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Original Article

Expression of proliferating cell nuclear antigen in the mouse germ line and surrounding somatic cells suggests both proliferation-dependent and -independent modes of function

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ABSTRACT The distribution of proliferating cell nuclear antigen (PCNA) in specific somatic and germ cells of the adult mouse ovary and testis was assessed using immunocytochemical staining and immunoblot analysis and was correlated with cellular proliferation and differentiation. In the adult ovary, immunocytochemical staining for PCNA within follicular cells varied depending on the stage of follicular growth. Since PCNA staining has proven to be a useful indicator of cells involved in DNA synthesis and repair, the pattern of PCNA staining in the ovary was compared to previous studies which used tritiated thymidine labeling as a marker for DNA synthesis. In the testis, PCNA was detected in the mitotically proliferating spermatogonia, but not in spermatocytes which had just entered meiosis. PCNA staining was again observed in spermatogenic cells in later stages of meiotic prophase, in particular zygotene and pachytene spermatocytes. As these cells are undergoing meiotic recombination, the presence of PCNA in these meiotic prophase cells could reflect a second function of PCNA, that of DNA excision repair.

KEY WORDS: *cell proliferation, spermatogenesis, folliculogenesis*

Introduction

PCNA is a highly conserved 36 kDa nuclear protein that serves as an auxiliary protein of DNA polymerase and functions during DNA replication (Bravo *et al.*, 1987; Prelich *et al.*, 1987a,b). The synthesis of PCNA increases during late G1 and S phase (Bravo and MacDonald-Bravo, 1987). During S phase there is a redistribution of PCNA: in early S phase, PCNA antibodies revealed a diffuse localization, whereas during the period of maximum DNA synthesis a more punctate pattern was observed (Bravo and MacDonald-Bravo, 1985; Celis and Celis, 1985). PCNA has also been found localized to the sites of DNA damage in the nuclei of non-S phase cells which have been exposed to ultraviolet irradiation (Celis and Madsen, 1986; Toschi and Bravo, 1988). In addition, recent *in vitro* studies have shown that PCNA is required for the DNA synthesis that converts nicked intermediates to completed repaired ends, thus demonstrating that PCNA is also required for DNA excision repair (Shivji *et al.*, 1992).

Treatment of exponentially growing murine 3T3 cells with antisense oligonucleotides to PCNA results in complete suppression of DNA synthesis and ensuing mitosis, suggesting that PCNA is important for DNA synthesis and cell cycle progression (Jaskulski *et al.*, 1988). In human fibroblast cell lines, PCNA has been shown to associate with several cell cycle-regulating proteins, including cyclins D1 and D3 and several cyclin-dependent kinases (Cdks) (Xiong *et al.*, 1992), which are believed to function during G1 or S phase (reviewed in Pines, 1993; Sherr, 1993). Association of PCNA with these cell cycle proteins further suggests a role for PCNA in cell cycle progression.

Recent studies from our laboratory have revealed that various cell cycle genes are expressed in distinct, developmentally regulated patterns during mammalian gametogenesis, including stages which are not undergoing active cell division. For example, *cdc2* is expressed predominantly in meiotic prophase, in pachytene spermatocytes, while *PCTAIRE-1* and *-3* mRNAs are most abundant in later stages of spermatids (Rhee and Wolgemuth, in preparation). The B-type cyclins, regulators of the Cdc2 and Cdk2 kinases, have also been found to be expressed in stage-restricted patterns during male germ cell development. *cycB1* is expressed at low levels in late pachytene spermatocytes and at highest levels in early round spermatids (Chapman and Wolgemuth, 1992), whereas *cycB2* is expressed at highest levels in late pachytene spermatocytes and at much reduced levels in early round spermatids (Chapman and Wolgemuth, 1993).

Abbreviations used in this paper: PCNA, proliferating cell nuclear antigen; cycB1, cyclin B1; cycB2, cyclin B2; cdks, cyclin-dependent kinases.

