Isolation of the murine cyclin B2 cDNA and characterization of the lineage and temporal specificity of expression of the B1 and B2 cyclins during oogenesis, spermatogenesis and early embryogenesis

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SUMMARY

A cDNA encoding the murine cyclin B2 (cycB2) was isolated from an adult mouse testis cDNA library as part of studies designed to identify cyclins involved in murine germ cell development. This cycB2 cDNA was then used to examine the pattern of cycB2 expression during male and female germ cell development and in early embryogenesis, and to compare this expression with the previously characterized expression of cycB1. A single 1.7 kb cycB2 transcript was detected by northern blot hybridization analysis of total RNA isolated from midgestation embryos and various adult tissues. Northern blot and in situ hybridization analyses revealed that cycB2 expression in the testis was most abundant in the germ cells, specifically in pachytene spermatocytes. This is in contrast to the highest levels of expression of cycB1 being present in early spermatids. In situ analysis of the ovary revealed cycB2 transcripts in both germ cells and somatic cells, specifically in the oocytes and granulosa cells of growing and mature follicles. The pattern of cycB1 and cycB2 expression in ovulated and fertilized eggs was also examined. While the steady state level of cycB1 and cycB2 signal remained constant in oocytes and ovulated eggs, signal of both appeared to decrease following fertilization. In addition, both cycB1 and cycB2 transcripts were detected in the cells of the inner cell mass and the trophectoderm of the blastocyst. These results demonstrate that there are lineage- and developmental-specific differences in the pattern of the B cyclins in mammalian germ cells, in contrast to the co-expression of B cyclins in the early conceptus.

Key words: cell cycle, spermatogenesis, oogenesis, cyclin B

INTRODUCTION

Germ cell development involves mitotic proliferation, meiotic recombination followed by reduction divisions and subsequent differentiation of highly specialized cells, the egg and the sperm. Although these events occur in both male and female germ cell development in mammals, the temporal pattern of their progression is quite different. In mouse, primordial germ cells (PGCs) enter the genital ridge by day 13.5 post-coitum (p. c.). Male germ cells arrest in mitosis and remain arrested until a few days after birth, at which time gonocytes resume mitosis. By day 8 of postnatal (p. n.) development, the first wave of spermatogenetic cells has entered meiosis I (Nebel et al., 1961). By day 22 p. n., these germ cells have completed both meiotic division I and II and have begun the complex morphological changes of spermiogenesis, which transform haploid spermatids into highly specialized spermatozoa (Nebel et al., 1961).

Female mammalian germ cells, on the other hand, enter into meiosis, arresting at prophase of meiosis I (MI). At birth, oocytes are found in primordial follicles, surrounded by a single layer of granulosa cells. Puberty is marked by the recruitment of a pool of oocytes into a growth period in response to cyclic variations in gonadotropins. During this growth period, the oocyte remains arrested in prophase of MI, but the oocyte itself increases in size. Concomitantly, the number of granulosa cells surrounding the oocyte increases. After completion of this growth and proliferative phase to form the mature follicle, the oocyte is stimulated to resume meiosis. MI is completed and the ovum is ovulated while arrested in MII, awaiting fertilization to complete the second meiotic division (rev. Peters, 1969; Buccione et al., 1990).

The genes involved in controlling the specialized mitotic and meiotic cycles of germ cell differentiation are only now being identified. Maturation promoting factor (MPF) was originally described as an activity found in unfertilized frog eggs capable of inducing germinal vesicle breakdown (GVBD) and resumption of meiosis when injected into immature oocytes (Masui and Markert, 1971). Cytoplasm
from eggs of various organisms, including mouse, has been shown to induce meiotic maturation of *Xenopus laevis* (Sorenson et al., 1985) or starfish oocytes (Hashimoto and Kishimoto, 1988), demonstrating that the meiosis-inducing function of MPF is evolutionarily conserved. Cytoplasm from mitotically active embryonic cells (Wasserman and Smith, 1978) and mammalian tissue culture cells at the G2 phase of the cell cycle (Sunkara et al., 1979) is also capable of inducing meiotic maturation of *Xenopus* oocytes, suggesting that a common factor is capable of driving cells into M phase of mitotic and meiotic cell cycles.

