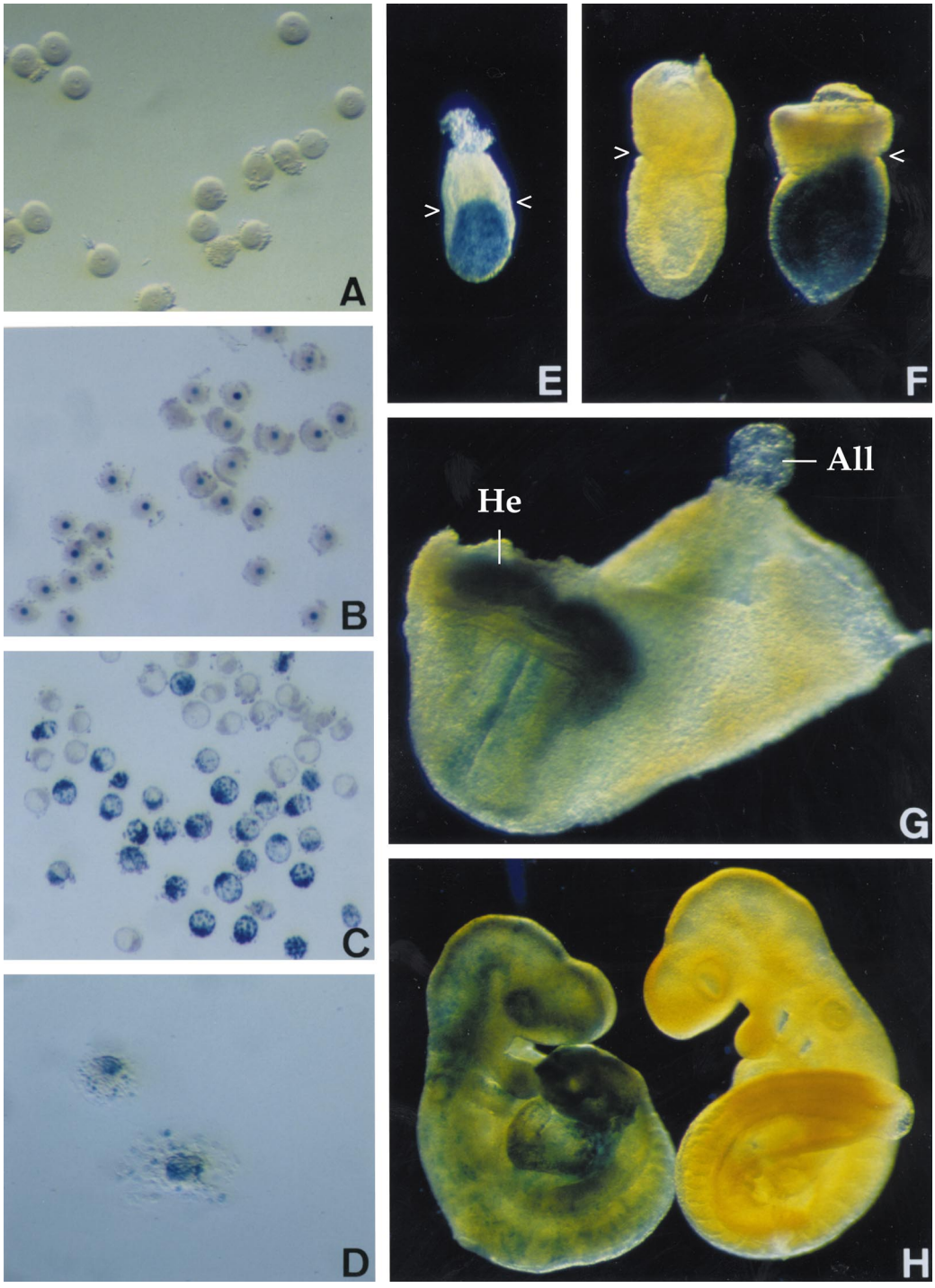

FIG. 1. Pattern of cyclin A2 gene transcription during early mouse development. Oocytes and embryos from mice heterozygous for cyclin A2+/− were stained for β-galactosidase activity (blue precipitate). Postimplantation embryos were staged according to Downs and Davies (1993) and Theiler (1989). (A) Absence of background staining in control GV oocytes from cyclin A2+/+ females. (B) GV oocytes recovered in the presence of dbcAMP to inhibit meiotic maturation. (C) Blastocysts from a heterozygote cross showing 75% of embryos heterozygous or homozygous null mutant for cyclin A2, with all nuclei stained positively for β-galactosidase activity, and 25% of wild-type embryos not stained. (D) Blastocyst outgrowths with stained nuclei in both the giant trophoblastic cells and the embryonic cell mass. (E) Early primitive streak stage (6.5 days pc) embryo stained in the embryonic zone (under the arrowheads). (F) Neural plate stage embryo with no allantoic bud (7.5 days pc) stained in the embryonic zone (under the arrowheads). Absence of staining in a wild-type littermate is shown on the left. (G) Late headfold stage embryo (8.5 days pc) stained in the headfolds (He) but not the allantois (All). (H) 13- to 20-somite stage embryo (9.5 days pc), showing a punctuate staining throughout the embryo. Unstained wild-type littermate is shown on the right. Original magnification ×50 (A–F), ×20 (G), ×16 (H).

