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Effects of W (c-*kit*) gene mutation on gametogenesis in male mice: agametic tubular segments in W^f/W^f testes

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Summary

Mutations of the W (c-*kit*) gene, which encodes a transmembrane tyrosine kinase receptor, affect the development and differentiation of many types of stem cell. Most homozygous W mutant mice are sterile, due to a lack of germ cells arising during embryonic development, but one of the notable exceptions is W^f/W^f mice, which are fully fertile in both sexes. In order to elucidate the effects of the W^f mutation on spermatogenesis, postnatal spermatogenesis in W^f/W^f mice was histologically examined. The number of gonocytes at birth was significantly reduced and small portions of agametic seminiferous tubule segments were observed in mutant mice. It is suggested that this is due to a deficiency of primordial germ cells (PGC). Other than the agametic tubules, there was no evidence of reduced spermatogenesis after birth. These results indicate that the function of the W (c-*kit*) gene is more necessary for the development of PGC than for postnatal germ cells.

Key words: mice, testis, spermatogenesis, germ cells, W (c-*kit*) gene.

Introduction

A number of independent mutations exist at the *dominant-white spotting* locus (W), which is located on chromosome 5 in mice. These mutations are known to produce the pleiotropic effects of sterility, macrocytic anemia, and depletion of melanocytes and mast cells (Russell, 1979; Kitamura et al., 1978; Green, 1990). The mechanisms underlying the depletion of erythrocytes, melanocytes and mast cells have been investigated by transplantation of cells and tissues (McCulloch et al., 1964; Russell and Bernstein, 1968; Kitamura et al., 1978) and absence of these three types of cells is attributable to a defect in their precursor cells. In recent years, W locus was determined to be allelic with the c-*kit* proto-oncogene (Chabot et al., 1988; Geissler et al., 1988; Tan et al., 1990; Reith et al., 1990; Nocka et al., 1989, 1990), which encodes a transmembrane tyrosine kinase receptor (Yarden et al., 1987; Qiu et al., 1988; Majumder et al., 1988).

Most of the W mutant alleles, such as W, W^v, W^j and W^{44}, cause severe impairment of fertility due to the almost absence of germ cells in the gonads when in the homozygous or double-dominant heterozygous states (Coulombre and Russell, 1954; Russell, 1979; Geissler et al., 1981). The germ cell defect is first apparent at day 9 of gestation, and results from a reduction in the proliferative capacity of the primordial germ cells (PGC) and a retardation in their migration from the yolk sac splanchnopleure to the genital ridge (Mintz and Russell, 1957; Mintz, 1957). Moreover, several severe alleles, such as W^{35}, W^{38}, W^{40}, W^{42} and W^{43}, which are almost homozygous lethal in utero, have deleterious effects on postnatal germ cell differentiation even in the heterozygotes (Geissler et al., 1981).

In contrast, a few alleles permit normal fertility in homozygotes. The W^f allele, which occurred spontaneously in a C3H/He stock, is one of the fertile alleles at the W locus of the mouse (Geissler et al., 1979). Homozygous W^f/W^f mice show very extensive white spotting and have macrocytic anemia, but they are fully fertile in both sexes. However, since further physiological and histological analyses have not been conducted, the possibility of undetected effect(s) of the mutation on gametogenesis cannot be ruled out. In fact, we recently demonstrated a suppression of the regenerative capacity of testicular germ cells in adult $W^f/+$ and W^f/W^f mice by using the experimental cryptorchidism and its surgical reversal; one of the stressed conditions that makes evident the masked effects of mutations at intact condition (Koshimizu et al., 1991).

In this study, the effect of the W^f mutation on spermatogenesis was examined histologically. In W^f/W^f testes, we found some agametic segments of seminiferous tubules lacking germ cells and a decrease in the number of gonocytes at birth. These results indicate

that the W^f allele does affect gametogenesis, at least in the male, although the effect is much less than with other W alleles.

Materials and methods

Mice

C57BL/6-W^f/W^f and congenic +/+ male mice were used in this study. The mutant stock was originally maintained on a C3H/He background at the Institut Pasteur, Paris (Guenet et al., 1979) and the mutant allele was subsequently backcrossed onto C57BL/6 by Dr Kitamura, Osaka University Medical School, Osaka (Nakayama et al., 1990). Mice have been maintained in our animal facilities and were kept in a controlled environment and provided with a commercial diet and tap water ad libitum.