MPF, now referred to as either the maturation or M phase promoting factor, is composed of two subunits, p34<sup>cdc2</sup> and cyclin B (Dunphy et al., 1988; Gautier et al., 1988; Labbe et al., 1989). *cdc2*, which was initially identified in the fission yeast *Schizosaccharomyces pombe*, encodes a 34×10<sup>3</sup> M<sub>r</sub> serine/threonine protein kinase (Simanis and Nurse, 1986). In fission yeast, p34<sup>cdc2</sup> kinase activity is required at two points in the cell cycle, the G1-S and the G2-M phase transitions (rev. Nurse, 1990). Cyclins, which were originally identified in marine invertebrates, have since been identified in a variety of species ranging from yeast to man (rev. Pines, 1991). The association of cyclin with p34<sup>cdc2</sup> is required for regulating the phosphorylation state of p34<sup>cdc2</sup> which in turn controls its kinase activity (rev. Clarke and Karsenti, 1991; Fleig and Gould, 1991). Cyclin may also be involved in conferring substrate specificity on the cdc2 kinase (rev. Hunt, 1991c).

Both cdc2 and cyclin B are members of gene families. cdc2-related or cyclin dependent kinases (cdk) have been identified in a number of different species, including human (Elledge and Spottwood, 1991; Tsai et al., 1991; Meyer son et al., 1992). *Xenopus* (Blow and Nurse, 1990; Paris et al., 1991; Gabrielli et al., 1992), *Drosophila* (Lehner and O’Farrell, 1990) and goldfish (Hirai et al., 1992). There is evidence that these cdk genes function earlier than cdc2 in the cell cycle, during G1-S phase (Fang and Newport, 1991; Elledge et al., 1992; Rosenblatt et al., 1992).

Cyclins have been divided into three major groups, G1, A and B, based on amino acid homology and timing of their appearance during the cell cycle (rev. Minshull et al. 1989b; Hunt, 1991b; Pines, 1991). Not only are there several groups of cyclins, but there are multiple members in at least two of the groups, including the B-type cyclins (rev. Pines, 1991). Multiple B-type cyclins have been identified in organisms as evolutionarily divergent as *Schizosaccharomyces pombe*. The fission yeast *Schizosaccharomyces pombe* encodes a 34×10<sup>3</sup> M<sub>r</sub> serine/threonine protein kinase (Simanis and Nurse, 1986). In fission yeast, p34<sup>cdc2</sup> kinase activity is required at two points in the cell cycle, the G1-S and the G2-M phase transitions (rev. Nurse, 1990). Cyclins, which were originally identified in marine invertebrates, have since been identified in a variety of species ranging from yeast to man (rev. Pines, 1991). The association of cyclin with p34<sup>cdc2</sup> is required for regulating the phosphorylation state of p34<sup>cdc2</sup> which in turn controls its kinase activity (rev. Clarke and Karsenti, 1991; Fleig and Gould, 1991). Cyclin may also be involved in conferring substrate specificity on the cdc2 kinase (rev. Hunt, 1991c).

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We have used mouse gametogenesis as a model system in which to study genes important in the in vivo regulation of the mammalian mitotic and meiotic cell cycles, in particular, the cyclins. Mouse spermatogenesis in particular provides an excellent system to study genes involved in cell cycle control. In the adult testis, one can examine cells in various stages of differentiation within a single lineage, from the self-renewing stem cells, the spermatogonia, to the meiotically dividing spermatocytes, as well as the post-meiotic germ cells, the spermatids. We have previously observed the developmentally regulated pattern of expression of murine cycB1 in both the male and female germ cells (Chapman and Wolgemuth, 1992). We now describe the isolation of a second murine B-type cyclin, cyclin B2 (cycB2). The pattern of cycB2 expression during male and female germ cell development was assessed by northern blot and in situ hybridization analyses. cycB2 is also expressed in a regulated pattern in the testis and ovary, but in a pattern quite distinct from that of cycB1. The fate of the cycB1 and cycB2 transcripts that accumulate in the fully grown oocytes, during meiotic maturation, at fertilization and during early embryogenesis was addressed by in situ hybridization analysis.

### MATERIALS AND METHODS

#### Source of tissues

Swiss Webster mice, obtained from Charles River (Wilmington, DE) or C57Bl/6J mice, obtained from Jackson Laboratory (Bar Harbor, ME), were used as the source of all adult tissues and cell populations. Adult testes and ovaries were obtained from mice older than 35 days. Ovaries obtained from mice 22 days p. n. were also used for in situ hybridization analysis as indicated. Embryos were collected at the days noted [day 0.5 post-coitum (p.c.) being the day the vaginal plug is detected] and dissected free of extra-embryonic membranes and placenta. The mouse mutant strain atrichosis (at) (ATEB/Le a/a dat/ebp) was obtained from the Jackson Laboratory (Bar Harbor, ME). Dissected tissue specimens were frozen in liquid nitrogen prior to RNA isolation. Dissected tissues for in situ analysis were fixed in 4% paraformaldehyde in 1× PBS (1× PBS: 130 mM NaCl, 7 mM Na<sub>2</sub>HPO<sub>4</sub>, 3 mM NaH<sub>2</sub>P<sub>4</sub>) overnight at 4°C.

