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Germ Cell Genotype Controls Cell Cycle during Spermatogenesis in the Rat[1]

Luiz R. França,[3] Takehiko Ogawa,[4] Mary R. Avarbock,[4] Ralph L. Brinster,[4] and Lonnie D. Russell[2,5]

Department of Morphology,[3] Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, Minas Gerais, Brazil 31270-901 CP 486
School of Veterinary Medicine,[4] University of Pennsylvania, Philadelphia, Pennsylvania 19104
Department of Physiology,[5] Southern Illinois University School of Medicine, Carbondale, Illinois 62901-6512

ABSTRACT

Spermatogenesis is one of the most productive self-renewing systems in the body: on the order of 10^7 spermatozoa are produced daily per gram of testis tissue. In each mammalian species, the time required for completion of the process is unique and unalterable. Because the process is supported by somatic Sertoli cells, it has generally been thought that cell-cell interaction between germ and Sertoli cells controls the duration of cell cycles and cellular organization. We have used the newly developed technique of spermatogonial transplantation to examine which cell type(s) determines the rate at which germ cells proceed through spermatogenesis. Rat germ cells were transplanted into a mouse testis, and the mouse was killed 12.9–13 days after administration of a single dose of [^{3}H]thymidine. The most advanced rat cell type labeled was the pachytene spermatocyte at stages VI–VIII of the spermatogenic cycle. In animals given only rat cells, some endogenous spermatogenesis of the mouse recovered. The most advanced labeled mouse cell types in recipients killed 12.9–13 days after administration of a single dose of [^{3}H]thymidine were meiotic cells or young spermatids, which is consistent with a spermatogenic cycle length comparable to the 8.6 days reported for the mouse. The same results were obtained if a mixture of rat and mouse cells were transplanted. There existed two separate timing regimens for germ cell development in the recipient mouse testis; one of rat and one of mouse duration. Rat germ cells that were supported by mouse Sertoli cells always differentiated with cell cycle timing characteristic of the rat and generated the spermatogenic structural pattern of the rat, demonstrating that the cell differentiation process of spermatogenesis is regulated by germ cells alone.

INTRODUCTION

Of the self-renewing cell systems of the body, spermatogenesis is thought to be capable of the greatest number of amplifications of cell divisions during differentiation [1, 2]. A single rat spermatogonial stem cell is theoretically capable of producing 4096 spermatozoa [2]; however, cell death during the process reduces final production substantially [3, 4]. In addition to high productivity, spermatogenesis is characterized by a remarkable maintenance of the cellular association among germ cells at each differentiation stage [2, 5]. As many as 50 different germ cells, which may arise from several individual clones, contact and/or are supported by a Sertoli cell extending from the basement membrane to the lumen of the testis seminiferous tubule, and most germ cells are associated with several Sertoli cells [6]. Specialized junctional complexes between the membranes of germ cells and Sertoli cells are numerous, and in late steps of differentiation the germ cell occupies a cavity or crypt formed by the invagination of the Sertoli cell [2, 7]. In addition, the morphological appearance of both the germ cell and Sertoli cell undergoes well-characterized and predictable changes during germ cell differentiation [2, 5, 8]. Because of this close physical and functional association, it has been impossible to determine whether the germ cell or somatic Sertoli cell controls the cell cycle timing of the germ cells.

At any given site in the seminiferous tubule, the onset of stem cell differentiation that initiates the spermatogenic process is cyclical, and it is followed by an orderly stepwise differentiation of progeny cells into mature spermatozoa. Because the cell cycle in this differentiation process is rigidly controlled, precise cellular associations for each species exist, and these relationships are constant [2, 5]. Thus, staging maps can be prepared for each species to characterize the cell associations present in histological cross sections of seminiferous tubules. The length of the spermatogenic process is different among species. In the Sprague-Dawley rat, recruitment of committed cells from stem cells occurs every 12.9 days [9], which also represents the time necessary for a cell to ascend one level above the original cell in a spermatogenic cycle map [2]. In the mouse, the spermatogenic cycle length is 8.6 days [10]. The spermatogenic cycle duration is typically determined from experiments in which [^{3}H]thymidine incorporated at the last S-phase of spermatogenesis (preleptotene spermatocyte) is identified by autoradiography at a later time in the most advanced labeled cell type. In rat, finding radioactive label in pachytene spermatocytes at stage VII of spermatogenesis in animals killed 12.9 days after injection of [^{3}H]thymidine indicates a cycle length of 12.9 days (Fig. 1A). The 12.9 days represent the time necessary for the cell to advance to the next vertical level of the same stage.