TABLE 2
Degradation of the Maternal Stock of Cyclin A2 mRNA in the Mouse Embryo at the Two-Cell Stage

Genotype of the parents		α-Amanitin treatment	Stage of cell cycle	Number of embryos analyzed	Number of positive embryos	% positive embryos	
♀	♂					Observed	Expected
+/+;+/−	+/−	No culture	Early 2-cell (G1)	130	124	95	100
+/+	+/−	−	4-cell (S/G2)	53	47	89	100
+/+	+/−	+	2-cell (G2)	75	18	24	0
+/−	+/−	−	4-cell (S/G2)	112	74	66	75
+/−	+/−	+	2-cell (G2)	102	29	28	0

Note. Cyclin A2 mRNA was detected in individual embryos by RT-PCR immediately after collection (early 2-cell) or after 24 h of culture in the absence (−) or presence (+) of α-amanitin. The number and percentage of cyclin A2 mRNA-positive embryos are indicated. The right-hand column gives the percentage of embryos expected to give a positive signal according to the genotype of the parents, assuming that the maternal stock of cyclin A2 mRNA is completely degraded at the late 2-cell stage.

individual oocytes and embryos derived from heterozygous parents. RT-PCR followed by Southern blotting of the DNA products was used to analyze single mouse oocytes and embryos. Since the cyclin A2 gene is actively transcribed during oocyte growth, all oocytes should inherit maternally derived cyclin A2 mRNA. Maternal cyclin A2 mRNA was clearly detected in all (14/14) MII oocytes and in 95% (124/130) of early two-cell embryos examined immediately after recovery (Table 2, line 1). Thus, the technique is reliable and reveals that maternal cyclin A2 mRNA does persist after fertilization.

Most maternally inherited mRNA is degraded following activation of the zygotic genome. If this were the case for the cyclin A2 maternal mRNA, embryos homozygous null for the cyclin A2 gene will give a negative result following RT-PCR analysis. The presence of cyclin A2 mRNA and β-tubulin mRNA as an internal control was assessed simultaneously in 49 individual blastocysts from crosses of mice heterozygous for cyclin A2, of which 25% are expected to be homozygous null mutants. Indeed 24.5% of embryos positive for β-tubulin gave no signal for cyclin A2 and presumably therefore will represent cyclin A2-null mutants. All control wild-type blastocysts ($n = 21$) gave a positive signal for both cyclin A2 and β-tubulin. These data thus show that by the blastocyst stage, maternal cyclin A2 mRNA was no longer detectable in null-mutant embryos, implying that it has been totally degraded by this stage.

Degradation of Maternal Cyclin A2 mRNA Occurs Upon Activation of the Zygotic Genome and Does Not Depend on Cell Division

The major activation of the zygotic genome at the two-cell stage is accompanied by a progressive decline in most but not all maternal mRNAs (Flach, 1982; Bolton, 1984). If cyclin A2 maternal mRNA is degraded at the late two-cell stage, embryos analyzed for the presence of cyclin A2 mRNA at the four-cell stage will give a positive signal only if they inherit at least one wild-type allele. This was indeed the case, as 89% of embryos with one wild-type parent gave a positive signal for cyclin A2 mRNA after 24 h of culture to the four-cell stage (expected proportion 100%; Table 2, line 2) and this fell to 66% when both parents were heterozygous for cyclin A2 (expected proportion 75%; Table 2, line 4).

To determine the fate of cyclin A2 maternal transcripts, groups of early two-cell embryos (G1 phase of the cell cycle) derived from both heterozygous and wild-type crosses were cultured for 24 h in the presence or absence of α-amanitin to inhibit transcription of the cyclin A2 zygotic gene (Table 2). Individual embryos were then analyzed by RT-PCR at the time of the two- to four-cell transition. After 24 h of culture in the presence of α-amanitin, embryos become blocked at the late two-cell stage (G2 phase of the cell cycle) and show reduced to undetectable levels of cyclin A2 mRNA (Fig. 2A). This result indicates that cyclin A2 mRNA degradation occurs at this stage and does not depend on cell division. In the α-amanitin-treated groups, the proportion of embryos positive for the cyclin A2 mRNA was significantly reduced (Table 2, lines 3 and 5). The discordance between the observed value (24 and 28% of cyclin A2-positive embryos) and the expected proportion (0%) can be explained by the asynchrony of development between individual embryos recovered from different female mice after fertilization *in vivo*. Developmentally more advanced embryos may hence already have a small stock of zygotic transcripts prior to their exposure to α-amanitin.

Finally, to confirm that α-amanitin treatment did not affect the stability of maternal cyclin A2 mRNA, we analyzed embryos recovered at the pronuclear stage and cultured to the early two-cell stage with or without α-amanitin. Individual embryos were assessed simultaneously for the presence of cdc25 mRNA, the levels of which show no significant reduction in the two-cell em-