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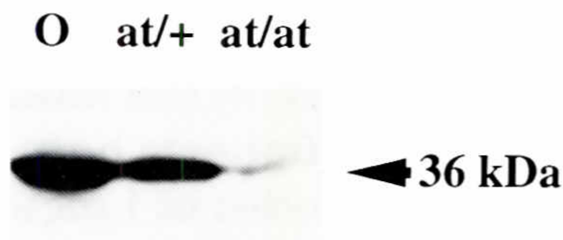

Fig. 1. Expression of PCNA in the testis and ovary. *Lysates from adult ovary (O) and from the testes of mice heterozygous (at/+) and homozygous (at/at) for the atrichosis locus were examined for the presence of PCNA proteins by immunoblotting with PCNA antibodies. The relative molecular weight of the polypeptide is indicated. 100 µg of total protein was loaded per lane.*

Given the distinct and surprising patterns of expression of cell cycle genes in the mouse germ cell lineage, we wished to determine whether PCNA was co-expressed with any of these cell cycle genes during specific stages of male germ cell development. Furthermore, it was of interest to determine whether the presence of PCNA was correlated with DNA synthesis and/or repair. A monoclonal antibody raised against rat PCNA has been shown to be useful for Western analysis and for immunocytochemical localization of the protein in paraffin embedded tissues. We therefore used this antibody to assess the distribution of PCNA in the adult mouse ovary and testis by both immunoblotting and immunocytochemistry.

Results

Immunoblot analysis of PCNA in the testis and ovary

The expression of PCNA in the testis and ovary was initially examined by immunoblot analysis of lysates from adult ovary and from the testes of mice homozygous and heterozygous for the *atrichosis* locus. A discrimination between expression in the somatic or germinal compartment of the testis can be made by utilizing the testes of mice homozygous for the *atrichosis* mutation, which are devoid of germ cells, but have the normal complement of somatic cells, including Leydig, Sertoli and peritubular myoid cells (Hummel, 1964). The testes of heterozygous littermates have the normal complement of germ and somatic cells and are fertile. Antibody to PCNA detected a 36 kDa protein in the adult mouse ovary as well as in the testes of *at/+* and *at/at* mice (Fig. 1). However, the level of PCNA was greatly reduced in the germ cell-deficient *(at/at)* testis sample as compared to the normal *(at/+)* testis sample. This result suggests that PCNA is present at low levels in the somatic cells of the testis, but is expressed at higher levels in the germ cell compartment.

To examine the distribution of PCNA in specific types of meiotic and post-meiotic germ cells, lysates from separated testicular cells were also analyzed for the presence of PCNA (Fig. 2). Testicular cells were separated into three enriched populations: pachytene spermatocytes, round spermatids and residual bodies and cytoplasmic fragments. Residual bodies are the cytoplasm that is naturally sloughed off from the elongating spermatids during spermiogenesis. Cytoplasmic fragments arise as artifacts of the cell separation technique and are the cytoplasm that is prematurely shed from the elongating spermatids. PCNA was detected in each of the enriched populations. Since equal amounts of protein were loaded in each lane, it would appear that the highest levels of PCNA are present in pachytene spermatocytes and that the levels decreased progressively in round spermatids and in the pool of residual bodies and cytoplasmic fragments. Immunoblotting thus revealed the presence of PCNA in both the germinal and somatic cell compartments of the testis.

Immunocytochemical localization of PCNA in the ovary and testis

To further localize PCNA to specific cell types within the testis and ovary and to correlate this localization with specific stages of the cell cycle, immunocytochemical detection was performed. In the testis, it is possible to recognize the particular cell cycle stage of many of the cells based on morphological criteria and their characteristic patterns of association within the seminiferous tubule. It is more difficult to determine this in the ovary, particularly in the somatic cells. This histological approach also yields additional information with regard to the form of PCNA present in the cells. There are two forms of the PCNA protein; organic solvent-sensitive and -insensitive forms (Bravo and MacDonald-Bravo, 1987). The nucleoplasmic form of PCNA, which can be extracted by organic solvents, is present in quiescent cells and is not involved in DNA synthesis. The organic solvent-insensitive form is localized to sites of DNA replication and is associated with ongoing DNA synthesis and repair. As the tissues used in these studies were treated with organic solvents, only the PCNA involved in DNA synthesis and repair will be detected with immunohistochemical analysis.