To determine the relative roles of the germ cell and somatic Sertoli cell in regulating the cell cycle during spermatogenesis, we employed the recently developed spermatogonial stem cell transplantation technique: testis cells from a fertile male are injected into the seminiferous tubules of an infertile recipient male in which donor cell-derived spermatogenesis is generated [11, 12]. Only stem cells in the injected testis cell population are capable of regenerating spermatogenesis [2, 7, 11, 12]. After transplantation of rat testis cells into the seminiferous tubules of immunodeficient mice, rat germ cell differentiation has al-

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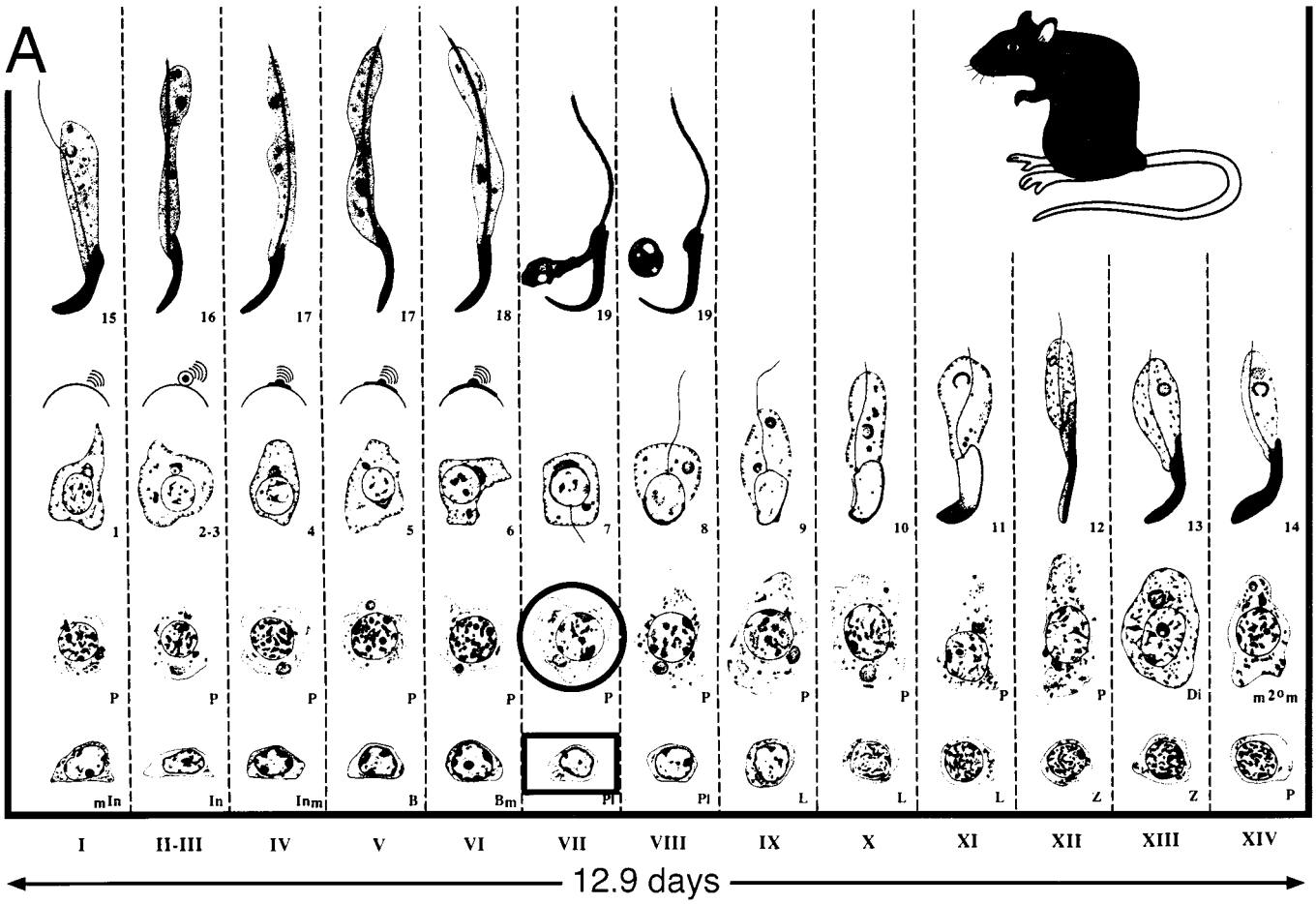
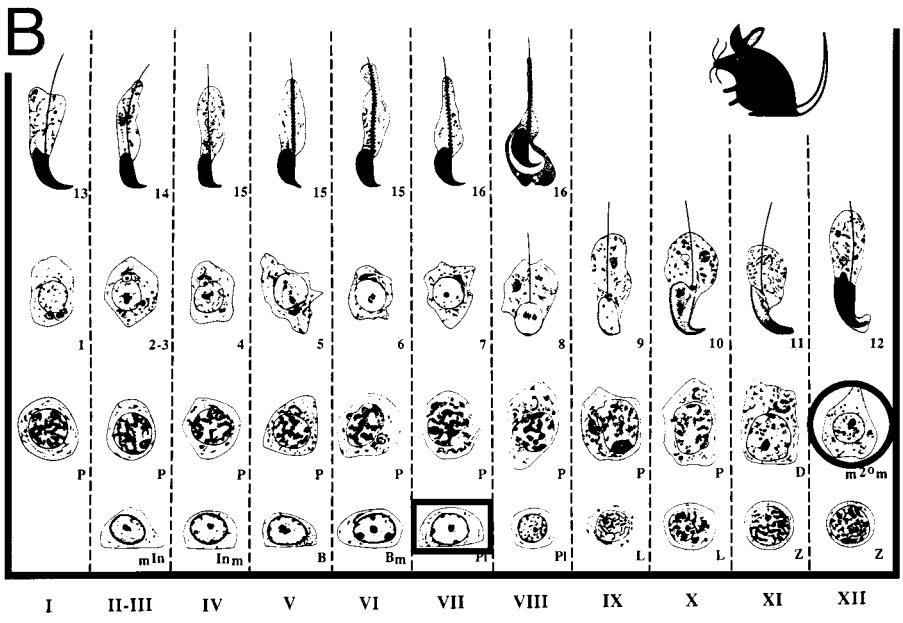
[2]Correspondence. FAX: 618 453 1517; e-mail: lrussell@som.siu.edu