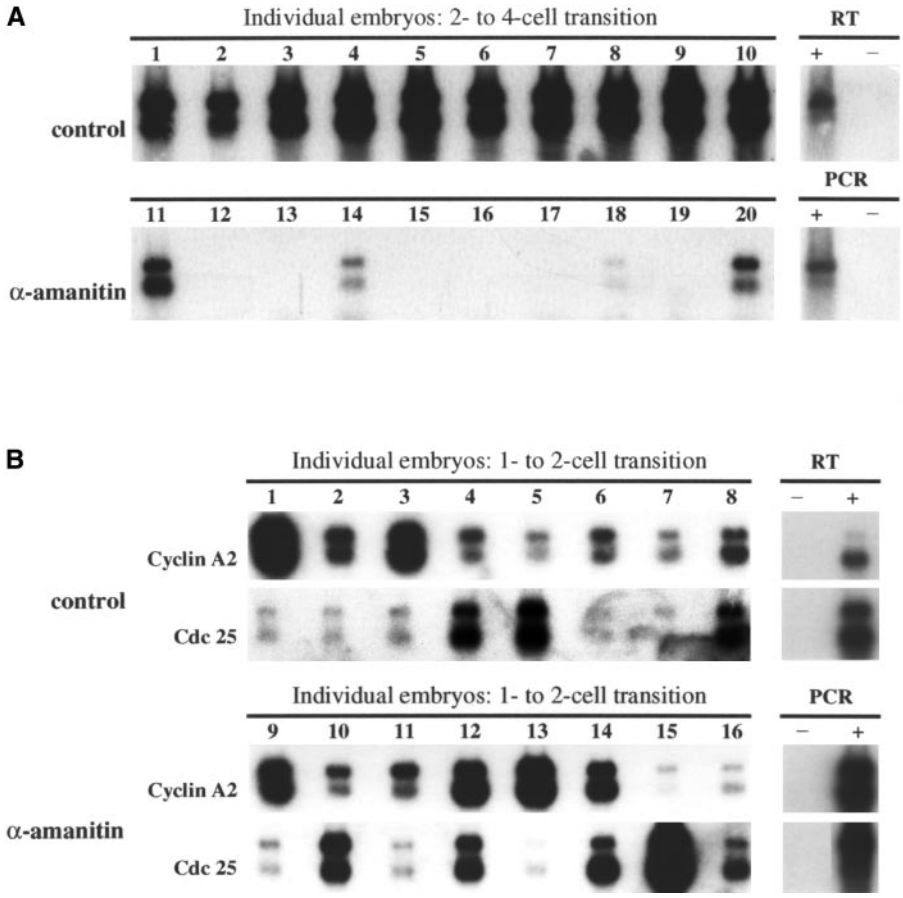

FIG. 2. Degradation of the maternal cyclin A2 mRNA at the 2- to 4-cell transition. Fertilized embryos in G1 phase of the cell cycle at the 2-cell (A) or 1-cell (B) stage were cultured in the presence (α-amanitin) or absence (control) of α-amanitin. Individual oocytes and embryos were collected and analyzed for the presence of cyclin A2 (A) or cyclin A2 and cdc25 (B) mRNAs using RT-PCR. Positive (+) and negative (−) control samples were added to the analysis during both the RT reaction and the PCR step. The amplification products were visualized after hybridization using probes specific for the cyclin A2 and the cdc25 cDNAs.

bryos (Yokoi, 1993) as an internal control. While a great variability in the signal intensity exists between individual embryos (Fig. 2B), 95% of α-amanitin-treated embryos and 92% of control embryos gave a positive signal for cyclin A2 mRNA. Of these embryos, 100% (23/23) and 91% (21/23), respectively, were also positive for cdc25 mRNA.

Together these data demonstrate for the first time in the mouse that maternally derived stocks of cyclin A2 mRNA persist in the fertilized oocyte through the first mitosis and are then degraded at the late two-cell stage, concurrent with the activation of the zygotic genome.

The Cyclin A2 Protein Is Not Completely Degraded during the First Mitotic Division

We next addressed the question of cyclin A2 protein stability at different stages of the meiotic and mitotic cell division cycles. Immunoblotting analysis revealed that cyclin A2 protein was already present in the oocyte. The protein was resolved in two forms, whose relative abundances differ between the stages analyzed (Fig. 3A). The lower form is predominant in GV oocytes and persists after GVBD, while in MII oocytes the proportion of the upper form is increased. After fertilization, steady-state cyclin A2 levels are significantly increased (six-fold) concurrent with the onset of S-phase (Fig. 3A, pronuclear stage PN), indicating that cyclin A2 can be synthesized actively from maternal mRNA. The cyclin A2 protein becomes principally restricted to the faster migrating lower band from this stage, although the upper band did not disappear completely until at least the two-cell stage (Fig. 3B, compare lanes 2C and 4C).

The stability of the cyclin A2 protein during the first and second mitosis was determined by culturing embryos collected at the late one-cell or two-cell stage (G2 phase of the cell cycle) in the presence or absence of the protein synthe-

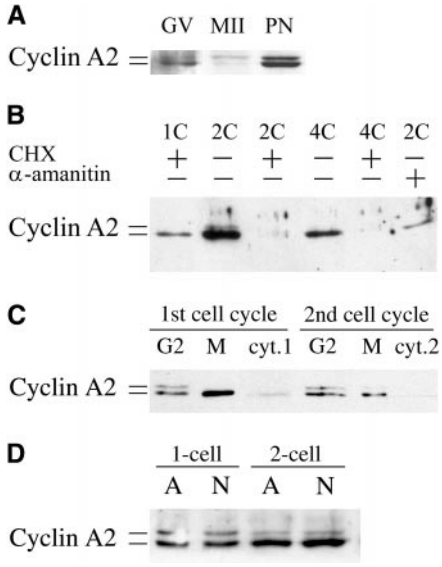

FIG. 3. Cyclin A2 protein levels during meiotic maturation and the first two embryonic cell cycles. Oocytes at the appropriate stage of maturation and embryos at different developmental stages were analyzed by immunoblotting with a polyclonal anti-cyclin A2 antibody. (A) Germinal vesicle (GV) and metaphase II (MII) oocytes. PN, fertilized oocytes at the pronuclear stage, 300 oocytes per sample. (B) Cycloheximide-blocked 1-cell stage embryos (1C + CHX) or divided CHX-treated 2-cells (2C + CHX) and control 2-cell embryos divided in the absence of CHX (2C), 50 embryos per sample. Four-cell embryos after division in the absence (4C) or presence (4C + CHX) of CHX and 2-cell α-amanitin-treated embryos blocked in the G2 phase of the cell cycle for 36 h (2C + α-amanitin), 34 embryos per sample. These experiments were repeated three times. (C) Embryos in G2 phase (G2), arrested in metaphase upon nocodazole treatment (M), or undergoing cytokinesis (cyt.1, cyt.2) of the first (1-cell stage) or second (2-cell stage) embryonic cell cycle, as indicated, 300 embryos per sample. (D) Distribution of cyclin A2 in bisected mouse embryos. A, anucleate and N, nucleate halves of 1- or 2-cell stage embryos, as indicated, 300 embryo halves (1-cell stage) and 600 embryo halves (2-cell stage) per sample.

sis inhibitor cycloheximide (CHX). Mouse embryos nearing mitosis have already synthesized all the proteins required to complete cell division and hence continued to progress through mitosis, even in the presence of CHX (Howlett, 1986). They are, however, unable to synthesize proteins *de novo* after dividing. In contrast, addition of CHX more than 2 h before nuclear envelope breakdown prevents mitosis. In CHX-treated embryos blocked at the late one-cell stage, cyclin A2 persisted but the upper band disappeared (Fig. 3B, lane 1C). Likewise, only the lower band persisted in embryos blocked upon treatment with α-amanitin for 36 h from the early two-cell stage (Fig. 3B, lane 2C + α-amanitin). Conversely, in embryos which had divided from one- to two-cells in the presence of CHX, cyclin A2 was degraded but a small amount of the protein remained detectable on immunoblots after a longer exposure (Fig. 3B, lane 2C + CHX). This suggests that degradation depends on cell division although it is not completed at first mitosis.

