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Developmental changes in cyclin B1 and cyclin-dependent kinase 1 (CDK1) levels in the different populations of spermatogenic cells of the post-natal rat testis

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Spermatogenesis is a highly ordered process which requires mitotic and meiotic divisions. In this work, we studied the relative changes in the levels of the two components of the M-phase promoting factor (MPF): the regulatory subunit cyclin B1 (CycB1) and its catalytic subunit cdk1, in spermatogenic cells of rats between 16 and 90 days of life. A multivariate flow cytometry analysis of forward scatter (FSC), side scatter (SSC) and DNA content was used to identify six populations of rat germ cells: spermatogonia with preleptotene spermatocytes, young pachytene spermatocytes, middle to late pachytene spermatocytes, secondary spermatocytes with doublets of round spermatids, round spermatids, and elongated spermatids. For any population studied no significant difference in the relative cellular content of CycB1 or cdk1 proteins between animals of different ages was observed. By contrast, CycB1 and cdk1 levels were different between the different populations of germ cells. CycB1 and cdk1 were rather high in young pachytene spermatocytes and culminated in late spermatocytes, i.e. just before the first meiotic division. The relative levels of the two proteins remained high in secondary spermatocytes then decreased in round spermatids at the exit of meiosis. Similar results were obtained by Western-blot analysis of total proteins obtained from lysates of elutriated fractions of spermatocytes and spermatids. MPF activity was assessed in lysates of germ cells from 32-day-old rats or adult animals using p13*^suc1^* agarose and histone H1 as an exogenous substrate. H1 kinase activity was higher in pachytene spermatocytes than in round spermatid fractions from both adult and young rats. These results indicate that the meiotic G2/M transition is associated to high levels of CycB1 and cdk1 leading to high MPF activity irrespective of the age of the animals.

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Introduction

During the proliferative phase of spermatogenesis, diploid spermatogonia divide mitotically several times to provide a population of spermatocytes. These latter proceed through meiosis to give birth to haploid spermatids. Thereafter, spermatids undergo a morphological differentiation leading to spermatozoa (Russel et al., 1990). Thus, spermatogenesis requires a coordinated series of cell cycle events involving both mitosis and meiosis together with subsequent differentiation. Cyclin-dependent kinases (CDKs) and their regulatory subunits, the cyclins, are key regulators of cell progression, catalysing transitions both into S phase and into mitosis (Nigg, 1995). The mitotic B-type cyclins activate the p34cdc2 cyclin-dependent protein kinase (cdk1) to form maturation-promoting factor (MPF), which is required for cells to undergo mitosis or meiosis. Indeed, MPF was originally identified by its role in meiosis, as an activity found in mature frog eggs which is able to induce germinal vesicle breakdown, when injected into immature oocytes (Masui and Markert, 1971). It was subsequently shown that the cytoplasm of mitotically dividing cells can also induce germinal vesicle breakdown of such oocytes (Sunkara et al., 1979; Wasserman and Smith, 1978).

Only two B-type cyclins, B1 (Chapman and Wolgemuth, 1992; Pines and Hunter, 1989) and B2 (Chapman and

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Wolgemuth, 1993; Minshull et al., 1989) have been identified so far in mammals. These two cyclins are expressed in testicular germ cells, but exhibit different patterns of expression during murine spermatogenesis. Cyclin B2 transcripts are most abundant in pachytene spermatocytes whereas the highest levels of expression of cyclin B1 are present in early spermatids (Chapman and Wolgemuth, 1993). However, cyclin B1 protein appears more abundant in pachytene spermatocytes than in the post-meiotic round spermatids (Chapman and Wolgemuth, 1994). Moreover, it has been shown recently that cyclin B2-null mice develop normally and are fertile, whereas cyclin B1-null mice die in utero (Brandeis et al., 1998). This indicates that cyclin B2 is dispensable for murine spermatogenesis and therefore suggests that cyclin B1 could have a role in this process. By contrast, Gromoll et al. (1997) in the rat testis found cyclin B1 mRNA expression in the cytoplasm of pachytene spermatocytes, secondary spermatocytes and round spermatids, but could observe cyclin B1 protein only in round spermatids, leading these authors to conclude that “a role for this cyclin in the second meiotic division is unlikely”.

In situ hybridization of cdk1 mRNA in the mouse testis has shown that this gene is expressed primarily in meiotic cells of the testis (Rhee and Wolgemuth, 1995). However, immunocytochemical detection of cdk1 has revealed a lower staining of meiotically dividing spermatocytes than of early pachytene spermatocytes (Ravnik and Wolgemuth, 1999). This latter result challenges somewhat the widely recognized role of cdk1 in the G2/M transition of both mitotically and meiotically dividing cells. By contrast, Cobb et al. (1999) observed similar amounts of cdk1 (and cyclin B1) in young and late spermatocytes. Hence, the first aim of the present study was to determine whether the timing of appearance of the cyclin B1 protein during the spermatogenic cycle is so different in the rat from that reported for the mouse and to compare the content in cdk1 (and cyclin B1) of early or late pachytene spermatocytes and secondary spermatocytes in an attempt to clarify the confused picture that had emerged when comparing the earlier literature in the field (reviewed in (Wolgemuth et al. (1995)) and the more recent studies (Cobb et al., 1999; Gromoll et al., 1997; Ravnik and Wolgemuth, 1999).