#### Collection of ovulated and fertilized eggs

C57Bl/6J females were superovulated by intraperitoneal injection with 5 I. U. pregnant mare serum gonadotropin (PMSG, Sigma) and 48 hours later with 5 I. U. human chorionic gonadotropin (HCG, Sigma). These females were then either (1) killed 24 hours later to obtain ovulated eggs, (2) mated to C57Bl/6J males and killed 24 hours later to obtain fertilized eggs or, (3) mated to B6 males and killed at day 3.5 p. c. to obtain blastocyst stage embryos. Ovulated eggs were dissected from the ampullae into M16 media (Hogan et al., 1986), cumulus cells were removed by treatment with 300 μg/ml hyaluronidase (Sigma) in M2 media (Hogan et al., 1986) and then moved to fresh M2 media to remove the hyaluronidase. Fertilized eggs were dissected from the ampullae into M2 media and checked visually for the presence of two pronuclei. At day 3.5 p. c., blastocysts were flushed from the uterus into 1× PBS. Ovulated eggs, fertilized eggs and blastocysts were collected with a micropipet and reinjected into host ampullae. The ampullae were then dissected and fixed in 4% paraformaldehyde overnight at 4°C before paraffin embedding for in situ analysis.

#### Probes

All DNA probes were labeled with [32P]dCTP (DuPont, Wilmington, DE) using Amersham’s Multiprime DNA labeling systems (Amersham, Arlington Heights, IL) to a specific activity greater
than \(10^9\) cts/minute/\(\mu\)g. The human cyclin B1 (HsCycB1) and cyclin B2 (pHsCycB2-2) cDNAs were generous gifts of B. Futcher (Cold Spring Harbor Laboratory). A 1465 bp BamHI-SacI insert of HsCycB1 and a 1300 bp SacII-BglII insert of pHsCycB2-2 were used as probes for screening a testis cDNA library. A 330 bp Pst I fragment of p3.3 (see Fig. 1A) was used as a probe for screening an embryo cDNA library. Sense and antisense RNA probes were transcribed from linearized plasmids using T3 or T7 RNA polymerase (Promega Biotech, Madison, WI), respectively, in the presence of \([\text{32P}]\)UTP (DuPont) or \([\text{35S}]\)UTP (DuPont) according to protocols suggested by the manufacturer. A \([\text{32P}]\)UTP-labeled antisense RNA probe generated from linearized p3.3 was used for northern blot hybridization analyses. Antisense and sense \([\text{35S}]\)UTP-labeled RNA probes were generated from linearized p3.3 and p18.2, hydrolyzed to 0.1 kb according to Cox et al. (1984) and were used for in situ hybridization analyses. p18.2 is the plasmid containing the entire coding region of murine cyclin B1 (Chapman and Wolgemuth, 1992).

**Screening of testis and embryo cDNA libraries**

The preparation of the adult mouse testis cDNA library has previously been described (Chapman and Wolgemuth, 1992). The day 10.5 mouse embryo library was a generous gift of J. Ruiz (Harvard University). Both cDNA libraries were prepared using Stratagene’s ZAP-cDNA Synthesis Kit (Stratagene, La Jolla, CA). For each library, \(1 \times 10^6\) recombinant plaques were screened in duplicate according to Maniatis et al. (1982). Filters were pre-washed in 50 mM Tris pH 8.0, 1 M NaCl, 1 mM EDTA, 0.1% SDS at 50°C for 1 hour and prehybridized in 5× SET (1× SET: 100 mM NaCl, 30 mM Tris-HCl pH 8.0, 2 mM EDTA), 1× Denhardt’s (0.02% Ficoll 400, 0.02% BSA (Fraction V), 0.02% polyvinylpyrrolodone), 20 mM NaPO₄ pH 7.0, 1% SDS, 0.1 mg/ml denatured and sheared salmon sperm DNA, 0.1 mg/ml *Escherichia coli* DNA at 50°C for 4 hours. Hybridization conditions were identical to those of prehybridization with the addition of \([\text{32P}]\)P-labeled probes to a final concentration of 1×10⁶ cts/minute/ml of hybridization mixture. Hybridizations were performed at 5°C for 16-20 hours. The filters were washed at a final stringency of 0.2× SSC, 1% SDS at 80°C for 1-2 hours and exposed to Kodak XAR films with intensifying screens at −70°C for the indicated time periods. Sizes of the transcripts were determined by comparison to the ribosomal RNA bands.