Stages of the Rat Cycle



Stages of the Mouse Cycle

TABLE 1. Details of animals used in the present study.

Experiment	Species/designation	Recipent body wt (g)	Interval between transplant and death (days)†	Species origin of transplanted cells†	% Surface tubules with dye† L	% Surface tubules with dye† R	Interval between [3H]thymidine injection and death	Right testis wt (mg)	Left testis wt (mg)
1097	mouse/5-1058	36	155	Rat & mouse	85	85	13 days	26	29
1097	mouse/6-1152	34	99	Rat	85	85	13 days	42	44
1097	mouse/7-1118	29	119	Rat	60	75	13 days	33	33
1097	mouse/9-1121	32	114	Rat & mouse	85	80	13 days	22	26
1097	mouse/11-1151	34	99	Rat	75	85	1 h, 16 min	47	44
1100	mouse/A-1167	39	232	Rat	70	65	13 days	116	114
1100	mouse/B-1180	35	225	Rat & mouse	85	85	13 days	44	98
1100	mouse/C-1181	36	225	Rat & mouse	75	75	13 days	108	90
1102	rat/1102	*	na	na	na		1 h, 25 min	adult size	adult size
1102	mouse/1102	*	na	na	na		1 h, 25 min	adult size	adult size

* Adult animals were used.
† na, Not applicable since transplantation was not conducted.

ways been found to be supported by mouse Sertoli cells [13, 14]. It was our objective to determine whether the timing of spermatogenesis is altered by the environment of the mouse testis, in which the cyclic development of comparable cells is inherently 50% faster.

MATERIALS AND METHODS

Sprague-Dawley rat testis cells from males 10–53 days of age were transplanted into the testes of immunodeficient nude (*nu*/*nu*; Taconic Farms, Germantown, NY) mice in which endogenous germ cells had been destroyed by treatment with 40 mg/kg busulfan. The transplantation procedure was performed according to the methods of Clouthier et al. [13] with additional methodological details, such as cell isolation techniques, provided by Ogawa et al. [15]. The cells used for transplantation probably include all major cell types found in the testis (unpublished observations by L.D.R. through electron microscopy of cell pellets). The route of transplantation was via injection of testis cells into the efferent ductules. The concentration of injected donor testis cells was 0.4 to 3.4 $\times$ 10^8 cells/ml, and approximately 10 μl were injected into the seminiferous tubules of a testis.

Four mice received only rat testis cells, and 4 mice received a mixture of rat plus mouse testis cells (Table 1). Because treatment of recipient mice with busulfan destroys most endogenous germ cells, in animals transplanted with rat cells alone, there may be only a variable and small degree of mouse spermatogenesis from a few stem cells that survive treatment [12, 16]. The transplantation of mouse plus rat testis cells in some experiments guaranteed the presence of mouse spermatogenesis to determine the duration of the mouse cycle within recipient seminiferous tubules. When both rat and mouse cells were injected, the concentration for each was approximately equal.

The recipient mice were maintained for 86-219 days to allow development of spermatogenesis from donor cells; then each mouse received an i.p. injection of 1 μCi [3H]thymidine per gram BW (80 Ci/mmol; Amersham, Arlington Heights, IL). A range of donor cell concentrations and times allowed for recipient mouse colonization were used to assure a suitable extent of rat and mouse spermatogenesis in multiple tubules of at least several recipients.