Apoptosis and cell cycle regulation are closely linked in proliferating cells (King and Cidlowski, 1995; Meikrantz and Schlegel, 1995). However, previous studies are not in agreement concerning the role of cdk1/cyclin B1 in apoptosis (Ongkeko et al., 1995; Shi et al., 1994). Taken together these results suggest that the role of cdk1/cyclin B1 kinase activity in apoptosis varies between cell types. The developing rat does not reach full spermatogenic efficiency until about 75 days of age (Robb et al., 1978). This may be accounted for by kinetic properties of spermatogenic stem cells, and/or by degeneration of cells. Russell et al. (1987) have shown that the degeneration rate of spermatogenic cells from normal pubertal animals is about 15, 10 and 2 times greater in 20-, 32- and 44-day-old animals, respectively, than in 75-day-old rats. More recently, Billig et al. (1995) reported that apoptotic internucleosomal fragmentation is age-dependent and peaks in 16 to 28-day-old rats. In both studies, spermatocytes and spermatids were the major cell types undergoing cellular degeneration. Therefore, the second aim of our work was to investigate whether the increasing efficiency of spermatogenesis in the developing rat testis could be related to changes in the levels of cyclin B1 and/or cdk1 in the different populations of spermatogenic cells.

Materials and methods

Reagents

Anti-human cyclin B1 (CycB1) monoclonal antibody (mAb) either or not fluorescein (FITC)-conjugated (clone GNS1) was obtained from Becton-Dickinson, Le Pont-de-Claix, France. This mAb was raised against amino acid residues 1–21 of the human CycB1. Anti-cdk1 antibody (clone 17) was obtained from Santa-Cruz (Tebu, Le Perray-en-Yvelines, France). This mAb, directed against *Xenopus* cdk1, does not cross-react with other cyclin-dependent kinases. Anti-vimentin (clone V9), phycoerythrin (PE)- or FITC-conjugated rabbit anti-mouse immunoglobulins (IgG) and rabbit anti-mouse IgG conjugated to horseradish peroxidase (HRP) were obtained from DAKO (Trappes, France). P13*suc1* agarose was obtained from Calbiochem (Meudon, France). Chemicals and Hoechst 33342 were obtained from Sigma (La Verpillière, France).

Animals

Male Sprague-Dawley 16 to 90-day-old rats were used in the experiments. Animals were killed by decapitation and their testes were removed.

Cell isolation

The preparation of germ cells for flow cytometric analysis was essentially as described by Weiss et al. (1997). Testes from several animals (4–12 pups at early post-natal stages, 2 mature animals) were pooled. Briefly, after decapsulation and elimination of interstitial cells by sedimentation, seminiferous tubules were digested for 30 min at 32 °C in PBS containing 0.8 mg/ml of porcine trypsin (Sigma). Then, seminiferous tubules were cut during 20 min with two lancets. Isolated cells were filtered through 100-µm then 20-µm nylon mesh to prepare total germinal cells or with a final filtration through glass fibers to remove most of the elongated spermatids and spermatozoa. Cells were fixed with ice-cold 70% ethanol for 24 h before staining. In order to check that these fixation conditions did not result in any selective loss of cyclin B1 or cdk1, in separate experiments, paraformaldehyde at 1% was used instead of 70% ethanol, with similar results (data not shown). In addition, cyclin B1 and cdk1 levels were assayed by Western blotting (see below).

Immunolabeling of cells for flow cytometry

After washing with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.71 mM KH_2PO_4 (pH 7.4)), fixed cells were resuspended in 150 µl of permeabilizing buffer: 0.25% Triton X-100 and 1% bovine serum albumin (BSA) in PBS for 20 min on ice. The cells were treated with blocking buffer: 2% BSA, 10% fetal calf serum (Gibco BRL Life Technologies, Cergy-Pontoise, France) in Ca^{2+}- and Mg^{2+}-free PBS and then exposed to anti-vimentin antibody diluted to 0.5 µg/ml in blocking buffer for 3 h at 4 °C. After three washes in PBS/BSA 1%, the cells were exposed to PE-labelled secondary antibody used at a dilution of 1:50 in blocking buffer for 1 h at 4 °C. After washing, the cells were incubated with 100 µl of anti-CycB1 FITC-conjugated mAb diluted as recommended by the manufacturer or with anti-cdk1 diluted to 2 µg/ml in PBS/BSA 1% and left overnight at 4 °C. After washing, cdk1-labelled cells were incubated with 60 µl of FITC-labelled secondary antibodies diluted to 1:50 in blocking buffer for 1 h at 4 °C. Before analysis, Hoechst was added to the suspensions of labelled cells at a final concentration of 0.12 µg/ml. Two negative controls were included: cells were labelled with anti-vimentin antibody prior to the addition of the FITC-conjugated mouse isotype control instead of the CycB1 FITC-conjugated antibody, or the FITC-conjugated secondary antibody alone (for cdk1). The specificity of cyclin B1 and cdk1 antibody binding was also assessed in a similar way by using cells fixed in 1% paraformaldehyde (not shown) and also by Western blotting (see below).