**RESULTS**

**Cloning and sequence analysis of mouse cyclin B2**

To identify the cyclins involved in the mitotic and meiotic stages of mouse germ cell development, an adult mouse total testis cDNA library was screened under reduced stringency using human cyclin B1 and B2 cDNAs as probes. Several cDNAs that hybridized with the human B1 cyclin were isolated and characterized (Chapman and Wolgemuth, 1992). Sequence analysis of a 1.2 kb clone, designated p3.3, which hybridized to both the human cyclin B1 and B2 probes, revealed homology to the human cyclin B2 protein (Xiong et al., personal communication; S. Reed, personal communication). Comparison of the sequence to the previously identified B2 cyclins and the absence of an in-frame initiating methionine in the sequence indicated that this cDNA was not full length. In addition, northern hybridization analysis using an antisense riboprobe generated from this 1.2 kb clone revealed a 1.7 kb transcript in total RNA isolated from both adult mouse testes and mid-gestation embryos (data not shown; see below).

To identify a cDNA encoding the entire murine cyclin B2 protein, a day 10.5 post-coitum (p.c.) mouse embryo cDNA library and the adult testis cDNA library were screened using a probe generated from the 5′ region of p3.3 (p3.3P1, Fig. 1A). The longest cDNA, p19.3, was isolated from the embryo library and was approximately 1.5 kb. Sequence analysis of p19.3 revealed an in-frame initiation codon in good agreement with the consensus sequence for the initiation of translation in vertebrates (Kozak, 1987). Restriction maps of p19.3 and p3.3 are shown in Fig. 1A. The nucleotide sequence of p19.3 and predicted amino acid sequence are shown in Fig. 1B. The open reading frame is 1194 nucleotides and is capable of encoding a protein of 398 amino acids with a predicted molecular weight of \(44.5 \times 10^3\) *M*.
The putative protein contains both the characteristic cyclin box and the destruction box. The cyclin box is the region of shared homology among all cyclins (Minshull et al., 1989b). The destruction box is characteristic of B-type cyclins and is believed to trigger the ubiquitin-mediated pathway of degradation (Glotzer et al., 1991; rev. Hunt, 1992).

**Fig. 1.** (A) Restriction map of p19.3. Location of restriction enzyme sites are shown in relation to the open reading frame (open box) of the mouse cycB2 cDNA, p19.3. The filled boxes represent the location of the ‘destruction box’ and the ‘cyclin box’ as indicated. The location of the probes used in these studies, p3.3 and p3.3P1, are also shown in relation to p19.3. Restriction enzymes are abbreviated as follows: EcoRI (E), HindII (H2), HindIII (H3), PstI (P) and XhoI (X). (B) Nucleotide sequence of p19.3. The nucleotide and deduced amino acid sequence of the mouse cycB2 cDNA are shown. A termination codon at the coding end region is indicated by an asterisk (*). The boxed region indicates the amino acid region that is most highly conserved among cyclins, the ‘cyclin box’. Two polyadenylation signals in the 3′ untranslated region are underlined. The sequence begins with an EcoRI linker and includes the 3′ untranslated region up to the position of the poly(A) tail.

The EMBL accession number for murine cycB2 is X60362. (C) Sequence comparison of murine cycB2 and cycB1 proteins. Alignment of the amino acid sequences of murine cycB2 (top line) and murine cycB1 (bottom line) are shown. (D) Sequence comparison of human cycB2 and murine cycB1 proteins. Alignment of the amino acid sequences of murine cycB2 (top line) and murine cycB1 (bottom line) are shown. [Diagram and figures are not included in this text-based format.]

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1991a). Where colinear, p19.3 and p3.3 are identical. Interestingly, p19.3 contains two consensus polyadenylation signals in its 3′ untranslated region. Both polyadenylation signals appear to be used in mRNAs from testis and embryos, since representative cDNAs of both classes were isolated from both libraries. The use of the alternative polyadenylation signals generates mRNAs that differ by approximately 100 nt in their 3′ untranslated regions.

