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The First and The Second Mitotic Phases of Spermatogonial Stage in *Xenopus laevis*: Secondary Spermatogonia Which Have Differentiated after Completion of The First Mitotic Phase Acquire an Ability of Mitosis to Meiosis Conversion

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ABSTRACT—In *Xenopus laevis*, the spermatogenic cells derived from a primary spermatogonium divide synchronously and form a cyst surrounded by Sertoli cells. Therefore, it is easy to know how many times the spermatogenic cells divide from the primary spermatogonium by counting the cells in a cyst. In the present report, the distinctive feature of mitotic divisions during the spermatogonial stage in *Xenopus laevis* is described as having two phases. The first phase took place during five mitotic divisions. During this phase, the nucleus of primary spermatogonium, which was identified by its large size and lobulated shape with weak stainability, progressively decreased in size and became round with heterochromatin patches. In the second phase, the spermatogonia, that had completed the fifth mitotic division and morphologically differentiated into the secondary spermatogonia, could undergo up to three additional mitotic divisions without any morphological change. From the observation of the cysts that consisted of approximately 2^5, 2^6, 2^7, or 2^8 primary spermatocytes, it was further suggested that the secondary spermatogonia at the second phase could also enter a meiotic phase. All primary spermatocytes were believed to have the ability to undergo the meiotic phase-specific gene expression and two meiotic divisions because the round spermatids in the cyst consisting of approximately 2^7, 2^8, 2^9, or 2^{10} cells expressed SP4 mRNA which was transcribed in the spermatogenic cells at mid-pachytene stage and thereafter. These observations suggested that after the fifth mitotic division, the spermatogenic cells differentiated into the secondary spermatogonia and acquired an ability for mitosis to meiosis conversion.

INTRODUCTION

Conversion from mitosis to meiosis is a characteristic feature in gametogenesis because a diploid chromosome set is reduced by meiosis to a haploid set which culminates in the successful development of fertilized eggs without chromosome multiplication from generation to generation. During the spermatogenesis, spermatogonia that have undergone several mitotic divisions enter the meiotic prophase and differentiate into the primary spermatocytes before two meiotic divisions take place. The cell proliferation and the initiation of meiosis is known to be regulated by several kinds of hormones (Steinberger, 1971; Russel *et al*., 1987; Skinner, 1991). Successful induction of cell proliferation and conversion from mitosis to meiosis was carried out with the cultivation of testes fragments in a chemically defined medium containing follicle stimulating hormone (FSH: Ji *et al*., 1992; Abe and Ji, 1994) or insulin-like growth factors (IGFs: Nakayama *et al*., 1994) in newts, human chorionic gonadotropin (HCG: Miura *et al*., 1991b) or 11-ketotestosterone (Miura *et al*., 1991c) in eels, and a mixture of several kinds of hormones such as FSH and insulin in *Xenopus laevis* (Risley *et al*., 1987).

In contrast with the thorough search for the substance(s) inducing the conversion from mitosis to meiosis, an analyses on the timing when the spermatogonia acquire an ability for conversion from mitosis to meiosis has never been carried out. Recently, Yazawa *et al*. (2000) observed the features of mitotic divisions during the spermatogonial stage and showed that there were two kinds of mitotic phases. The nuclear diameter of spermatogonia progressively decreased after the division during early phase and then remained constant throughout late phase. Since the spermatogonia at the late phase (secondary spermatogonia) did not change their morphology before and after the mitotic divisions until they en-

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tered the meiotic phase, it may be natural for us to expect that these cells have acquired an ability for conversion from mitosis to meiosis. The present observations were attempted to understand the correct features of the mitotic divisions prior to the conversion from mitotic to meiotic phase in *Xenopus laevis* and showed that the spermatogonial stage in *Xenopus* spermatogenesis could be divided into two phases similar to that in newt spermatogenesis based on the transition (in the first phase) and the constancy (in the second phase) of spermatogonial morphology before and after the mitotic division. We also found primary spermatocytes which were believed to have differentiated from the spermatogonia immediately after the completion of mitotic divisions at the first phase based on the results of the cell number in a cyst and suggested that all secondary spermatogonia, which were morphologically distinguished from the spermatogonia at the first phase, possessed the ability of the mitosis to meiosis conversion.