Flow cytometric analysis

Experiments performed at the various developmental stages were repeated at least three times. A single experiment corresponds to the analysis of all populations at the eleven ages tested. After immunolabelling, cells were analysed using a FACSTAR Plus cytometer (Becton Dickinson) equipped with a 50-mW argon laser, tuned to 488 nm, and an INNOVA 300 ion multilined/UV laser tuned to UV. Emission fluorescence was measured with a DF 530/30 filter for FITC, a DF 575/26 filter for PE and DF 424/44 filter for Hoechst. Data acquisition and analysis were performed with "CellQuest" software (Becton Dickinson). Six data parameters were acquired and stored in list mode files: linear forward light scatter (FSC) and linear side angle light scatter (SSC) which roughly represent cell size and cellular granularity, respectively; logarithmic (log) PE and linear or log FITC to detect the immunolabelling and linear Hoechst to measure the DNA content of the different populations of cells. Contaminating events such as debris and clumped cells were eliminated from the analysis. Each acquisition was performed on 70 000 events.

Computer analysis

The vimentin-positive somatic cells were eliminated with the bivariate analysis: DNA content/vimentine. Three populations with 4C, 2C and C DNA content were selected. For each of these three populations, the bivariate FSC/SSC analysis led to the identification of the six populations depicted in Fig. 1. The CycB1 content for each of these six populations of CycB1-positive cells, was obtained by subtracting the fluorescence background from the mean fluorescence of positive cells. The same procedure was applied to determine the cdk1 content of the seven populations identified within cdk1-labelled germ cells. The linearity of these assays was checked by plotting the contents in cyclin B1 of single, doublets and triplets of round spermatids ($r = 0.930$, $p < 0.0001$) or the contents in cdk1 of single, doublets and triplets of round spermatids and of late pachytene spermatocytes ($r = 0.818$, $p < 0.0001$).

Microscopy and photography

Electronic windows were placed around defined regions as previously described, and 1 000 cells from each fraction were sorted directly onto microscopic slides. Hematoxylin/eosin-stained cells were examined with an Axioskop microscope (Carl Zeiss, Oberkochen, Germany) connected to a 3 CCD Color video camera (Sony, Tokyo, Japan). Sorted cells were identified according to their nuclear shape and cellular size utilizing the description of the XIV steps of rat spermatogenesis by Russel et al. (1990).

Preparation of cell samples

Pachytene spermatocytes (PS) or round spermatids (RS), obtained from adult (90-day-old) or 32-day-old rats by centrifugal elutriation (Weiss et al., 1997), were centrifuged and stored in liquid nitrogen. Elutriated cells were solubilized in ice-cold lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20, 0.1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, and protease inhibitor cocktail (Roche Diagnostics, Meylan, France)). After 2 h at 4 °C, the cell extracts were centrifuged at 12 000*g* for 20 min. The protein concentrations of lysates were determined with the BCA protein assay kit (Pierce, Perbio Science, Bezon, France).

Histone H1 kinase assay

An equal amount of total protein (500 µg) from each fraction of elutriated cells was precipitated with agarose p13^{suc1} (Calbiochem, Meudon, France) and tested for the presence of histone H1 kinase activity. For precipitation, 40 µl of a 50% (v/v) suspension of agarose p13^{suc1} beads per assay were incubated with cell lysate for 3 h at 4 °C. The beads were collected by centrifugation and washed four times with lysis buffer. The precipitated beads were equilibrated in kinase buffer (80 mM β-glycerophosphate, 0.1 mM sodium orthovanadate, 15 mM $MgCl_2$, pH 7.3) and collected by centrifugation. Histone H1 kinase reaction was performed in 20 µl final of kinase buffer with 1 mM DTT, 10 mM EGTA, 20 mM ATP, 10 µCi [γ-^{32}P]ATP 3000 Ci/mmol (Amersham, Orsay, France), calf thymus histone H1 (Roche Diagnostics, Meylan, France) for 1 h at room temperature. The reaction was stopped by addition of 5 µl of 5× sample buffer (Laemmli, 1970). Samples were denatured at 100 °C for 5 min and loaded onto denaturing 10% polyacrylamide gels (SDS-PAGE; (Laemmli, 1970)). Following electrophoresis with the mini-Protean II system (BioRad, Ivry-sur-Seine, France), gels were fixed, air dried and exposed to a PhosphoImager screen. Histone H1 kinase activity was analysed and quantified with a PhosphoImager STORM 840 (Molecular Dynamics, Bondoufle, France).

Western blots

Equal amounts of total protein (50 µg) were denatured in Laemmli sample buffer by boiling for 5 min and separated by 10% SDS-PAGE. Immunoblotting was performed according to the method of Towbin and Gordon (1984), using nitrocellulose membranes (Schleicher and Schuell, Nanterre, France) and a BioRad transfer apparatus. Blots were blocked for 1 h in Blotto (5% nonfat dry milk in Tris-buffered saline (TBS: 20 mM Tris-HCl, 137 mM NaCl, pH 7.5)) at 4 °C and incubated with anti-CycB1 (1:100) or with anti-cdk1 (1:500) in TBS, 0.1% Tween 20. After a 5-min wash in TBS and two 10-min washes in TBS, 0.5% Tween 20, blots were subsequently incubated with the HRP-conjugated rabbit anti-mouse IgG antibody (1:1 000) in TBS, 0.1% Tween 20, for 3 h at 4 °C to detect primary antibody binding. An enhanced chemiluminescence kit (ECL-kit, DAKO) was used to detect immune complexes, and blots were exposed to Kodak autoradiographic films.