The growth phase of follicular development has been characterized and divided into stages, based on the size of the oocyte and the number of granulosa cells surrounding the oocyte (Pedersen and Peters, 1968). Resting oocytes are found in small follicles (type 1 to 3b), while growing oocytes are found in medium (type 3b to 5a) and large follicles (type 5b to 8). PCNA staining was observed in the granulosa cells of growing follicles (Fig. 3A-C), while little or no staining was detected in the granulosa cells of small follicles (Fig. 3A). In medium follicles, less than 10% of the granulosa cells

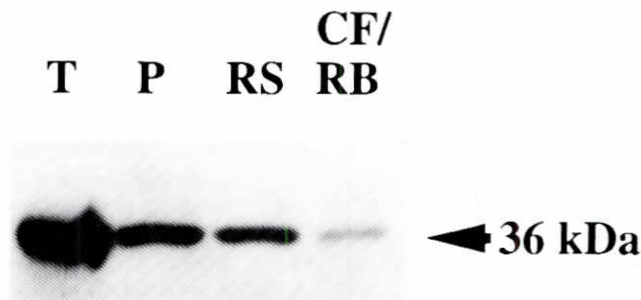

Fig. 2. Immunoblot analysis of PCNA in separated testicular cells. *Testicular cells were separated into purified pools of pachytene spermatocytes (P), round spermatids (RS), and cytoplasmic fragments and residual bodies (CF/RB). Lysates from the pooled cells, as well as from total testis (T), were analyzed for the presence of PCNA proteins by immunoblotting with a PCNA antibody. The relative molecular weight of the polypeptide is indicated. 50 µg of total protein was loaded per lane.*