One control Sprague-Dawley rat and one control mouse (C57BL/6) received injections of [3H]thymidine to verify labeling of cells at the last S-phase (preleptotene spermatocyte) during spermatogenesis (experiment 1102 of Table 1). In addition, one recipient mouse that had received rat donor cells 99 days previously received an injection of [3H]thymidine and was killed approximately 1 h later (mouse 11–1151 of Table 1) to show that the transplantation protocol did not alter the labeling of preleptotene spermatocytes.

At the time they were killed, mice were anesthetized with pentobarbital and perfused with 5% glutaraldehyde; this was followed by postfixation in an osmium:ferrocyanide mixture [17]. Testis tissue was embedded in plastic resin (Araldite CY212; Ladd Research Industries, Burlington, VT) and sectioned at 1-μm thickness for microscopic examination. Cells were considered labeled when five or more grains were present over the nucleus or within 1 μm of the nucleus in the presence of low-to-moderate background. The staging criteria of Leblond and Clermont [5] as modified by Russell et al. [2] were used to determine the cell association in which labeled cells were present.

Tissue sections in which tubules were identified as containing rat germ cells and which also demonstrated the labeling pattern characteristic of the rat as described in the *Results* section below were re-sectioned to show that the germ cells were of rat phenotype. To do this, autoradiographs showing the desired labeling pattern were first identified, and images were recorded at low magnification with an image analysis system. Cover slips were removed by

FIG. 1. Spermatogenic cycle of rat and mouse. The diagrams show the histological relationship of cells in the rat (**A**) and mouse (**B**) seminiferous tubules of the testis during spermatogenesis. In each diagram, the lower row of cells rests closer to the basement membrane, and the upper row of cells is generally closer to the lumen of the seminiferous tubule (undifferentiated and young differentiated type A spermatogonia are not depicted). Because of precise timing of the differentiation process, the same cell types are always aligned from the basement membrane to the lumen and in association within a cross section of a seminiferous tubule. In the rat, there are 14 stages (cell associations) that repeat at the same location in the tubule at a 12.9-day interval. In the mouse, there are 12 stages that repeat at an 8.6-day interval. When a preleptotene (Pl) spermatocyte is labeled during S-phase of mitosis with [3H]thymidine (box) and the testis is examined 13 days later, the radioactivity is found in the pachytene (P) spermatocytes of the rat (circle) and the diplotene meiotic ($_m2°_m$) spermatocytes of the mouse. Rat germ cells transplanted to mouse testes and supported by mouse somatic Sertoli cells differentiate with the spermatogenic cycle of the rat (12.9 days), indicating that the germ cells totally control the cell cycles of the complex spermatogenic differentiation process. Roman numerals I to XIV indicate spermatogenic cycle stages of the rat or mouse. Details of symbols used in the staging maps shown can be found in Russell et al. [2].