After division from two to four cells, the lower form of cyclin A2 was clearly detected in four-cell embryos in S/G2 phase cultured without CHX (Fig. 3B, lane 4C). This signal results from neosynthesis, as four-cell embryos that had divided in the presence of CHX showed no detectable cyclin A2 (Fig. 3B, lane 4C + CHX).

To confirm the fate of cyclin A2 protein during the first and second mitosis, we also analyzed synchronized embryos that were blocked in metaphase in the presence of nocodazole and then released without the drug until cytokinesis (Fig. 3C). Cyclin A2 was present in G2 phase embryos at both the one-cell and the two-cell stages. Upon treatment with nocodazole, immunoblotting demonstrated that cyclin A2 was redistributed between the upper and the lower form at metaphase (Fig. 3C, M). It remained detectable during the first cytokinesis (cyt.1), in contrast to the second cytokinesis (cyt.2), when it was degraded to undetectable levels.

These results demonstrate that degradation of the maternal cyclin A2 is not complete during the first mitosis in mouse embryos. However, complete degradation occurs during the second mitosis.

Cyclin A2 Undergoes Intracellular Relocalization

To further characterize the expression pattern of cyclin A2, we examined its intracellular localization by immunofluorescence. Cyclin A2 was detected in GV oocytes particularly at the nuclear membrane (Fig. 4A) and appeared essentially cytoplasmic after GVBD (Fig. 4B). In MII stage oocytes, cyclin A2 was also concentrated at two distinct intensely stained groups of points located at an equal distance either side of the metaphase chromatin (Fig. 4C, indicated by an arrowhead). These labeled structures strongly resemble the pericentriolar material situated at the periphery of the spindle. Other cyclin A2-labeled points, possibly microtubule-organizing centers (Maro, 1985), were also observed in the cytoplasm (Fig. 4C, indicated by arrows).

After fertilization, cyclin A2 accumulates in the pronuclei (Fig. 4D). This nuclear labeling was also observed at later stages in S and G2 phases (Figs. 4H and 4K), but not in G1 (Figs. 4G, 4I, and 4J). Although cyclin A2 is detected using immunofluorescence essentially in the nucleus during the first two cell cycles, a large pool of the cyclin A2 protein persists in the cytoplasm. This was confirmed by Western blotting (Fig. 3D), using embryos at the late one-cell or two-cell stage (G2 phase) that had been cut in two halves, only one of which contained the nucleus. Cyclin A2 was detected at similar levels as a doublet in both nucleated and enucleated halves, indicating that there is at least as much cyclin A2 in the cytoplasm as in the nucleus. Interestingly, the pool of cyclin A2 that is resistant to degradation during the first mitotic cleavage persists around the