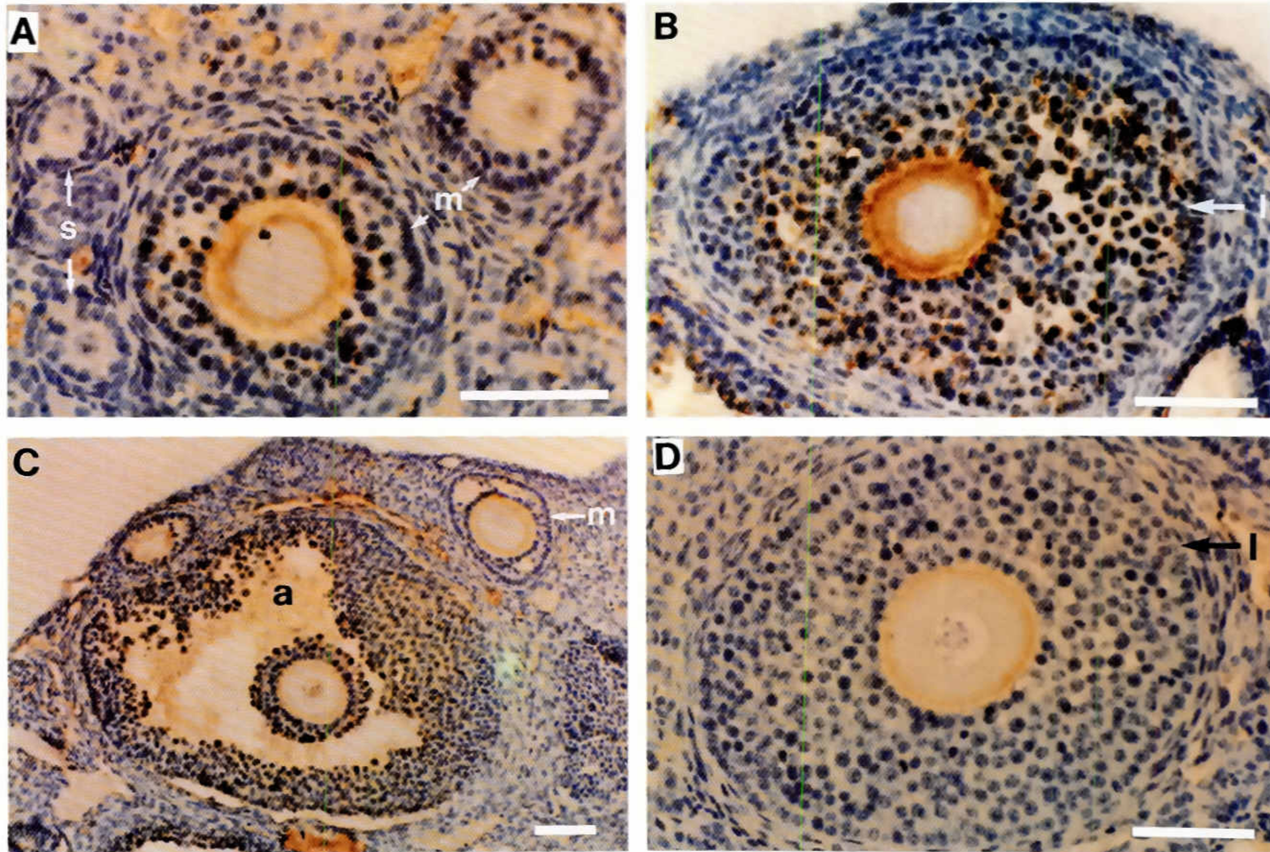

Fig. 3. Immunocytochemical localization of PCNA in the adult ovary. *Paraffin-embedded ovary sections were stained with immunoperoxidase using antibodies against PCNA* **(A-C)** *or normal mouse serum as the primary antibody* **(D)**. *Follicle types are designated as follows: small (s), medium (m), large (l), and antral (a). The sections were counter-stained with hematoxylin, which stains the nucleus blue. Immunoperoxidase staining is brown. Bars A-D, 100 μm.*

stained for PCNA, while 40 to 50% of the granulosa cells of large follicles stained. In antral follicles, more of the granulosa cells closest to the oocyte, as compared to those near the basement membrane, stained for PCNA (Fig. 3C). Occasionally, the nuclei of oocytes found in medium and large follicles stained for PCNA (Fig. 3A-C), as did a few of the thecal cells surrounding the follicles (data not shown). No staining was detected in the cells of the corpus luteum, although the granulosa cells of atretic follicles continued to stain for PCNA (data not shown). Negative controls, using no primary antibody or using purified mouse IgG as a primary antibody, were run in parallel to determine non-specific staining. Non-specific staining was detected in the zona pellucida surrounding the oocyte and in the blood vessels (Fig. 3D).

In the testis, PCNA-specific staining was observed in the nuclei of the mitotically dividing spermatogonia and in a subset of the spermatocytes (Fig. 4A). Staining was detected in the interstitial Leydig cells in both the normal (Fig. 4A) and the germ cell-deficient testes (data not shown); however, this was non-specific since it was also observed in negative controls using normal mouse serum as the primary antibody (Fig. 4B). A detailed analysis of PCNA localization in specific stages of spermatogenesis was undertaken by determining the stages of the seminiferous tubules containing stained cells. Staging of the tubules was performed according to Oakberg (1956b) and Russell *et al.* (1990) and is indicated in Fig. 4C-J. PCNA staining was observed in spermatogonia, most easily observed in tubule stages II-VI (panels C-E). PCNA staining was also observed in spermatocytes in leptotene and zygotene of prophase I (stages IX-XII, panels H-J), as well as in early pachytene spermatocytes (stages I-V, panels C-E). However, by mid to late pachytene (stages VIII-XII, panels G-J), PCNA was no longer detectable by immunohistochemistry. Surprisingly, no PCNA staining was detected in preleptotene spermatocytes (stages VII-VIII, panels F-G). A summary of PCNA staining in the adult testis is presented schematically in Fig. 5.

Discussion

PCNA expression correlates with cellular DNA synthesis during folliculogenesis

There are several methods available for identifying cells active in DNA synthesis at the histological level. Cells can incorporate tritiated thymidine which can be detected by autoradiography. Cells can also be labeled with bromodeoxyuridine, which is incorporated into the DNA instead of thymidine. The cells can then be