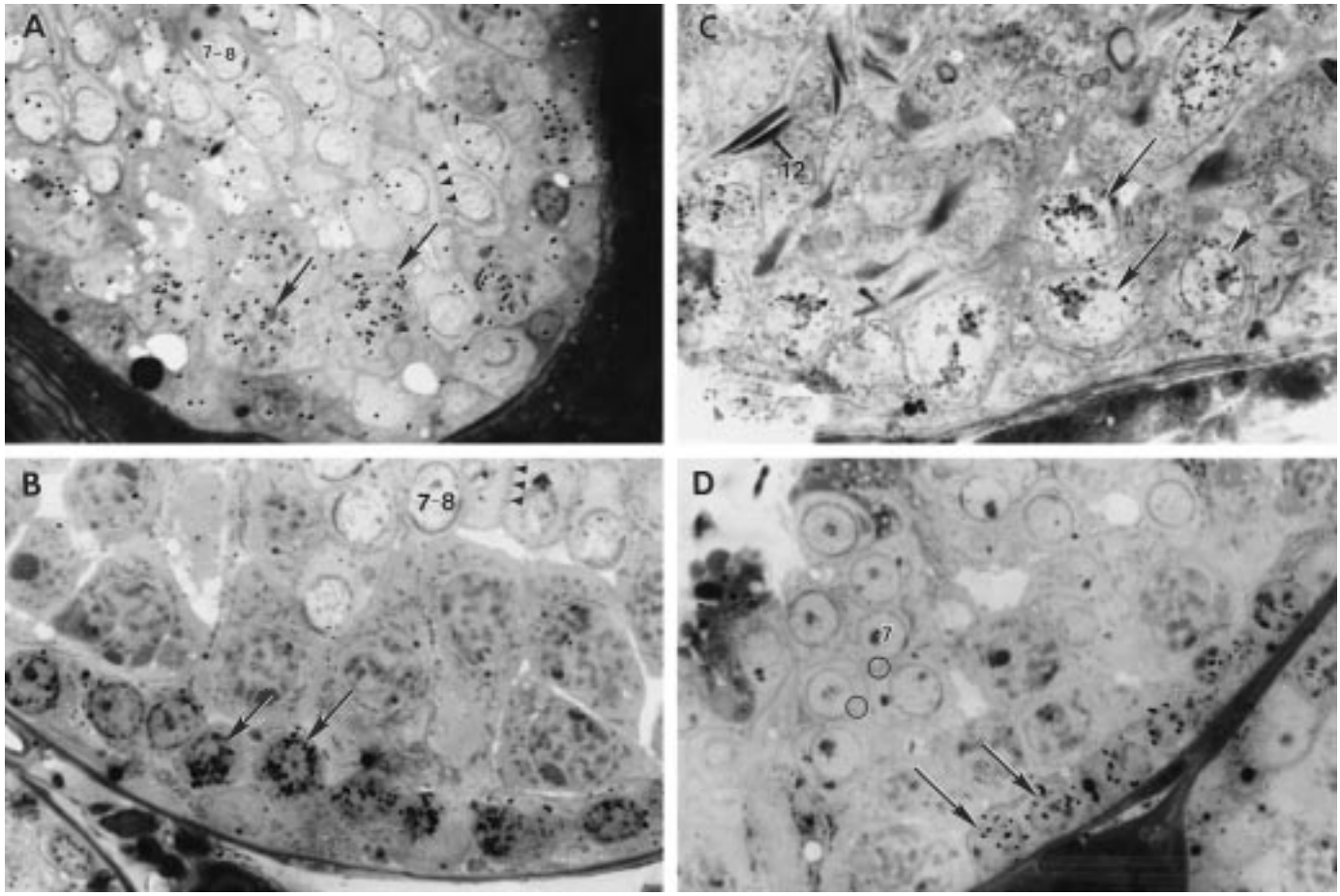


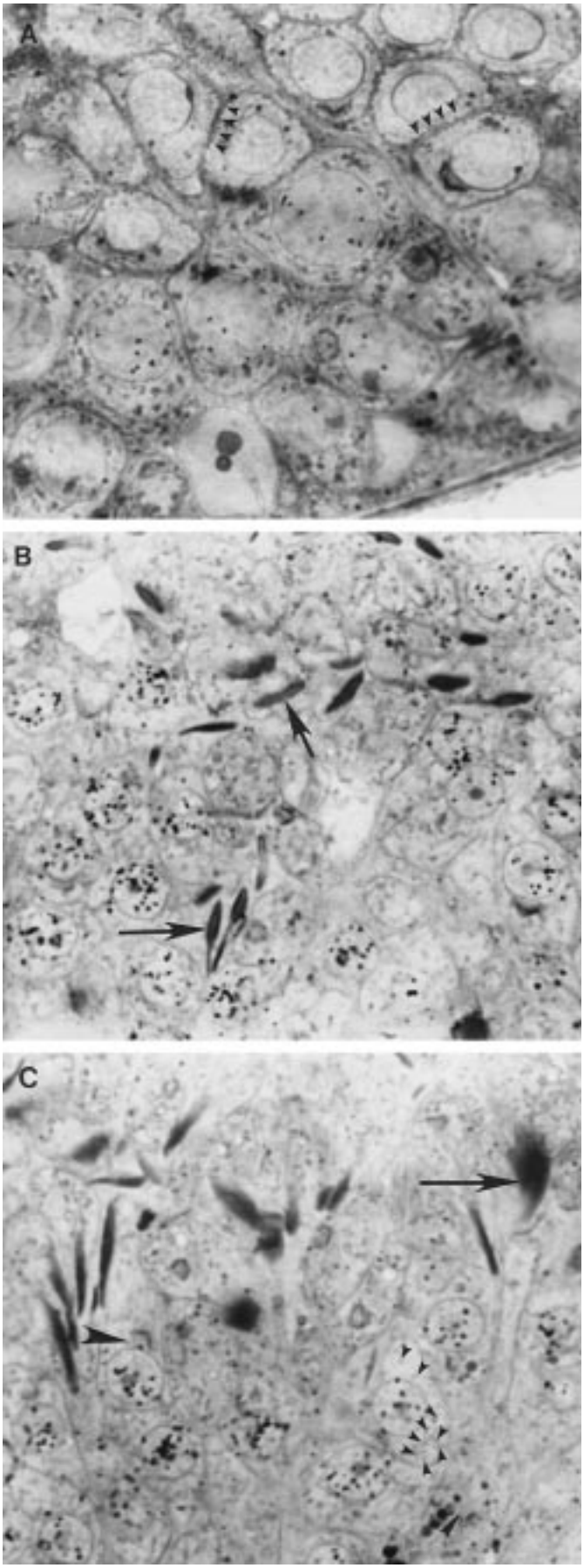
FIG. 2. Radioactive labeling of germ cells (other than spermatogonia) after administration of [^{3}H]thymidine to a recipient mouse into which rat germ cells had been transplanted. **A**) At 13 days after injection of [^{3}H]thymidine, rat pachytene spermatocytes in stage VII–VIII are labeled (arrows); **B**) At approximately 1 h after injection, rat preleptotene/leptotene transition spermatocytes are labeled (arrows) in late stage VII–VIII (see Fig. 1A). **A** and **B** are identified as stage VII and/or VIII by the presence of step 7-8 spermatids (7-8). The germ cells in **A** and **B** are of rat origin as indicated by the alignment of mitochondria along the plasma membrane (arrowheads), making the cell surface distinctive (see also Fig. 3), and by the characteristic shape of the elongated spermatid heads (not shown). **C**) At 13 days after injection of [^{3}H]thymidine, mouse meiosis I metaphases (arrows), and secondary spermatocytes (arrowheads) in stage XII are labeled. The germ cells in **C** are identified as being from the mouse by the presence of partially condensed step 12 spermatids (12) in the cell association and by the characteristic shape of their heads. **D**) At approximately 1 h postinjection, preleptotene spermatocytes of the mouse are labeled (arrows) in late stage VII (see Fig. 1B). The presence of step 7 spermatids (7) identifies stage VII. The germ cells in **D** are from the mouse as indicated by the position of mitochondria (encircled) scattered throughout the cytoplasm in step 7 spermatids, making the surface of the cell indistinct compared with that of the rat (see also Fig. 3). All micrographs are oriented with the periphery or base of the tubule depicted below. Magnification ×1500 (reproduced at 60%).