Statistical analysis

Analysis of variance followed by the Bonferoni/Dunn test was used throughout. Differences were considered significant at $p < 0.05$.

Results

Separation of testis germ cells (after elimination of somatic cells of the testis on the basis of their positive reaction with an anti-vimentin antibody (see Materials and methods section)) (Suter et al., 1997) by flow cytometry using three different parameters (ploidy, forward scatter and side scatter) allowed the identification of 6 different populations which were further characterized by microscopic examination (Fig. 1A, B and C). Young spermatocytes (Fig. 1, R1), middle to late pachytene spermatocytes (Fig. 1, R2), round spermatids (Fig. 1, R6), and elongated spermatids (Fig. 1, R5) were obtained in pure fractions, whereas spermatogonia (Fig. 1, R3) could not be separated from preleptotene spermatocytes, and secondary spermatocytes (Fig. 1, R4) were contaminated by doublets of round spermatids. The number of cells in the different populations identified varied greatly with the age of the animals as shown in Fig. 2.

Hence, in the next series of experiments we studied the relative levels of cyclin B1 and cdk1 in these populations throughout the pubertal period of the rat. For any population studied, no significant difference in the relative cellular content of cyclin B1 between animals of different ages was ever observed (Fig. 3). By contrast, the mean levels of cyclin B1 per cell were quite different between these populations. Very low levels were observed in spermatogonia plus preleptotene spermatocytes (73 ± 4, mean ± SEM), whereas higher cyclin B1 contents were present in young spermatocytes: 163 ± 8 ($p < 0.05$, vs 73 ± 4). Cyclin B1 levels continued to increase during the meiotic prophase to reach 508 ± 21 in the population of middle to late pachytene spermatocytes. Then, they decreased slightly but significantly after meiosis