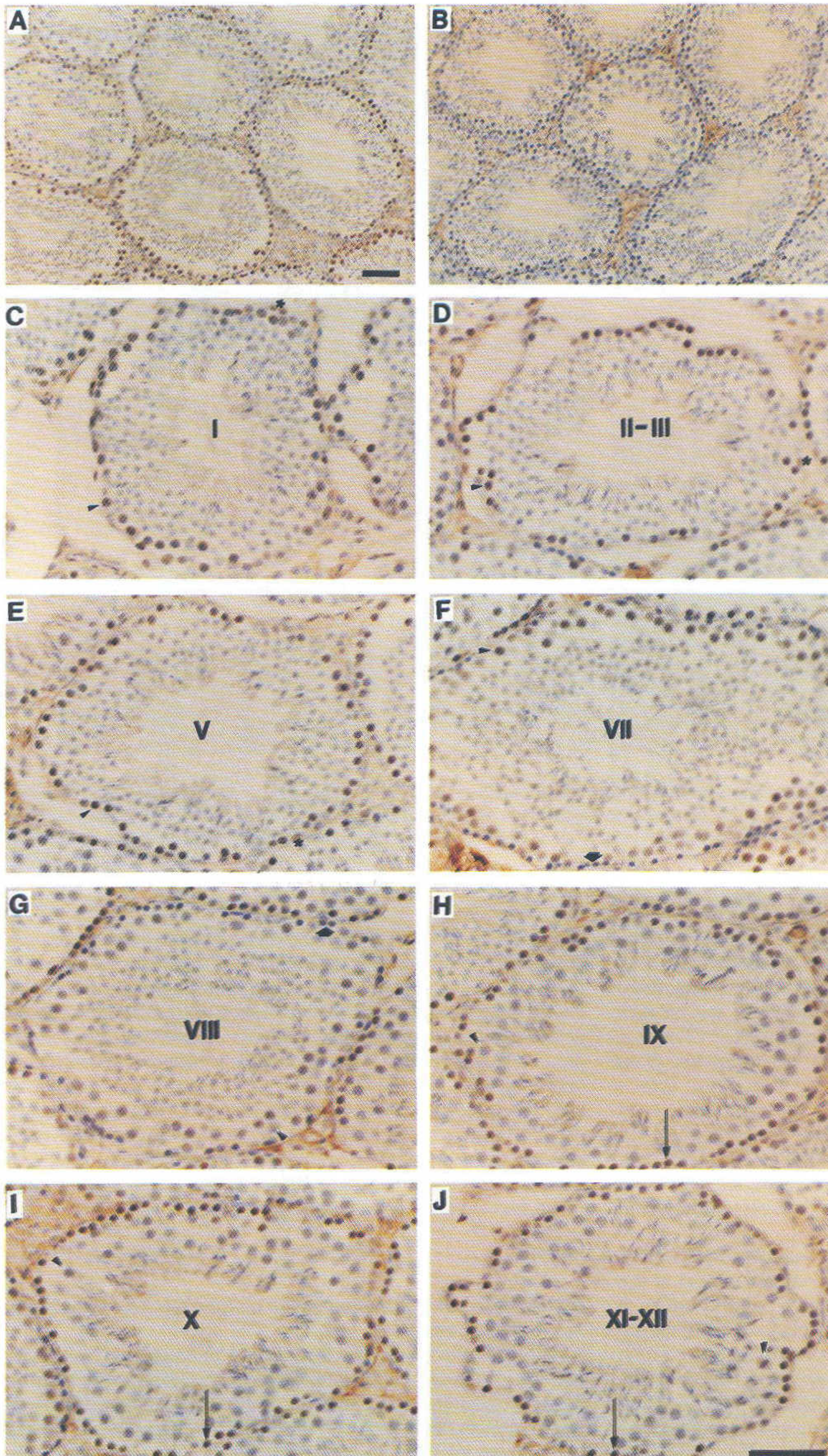

Fig. 4. Immunocytochemical localization of PCNA in the testis. *Paraffin embedded at/+ testis sections were stained with immunoperoxidase using a PCNA monoclonal antibody* **(A, C-J)** *or normal mouse serum as the primary antibody* **(B)**. *The sections were counterstained with hematoxylin, which stains the nucleus blue. Immunoperoxidase staining is brown. Staging of the tubules was according to (Oakberg, 1956b and Russell et al., 1990) and are indicated by Roman numerals in* **C-J**. *Representative cell types are indicated as follows: (L) Leydig cells* **(A)**; *(*) spermatogonia* **(C-E)**; *(short arrow) preleptotene spermatocytes* **(F-G)**; *(long arrow) leptotene spermatocytes* **(H and I)**; *(long arrow) zygotene spermatocytes* **(J)**; *(arrow heads) pachytene spermatocytes* **(C-I)**; *(arrow head) diplotene/secondary spermatocytes* **(J)**. *Bars: A, 100 µm and is identical for B; J, 100 µm and is identical for C-J.*