MATERIALS AND METHODS

Animal

Sexually mature male South African clawed frogs, *Xenopus laevis* (DAUDIN), were purchased from a dealer in Aomori (JAPAN).

Light Microscopic Observations

The testes were fixed with Bouin's fixative for more than 12 hr, dehydrated through a graded ethanol series, embedded in paraffin, and sectioned serially at 7 μm in thickness. The sections were spread onto slides (Matsunami Glass Ind. LTD., Japan) with warm H_2O at 50°C and fixed completely on the slides by drying overnight at 37°C. After removal of the paraffin from the sections with xylene, specimens were hydrated and stained with Delafield hematoxylin and eosin, according to the conventional methods. When the number of spermatogenic cells in a cyst was estimated to be few, i.e., less than 16 cells, we counted them directly taking care not to count the same cells in adjacent sections twice. For the cyst consisting of a large number of secondary spermatogonia, primary spermatocytes, or round spermatids, we adopted Abercrombie's method (Abercrombie, 1946) to estimate the number of cells. We counted the number of the nuclei (n) in a cyst in all sections that contained the cyst, measured the diameter of five to ten of the largest nuclei (d: average), and calculated the estimated number of cells (N) by the following equation to avoid repeated counting of the same nuclei in adjacent sections:

$$N = n \times 7/(7 + d)$$

wherein 7 is the thickness of the section (μm).

in situ Hybridization Analyses

SP4 cDNA in pUC 18 vector (pXSP531; Hiyoshi *et al.*, 1991) was digested with *Eco* RI and inserted into the *Eco* RI site of pBluescript SK II+ (Stratagene). This construct was used for synthesizing sense and antisense RNA probes labeled with digoxygenin (DIG) 11-UTP (Boehringer Mannheim), using T7 RNA polymerase (Boehringer Mannheim) for sense probe and T3 RNA polymerase (Boehringer Mannheim) for antisense probe. Labeled RNA probes were dissolved in the hybridization buffer (1x Denhardt's solution, 600 mM NaCl, 50% formamide, 200 μg/ml *E. coli* tRNA, 10% dextran sulfate, 0.25% SDS, 1 mM EDTA, 10 mM Tris-HCl; pH 7.5) to a final concentration of 1.0 μg/ml. Testis was fixed with the mixture of 1/4 vol. of acetic acid and 3/4 vol. of 4% paraformaldehyde in phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4; pH 7.4) on ice for 4 hr, dehydrated through a graded ethanol series, embedded in paraffin, and sectioned serially at 7 μm in thickness. The 1st to 14th or 16th to 29th sections were spread onto slides (Matsunami Glass Ind. LTD., Japan) and stained with Delafield hematoxylin and eosin as described above. The 15th and 30th sections were spread on warm water at 50°C and picked up onto silanized slides (Dako Japan). After drying the section at 40°C for 4 hr and removing the paraffin from the section by treatment with xylene, the specimens were successively treated with 10 μg/ml Proteinase K (Boehringer Mannheim) at 37°C for 7 min, 4% paraformaldehyde in PBS at room temperature (RT) for 10 min, 0.1 N HCl at RT for 10 min, and 0.1 M triethanolamine (pH 8.0) / 0.25% acetic anhydride at RT for 10 min. After hybridization with sense or antisense probe at 50°C for 16 hr, each specimen was treated with 2 μg/ml RNase A (Sigma) in 500 mM NaCl/ 1 mM EDTA/ 10 mM Tris-HCl (pH 7.5) at 37 °C for 30 min and washed successively in 2 × SSC (0.3 M NaCl / 0.03 M sodium citrate) at 50°C for 20 min, and twice in 0.2 × SSC at 50°C for 20 min. For immunological detection of the DIG, the specimens were incubated in DIG II buffer (1.5% blocking reagent (Boehringer Mannheim) in DIG I buffer (150 mM NaCl, 100 mM maleic acid; pH 7.5)) at RT for 1 hr, followed by treatment with 1:500 diluted alkaline phosphatase-conjugated anti-DIG antibody (Boehringer Mannheim) in DIG II buffer overnight at 12°C. After washing the specimens in DIG I buffer for 30 min and then in DIG III buffer (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris-HCl; pH 9.5), localization of the DIG in the specimens was detected by treatment with 1:50 diluted NBT/BCIP Stock Solution (Boehringer Mannheim) in DIG III buffer.