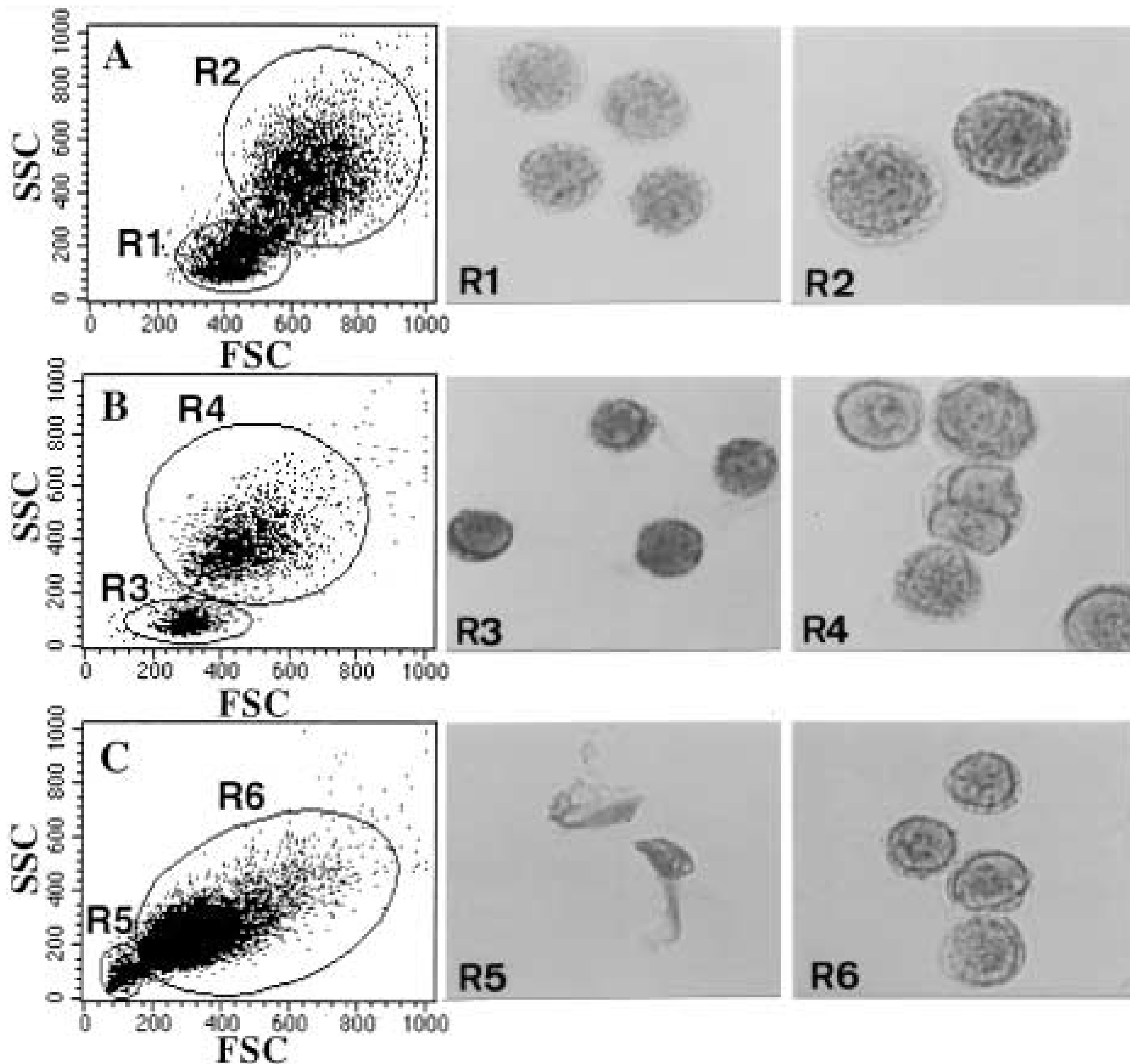


Fig. 1. Representative flow cytometry analysis and flow sort data of populations of testis germ cells obtained from adult rats. Distribution of tetraploid (**A**) diploid (**B**) or haploid (**C**) populations of testis germ cells (see Materials and methods) according to their forward scatter (FSC) and side scatter (SSC). Gated populations were sorted, stained with hematoxylin/eosine and identified as young spermatocytes (R1) spermatogonia and preleptotene spermatocytes (R3) elongated spermatids (R5) middle to late pachytene spermatocytes (R2) secondary spermatocytes and doublets of spermatids (R4) round spermatids (R6). Magnifiction: $\times 500$. Identical results were obtained in two separate experiments.

(403 ± 19 in secondary spermatocytes plus doublets of round spermatids) diminishing further in round spermatids (197 ± 10) and elongated spermatids (55 ± 8).

The relative cellular content of cdk1 was similarly evaluated (Fig. 4B and C). However, it is important to note that with the anti-cdk1 antibody, secondary spermatocytes (lower right panel: R8) could be separated from doublets of round spermatids (lower left panel: R7) as shown in Fig. 4A. Very small variations in cdk1 were observed for a given cell population obtained from animals of different ages, whereas differences did exist between these populations (Fig. 4B).

Spermatogonia plus preleptotene spermatocytes exhibited low levels of cdk1 (33 ± 1). These levels increased significantly in young spermatocytes (49 ± 1) to culminate in middle to late pachytene spermatocytes (179 ± 4). The first meiotic division resulted in a small decrease in the cdk1 content of secondary spermatocytes (124 ± 3), whereas after the second meiotic division very low values were observed in round spermatids (30 ± 2) becoming even lower in elongated spermatids (Fig. 4C).

Since both cyclin B1 and cdk1 were present at high levels at the time of the meiotic divisions, and particularly in premeiotic spermatocytes, and lower after the two divisions, i. e. in round spermatids, it was important to determine if the kinase activity of MPF was correlated to these levels. Moreover, it was of interest to determine if the kinase activity was similar in pachytene spermatocytes or round spermatids from young and adult rats. Therefore, populations of middle to late pachytene

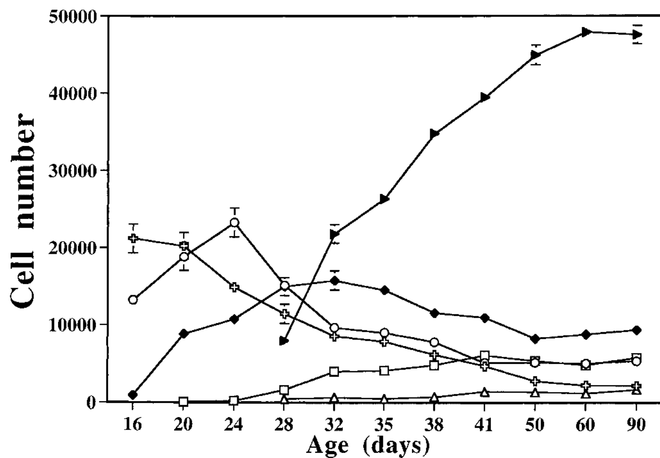


Fig. 2. The number of germ cells in the different populations identified as a function of age. (✙) spermatogonia and preleptotene spermatocytes; (○) young spermatocytes; (◆) middle to late pachytene spermatocytes; (□) secondary spermatocytes and doublets of spermatids; (▶) round spermatids; (△) elongated spermatids (most elongated spermatids and spermatozoa were removed by filtration through glass fiber as described in the Materials and methods section). Each point is the mean ± SEM of three experiments.