Analysis of the predicted protein product of p19.3 revealed that it is more closely related to the B2 cyclins than to the B1 cyclins. Similarities to the B2 cyclins of *Xenopus* (Minshull et al., 1989a), chicken (Gallant and Nigg, 1992) and human, were 76%, 79% and 90%, respectively, at the amino acid level. This is in contrast to the similarities of 67.5%, 68% and 68% when compared to the cyclin B1 of *Xenopus* (Minshull et al., 1989a), mouse (Chapman and Wolgemuth, 1992) and human (Pines and Hunter, 1989), respectively. Comparisons of the murine cysB2 and cycB1 proteins and of the murine and human cycB2 proteins are shown in Fig. 1C and D, respectively. The gene encoding p19.3 has thus been designated murine *cyclin B2*, cycB2.

**Expression of murine cycB2 in adult and embryonic samples**

Northern blot hybridization analyses, using an antisense riboprobe generated from p3.3, were performed to determine the tissue distribution of cycB2 transcripts. A 1.7 kb transcript was detected in total RNA isolated from adult spleen and intestine, but was not observed in total RNA isolated from adult brain, heart, lung, liver or kidney (data not shown). A 1.7 kb cycB2 transcript was also detected in the two germ cell-containing tissues, the ovary and testis, as well as in day 12.5 p.c. embryos (Fig. 2). A 1.7 kb transcript was also detected by northern analysis of total RNA isolated from mid-gestation embryos, from day 10.5 to day 17.5 p.c. and from day 11.5 p.c. placenta (data not shown).

To determine the cellular specificity of cycB2 expression in the adult testis, whether germinal or somatic, total RNA was isolated from the testes of mice homoygous and heterozygous for the *attrichosis (at)* locus. Mice homozygous for the *at* locus are devoid of germ cells, but have the normal complement of somatic cell types, including Leydig, Sertoli and peritubular myoid cells (Hummel, 1964). The heterozygous littersmtes have the normal somatic and germ cell complements and are fertile. By northern blot hybridization, a 1.7 kb transcript was detected in total RNA from *at/+* testes, but not in RNA isolated from the testes of *at/at* mice (Fig. 2). The absence of a cycB2 transcript in the homozygous mutant testis indicated that the cycB2 mRNA was most likely localized to the germ cell compartment of the testis, as has been demonstrated for a number of genes expressed in the testis, i.e. *Hox 1.4* (Wolgemuth et al., 1987) and Zfp-37 (Burke and Wolgemuth, 1992). Alternatively, it is possible that cycB2 is also expressed in the somatic cells but requires the presence of germ cells for its proper expression, as was observed for the level of POMC expression in the somatic cell compartment, which was influenced by the presence or absence of germ cells (Gizang-Ginsberg and Wolgemuth, 1987).

![Fig. 2. Distribution of cycB2 transcripts in mouse tissues. Each sample contained 20 µg of total RNA from day 12.5 p.c. embryos, ovary and from the testes of adult SW, *at/+* and *at/at* mice as indicated. Hybridization was with a 32P-labeled p3.3 antisense riboprobe. Exposure time: 24 hours.](image)

**In situ hybridization localizes cycB2 transcripts to spermatocytes and early spermatids**

To localize cycB2 expression further to particular testicular cells, in situ hybridization using 35S-labeled sense and antisense RNA probes was performed. The results of in situ hybridization analysis of *at/+* and *at/at* testes are shown in Fig. 3. In agreement with the results from northern blot hybridization experiments, no signal above background was observed over the somatic cells in the germ-cell-deficient (*at/at*) testes (Fig. 3A,B). Furthermore, no signal was detected over the somatic cells in the heterozygous, fully fertile *at/+* sections (Fig. 3C-H). This indicated that the cycB2 transcript detected by northern analysis in the testes samples was indeed due to germ cell and not somatic cell expression. No signal above background was detected in either *at/+* or *at/at* testis sections using the sense probe (data not shown).

Expression of cycB2 was readily detected within tubules from the testes of the fertile heterozygous mice (Fig. 3C-H). However, the most intense labeling was observed over only a subset of these tubules. Analyses of histological cross-sections of seminiferous tubules have revealed that germ cells at distinct stages of spermatogenesis are always found in specific patterns of association with one another (Oakberg, 1956; rev. Russell et al., 1990). These defined groups of cellular associations arise because a new round of spermatogenesis is initiated every 12 days, but the entire process lasts 35 days. The various cellular associations can be divided into twelve distinct stages, which together make up a complete cycle of the seminiferous epithelium (Oakberg, 1956; rev. Russell et al., 1990). Determination of the stage of a specific tubule allows for more precise identification of the spermatogenic cells present in the tubule.