RESULTS

During spermatogenesis in *Xenopus laevis*, primary spermatogonium undergoes mitotic divisions and develops to secondary spermatogonium followed by entering the meiotic phase and differentiating into primary spermatocyte. By the criteria given by Kalt (1976), the primary spermatogonium was distinguished easily in section as a cell which contained lobulated nucleus with weak stainability (Fig. 1, A) from secondary spermatogonium which had a round nucleus with heterochromatin patches (Fig. 1, F and G) and typical primary spermatocyte (zygotene to diplotene stage) which was characterized by the presence of a large round nucleus (Fig. 1, H). These observations revealed that the differentiation of primary spermatogonium to primary spermatocyte is accompanied with drastical morphological alterations in size, shape, and stainability of nucleus. In contrast with the detailed observations on the morphological changes from secondary spermatogonium to primary spermatocyte (Takamune *et al.*, 1995), it is unclear how the primary spermatogonium differentiates into the secondary spermatogonium with morphological alterations. The spermatogenic cells derived from one primary spermatogonium develop synchronously and form a cyst with Sertoli cells around them (Lofts, 1974). Therefore, we can easily know how many times the spermatogenic cells divide mitotically by counting the cell number in a cyst. When we observed cysts consisting of two spermatogenic cells, which were produced by one mitotic division of the primary spermatogonium, the nuclei of the cells became smaller than that of the primary spermatogonium but otherwise were similar to the primary spermatogonium (Fig. 1, B). When the number of spermatogenic cells in a cyst increased to four to sixteen as a result of two to four mitotic divisions, the nuclei of the sper-