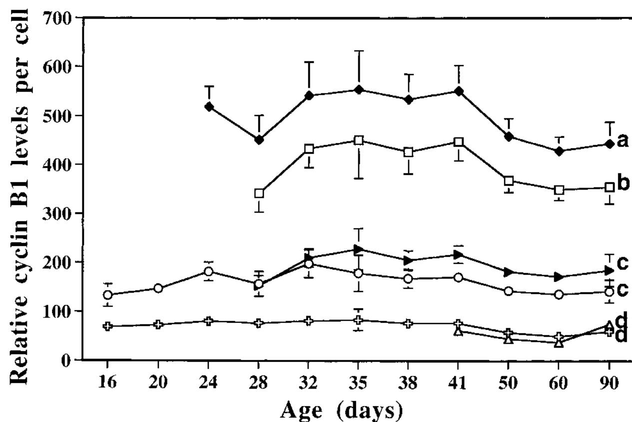
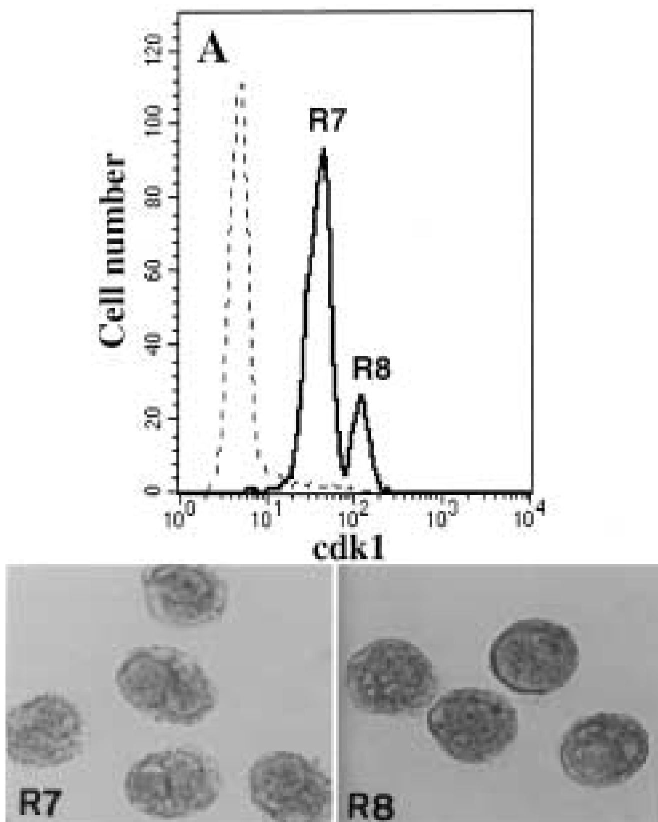


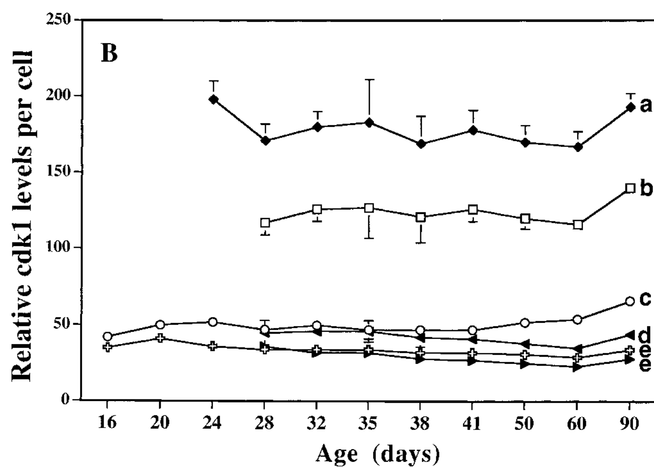
Fig. 3. Developmental changes in cyclin B1 levels in the different populations of rat testis germ cells from 16 to 90 days of age. Each point is the mean ± SEM of three experiments except for elongated spermatids (two experiments). (✙) spermatogonia and preleptotene spermatocytes; (○) young spermatocytes; (◆) middle to late pachytene spermatocytes; (□) secondary spermatocytes and doublets of spermatids; (▶) round spermatids; (△) elongated spermatids. Curves labelled with different letters are significantly different ($p < 0.05$).

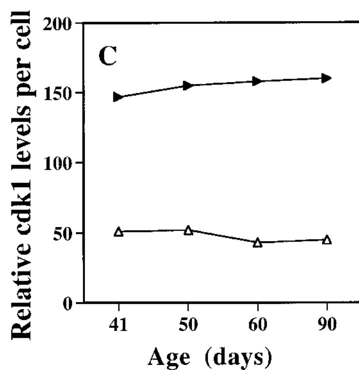
spermatocytes and of round spermatids were isolated by elutriation (see Materials and methods section) from either 32-day-old rats or from adult rats. Then lysates of the cells were analysed by immunoblotting for the presence of cyclin B1 and cdk1 or precipitated with p13*suc1* agarose; and precipitates were analysed for their ability to phosphorylate histone H1. Cyclin B1 was detected in pachytene spermatocytes and at lower levels in round spermatids (Fig. 5A). Likewise, the amount of cdk1 was higher in lysates from pachytene spermatocytes than from round spermatids (Fig. 5B). For both proteins very similar expression levels were observed in adult or 32-day-old rats. Similarly, H1 kinase activity (Fig. 5C) was higher in pachytene spermatocytes than in round spermatid fractions from both adult and young rats. However, it has to be stressed that whereas purity of pachytene fractions was 97% for both adult and young rats, and purity for round spermatids from adult rats was 84%, it was only 58% for round spermatids from 32-day-old rats; hence this last result should be considered only indicative.