probed with an antibody against bromodeoxyuridine. Finally, paraffin-embedded tissues can be stained using an antibody against PCNA. There are two major advantages for using PCNA as a marker for cells involved in DNA synthesis (reviewed in Yu *et al.*, 1992). First, immunocytochemical staining for PCNA does not require the use of fresh or frozen tissues, thereby enabling retrospective studies of paraffin embedded tissues. Second, this staining procedure does not require the use of radioactive isotopes. Presented below is a discussion of PCNA immunocytochemical staining in the context of previous results using pulse-labeling of ovaries with tritiated thymidine followed by autoradiography to monitor follicular growth (reviewed by Pedersen, 1972).

Cells within the single layer of granulosa cells in follicles containing resting oocytes are non-proliferating (Pedersen, 1972); correspondingly, the granulosa cells of these small follicles do not stain for PCNA (Fig. 3A). In agreement with tritiated thymidine labeling, less than 10% of the granulosa cells in medium-sized growing follicles stained for PCNA (Fig. 3A), while approximately 40-50% of granulosa cells in large follicles (Type 5b-8) stained for PCNA (Fig. 3B-C). Studies using tritiated thymidine labeling also revealed that thecal cells surrounding large follicles proliferate at this time (Pedersen, 1972). Although thecal cells surrounding the medium and large follicles were occasionally stained for PCNA (data not shown), this staining was not an obvious feature.

The granulosa cells in antral follicles can be divided into two groups, the cumulus and mural granulosa cells (reviewed in Buccione *et al.*, 1990). The cumulus granulosa cells associate with the oocyte before and after ovulation. The mural granulosa cells are more peripheral and are attached to the basement membrane which encloses the follicle. Studies in which rat ovaries were labeled with tritiated thymidine revealed that these two different types of granulosa cells display distinct patterns in that the cumulus granulosa cells were shown to divide more frequently than the mural granulosa cells (Hirshfield, 1986). Correspondingly, our studies with PCNA localization revealed more cumulus granulosa cells staining for PCNA as compared to the mural granulosa cells (Fig. 3C).

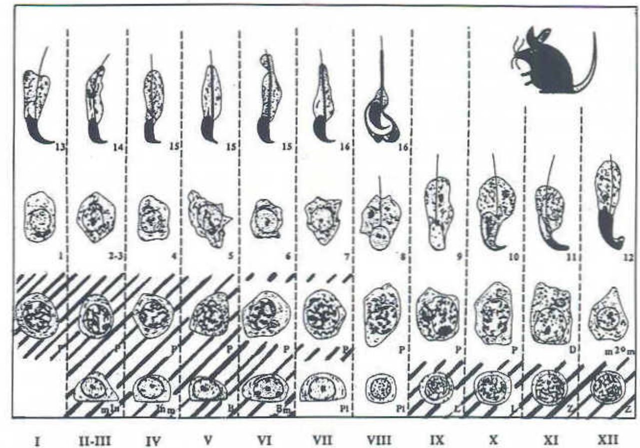

Fig. 5. Summary of PCNA expression in the testis. *Staging of the seminiferous tubules is adapted from Russell et al. (1990). Cell types are indicated as follows: intermediate (In) and type B (B) spermatogonia; preleptotene (Pl), leptotene (L), zygotene (Z), pachytene (P), diplotene (D), and secondary (2°) spermatocytes. The 16 stages of spermiogenesis (1-16) show a range of spermatids from round to elongating spermatids. Mitotic or meiotic cell divisions are indicated by (m). The cross-hatching indicates the cell types expressing PCNA.*