W^f/W^f mice have normal prenatal and postnatal viabilities as compared to +/+. Testes of W^f/W^f mice were reduced in weight (Mean ± s.e.m., 88.3±4.3 mg in +/+ versus 73.8±2.2 mg in W^f/W^f; $P<0.01$). Nevertheless, almost all of the mutant males showed normal fertility (Koshimizu et al., 1991).

Histological examination

Testes were removed and fixed in Bouin's solution, embedded in paraffin, serially cut at 5 μm, and stained with hematoxylin and eosin. Serial cross-sections were examined histologically, and each seminiferous tubule was followed and reconstructed under the light microscope. Identification of spermatogenic cell was according to the criteria of Leblond and Clermont (1952) and Oakberg (1956).

On the day of birth, the number of gonocytes and immature Sertoli cells were counted in more than 200 tubular cross-sections per testis and expressed as the number per 50 tubular cross-sections. The total number of gonocytes per testis, T(NG), was calculated by the formula (Carnes et al., 1991):

$$T(NG) = \frac{N \times L}{T} = \frac{N \times W \times A}{T \times \pi \times R^2}$$

where N was the average NG per tubules; L, the length of tubule; T, the section thickness; W, the testicular weight; A, the relative tubule area, viz, the area of the testis occupied by seminiferous tubules; and R, the radius of the tubule.

Statistics

For each parameter, statistical significance of differences between the two genotypes was determined by Student's *t*-test or Chi-square test.

Results

Histological description of mutant testis

The tunica albuginea, rete testis and seminiferous tubules were all well developed in the testes of W^f/W^f mice as in +/+ mice. As shown in Fig. 1, the seminiferous system was composed mainly of gametic tubules with a small number of agametic tubules. The gametic tubules were populated with spermatogenic cells of all stages, indicating normal spermatogenesis and well-established spermatogenic cycle. In these tubules, no difference was found in the tubule diameter and the number of germ cells at each stage, when compared to +/+ seminiferous tubules (data not shown).

In all W^f/W^f mice examined (more than 40 mice), some agametic tubular cross-sections could be observed. Agametic tubules were reduced in diameter and had very few germ cells; those present, either sperm and/or other types of germ cells, in the central part of the lumen seemed to be an overflow from flanking gametic segments (Fig. 1C,D,E). Although the proportion of agametic tubular cross-sections in the largest testicular sections varied among individuals (1% to more than 10%), there was no difference in the frequency of agametic tubules observed among different age groups (Fig. 2). Such agametic tubules were never observed in +/+ mice. The somatic components of the testis, such as Sertoli cells and Leydig cells, showed normal size, shape and number when examined under the light microscope.

Structure of agametic tubules

We reconstructed the whole seminiferous tubules from complete serial cross-sections under the light microscope, and found that a group of agametic cross-sections existing in a limited area was derived from a single tubular segment. The number of agametic segments per testis was variable from one to more than ten (Means±s.e.m., 4.6±0.8). These segments could be found in any part of the testis. Furthermore, all of the agametic segments were connected at both ends with normal gametic tubules that opened into the rete testis (Fig. 3B). We never observed gametic and agametic tubular segments alternately in a single tubule (Fig. 3C), and agametic segments never opened directly into the rete testis (Fig. 3D). 'Intermediate' segments, less than 0.2 mm in length, containing a few spermatogonia but no differentiated germ cells were observed in the flanking portion of gametic and agametic segments. The length of agametic segments was estimated for reconstructed tubules of 20-, 40- and 60-day-old mice. The length varied from 0.7 to 6 mm, 0.8 to 14 mm and 0.7 to more than 20 mm, respectively (Fig. 4). Although the maximum length of agametic segments was usually longer in older testes, the minimum length was almost constant (0.7-0.8 mm).

Spermatogenesis in prepuberal mice

To understand the developmental origin of the agametic segments, we examined perinatal and prepuberal testes of W^f/W^f mice. In seminiferous cords of 1-day-old mice, two different types of cells are observed, large round cells at the center of the lumen and smaller cells arranged at the periphery of the tubules (Fig. 5A). The former are the gonocytes, the progenitors of type A spermatogonia, and the latter are immature Sertoli cells (Clermont and Perey, 1957; Gondos, 1970; Rugh, 1991). Although no difference was detected in the number of immature Sertoli cells per tubule between +/+ and W^f/W^f testes, the number of gonocytes in W^f/W^f testes was significantly ($P<0.01$) smaller than that in +/+ testes (Table 1, Fig. 5B) and estimated to be approximately 50% of the control value.