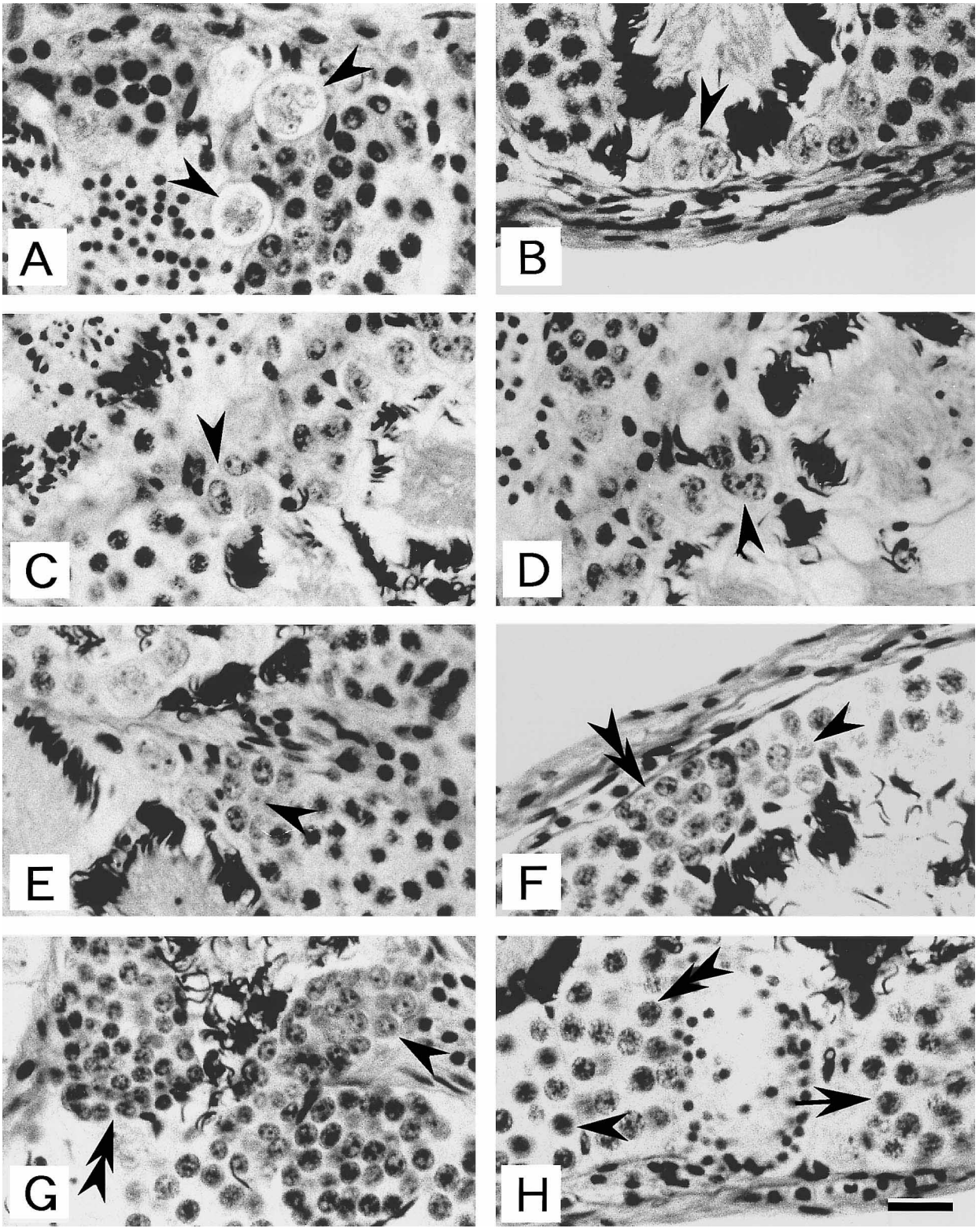

Fig. 1. Photomicrographs of sections of *Xenopus* testis showing various stages from primary spermatogonium to primary spermatocytes. Arrows, arrowheads, or double arrowheads show the cysts consisting of a primary spermatogonium (A: arrowheads), 2 (2^1) spermatogonia (B:arrowhead), 4 (2^2) spermatogonia (C: arrowhead), 8 (2^3) spermatogonia (D: arrowhead), 16 (2^4) spermatogonia (E: arrowhead), 32 (2^5) secondary spermatogonia (F: arrowhead), 56 (approximately 2^6) secondary spermatogonia (F: double arrowhead), 112 (approximately 2^7) secondary spermatogonia (G: arrowhead), 232 (approximately 2^8) secondary spermatogonia (G: double arrowhead), 32 (2^5) primary spermatocytes (H: arrowhead), 108 (approximately 2^7) primary spermatocytes (H: double arrowhead), and 202 (approximately 2^8) primary spermatocytes (H: arrow). Scale bar represents 20 μm.

matogenic cells became smaller and round in shape with heterochromatin patches (Fig. 1, C–E). The spermatogenic cells in a cyst consisting of 30.4±2.9 (S.E.) cells, which had undergone five mitotic divisions, showed the morphology as typical secondary spermatogonia (Fig. 1, F, arrowhead). The cysts consisting of the cells which showed the morphology of the typical secondary spermatogonia were classified into the other three groups in terms of the number of cells in a cyst; 55.2± 5.5 (S.E.) (Fig. 1, F, double arrowhead), 103.7±12.3 (S.E.) (Fig. 1, G, arrowhead), and 213.9±25.9 (S.E.) (Fig. 1, G, double arrowhead) cells, which were approximately 2^6, 2^7, and 2^8, respectively. These results indicate that after the completion of morphological alterations from primary spermatogonium to secondary spermatogonium by five mitotic divisions, the secondary spermatogonia underwent an additional three mitotic divisions without morphological changes. Fig. 2 shows the relationship between the number of cells in a cyst and the size of the nucleus. It clearly indicates the existence of two types of mitotic divisions at the spermatogonial stage; the first to the fifth mitotic divisions were accompanied with changes in nuclear size while the sixth to the eighth mitotic divisions coincided with neither change in nuclear size nor in shape nor in stainability (Fig. 2, closed triangles).