placing slides in xylene for three days. Plastic capsules containing unpolymerized epoxy were placed over the tissue section, and the plastic was polymerized in an oven at 60°C for 20 h. The slide containing the polymerized epoxy was removed from the oven and placed on a hotplate heated to 80°C for 1 min. Rapid lateral pressure exerted on the block snapped off the block and the section from the glass slide. The digitized image was used as a guide to trim the block to remove all epoxy except that containing the tubules of interest. The trimmed block was sectioned for electron microscopy. Cells from a tubule at the appropriate stage of the cycle were identified and photographed using a Hitachi 500C electron microscope (Hitachi, Ltd., Tokyo, Japan).

The criteria used to identify rat and mouse spermatogenesis in autoradiographs from mouse recipients were morphological and were four in number. 1) Mitochondrial position in round spermatids of the rat and mouse differed, and this difference could be detected by both light and electron microscopy. By electron microscopy [14], mitochondria have been shown to remain randomly scattered within the cytoplasm of mouse round spermatids. On the other hand, rat round spermatids possess mitochondria that have aligned to the cell surface. This feature is readily visualized by electron emicroscopy but can also be detected by light microscopy [14]. 2) In some tubules the shape of elongated spermatids reveals the species origin of particular germ cells since rat and mouse spermatogenesis results in sperm head shapes that are different [2]. 3) Mouse and rat spermatogenesis can be distinguished on the basis of the timing of the nuclear condensation of elongating spermatids. Rat spermatids begin pronounced nuclear condensation in stage XI whereas mouse spermatids condense primarily at stage I. Thus stages XI through I can be distinguished by whether or not elongating spermatid condensation has begun. 4) Acrosomal morphology in steps 3–6 in the mouse differ [2]; in the mouse the acrosomes indent the nucleus, and in the rat they simply flatten the nucleus.

RESULTS

The general pattern of donor cell colonization for all 8 recipient mice was similar. Testis tissue examined in xen-



ogeneic transplants showed tubules containing both mouse and rat spermatogenesis. Regardless of whether rat or rat plus mouse donor cells were used, testes showed prominent rat spermatogenesis. Intermingling of rat and mouse germ cells in a single cross-sectioned tubule was never seen. In some tubules, spermatogenesis was not complete and lacked a layer of differentiating cells. The longer the interval after transplantation, the more complete was spermatogenesis. For analysis of cell cycle timing by autoradiography, only seminiferous tubules with sufficient cells to clearly identify cell associations and species characteristics were used.