In contrast, the first wave of spermatogenesis, characterized by defined accumulation of germ cells at

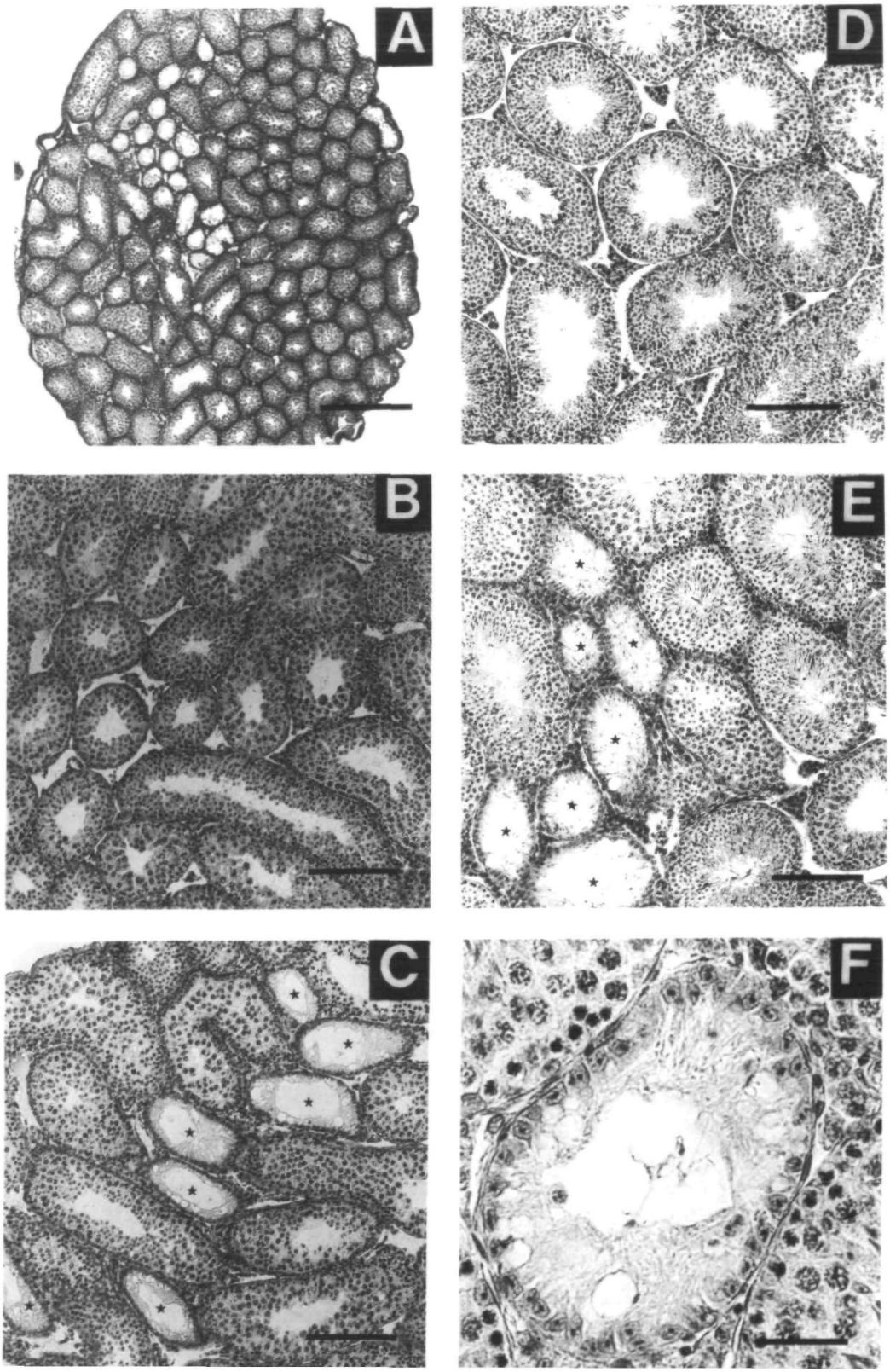

Fig. 1. Cross-sections of seminiferous tubules in +/+ and W^f/W^f mice. (A) A low-power photomicrograph of W^f/W^f testis on day 20 showing both gametic and agametic tubules. (B,C) +/+ and W^f/W^f testis on day 20, respectively. (D,E) +/+ and W^f/W^f testis on day 60, respectively. In C and E, reconstruction of agametic tubules (asterisks) under the light microscope resulted in a single segment. (F) Agametic tubules containing only Sertoli cells. Scale bars=500 μm, 100 μm, and 50 μm in A, B-E, and F, respectively.