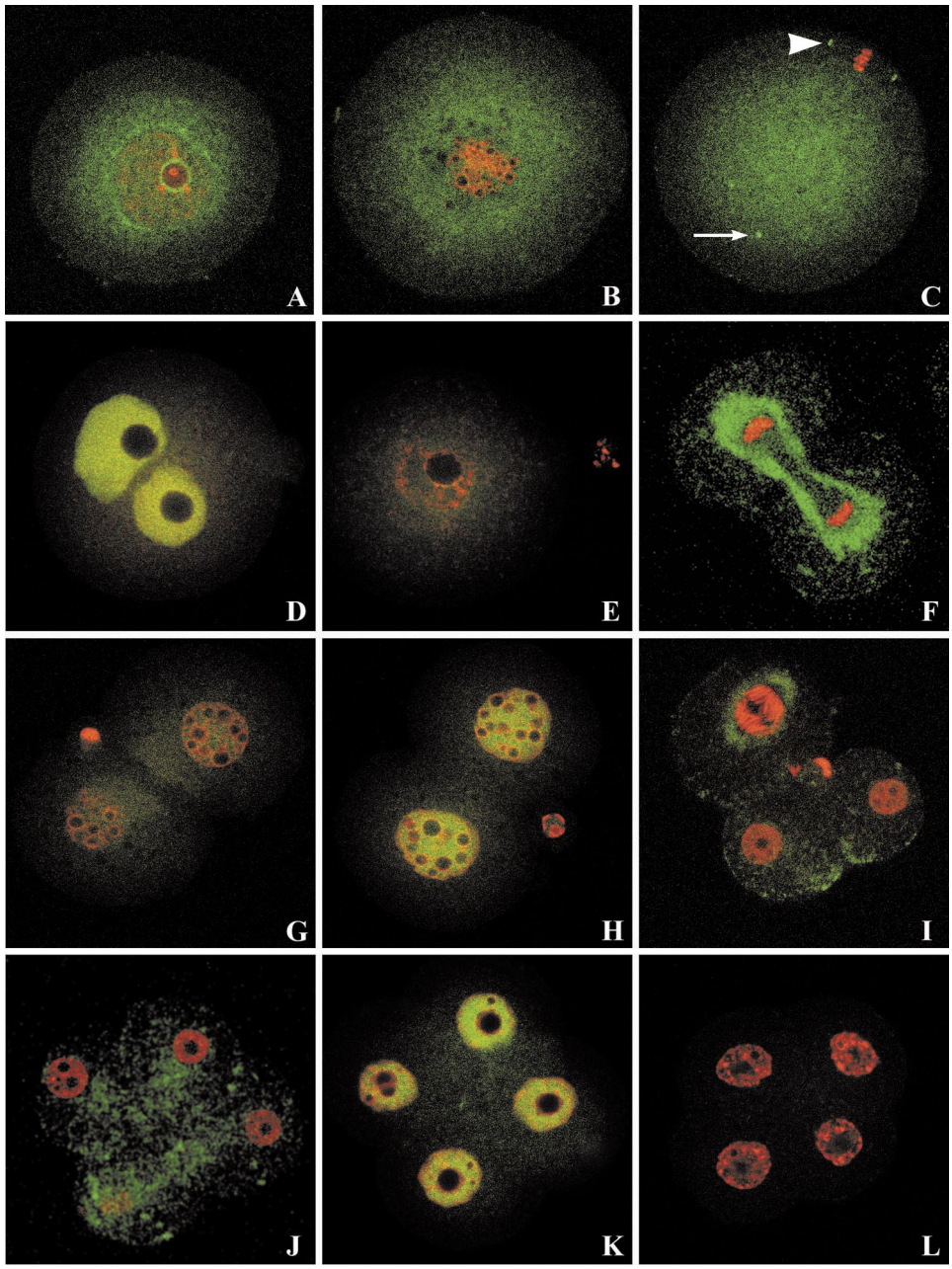

FIG. 4. Cyclin A2 distribution during meiotic maturation and the first two embryonic cell cycles. Maturing oocytes and fertilized embryos were fixed, immunolabeled with a rabbit antiserum specific for cyclin A2 (fluorescein, green), and stained with propidium iodide to show the chromatin (red). Oocytes at GV stage (A), at the onset of GVBD (B), and in metaphase II (C). The centrosome region (arrowheads) and microtubule organizing centers (arrow) are labeled. Immunolabeled pronuclei in a fertilized oocyte (D) and no labeling with antigen competition (E). Anaphase of the 1- to 2-cell stage transition (F). Two-cell stage embryo in G1, 30 min after mitosis (G) and in S phase, 6 h after mitosis (H). Dividing 3-cell (I) and recently divided 4-cell (J) stage embryos; note the absence of nuclear labeling in recently divided blastomeres. Four-cell stage embryos in S/G2 (K) or after division in the presence of CHX (L).

spindle and is excluded from the chromosomes at anaphase (Fig. 4F). During the second mitotic cleavage, a transient cytoplasmic labeling was also observed at anaphase (Fig.4I), corresponding to the protein that will be completely degraded before the completion of cytokinesis (Fig. 3C, lane cyt.2). Accordingly, no detectable staining was found in

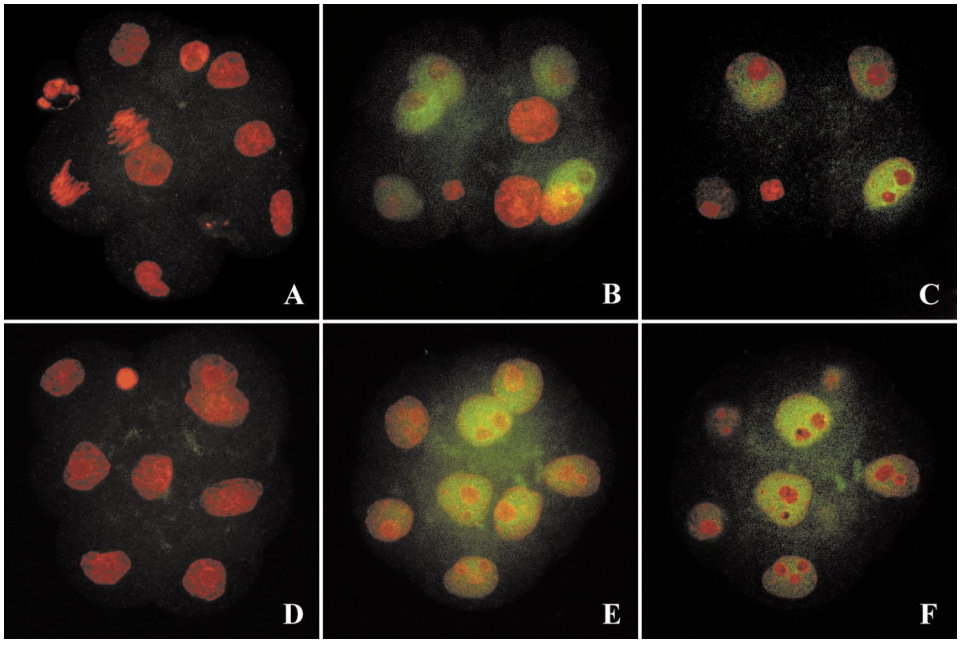

FIG. 5. Cyclin A2 labeling in 8-cell stage embryos from heterozygote crosses. Eight-cell stage embryos were collected from heterozygote crosses, immunolabeled with a rabbit antiserum specific for cyclin A2 (fluorescein), and stained with propidium iodide (red). Confocal microscopy sections were projected to reconstitute the entire embryos (A, B, D, E). Cyclin A2-negative (A, D) and positive (B, E) embryos are shown. The cyclin A2 immunolabel was restricted to interphase nuclei (C and F, single confocal microscopy sections from the embryos shown in B and E, respectively). Dividing and recently divided cells with condensed or decondensing chromatin and no nuclear envelope are not labeled (B, red nuclei).

four-cell embryos that have divided in the presence of CHX (Fig. 4L).

In summary, the cyclin A2 protein is located in both the nucleus and the cytoplasm at varying levels resulting from its relocalization at specific stages of the meiotic and the mitotic cell cycle.

Cyclin A2-Null Embryos Develop beyond the Four-Cell Stage in the Absence of Detectable Maternal Cyclin A2

The data above show that little or no maternal cyclin A2 persists after the second mitotic cell division, indicating that cyclin A2-null mutant embryos reach postimplantation stages in the absence of cyclin A2. This hypothesis was confirmed by immunolabeling experiments in eight-cell stage embryos. To this end, we modified our initial methods of fixation and staining by adding a permeabilization step to avoid the trapping of unbound antibody in the cytoplasm. This technical approach was the only one sufficiently sensitive to work on individual cells. However, cytoplasmic staining would be extracted under these conditions. We thus used the presence or absence of nuclear staining as an indicator of the state of the whole cell. We also used confocal analysis to determine precisely the location of the immunofluorescence signal. The reliability of the technique was assessed using embryos from wild-type females crossed with heterozygote males. All embryos (25/25 eight-cell stage and 25/25 blastocysts) were cyclin A2 positive (not shown), with a strictly nuclear labeling in at least one interphasic blastomere and no detectable labeling in mitotic cells (similar to Figs. 5B and 5C). Our initial observations (Murphy, 1997) suggesting the persistence of cyclin A2 protein in cyclin A2-null mutant blastocysts were not reproduced under these improved experimental conditions (Murphy, 1999). Twelve percent (5/37) of eight-cell embryos (Figs. 5D–5F) and 16% (6/31) of morulae and blastocysts (not shown) from heterozygote crosses showed no cyclin A2-specific staining, even when the nuclear material was in interphase, and will hence represent cyclin A2-null mutants. They were, however, morphologically normal and able to perform mitosis at all stages analyzed (Figs. 5A and 5D).