The stages of individual tubule indicated by Roman numerals in Fig. 3C-H were classified according to Oakberg (1956) and Russel et al. (1990). A stage I tubule, which contains primarily early round and elongating spermatids, is shown in Fig. 3C and D. The darkly staining cells in the basal compartment of the tubule are spermatocytes that have just entered the pachytene stage of meiosis I. No signal above background was detected in these early pachytene spermatocytes. cycB2 transcripts were, however, detected in the early round spermatids, located in the middle layer of the tubule. By stage VII, a low level of signal was
detected in the spermatocytes that had reached the mid-pachytene stage (Fig. 3E,F). No cycB2 signal was observed in the preleptotene spermatocytes that lie in the basal compartment of the tubule. The highest level of cycB2 signal was detected in stage X-XI tubules, which contain predominantly pachytene and diplotene spermatocytes in the

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**Fig. 3.** Cellular localization of cycB2 transcripts in the testis by in situ hybridization. Testis sections were photographed using bright-field (A, C, E and G) and epiluminescence (B, D, F and H) optics. Hybridization was with a 35S-labeled antisense p3.3 riboprobe. (A,B) Testis sections from at/at mice; (C-H) Testis sections from at/+ mice. The stages of the seminiferous epithelium are designated by Roman numerals in C-H. Cell types are designated in A as follows: Leydig cells (LC), Sertoli cells (SC) and peritubular myoid cells (MC). Exposure time: 10 days. Size bar, 100 µm.
center layer of the tubule. A stage XI tubule is shown in Fig. 3G,H. Spermatocytes in leptomene and zygote of meiosis I are also present in stage X-XI tubules; however, no signal above background was detected. In situ hybridization analysis of testes from day 7 p. n. mice, where the spermatogonia are clearly seen, did not reveal any cycB2 signal above background in these mitotically dividing germ cells (data not shown). Secondary spermatocytes are found in only a very small portion of the tubules present in the adult testis due to the brevity of this particular stage. Nonetheless cycB2 transcripts were also detected in these cell types (data not shown). No signal above background was detected over the elongating spermatids found in the adluminal region of the tubules (Fig. 3C-H). This pattern of cycB2 expression is in striking contrast with that of cycB1 expression, wherein cycB1 transcripts were detected at low levels in late pachytene spermatocytes and at highest levels in the round spermatids (Chapman and Wolgemuth, 1992). These distinct patterns of expression in male germ cells are summarized in the diagram in Fig. 4.

**Localization of cycB2 signal to both germinal and somatic cells in the ovary**

Northern blot hybridization analysis revealed that cycB2 transcripts were also present in the ovary. To determine if cycB2 was expressed in the germ cells, as in the testis, or in the somatic cells, sections of ovaries were examined by in situ hybridization (Fig. 5). Developing ovarian follicles were staged by the size of the oocyte and by the number of layers of granulosa cells surrounding the oocyte according to Pedersen and Peters (1968). Oocytes that have not entered the growth phase, called resting oocytes, are found in follicles staged 1 through 3a, while growing oocytes are found in follicles staged 3b through 6. Fully grown oocytes are found in follicles of stages 7 and 8, in which meiosis resumes. No cycB2 signal was detected in the resting oocytes (Fig. 5A,B). cycB2 signal was first detected at low levels in growing oocytes of follicles staged 3b. cycB2 transcripts were detected in oocytes as they continued to grow, through to the antral stages (Fig. 5A-D). No obvious difference was noted in the level of cycB2 transcripts in oocytes in later stage follicles. The pattern of accumulation of cycB2 transcripts in the growing oocytes was thus quite similar to that observed for cycB1 transcripts (Chapman and Wolgemuth, 1992).

cycB2 expression was also readily detected in somatic cells of developing follicles, the granulosa cells. This is in contrast to cycB1, which is expressed most abundantly in the germ cells of the ovary and at very low levels in the granulosa cells of larger follicles (Chapman and Wolgemuth, 1992). cycB2 transcripts were detected over the granulosa cells, in particular granulosa cells of follicles in stage 5 and larger (Fig. 5A-D). Within the granulosa cells of stage 7 and 8 follicles (antral follicles), cycB2 transcripts were not evenly distributed. Rather, cycB2 transcripts were most abundant over those granulosa cells closest to the oocyte, the cumulus granulosa cells (Fig. 5C,D). Within the adult ovary, there are also follicles that stop growing and begin to degenerate. These are known as atretic follicles (Peters, 1969). When compared to a healthy follicle, the level of cycB2 signal over the granulosa cells in atretic follicles appeared to be diminished while a low level of cycB2 signal was still detectable in the oocytes (data not shown).