Recipient mice receiving rat or rat plus mouse germ cells and killed 13 days after [^{3}H]thymidine injection showed two patterns of germ cell labeling as depicted in the cycle maps in Figure 1 and in the autoradiographs of Figures 2 and 3. In one pattern, the most advanced cell types labeled beyond preleptotene spermatocytes were pachytene spermatocytes in tubules from Stage VI to VIII, with the most labeling in stage VII (Fig. 2A). This cell association was identified as rat spermatogenesis because of the presence of 1) elongated spermatids with a shape characteristic of the rat and 2) round spermatids in which mitochondria were at the periphery of the cell. Rat spermatogenesis is distinguished by the alignment of mitochondria along the periphery of the round spermatids [14], a feature that is seen readily by light microscopy (Fig. 2, A and B, and Fig. 3A). The pattern of labeling was also confirmed by electron microscopy by resectioning an autoradiograph thick section for electron microscopy (Fig. 4). This pattern of labeling is characteristic of rat spermatogenesis and results in a developmental period of 52 days from differentiated type A_1 spermatogonia to spermatozoa. In the second pattern of labeling, the most advanced cell types labeled beyond preleptotene spermatocytes were diplotene spermatocytes, meiotic figures, and occasional step 1–3 spermatids (Fig. 2C). These were verified as germ cells of the mouse because occasional uncondensed or semicondensed spermatids, which are not seen in rat spermatogenesis, were seen as part of the cell association (Fig. 2C and Fig. 3, B and C). This pattern of labeling is characteristic of mouse spermatogenesis and results in a developmental period of 35 days from differentiated type A_1 spermatogonia to spermatozoa. All 7 recipient mice with transplanted cells showed a similar pattern of [^{3}H]thymidine labeling for both rat and mouse spermatogenesis 13 days after injection.

A control rat and a control mouse administered [^{3}H]thymidine and killed approximately 1 h later showed the most advanced labeling over preleptotene spermato-

FIG. 3. Radioactive labeling of germ cells (other than spermatogonia) after administration of [^{3}H]thymidine. Rat and mouse germ cells were transplanted into a recipient mouse that was killed 12.9 days after a single injection of [^{3}H]thymidine. The photomicrographs show the morphological characteristics of rat and mouse germ cells that allowed for their identification as either being rat or mouse. In **A**, in which pachytene cells of stage VII were labeled autoradiographically, the round spermatids are identified as having characteristics of the rat by the alignment of mitochondria along the cell surface (small arrowheads). In **B**, the cells are from a mouse stage I tubule and show the labeled step 1 spermatids and step 13 spermatids. The step 13 spermatid nuclei have not fully condensed (arrow), a feature characteristic of the mouse and not the rat. In **C**, mouse step 2-3 spermatids are labeled (arrowhead) in a stage II–III tubule containing an elongated spermatid in the shape characteristic of the mouse and not the rat. Note that the mitochondria of the step 2-3 spermatids are scattered throughout the cytoplasm (arrowheads). Magnification ×1500 (reproduced at 80%).

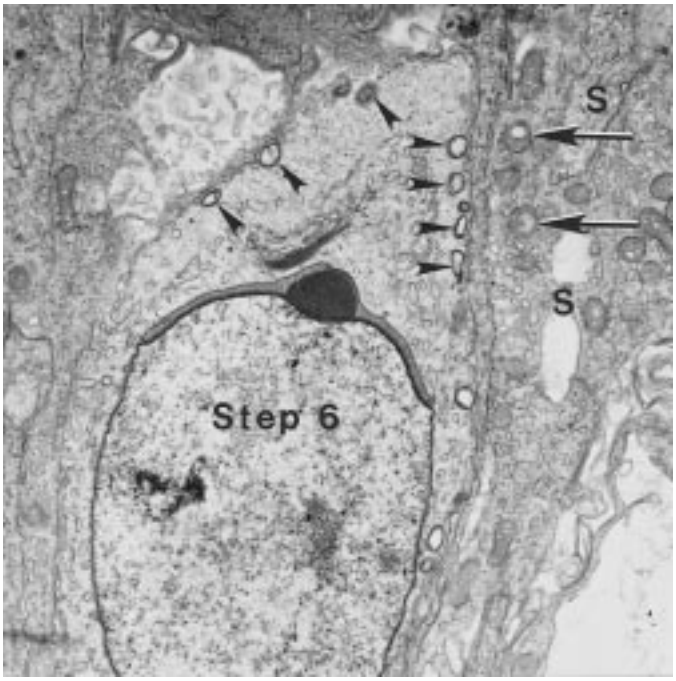


FIG. 4. Rat germ cell associated with mouse Sertoli cell. The electron micrograph was cut from a 1.0-μm-thick section that showed labeling of pachytene spermatocytes 13 days after injection of [^{3}H]thymidine similar to the labeling shown in Figure 2A. The late step 6 spermatid shown is of rat origin, as evidenced by the peripheral positioning (arrowheads) of the mitochondria (see [14]). This spermatid is developing in association with a mouse Sertoli cell (S) as evidenced by the characteristic expanded cristae (arrows) of the mouse Sertoli cell mitochondria (see [14]). Magnification ×9000.