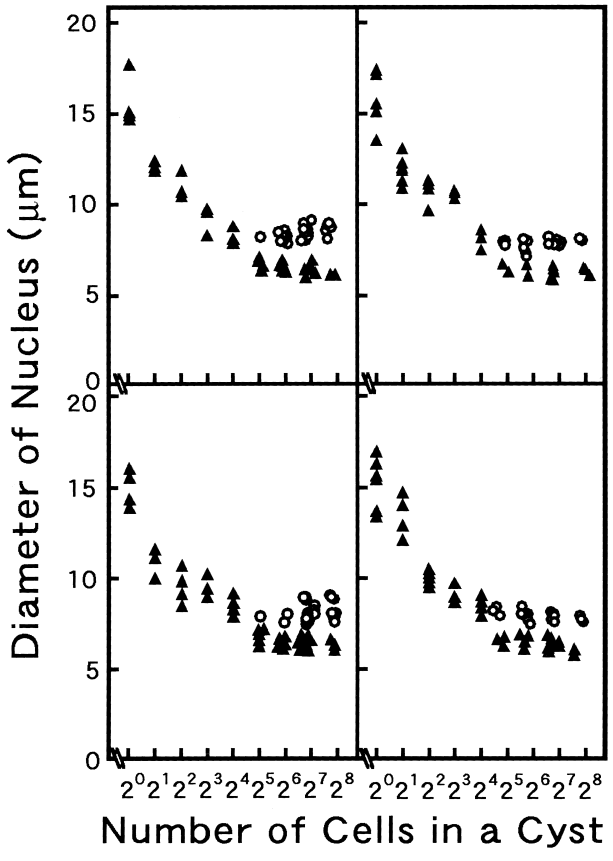

Fig. 2. Relationship between the number of cells in a cysts (abscissa) consisting of cells identical to spermatogonia (closed triangles) and primary spermatocytes (open circles) and the diameter of nucleus of those cells (ordinate). Results from four individuals are shown separately.

In a similar way to the cysts consisting of the secondary spermatogonia, the cysts consisting of primary spermatocytes were classified into four groups in terms of the number of cells in a cyst; 27.6±3.2 (S.E.), 54.2±6.2 (S.E.), 104.1±10.2 (S.E.), and 203.7±17.2 (S.E.) cells, which were approximately 2^5, 2^6, 2^7, and 2^8, respectively (Fig. 1, H; Fig. 2, open circles). These results indicate that the secondary spermatogonia which had undergone at least five mitotic divisions, could enter the meiotic phase and differentiate into the primary spermatocytes, even though they did not undergo the full (eight) mitotic divisions. In order to clarify whether the primary spermatocytes which have differentiated after a few (five to seven) mitotic divisions can proceed to develop normally, we investigated the capability of those cells in the meiotic phase-specific gene expression and the progression of two meiotic divisions. SP4 gene is known to be expressed in the primary spermatocytes at the mid-pachytene stage and its transcripts remain until completion of the spermiogenesis (Mita *et al.*, 1991). If all primary spermatocytes can proceed to develop normally, irrespectively of the number of mitotic divisions before the differentiation to these cells, the cysts consisting of 2^7 to 2^{10} round spermatids that contain SP4 mRNA should be found. Therefore, we performed in situ hybridization using antisense RNA for SP4 as a probe and counted the number of the cells in a cyst consisting of the round spermatids which contained the SP4 mRNA (Fig. 3). Consistent with a previous report (Mita *et al.* 1991), all round spermatids as well as groups of spermatocytes and other post meiotic cells, including elongating spermatids and the residual body of mature sperm exhibited a