Together, these data show that mouse embryos lacking detectable nuclear levels of the maternal cyclin A2 protein are nonetheless able to divide and develop normally to the blastocyst stage. It is thus reasonable to infer from these data along with those presented above that development of null mutants also occurs in the complete absence of cyclin A2.

Cyclin A2-Null Mutant Embryos Are Able to Implant and to Perform DNA Replication

We showed previously that cyclin A2-null mutant embryos stop development immediately after implantation, with a size and morphology similar to those of the implanted blastocyst (Murphy, 1997). To better understand the events leading to this arrest, we examined in parallel postimplantation stage embryos from heterozygote crosses developing either *in vivo* or *in vitro* for (i) their ability to implant and (ii) their capacity to replicate DNA by the incorporation of BrdU.

The ability of mutant embryos to implant was monitored *in vitro* using living blastocysts cultured for 3–4 days. Blastocysts first attach to the culture dish and then hatch, developing both an inner embryonic cell mass and migratory trophoblast giant cells, easily identifiable by their large nuclei which result from genome endoreduplication. No difference was seen in the number of blastocysts from heterozygous or wild-type females crossed with heterozygous males, which were able to attach and form outgrowths (mean of six experiments, more than 300 blastocysts analyzed). In addition, the presence of viable, morphologically normal, homozygous mutant outgrowths was confirmed by PCR genotyping (data not shown). The ability of mutant embryos to implant was also investigated *in vivo* using the *lacZ* gene as a genetic marker for the cyclin A2-null allele. β-Galactosidase staining of embryos from different parental crosses reveals an approximately appropriate proportion of positively stained mutant embryos, although a technical bias exists owing to the small size of cyclin A2-null mutant embryos and resorption of deciduae (Table 1).

The question remains as to when cellular arrest occurs. The capacity of implanted embryos to perform DNA replication was studied both *in vitro* and *in vivo*. Blastocysts were cultured for 4 days and BrdU was added 50 min prior to fixing and immunolabeling. S-phase BrdU-positive nuclei were observed in both inner cell mass and trophoblastic cells in 100% of blastocyst outgrowths independent of their genotype (obtained from both heterozygous and wild-type females crossed with heterozygous males) (Table 3 and Fig. 6A). A similar observation was made *in vivo*. Pregnant mice (6.5 days pc) were injected with BrdU and the embryos were removed 3 h later. We detected the incorporation of BrdU in 45% of nuclei in morphologically mutant embryos, although they were highly disorganized (Fig. 6B). By comparison, wild-type embryos contained around 60% of BrdU-positive nuclei (Fig. 6C). Therefore, the cyclin A2-null mutant embryos that implanted successfully were able to replicate their DNA without any requirement for cyclin A2.

TABLE 3

BrdU Incorporation in Blastocyst Outgrowths

Length of the culture period	♀ +/+	♀ +/−
3 days	*n* = 210 (100%)	*n* = 197 (99.5%)
4 days	*n* = 59 (100%)	*n* = 30 (100%)

Note. Blastocysts from cyclin A2 +/+ wild-type or cyclin A2 +/− heterozygous females crossed with a heterozygous male were cultured *in vitro* for 3 or 4 days and then labeled with BrdU. The number (*n*) of embryos analyzed and the percentage of BrdU-positive embryos are indicated.

DISCUSSION

In this report, we have investigated the fate of maternally derived cyclin A2 in the mouse early embryo. Maternal cyclin A2 mRNAs are laid down in the growing oocyte and persist until the two-cell stage, at which they are degraded independent of cell division. The maternal cyclin A2 protein is synthesized after fertilization at both the one- and the two-cell stages and is then degraded at each mitosis. However, the degradation process is not fully operational during the first mitosis, as a fraction of cyclin A2 protein persists in the cytoplasm of dividing embryos. A clear and decisive changeover from the use of maternal to embryonic cyclin A2 mRNA occurs at the late two-cell stage, when the cyclin A2 zygotic gene is switched on in parallel with the major activation of the zygotic genome and the degradation of the maternal stock. These results imply that cyclin A2-null mutant embryos develop from the four-cell stage to the implanted blastocyst in the absence of detectable maternal cyclin A2. The early embryonic cell division cycles thus appear to differ from the somatic cycles in their requirements for cyclin A2.

Stability of the Maternal Cyclin A2 mRNA

We show that the maternal cyclin A2 transcripts are degraded concurrent with the activation of the zygotic genome and the cyclin A2 gene itself. This is in good agreement with previous studies showing that cyclin A2 is expressed as both maternal and zygotic transcripts in the two-cell stage embryo (Moore, 1996; Domashenko, 1997). Thus, while a positive signal for maternal cyclin A2 mRNA was obtained in virtually all early two-cell embryos, it was no longer detectable in the majority of embryos when transcription was inhibited in the presence of α-amanitin. In addition, the cyclin A2 mRNA degradation pathway functions in embryos treated with α-amanitin, even though they are blocked at the late two-cell stage, suggesting that neither cell division nor gene transcription is essential for its activation. A similar observation has been made in the *Xenopus* embryo (Howe, 1995). This is in sharp contrast with the situation in adult somatic cells, in which cyclin A2 mRNAs are stable in S and G2 phases and then are degraded at mitosis and during G1 (Maity, 1995).