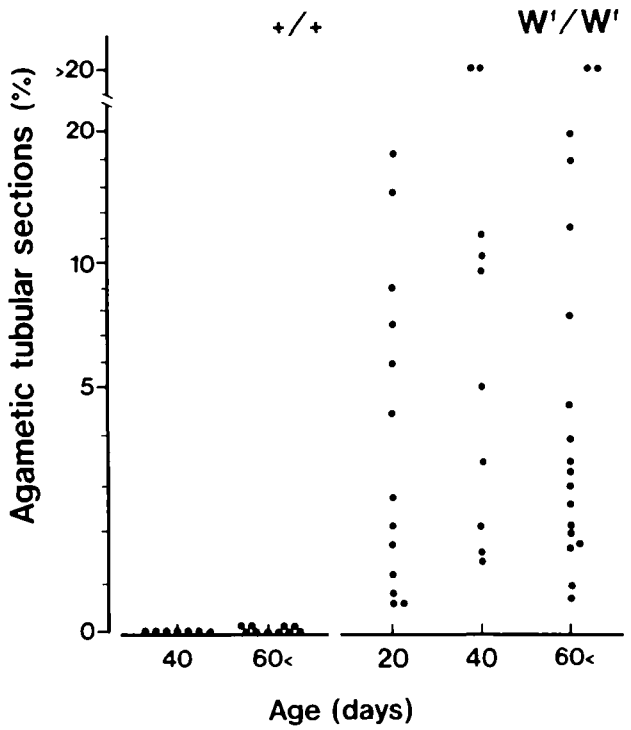

Fig. 2. Frequency distribution of agametic tubules in testes. All of the gametic and agametic tubular cross-sections were counted in the largest testicular sections. The proportion of agametic tubule sections is expressed as a percentage of the total. Each point represents a value for a single testis.

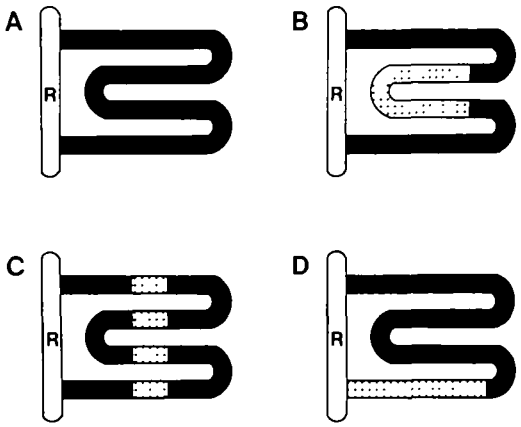

Fig. 3. Schematic representation of seminiferous tubules with agametic segments. Closed and spotted bars indicate the gametic and agametic segments of tubules, respectively. (A) Gametic tubules observed in +/+ and W^f/W^f testis. (B) Tubules having agametic segments observed in W^f/W^f testis. (C,D) Types of tubules not observed in our present study. R, Rete testis.

progressively more advanced stages of differentiation (Nebel et al., 1961; Bellv'e et al., 1977; Rugh, 1991), was not impaired by the W^f mutation (Fig. 1C). There was no difference between genotypes in the proportion of differentiated tubules which both have meiotic germ cells in 12-day-old testes and haploid germ cells in 24-day-old testes (Table 2).

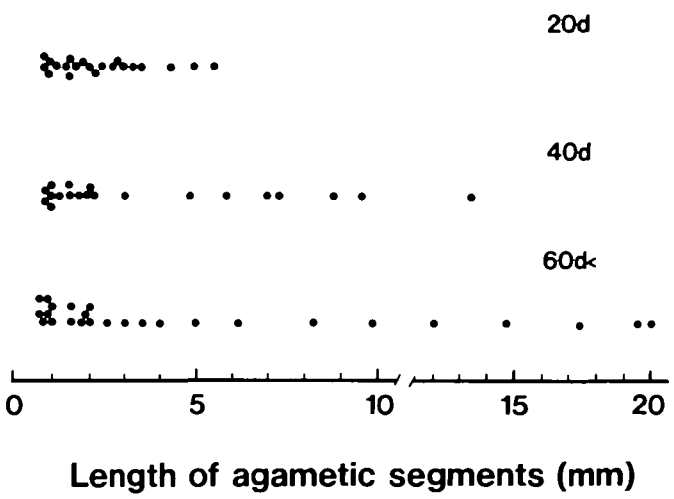

Fig. 4. Length of agametic segments of W^f/W^f mice in various ages. Length of tubular segments having no spermatogenic cells was estimated from serial testicular sections of mice aged 20, 40, and more than 60 days. Data from more than 5 testes in each age (more than 20 agametic segments) are plotted.