Distinct patterns of PCNA expression during spermatogenesis

Immunocytochemical detection specifically localized PCNA to spermatogonia and to particular stages of spermatocytes. In contrast, immunoblot analysis revealed the presence of the PCNA protein in both the somatic and germ cell compartments of the testis. Within the germinal compartment, the 36 kDa protein was detected in later stages of spermatocytes and in spermatids (although the level of the protein decreased in round spermatids and thereafter). One possible reason for these differences using the two different detection techniques is the fact that, as discussed above, there are two distinct forms of PCNA, organic solvent-sensitive and -insensitive. Both the organic solvent-sensitive and -insensitive forms will be detected by Western analysis, while only the organic solvent-insensitive form will be detected by the immunocytochemistry (Bravo and MacD onald-Bravo, 1987).

Hall *et al.* (1990) had previously reported PCNA staining in the majority of spermatogonia and no staining present in spermatids or sperm in the human testis. However, these studies did not determine the specific stages of spermatogenesis in which PCNA was detected, perhaps due to the fact that it is much more difficult to stage human as opposed to rodent spermatogenic cells by their association patterns. In the present study, we have more accurately described the localization of PCNA to specific spermatogenic stages and in so doing, have observed intriguing patterns of PCNA expression.

PCNA was detected in the mitotically dividing spermatogonia, as also noted by Hall *et al.* (1990). Curiously, PCNA was not detected in the pre-leptotene spermatocytes (Fig. 4, panels F-G), the stage at which pre-meiotic DNA synthesis is supposedly occurring. It is possible that DNA synthesis in pre-meiotic cells, as opposed to pre-mitotic, does not involve PCNA. Alternatively, the replication complex could involve association with a meiosis-specific protein that masks PCNA, since the antibody is a monoclonal. It was also surprising that PCNA was found in leptotene, zygotene and pachytene spermatocytes, stages following DNA synthesis in the pre-leptotene stage. Since PCNA is a stable protein and can be detected in quiescent cells 24 to 48 h after cells stop dividing (Bravo and MacDonald-Bravo, 1987), one possibility was that the PCNA observed in meiotic prophase cells was due to persistence of PCNA synthesized during mitotic proliferation of the spermatogonia. However, PCNA staining was not detected in the pre-leptotene spermatocyte stage, which comes between the last mitotic division and the stages of meiotic prophase in which PCNA was again detected. If the absence of PCNA staining in the pre-leptotene spermatocytes was due to redistribution of PCNA to the nucleoplasm, then the presence of PCNA in leptotene spermatocytes may suggest that PCNA becomes reassociated with the DNA at this stage, and therefore becomes or is insensitive to organic solvents.

While this is plausible, it does not take into account the amount of time that has transpired from the last mitotic stages in which

PCNA is detected to the meiotic stages in which it is once again observed. Although PCNA has been estimated to have a 20-h half life (in mitotic cells), approximately 41 hours have passed during this time interval (duration of the cycle of the seminiferous tubule according to Oakberg, 1956a). Alternatively, the PCNA found in meiotic prophase may be newly synthesized. The presence of PCNA in the meiotic prophase compartment of the testis could involve the second function of PCNA, that of DNA excision repair. PCNA is required for the DNA synthesis that converts nicked intermediates into repaired products (Shivji *et al.*, 1992). As these cells are undergoing recombination, it is possible that PCNA in these meiotic cells functions in the DNA excision repair that is part of the recombination process.

Materials and Methods

Source of tissues

Swiss Webster mice, obtained from Charles River (Wilmington, DE, USA) and C57Bl/6J mice, obtained from Jackson Laboratory (Bar Harbor, ME, USA) were used as the source of adult testes and ovaries, respectively. Testes were obtained from mice older than 35 days. Ovaries were obtained from mice older than 21 days. Mice of the strain *atrichosis (at) (ATEB/Le a/a dat/deb)* were obtained from the Jackson Laboratory. Dissected tissues for immunocytochemistry were fixed in 4% paraformaldehyde in 1xPBS (1xPBS: 130 mM NaCl, 7 mM Na_2HPO_4, 3 mM NaH_2PO_4) overnight (ON) at 4°C.