α-Amanitin is known to inhibit selectively mRNA synthesis (Levey and Brinster, 1978), and the synthesis of most polypeptides is unaffected quantitatively by up to 24 h of exposure (Braude, 1979). However, we performed experi-

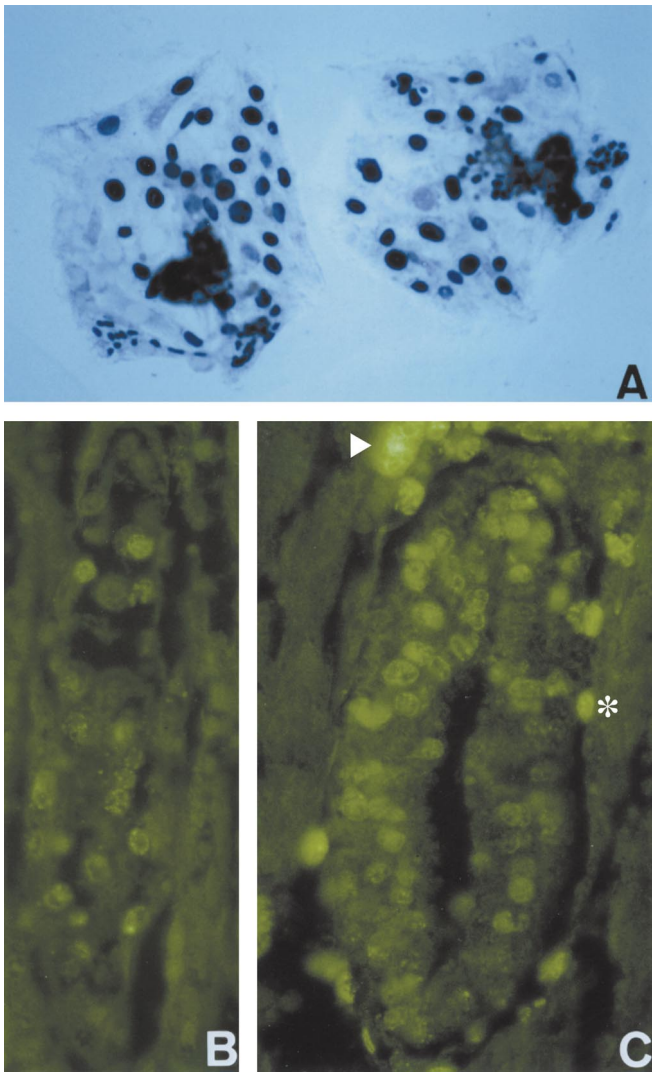

FIG. 6. BrdU incorporation in embryos from heterozygote crosses after development *in vitro* or *in vivo*. (A) Embryos were recovered at the blastocyst stage and allowed to develop *in vitro* for a further 4 days. BrdU labeling (brown precipitate) was detected in most of the giant cells in the trophectoderm, in cells located at the surface of the mass of embryonic cells, and in small cells presumably corresponding to visceral endoderm. (B and C) Sagittal sections of *in vivo* implanted embryos 6.5 days pc. Both morphologically mutant (B) and normal (C) embryos were labeled for BrdU (FITC, green). Original magnification ×667. Note the absence of labeling in the maternal tissue immediately surrounding the embryo, except the ectoplacental cone (> in C). The trophectoderm is also labeled (* in C). No ectoplacental cone was seen adjacent to the mutant embryo (B).

ments to control for possible side effects of α-amanitin on the stability of the maternal cyclin A2 mRNA. No difference was observed between control and α-amanitin-treated one-cell and early two-cell embryos, indicating that the cyclin A2 mRNA degradation pathway does not operate during this period. From these data, one would therefore predict that cyclin A2 homozygous-null embryos, which lack a functional cyclin A2 embryonic gene, will give no signal using RT-PCR analysis from the four-cell stage, after the maternal stocks of cyclin A2 mRNA have been degraded. This was indeed the case. About one-quarter of four-cell embryos and blastocysts from heterozygote crosses gave no signal for cyclin A2 (Table 1) and will hence represent, at least for the most part, cyclin A2-null mutants.

Stability and Localization of the Cyclin A2 Protein

Low levels of the maternal cyclin A2 protein can be detected in immature GV mouse oocytes and throughout meiosis. Fertilization triggers a sixfold increase in the levels of cyclin A2 protein, which becomes cell cycle regulated. Therefore the maternal transcripts are fully functional. Interestingly, cyclin A2 was resolved as a doublet by Western blotting. The nature of these two forms is currently under investigation. Preliminary results indicate that the lower form has the same apparent molecular weight as the primary translation product, suggesting that the upper form is the result of posttranslational modifications. The relative abundance of the two forms switches between the oocyte and the embryonic stages. In MII oocytes, this switch coincides with the relocalization of some cyclin A2 protein to cytoplasmic structures resembling the centrosomes and the microtubule organizing centers described by Maro (1985). It is therefore possible that cellular relocalization of cyclin A2 may be linked to posttranslational modifications and to its potential role in microtubule dynamics (Buendia, 1992). A transient association of cyclin A2 with the centrosome has been described previously from preprophase to metaphase in HeLa cells (Bailly, 1992). In addition, the cyclin A2/Cdk2 kinase has been implicated recently in centrosome duplication in mammalian adult cells, arguing for a direct role of cyclin A2 in this activity (Meraldi, 1999).

In contrast to the situation in somatic cells, a proportion of the maternal cyclin A2 protein escapes degradation during the first mitosis in the mouse embryo. This stable subpopulation corresponds to the primary translation product on immunoblots (Fig. 3D) and may be equivalent to that immunolabeled around the spindle during the first mitosis (Fig. 4F). This result suggests that the mitotic machinery which completely degrades cyclin A2 subsequently at the second mitosis is not yet fully operational or recognizes preferentially the upper modified form of cyclin A2. Of interest is the long duration of the first mitosis that lasts in the region of 119 min, compared to 70 min for the second and 60 min for the third mitoses (Ciemerych, 1999). The cyclin A2 protein might hence play a role in the prolongation of the first mitosis to allow for proper mixing of the chromosomes originating from the male and female pronuclei. Following mitosis, increases in the level of cyclin A2 protein were observed as mouse embryos proceeded

through the S and G2 phases of the second mitotic cell cycle both on immunoblots and by immunolabeling (Figs. 4H, 4K, and 3). The protein is thus resynthesized either from maternal stocks of cyclin A2 mRNA or *de novo*, requiring the presence of zygotic mRNA.

Howe (1995) demonstrated that cyclin A protein degradation is not directly attributable to the degradation of its mRNA in *Xenopus* embryos. We observed that mouse embryos blocked in G2 of the second mitotic cell cycle (late two-cell stage) by α-amanitin also maintain a stable stock of cyclin A2 protein, while the maternal stock of cyclin A2 mRNA was degraded (Fig. 4).