Table 1. *Number of immature Sertoli cells and gonocytes in 1-day-old mice (Mean±s.e.m.)*

	Genotypes	
	+/+	W^f/W^f
No. of testes	4	6
No. of immature Sertoli cells per 50 tubules	840±11	845±27
No. of gonocytes per 50 tubules	118±5	87±7*
Calculated no. of gonocytes per testis ($\times 10^4$)	8.4±0.7	4.3±1.1*

*; Significantly different at $P<0.01$ when compared to +/+. No. of gonocytes per testis were calculated as described in Materials and methods.

Table 2. *Percentage of differentiated testicular tubules in prepuberal mice*

Age (days)	Genotypes	No. of testis	Type of tubules			
			I	II	III	IV
12	+/+	4	12.4	71.4	16.2	0
	W^f/W^f	5	18.9	64.0	17.1	0
24	+/+	3	0	0	44.8	55.2
	W^f/W^f	4	7.2*	1.5	42.9	48.4

The number of tubules counted was more than 100 in each testis. Tubular cross-sections were classified into 4 types as follows; Type I, undifferentiated tubules containing only spermatogonia as germ cells or agametic tubules. Type II, tubules containing preleptotene, leptotene, and zygotene spermatocytes, as most advanced type of germ cells. Type III, tubules containing pachytene spermatocytes, as most advanced type of germ cells. Type IV, tubules containing secondary spermatocytes and spermatids. *; Significantly different at $P<0.01$ when compared to +/+ by Chi-square test.

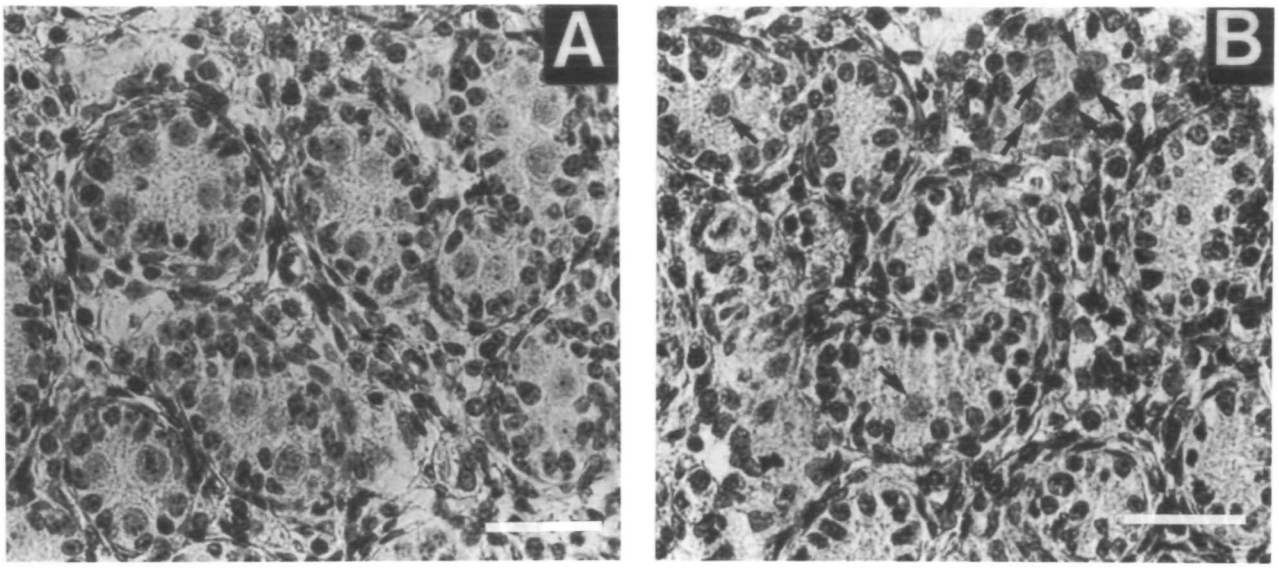

Fig. 5. Cross-sections of seminiferous tubules of mice at birth. (A) +/+ testis. (B) W^f/W^f testis. Note that gonocytes (arrowheads) are less frequent. Scale bar=50 μm.