The paraformaldehyde was replaced by 1xPBS, followed by washes of 0.85% saline, 0.85% saline/ethanol (1:1), and 70% ethanol. Tissues were dehydrated by transfer through graded ethanols, followed by toluene and were then embedded in paraffin wax (1:1 mixture of Paraplast and TissuePrep2, Fisher Scientific, Fairlawn, NJ, USA). Sections were cut at 8 µm, collected onto Tespa-treated slides (Rentrop *et al.*, 1986) and stored at 4°C until needed.

Separation of testicular cells

Testicular cells of Swiss Webster mice were separated on a 2-4% BSA gradient at unit gravity as described by Wolgemuth *et al.* (1985). Pools of pachytene spermatocytes, round spermatids, and residual bodies and cytoplasmic fragments were isolated with average purities of 76, 78, and 73%, respectively.

Tissue preparation and protein quantitation

Fresh or frozen tissues and cells were homogenized in 2x sample buffer (Laemmli, 1970), denatured for 5 min at 100°C and clarified by centrifugation. Homogenates were either used immediately or stored at -70°C. The amount of protein in each sample was determined by the Bradford dye assay (Bradford, 1976).

Description of antibodies

The PCNA monoclonal antibody, PC10, was obtained from Oncogene Science, Inc. (Uniondale, NY, USA). The mouse monoclonal antibody was generated against rat PCNA and was used at a concentration of 10 µg/ml for Western blot analysis and 20 µg/ml for immunocytochemistry.

Western blot analysis

Protein lysates were run on SDS-polyacrylamide gels and blotted onto nitrocellulose using a Hoeffer tank transfer apparatus. Blots were blocked for 2 h in Blotto — 6% non-fat dry milk in Tris buffered saline (1xTBS: 20 mM Tris, pH 7.5, 150 mM NaCl) — at room temperature (RT). Blots were incubated with primary antibodies in Blotto for 1-2 h at RT and then rinsed three times for 10 min in 1xTBS. The blots were then incubated with the secondary antibody, goat anti-mouse IgG (Pierce, Rockford, IL, USA) conjugated to horseradish peroxidase (1:20,000), for 1-2 h at RT, followed by three 10-min washes in 1xTBS. An Enhanced ChemiLuminescence Kit (ECL Kit, Amersham, Chicago, IL, USA) followed by autoradiography was used to detect immune complexes.

Immunocytochemistry

The avidin-biotinylated-peroxidase complex (ABC) method was used for immunocytochemistry (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA). Prior to incubation with the primary antibody, paraffin-embedded sections were deparaffinized by two 10-min incubations in xylene. Tissues were rehydrated by passage through graded ethanols and then washed in 1xPBS containing 0.1% Triton X-100 (1xPBST). Endogenous peroxidase activity was abolished by incubating sections in methanol containing 0.3% hydrogen peroxide for 20 min. Sections were then rehydrated by two 10-min washes in 1xPBST. All of the following incubations were performed in a humidified container. Sections were blocked by pre-incubating with dilution solution — 1xPBST, 1% normal goat serum — with the addition of normal horse serum for 1 h at 4°C. Sections were incubated with the primary antibody (20 µg/ml) in dilution solution plus normal horse serum ON at 4°C. The sections were rinsed by three 10-min changes of 1xPBST, followed by incubation with biotinylated horse anti-mouse IgG (1:200) in dilution solution for two h at RT. Sections were washed by three 10-min rinses in 1xPBST. Sections were incubated in ABC reagent in dilution solution for 2 h at RT, followed by three 10-min washes in 1xPBST. Sections were then equilibrated in 0.1 M Tris, pH 7.2 for 5 min. Immunostaining was visualized using 0.2 mg/ml diaminobenzadine and 0.01% hydrogen peroxide in 0.1 M Tris, pH 7.2. The sections were counter-stained with hematoxylin and coverslipped using Protexx mounting media (Baxter). Parallel controls were performed using either normal mouse serum (SIGMA, cat. # I-5381) as the primary antibody or no primary antibody. Staining in the experimental was interpreted as specific if there was an absence of staining in the particular cells in the controls. Slides were viewed on a Leitz photomicroscope under bright field optics and photomicrographs were taken with Kodak Ektar film.

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