From these observations, one would predict that from the four-cell stage, cyclin A2 homozygous null embryos will give no signal using immunofluorescence. In our previous report, it was suggested that cyclin A2 protein could be detected in cyclin A2-null mutant blastocyst stage embryos (Murphy, 1997). However, this signal was found to be due to a nonspecific retention of the anti-cyclin A2 antibody in the blastocyst that was, however, abolished upon competition with the antigen. This observation has now been corrected (Murphy, 1999). In this report, we have greatly modified both the method of fixation and the staining protocol and have also used confocal analysis to determine precisely the location of the immunofluorescence signal. Under these improved conditions, cyclin A2 is detected as a nuclear protein in interphase cells. However, a large pool of cyclin A2 protein also exists in the cytoplasm, as confirmed by Western blotting. Indeed, similar amounts of protein were detected in both nucleated and enucleated halves of one- and two-cell stage embryos (Fig. 3D). We observed that all eight-cell embryos obtained from crossing wild-type and heterozygous mice are stained positively for the presence of the cyclin A2 protein in at least one nucleus. By comparison, some embryos obtained from heterozygote crosses show no nuclear staining, even when the nuclear material is in interphase (Fig. 5), and will hence represent cyclin A2-null mutants. These data therefore confirm that the maternal cyclin A2 protein is undetectable from the four-cell stage.

DNA Synthesis in Cyclin A2-Null Mutants

The data we present here provide important information regarding the role of cyclin A2 in early mouse embryonic cell cycles by showing that the persistence of cyclin A2-null mutant embryos to postimplantation stages occurs in the absence of cyclin A2. This is in sharp contrast with the situation in *Drosophila*, in which cyclin A-deficient embryos stop developing when the maternal stock of protein is exhausted (Lehner and O'Farrell, 1989). However, the questions remain as to why and how do cyclin A2-deficient mouse embryos cease to develop after implantation, which by definition represents the time when cyclin A2 becomes essential. This dependence occurs at the time when cyclin A2 transcription becomes restricted to the embryonic region (Fig. 1) and cellular proliferation dramatically increases (Snow, 1977).

We observed that the absence of the cyclin A2 gene does not affect the ability of blastocysts to hatch from the zona pellucida and form outgrowths *in vitro*. In addition, mutant embryos which cease to develop postimplantation *in vivo* maintain a recognizable structure reminiscent of a blastocyst, suggesting that the absence of the cyclin A2 gene simply leads to an abrupt arrest of cellular proliferation and hence of development. Our analysis of cellular proliferation showed that all cell types in day 6.5 pc wild-type or mutant embryos were able to enter S phase, indicating that DNA replication is not impaired in the absence of cyclin A2. In addition, trophoblastic cells appear to perform endoreplication since large nuclei were observed similarly in wild-type and mutant blastocyst outgrowths. This observation suggests that cyclin A2 is not required for endoreplication. Interestingly, its degradation appears to be required to allow reinitiation of the endocycle during each endocycle (MacAuley, 1998).

Cyclin A2 is known to be rate limiting for cellular progression in S phase and mitosis in cultured mammalian somatic cells (Resnitzky, 1995; Rosenberg, 1995), with recent evidence indicating that it plays an essential role until prophase in human cells (Furuno, 1999). This is consistent with our observation that all embryonic cells, irrespective of their genotype, can enter S phase and suggests that cellular arrest is occurring later in S phase or in G2. In addition, cyclin A2 has recently been implicated in the coordination between S and M (Petersen, 1999; Lukas, 1999). Our results indicate that mammalian early embryonic cells differ from somatic cells in their requirement for cyclin A2 to complete the cycle. However, postimplantation, while cyclin A2$-/-$ mutants appear to be able to at least commence S phase, they may not be able to ensure the completion of the cell cycle and mitosis. This arrest does not trigger the cell death program (our unpublished observations). If this explanation is correct it suggests that cyclin A2 becomes essential for the first time as a mitotic cyclin in the early mouse embryo, rather than at the G1/S transition as the onset of DNA replication is clearly unaffected. A likely explanation for their developmental arrest is therefore failure to ensure sufficient levels of cellular proliferation at the onset of organogenesis. This stage of development appears as a critical "checkpoint" of cell cycle regulators. Interestingly, embryos mutant for the components of ubiquitin ligases (E3 enzymes), cullin-1 or -3, are also developmentally arrested at this stage with accumulation of cyclin E (Singer, 1999; Dealy, 1999; Wang, 1999), but cullin-1-null mutants undergo a massive apoptosis.

The data presented in this report have substantiated that the persistence of residual stocks of maternal cyclin A2 cannot explain the continued development of embryos mutant for the gene. One hypothesis to explain this might be that cyclin A2 is redundant with another protein during the nongrowth period of preimplantation mouse embryo development. While it is not known whether cyclins A1

and A2 are functionally interchangeable *in vivo*, they share the capacity to bind and activate the same kinases, Cdc2 and Cdk2, and are both localized to the centrosomal region in metaphase-arrested oocytes (Sweeney, 1996, and this report). Mice mutant for the cyclin A1 gene are viable (Liu, 1998), which means that cyclin A1 is dispensable for early embryonic development. However, while null mutant female progeny are able to reproduce, males are sterile (Liu, 1998). Both cyclin A1 and A2 proteins are present simultaneously in the oocyte (Sweeney, 1996, and this report), but their expression does not overlap during spermatogenesis (Ravnik and Wolgemuth, 1999; our unpublished observations). Thus, functional compensation between the two proteins might be occurring in the oocyte. Furthermore, in addition to its functions in meiosis, cyclin A1 may play a role in the mitotic cell cycle in certain cells (Yang, 1999). While A-type cyclins do not show any functional overlap with known B-type cyclins, the novel cyclin B3, which exhibits similarities with both A- and B-type cyclins (Gallant and Nigg, 1994; Jacobs, 1998), may also be a possible candidate to compensate for the absence of cyclin A2 during early embryonic cell cycles, although it has as yet to be identified in mammalian cells. The study of functional redundancy between these cyclins will form the subject of future investigations.

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