**cycB1 and cycB2 transcripts are present following ovulation and in the early embryo**

The above results on cycB2 and our previous observations on cycB1 expression (Chapman and Wolgemuth, 1992) demonstrate that these murine B-type cyclins are expressed in a developmentally regulated manner in the adult ovary, but in quite distinct patterns. cycB1 is expressed most abundantly in the germ cells, particularly in growing oocytes, while cycB2 is expressed at apparently comparable levels in both the growing oocyte and in a subset of the somatic cells of the developing follicle. To determine whether the relative levels of cycB1 and cycB2 mRNAs change as oocytes undergo meiotic maturation and fertilization, in situ hybridization was used to examine cycB1 and cycB2 expression in histological sections of ovarian oocytes, ovulated eggs and fertilized eggs (Fig. 6). As previously observed, cycB1 and cycB2 transcripts were detected in oocytes (Fig. 6A,D). While both cycB1 and cycB2 transcripts were detected in ovulated (Fig. 6B,E) and fertilized eggs (Fig. 6C,F), the relative levels of both appeared to decrease in the fertilized eggs.

To determine whether both cycB1 and cycB2 are expressed during early embryonic development, blastocyst-staged embryos were examined by in situ hybridization analysis. cycB1 and cycB2 transcripts were detected in cells constituting both the inner cell mass and the trophectoderm of the blastocyst (Fig. 7). Thus while cycB1 and cycB2 are expressed differentially in specific somatic and germinal lineages during male and female germ cell development, they are co-expressed in both the embryonic and extraembryonic cell lineages at apparently comparable levels during the early stages of embryogenesis.
DISCUSSION

Isolation of murine cYcB2

Given the distinct temporal and chronological progression of mitosis and meiosis in female and male germ cells in mammals, it is likely that there are unique cell cycle control points in each lineage. Although the adult testis contains germ cells undergoing both mitotic and meiotic cell divisions, little or no attention has been focused on the role of the cell cycle genes in these processes. The high degree of evolutionary conservation of the genes that control the cell cycle has facilitated the isolation of their homologs in various species. We were interested in examining cyclin B expression during the mitotic and meiotic cell cycles of mouse germ cell development. In this paper, we present the isolation and sequence of a cDNA encoding a new murine B-type cyclin, cYcB2. The putative cYcB2 protein contains the characteristic ‘cyclin box’ and ‘destruction box’ and shows higher homology to the previously identified cyclin B2 proteins than the cyclin B1 proteins. cYcB2 encodes a 1.7 kb transcript that was detected in both the adult ovary and testis by northern blot analysis. Since cyclinB1 had previously been shown to be expressed in the testis and ovary, specifically in germ cells, the pattern cyclinB2 expression during germ cell development was determined, with particular focus on whether its expression was similar to or distinct from that of cyclinB1.

Cyclin B expression in the testis

Northern blot and in situ hybridization analysis revealed that, in the testis, cyclinB2 was present at highest levels in the meiotically dividing spermatocytes. This is in contrast to the distribution of cyclinB1 transcripts, which were most abundant in the post-meiotic germ cells in the testis, the spermatids. Lower levels of cyclinB2 mRNAs were also detected in the early round spermatids. It is curious that neither cyclinB2 nor cyclinB1 signal was detected in the mitotically dividing spermatogonia. This could be because the level of cyclinB1 and cyclinB2 transcripts were simply below the level of sensitivity of the in situ hybridization procedure. Alternatively, since at least nine cyclin B loci have been localized in the mouse genome (Lock et al., 1992; Hanley-Hyde et al., 1992), a different cyclin B could be responsible for activating p34cdc2 in these cells.

Cyclins function as the regulatory subunit of the p34cdc2/cyclin complex. Synthesis of cyclin B is capable of driving cells into M phase of mitotic and meiotic cell cycles (Minshull et al., 1989a; Murray and Kirschner, 1989; Murray et al., 1989; Westendorf et al., 1989). Based on this knowledge of cyclin function in cell cycle progression, the
pattern of \textit{cycB2} expression in the testis more closely follows a predicted pattern of expression than does that of \textit{cycB1}. High levels of \textit{cycB2} transcripts in the meiotically dividing germ cells lends further support for a \textit{cycB2} role in the meiotic cell divisions of germ cell development. Our finding of high levels of \textit{cycB1} transcripts in the post-meiotic male germ cells was surprising (Chapman and Wolgemuth, 1992). However, since we are only now beginning