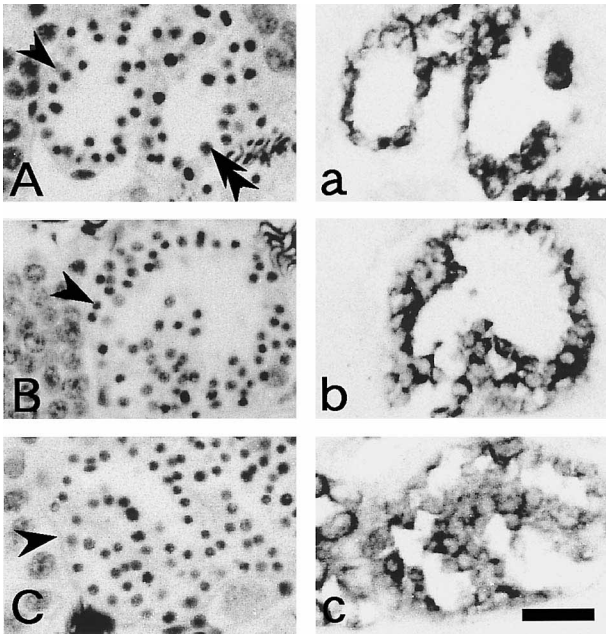

Fig. 3. Photomicrographs of sections of *Xenopus* testis, stained with hematoxylin and eosin (A, B, and C) and hybridized in situ with digoxigenin-labeled SP4 antisense RNA (a, b, and c). Arrows or arrowheads show the cysts consisting of 122 (approximately 2^7; A: arrowhead), 253 (approximately 2^8; A: double arrowhead), 472 (approximately 2^9; B: arrowhead), and 1000 (approximately 2^{10}; C: arrowhead) round spermatids. Scale bar represents 20 μm.

significant labeling over the background level in the cytoplasmic area, but there was no signal above the background in the areas occupied by spermatogonia, primary spermatocytes at leptotene-zygotene stage, and somatic cells, including Sertoli cells and other sustentacular cells. After confirmation of the expression of SP4 gene in the round spermatids, we enumerated the number of cells in the cysts consisting of those round spermatids using the serial sections before and behind the section which was used for in situ hybridization. As Fig. 3 shows, there were cysts consisting of 122 cells (Fig. 3, A and a, arrowhead), 253 cells (Fig. 3, A and a, double arrowhead), 472 cells (Fig. 3, B and b, arrowhead), and 1000 cells (Fig. 3, C and c, arrowhead), which were approximately 2^7, 2^8, 2^9, and 2^{10}, respectively. These results clearly indicate that the primary spermatocytes which have undergone a few (especially five) mitotic divisions prior to entering the meiotic prophase have normal ability to cause the stage-specific gene expression and proceed to two meiotic divisions to differentiate into round spermatids.

DISCUSSION

Figure 4 shows the schematic illustration of spermatogenesis in *Xenopus laevis*, which is based on the results of this study. The primary spermatogonium, which was one of two daughter cells produced by the division of a stem cell, divided mitotically five times with the decrease in nucleus size and differentiated into secondary spermatogonia. Some of the secondary spermatogonia entered the meiotic phase and differentiated into the primary spermatocytes and others repeated the mitotic division up to three more times prior to entering the meiotic phase. The primary spermatocytes underwent two meiotic divisions, followed by spermiogenesis to form the mature sperm. As it is clear from this figure, there were two types of mitotic phases during the spermatogonial stage; the first (the first to the fifth mitotic divisions) and the second (the sixth to the eighth mitotic divisions) phases. During the first phase, the morphology of the spermatogonial nuclei drastically changed after each division, while the mitotic division at the second phase was not accompanied with a morphological alteration of the secondary spermatogonia. It should be noticed that the secondary spermatogonia at the second phase could either enter the meiotic phase or undergo further mitotic divisions. These observations suggested that after the fifth mitotic division, the secondary spermatogonia acquired the ability to enter the meiotic phase and that conversion of these cells from mitosis to meiosis was regulated by an external signal(s) but not by cells themselves. With regard to this acquisition of ability for mitotic to meiotic phase conversion, Miura *et al*. (1997) have also suggested the possibility of the existence of a regulatory point prior to entering the meiotic phase, based on the observations of impaired spermatogenesis in Japanese eels. In general, the type A spermatogonium (corresponds to the primary spermatogonium in *Xenopus*) of eel undergoes ten mitotic divisions prior to entering the meiotic phase when the spermatogenesis is induced by HCG injection into male eel (Miura *et al*., 1991a). In some cases, however, the spermatogenesis stopped after four or five mitotic divisions and the late type B spermatogonia (correspond to the secondary spermatogonia in *Xenopus*) in these eels could not differentiate into primary spermatocytes (Miura *et al*., 1997). From the results of differential mRNA display to search for mRNA that was expressed prior to entering the meiotic phase, Miura *et al*. (1999) concluded that PCNA mRNA was expressed only in the late type B spermatogonia which had undergone five to six mitotic divisions. These results strongly pointed out the existence of a regulatory point at which spermatogenic cells had to go through before entering the meiotic phase and proceeding to a later stage of spermatogenesis. As the fifth mitotic division in *Xenopus* spermatogenesis seems to correspond to this regulatory point seen in eels, the stage-specific expression of PCNA mRNA during *Xenopus* spermatogenesis was anticipated. However, we detected the PCNA mRNA in all spermatogonia except for the primary spermatogonium from in situ hybridization, in contrast with the results seen in eels (data not shown). PCNA is a DNA polymerase auxiliary protein that functions during DNA replication and repair (Waga and Stillman, 1994). Therefore, it is no wonder that spermatogonia undergoing DNA replication followed by mitotic division express the PCNA mRNA. More recently, we