Discussion

The use of flow cytometry to separate the different types of spermatogenic cells has allowed a quantification of their relative content in cyclin B1 and cdk1 much more precisely than in all previous works (Chapman and Wolgemuth, 1994; Cobb et al., 1999; Gromoll et al., 1997; Wang and Kim, 1993). Thus, it has been possible to study the relative changes in the cellular levels of these proteins at almost every stage of differentiation of spermatogenic cells of the rat, including secondary spermatocytes which cannot be isolated by other methods. Moreover, each measure was obtained from the analysis of 70000 events thus increasing its reliability. However, only relative values were obtained, and on the condition that every batch of cells was managed at the same time as the other ones, no absolute quantification of either protein could be performed. Hence, we have shown that, throughout the long meiotic prophase of spermatogenesis in the rat, there is an accumulation of both the catalytic subunit and the regulatory subunit of MPF which culminates before the first meiotic division. In this way, this process looks like what occurs in a mouse (Chesnel and Eppig, 1995) or goat (Dedieu et al., 1998) oocyte during its acquisition of competence to resume meiosis. The high cyclin B1 and cdk1 contents of middle to late pachytene spermatocytes found here are in rather good agreement with the results found in the mouse for cyclin B1 (Chapman and Wolgemuth, 1994), but not with a more recent report by Ravnik and Wolgemuth (1999) claiming that cdk1 was most apparent in early pachytene spermatocytes and diminished in diplotene and meiotically dividing spermatocytes. Moreover, the present results are at variance with those of Gromoll et al. (1997) in the rat, who could not detect cyclin B1 protein in spermatocytes but only in round spermatids by immunocytochemistry. Taken together, the results of Ravnik and Wolgemuth (1999) and those of Gromoll et al. (1997) indicate that cyclin B1 and cdk1 are not the main components of MPF in meiotic spermatogenic cells. By contrast, our results showing a strong correlation between the cellular levels of both cyclin B1 and cdk1 and the G2/M phase transition strongly suggest that these proteins are indeed important components of MPF activity. The reason for the discrepancy between the results of Gromoll et al. and our results is not clear. Using a rabbit polyclonal antibody, raised against full-length human cyclin B1, from Santa Cruz Biotechnology, as did Gromoll et al. (1997), on Western blots we got similar results to those with the GNS1 antibody we used throughout (unpublished results). However, antigen accessibility and/or threshold of detection may be quite different in preparations as different as protein extracts (our unpublished work) and slices of paraffin-embedded tissues (Gromoll et al., 1997). As for the differences in the levels of cdk1 in late pachytene spermatocytes found in our work and in that of Ravnik and Wolgemuth (1999), it may be due to modifications that affect the detection of this protein by immunohistochemistry, as







◀ **Fig. 4.** Identification of a pure population of secondary spermatocytes and developmental changes in cdk1 levels in different populations of rat testis germ cells. (**A**) Histogram of cdk1 fluorescence of the R4 population (Fig. 1) composed of secondary spermatocytes (*lower right panel*) and doublets of spermatids (*lower left panel*) labelled with anti-cdk1 antibody (*continuous line*) or with control antibody (*dotted line*). Two new distinct populations: R7 and R8, were observed based on their cdk1 fluorescence. Gated cells were sorted and identified. The R8 population with the highest fluorescence level was composed exclusively of secondary spermatocytes, and R7 of doublets of spermatids. (**B**) Developmental changes in cdk1 levels in the different populations of rat testis germ cells from 16 to 90 days of age. Each point is the mean ± SEM of three experiments. (✣) spermatogonia and preleptotene spermatocytes; (○) young spermatocytes; (◆) middle to late pachytene spermatocytes; (□) secondary spermatocytes; (◀) doublets of spermatids; (▶) round spermatids. Curves labelled with different letters are significantly different ($p < 0.05$) (**C**) Developmental changes in cdk1 expression in (▶) round and (△) elongated spermatids from 41 to 90 days of age. Each point is the mean of two experiments.

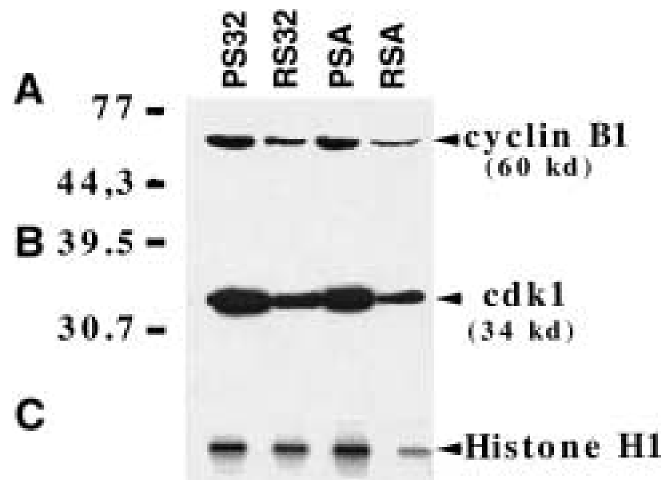


Fig. 5. Immunoblot analysis of cyclin B1 and cdk1 proteins, and histone H1 kinase activity in lysates from pachytene spermatocytes (PS) and round spermatids (RS). Equal amounts of protein lysates of elutriated germ cells (PS from 32-day-old (PS32) or adult (PSA) rats and RS from 32-day-old (RS32) or adult (RSA) rats) were separated by SDS-PAGE, transferred to nitrocellulose and incubated with anti-cyclin B1 (**A**) or anti-cdk1 (**B**) antibodies. Molecular mass markers are indicated in kilodaltons on the left. The positions of migration of cyclin B1 (**A**) and cdk1 (**B**) are indicated on the right. 50 µg of total protein was loaded per lane. (**C**) Histone H1 kinase activity of cyclin B1-cdk1 complex precipitated with p13^{suc1} agarose beads. Precipitates were analysed for H1 kinase activity by incubation with histone H1 as substrate and [γ-^{32}P]ATP (see Materials and methods section). Similar results were obtained in two other experiments.

suggested also by these authors. Anyhow, immunohistochemistry is not a "quantitative" method.