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig6.png}
\caption{\textit{cycB1} and \textit{cycB2} transcripts in oocytes, ovulated eggs and fertilized eggs. Photomicrographs were taken using epiluminescence optics. Hybridization was with $^{35}$S-labeled antisense p18.2 riboprobe representing \textit{cycB1} (A-C) and $^{35}$S-labeled antisense p3.3 riboprobe representing \textit{cycB2} (D-F). Exposure time: 21 days. Size bar, 100 \text{µm}.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{\textit{cycB1} and \textit{cycB2} signal in blastocysts. Photomicrographs were taken using bright-field (A,C) and epiluminescence (B,D) optics. (A,B) Hybridized with a $^{35}$S-labeled antisense p18.2 (\textit{cycB1}); (C,D) hybridized with a $^{35}$S-labeled antisense p3.3 (\textit{cycB2}). Exposure time: 21 days. Size bar, 100 \text{µm}.}
\end{figure}
to understand the way in which cyclins regulate cdc2 kinase activity, these results may implicate a function for cycB1 which does not involve cell cycle progression. Instead, cycB1 could be involved in directing kinase activity to specific substrates which themselves are involved in germ cell differentiation.

**Cyclin expression during oogenesis and early embryogenesis**

Our results on cycB1 and cycB2 expression during murine oogenesis also revealed surprisingly different patterns. In situ analysis revealed that, whereas cycB1 transcripts were most abundant in the growing oocytes, cycB2 transcripts were present in the granulosa cells of growing and mature follicles, as well as in growing oocytes. The steady state levels of cycB1 and cycB2 did not change dramatically following ovulation; however, the levels of both appeared to drop following fertilization. In stage 7 and 8 follicles, cycB2 transcripts were most abundant over the granulosa cells closest to the oocyte. In these antral follicles, the granulosa cells can be divided into two groups. The mural granulosa cells are attached to the basement membrane that encloses the follicle and the cumulus granulosa cells are attached to the oocyte (rev. Buccione et al., 1990). In the rat ovary, the cumulus granulosa cells have been shown to divide more frequently than the mural granulosa cells in these antral follicles (Hirshfield, 1986). Our results are therefore consistent with a role for cycB2 in mitotic proliferation.

It is not yet known whether cycB1 and cycB2 are functionally interchangeable in vivo. cycB1 and cycB2 cDNAs have been identified in only a few species and studies on the regulation of the cell cycle in higher eukaryotes has been largely confined to vertebrate cell lines in vitro. In one of the few in vivo studies, expression of both cycB1 and cycB2 was shown to peak around the G2-M phase transition in regenerating rat liver (Lu et al., 1992). Mouse cycB1 and cycB2 have been shown to be co-expressed in both embryonic and extraembryonic cell lineages in early embryogenesis (our above observations) as well as in a variety of adult tissues, including spleen, intestine, ovary and testis (Chapman and Wolgemuth, unpublished observations). By determining the lineage and stage-specific sites of expression of cycB1 and cycB2 in the ovary and testis, we have discovered a surprising uncoupled expression of the two genes in specific cells. We observed that cycB1 and cycB2 are co-expressed but are differentially modulated in the two species. In Xenopus, zygotic transcription does not begin until after the twelfth mitotic division, at the time of the midblastula transition (Newport and Kirschner, 1982a,b). Mouse zygotic transcription, however, begins by the two-cell stage (Braude et al., 1979; Flach et al., 1982). Therefore the accumulation of maternal RNAs and proteins in the egg is critical for the early stages of amphibian development, whereas it may not play such a vital role in mouse development.

While the levels of cycB1 and cycB2 transcripts did not change during Xenopus oogenesis, the levels of protein did (Kobayashi et al., 1991). cycB2 was the major cyclin protein during oogenesis. The level of cycB1 protein reached that of cycB2 in the matured egg. An interesting possibility suggested by these observations was that the MPF kinase prior to MI was different than that present in the MII-arrested eggs. We have shown that both cycB1 and cycB2 transcripts are present in mouse oocytes, ovulated eggs and fertilized eggs. Whether the levels of cycB1 and cycB2 transcripts reflect the corresponding amounts of protein remains to be determined. It will be interesting to see whether different MFP species exist during mouse oogenesis as exist in Xenopus. It is not clear whether different cyclins can direct p34^cdk2 to specific substrates (rev. Hunt, 1991c). However, it is interesting to speculate that the differential pattern of cycB1 and cycB2 expression observed in the germ and somatic cell lineages of the testis and ovary may reflect differences in the preferred p34^cdk2 substrate. Furthermore, some of these substrates may not be involved with cell cycle progression per se, but rather with other cellular events such as chromatin remodeling and nuclear condensation.

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