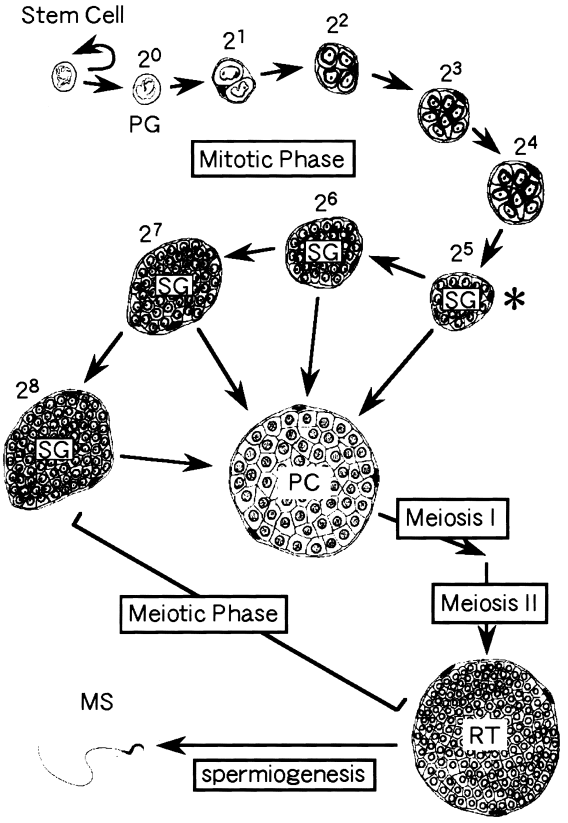

Fig. 4. Schematic illustration of spermatogenesis in *Xenopus laevis*. PG; primary spermatogonium. SG; secondary spermatogonia. PC; primary spermatocytes. RT; round spermatids. MS; mature sperm. Asterisk shows the timing of acquisition of an ability for conversion from mitosis to meiosis.

identified a novel gene which was expressed only in germ cells and cleavage stage embryos in *Xenopus laevis* and showed by in situ hybridization that the amount of this transcript clearly increased in secondary spermatogonia (Ikema *et al.*, unpublished data). The up-regulation of this gene seemed to be closely related with the acquisition of the ability for mitotic to meiotic phase conversion. It should be focused to clarify the regulatory mechanisms of this gene expression for understanding the mitotic to meiotic phase conversion mechanisms.