cytes of stages VI–VII and young leptotene spermatocytes of stage VIII (Fig. 2, B and D). In addition, a recipient mouse that had received rat donor cells 99 days previously and was killed approximately 1 h after injection with [^{3}H]thymidine showed only preleptotene spermatocytes as the most advanced labeled cell type (data not shown). The fact that in both rat and mouse, as well as in a recipient mouse transplanted with rat cells, the most advanced labeled cell after 1 h was the preleptotene spermatocyte is in accord with previous findings for these species [9, 10].

DISCUSSION

The results demonstrate that rat spermatogenesis in mouse seminiferous tubules proceeds with cell cycles characteristic of rat germ cells. Although rat germ cells arising from transplanted stem cells are supported by and associated with only mouse Sertoli cells ([14]; Fig. 4), their cell cycle is unaltered. This is a remarkable finding considering the close membrane association and specialized junctional complexes that exist between germ cells and Sertoli cells. Furthermore, cyclic morphological changes are known to occur in Sertoli cells associated with the different stages of the spermatogenic cycle [8]. These phenomena have suggested that the Sertoli cell plays a key, if not primary, role in regulating the spermatogenic cycle. Thus, the total dominance of the germ cell genotype in controlling this process is surprising.

The Sertoli cell is the somatic cell closely associated with the developing germ cells. The cyclic changes noted in germ cells are the most noticeable, but it is also well known that the Sertoli cell demonstrates cyclic biochemical [8] features as well as a cyclic structural pattern [18]. We do not know whether the Sertoli cycle timing in the mouse is altered in the mouse tubules containing rat germ cells to match the rat germ cell cycle or the Sertoli cell cycle continues at a timing characteristic of the mouse.

In the rat, there are believed to be 12 cell divisions leading from stem cell to mature spermatozoa, which is more than for other self-renewing tissues in the body [1, 2]. Furthermore, male germ cell meiosis and morphological changes associated with spermatozoa shaping introduce a complexity to this process not present in other self-renewing tissues [2, 7]. Perhaps this long development period and functional complexity require rigid control of the cell cycle and result in the typical cell associations and cycle maps seen in seminiferous tubules of various species. We know that the length of the cell cycle and pattern of cell associations, as well as the time necessary to produce spermatozoa, vary greatly among species [2]. However, within any species, the cell cycle of male germ cells is fixed and cannot be altered by natural phenomena or experimental manipulations, including, as described in this report, the movement of rat germ cells to a different species. Thus, there are two cell cycle kinetic characteristics of spermatogenesis. One is the absolute length of the cell cycle, which dictates the time necessary to produce a spermatozoon. This varies among species and, therefore, shows little conservation during evolution of individual species from common ancestors. Mouse and rat diverged 10–11 million years ago, and now spermatogenesis takes 50% longer in the rat than in the mouse [19]. The second characteristic is that within a species, the cell cycles of the differentiating cells are unalterable. This aspect of cell cycle kinetics appears to be highly conserved in spermatogenesis as species diverge. Mouse Sertoli cells cannot alter the rat germ cell cycle from its inherent genetic determinants. The reason for inflexible cell cycle duration in germ cell differentiation within a species is not clear, but biological phenomena characterized by a high level of evolutionary conservation have fundamental importance to the system and species. Perhaps there are cellular relationships between the differentiating germ cells that are critical for their survival, and these relationships account for the unalterable germ cell cycle kinetics in a species.

While the current studies demonstrate the complete domination of rat germ cell genotype in differentiation timing, it is difficult to know whether a similar control of differentiation steps exists in other self-renewing tissues. All these tissues are characterized by a close association of differentiating and supporting cells, which has made it difficult to assess accurately the cell that exercises primary control of maturation events, such as cell cycle duration. The spermatogonial transplantation technique has provided insight into this regulation in the testis, and these results may have relevance to cell cycle kinetics in other tissues in at least two ways. First, similar experiments with transgene marked stem cells may allow more accurate timing of the differentiation steps in other self-renewing tissues such as skin, bone marrow, and intestinal epithelium. Second, the evolutionary conservation of cell cycle control in the differentiating cells of spermatogenesis may provide a paradigm for other self-renewing tissues. Thus, cells in early stages of differentiation in skin, hematopoiesis, and intestine may likewise have constant cell cycle kinetics that is tissue- and species-specific. These self-renewing tissues generate some of the most interesting and important cells of the body, and new techniques of transgenesis and stem cell transplanta-

tion may allow us to address fundamental questions regarding their renewal and productivity.

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