It has been shown recently (Cobb et al., 1999; Tarsounas et al., 1999) that young spermatocytes are not competent to respond to okadaic acid whereas treatment of pachytene spermatocytes causes precocious entry into metaphase I of meiosis. Cobb et al. (1999) reported that both young and late spermatocytes have the molecular components of metaphase promoting factor cdk1 and cyclin B1, as assessed by immunoblot analysis. Our results, however, show that young spermatocytes have a much lower content of both cyclin B1 and cdk1

than late pachytene spermatocytes. The differences between our results and those of Cobb et al. are most likely explained by a difference in the sensitivity of the methods used in these two works. Hence, we propose that meiotic competence of spermatocytes could indeed be related not only to the first appearance of testicular histone H1t and CDC25 protein phosphatase, as suggested by Cobb et al., but also to a threshold content of MPF components. This hypothesis is supported by the observation that after 2 days of culture, germinal vesicle breakdown-incompetent mouse oocytes in vitro accumulate $p34^{cdc2}$ autonomously and become progressively more responsive to okadaic acid (Chesnel et al., 1994).

After the first meiotic division, cyclin B1 and cdk1 contents of spermatocytes II were still 80% and 70%, respectively, of those of middle to late pachytene spermatocytes, whereas after the second meiotic division, the contents of round spermatids in these proteins were only 39% and 17%, respectively, of the levels before the first division. That is why under our experimental conditions, secondary spermatocytes could be easily distinguished from doublets of round spermatids on the basis of their content in cdk1 but not on the basis of their content in cyclin B1. Indeed, if the content in cyclin B1 of a doublet of round spermatids is two-fold that of a single round spermatid, cyclin B1 content of a doublet should be identical to that of a secondary spermatocyte; this fits closely with the results of our experiments. By contrast, the content in cdk1 of the doublet of round spermatids should not exceed half that of the secondary spermatocyte. If so, it would mean that after the second meiotic division, cdk1 is destroyed more quickly than cyclin B1. This is a little unexpected, but Chapman and Wolgemuth (1994) claimed that a low level of 34-kDa cdc2 protein was "occasionally" detected in round spermatids, and Wang and Kim (1993) did not detect this protein in these cells. The fact that secondary spermatocytes exhibited cyclin B1 and cdk1 contents higher than half of those found in middle to late pachytene spermatocytes suggests that cyclin B1 and cdk1 synthesis occurs between the two meiotic divisions. However, additional experiments with cultured cells (Weiss et al., 1997) are required to answer this question definitely.

Similar levels of both cyclin B1 and cdk1 proteins were measured in each spermatogenic cell population present between 16 and 90 days post-partum, despite an efficiency of the spermatogenic process much lower in young than in mature ($\approx$70-day-old) rats (Russell et al., 1987). This indicates that the content in MPF components of rat spermatogenic cells is a characteristic of the different cell populations and is probably not related to the age-dependent changes in apoptosis observed during the prepubertal period (Billig et al., 1995).

In the present study, very low contents of cyclin B1 were measured in spermatogonia. Gromoll et al. (1997) could not detect a specific immunostaining for cyclin B1 in rat spermatogonia, whereas Wang and Kim (1993) found that the replenishment of retinol to vitamin A-deficient rats resulted in an unchanged level of cyclin B1, as assessed by immunoblotting. Taken together these results indicate that cyclin B1 levels in spermatogonia are indeed much lower than in pachytene spermatocytes, and that this low level does not reflect a dilution of the dividing spermatogonia into the total population. Similarly, low levels of cdk1 were present in these cells. Wang and Kim (1993) reported that the amount of p34cdc2 was roughly similar in retinol-depleted rat testes and in retinol-treated testes but that the H1 histone kinase activity was low in the testis deficient in vitamin A compared to activity at 24 h after retinol treatment. Taken together, these results suggest strongly that the meiotic G2/M transition requires much higher levels in both cyclin B1 and cdk1 than does the mitotic G2/M transition of spermatogenic cells.

At least, in pachytene spermatocytes and in round spermatids, the presence of cyclin B1 and cdk1 was associated to histone H1 kinase activity. However, the specific activity of round spermatid fractions was 3 to 4-fold lower than that of pachytene spermatocyte fractions in adult testes. This fits well with the results of Chapman and Wolgemuth (1994) in the mouse and those of Wang and Kim (1993) in the rat. More importantly, our results show that at least for pachytene spermatocytes, similar contents in both cyclin B1 and cdk1 were associated to a similar histone H1 kinase activity either at 32 or 90 days of life.

In summary, our study using flow cytometric analysis of rat spermatogenic cells allowed a precise evaluation of the changes in the relative cyclin B1 and cdk1 contents of spermatogenic cells through their different stages of differentiation from spermatogonia to elongating spermatids. As opposed to other studies (Cobb et al., 1999; Gromoll et al., 1997; Ravnik and Wolgemuth, 1999), our results showed a strong correlation of MPF components with the meiotic G2/M phase transition in spermatogenic cells; hence they suggest that the cellular levels of both cyclin B1 and cdk1 are among the limiting factors for meiosis of spermatogenic cells. It has been shown also that the increasing efficiency of spermatogenesis during pubertal development is most probably not related to changes in the content of germinal cells in MPF components. Studies are in progress in our laboratory to determine the effects of inhibiting MPF activity in cocultures of testicular germinal cells and somatic cells (Weiss et al., 1997).

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