After the fifth mitotic division, the secondary spermatogonia could either enter the meiotic phase or undergo further mitotic divisions. This observation clearly indicates that the timing of the mitotic to meiotic phase conversion is not regulated by the number of mitotic divisions and leads us to suspect the necessity of an external signal(s) for conversion of mitotic phase to meiotic phase. Our presumption may be supported by the following experimental results: Human recombinant activin A and B could not induce the mitotic to meiotic phase conversion in eels although it could successfully induce the spermatogonial proliferation in the same manner with 11-ketotestosterone (Miura *et al.*, 1995), which could induce both proliferation of spermatogonia and mitotic to meiotic phase conversion through the activation of Sertoli cells (Miura *et al.*, 1991c). Experimental induction both of spermatogonial proliferation and mitotic to meiotic phase conversion were also successful when newt testes fragments rich in secondary spermatogonia were cultured in a medium containing FSH (Ji *et al.*, 1992; Abe and Ji, 1994) or IGFs (Nakayama *et al.*, 1999). Since the FSH receptor exists only in the Sertoli cells, FSH is believed to act through the activation of Sertoli cells (Ji *et al.*, 1995; Maekawa *et al.*, 1995). On the other hand, since IGF receptors exist in germ cells as well as Sertoli cells, IGFs are thought to be a candidate for the signals inducing mitotic to meiotic phase conversion by directly interacting with germ cells. Spermatogenesis is also maintained in *Xenopus* testes fragments cultured in serum free media supplemented with several kinds of hormones and vitamins, such as FSH, insulin and retinol (Risley *et al.*, 1987). Our preliminary observations with a culture system showed the possibility that the differentiation of secondary spermatogonia to primary spermatocytes in *Xenopus* could be induced by treatment of testes fragments solely with FSH (Kawasaki *et al.*, unpublished data). As the effect of FSH in *Xenopus* spermatogenesis was the same as that in newt spermatogenesis, we assumed that the initiation mechanisms of meiosis in *Xenopus* were similar to that in newt.

Recently, Yazawa *et al.* (2000) revealed that the spermatogonial stage in the newt could be divided into the following two phases similar to that in *Xenopus*; early (the first to the fourth generations) and late (the fifth to the eighth generations) phases. In contrast with *Xenopus* secondary spermatogonia which are able to convert mitotic phase to meiotic phase after the merely five mitotic divisions, the newt spermatogonia usually do not enter the meiotic phase until the completion of the eight mitotic divisions. In general, the spermatogonia enter the meiotic phase after the species-specific fixed number of mitotic divisions (Roosen-Runge, 1977; Miura *et al.*, 1991a; de Rooji and Grootegoed, 1998; Ando *et al.*, 2000) although there are a few species in which number of mitotic divisions prior to entering meiosis is not fixed (nine or ten in the medaka, Sibata and Hamaguchi, 1988; five or six in the zebrafish, Ewing, 1972; Ando *et al.*, 2000). We also reported previously that the differentiation of the secondary spermatogonia into the primary spermatocytes occurred after eight mitotic divisions in *Xenopus* (Takamune *et al.*, 1995). In this study, however, we observed the primary spermatocytes which had divided mitotically fewer times (five to seven) before entering the meiotic phase. In contrast with the previous study in which we fixed the testes obtained from males which were injected with HCG and kept for a week before use for reinitiating the spermatogenesis following the release of the mature sperm from the lumen of the seminiferous tubules, we used the testes from the sexually mature males which were not injected with HCG. Therefore, lumen in the seminiferous tubules was filled with mature sperm. With regard to this point, almost all cysts consisting of primary spermatocytes in the testis from juvenile at five weeks after metamorphosis, in which the space of lumen had not fully formed yet, contained approximately 2^5 spermatogenic cells (data not shown). The number of mitotic divisions of the secondary spermatogonia after the fifth division may be a result of spatial restriction in the lumen where mature sperm are stored until ejaculation.

In summary, the present study suggested that the conversion from mitosis to meiosis was regulated at two points; acquisition of an ability for the conversion and initiation of the meiotic phase. In *Xenopus*, the secondary spermatogonia possessed the ability for both proceeding to further mitotic divisions and entering the meiotic phase. We are expecting to elucidate the mechanisms for the mitotic to meiotic phase conversion through investigating the molecular basis for the differentiation into the secondary spermatogonia.

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