Discussion

In general, most mutations at the W locus affect the hematopoietic stem cells, melanocytes, mast cells and germ cells. However, in some W mutations, such as W^f, W^{sh} and W^{41}, fertility is not affected, even in the homozygotes, although severe impairments of pigmentation and/or hematopoiesis are observed (Guenet et al., 1979; Geissler et al., 1981; Lyon and Glenister, 1982). Although several possible explanations for such a non-parallel display of mutant characteristics have been discussed (see Discussion in Reith et al., 1990; Nocka et al., 1990), little information on the effects of these mutations on germ cell development was available. In this study, we demonstrate for the first time that homozygous W^f/W^f mice, even though they are fully fertile, had impaired gametogenesis. In W^f/W^f testes, a small number of agametic segments of seminiferous tubules were observed (Fig. 1). These segments lacked all stages of spermatogenic cells, reflecting a complete absence of stem cells. Furthermore, the number of gonocytes at birth was significantly reduced in mutants (Table 1, Fig. 5), indicating either a reduction in the number of PGC colonizing the W^f/W^f gonad or in their subsequent proliferation and/or survival. It has already been reported that c-*kit* transcripts are expressed not only in PGC in fetal testis (Orr-Utreger et al., 1990; Keshet et al., 1991; Manova and Bachvarova, 1991) but also in germ cells in the postnatal testis (Manova et al., 1990; Sorrentino et al., 1991). Although cytological evidence has suggested that the W (c-*kit*) and Sl (c-*kit* ligand, for review, see Witte, 1990) genes play an important role in spermatogenesis even after birth (Nishimune et al., 1980; Tajima et al., 1991; Koshimizu et al., 1991; Sawada et al., 1991; Yoshinaga et al., 1991), the presence of agametic segments of tubules in W^f/W^f seems to result from a prenatal reduction in germ cells and not impaired spermatogenesis after birth. At first, W^f/W^f mice had normal fertility as compared to +/+ and well-established spermatogenesis through most portions of the testis (Fig. 1). Secondly, agametic tubules in W^f/W^f mice were observed at all ages examined and the proportion of these agametic tubules did not increase with age (Fig. 2). Furthermore, no retardation of the first wave of spermatogenesis was observed in prepuberal W^f/W^f mice (Fig. 1D, Table 2). In addition, we previously reported the normal proliferative activity of type A spermatogonia in this mutant testis (Koshimizu et al., 1991). Judging from these results, it is suggested that W^f mutation, like other W series of mutants, primarily affects the migration and/or proliferation of PGC, resulting in a decrease in the number of stem cells, but hardly impairs spermatogenesis after birth. We have obtained similar results in W^{sh}/W^{sh} (data not shown), another fertile W mutant mice (Lyon and Glenister, 1982). These results indicate that the W (c-*kit*) gene is more indispensable for the development of PGC than that of postnatal germ cells. A similar result was also reported by Baker and McFarland (1988) for the hematopoietic cell lineage in W^{44}/W^{44} mice (Geissler et al., 1981) which are nonanemic but stem-cell-deficient. During hematopoiesis, an erythroid progenitor for in vitro burst (BFU-E) is not a primary site of mutant gene action and a progenitor less mature than BFU-E is one of the cells most severely affected.

The important conclusion from our study is that the W (c-*kit*) gene has its primary effect on PGC, but has less effect on more differentiated stages of germ cells. Such phenomenon is not observable in other W mutants, such as W, W^{19H}, W^{37} and W^{42}, in which c-*kit* kinase activity is severely reduced (Tan et al., 1990; Reith et al., 1990; Nocka et al., 1989, 1990), because of death in utero or the complete loss of germ cells in homozygous condition. The residual level of c-*kit* kinase activity present in W^f may be sufficient for

postnatal germ cell development, but inadequate for normal development of PGC, melanogenesis and hematopoiesis. In fact, W^{41}, the phenotypes of which are very similar to W^f (Geissler et al., 1981), has a point mutation within the kinase domain of c-*kit* that partially impairs the kinase activity in cultured mast cells (Nocka et al., 1990). The molecular characterization of the W^f allele, which is unknown at present, will be useful in elucidating the mechanism of the W (c-*kit*) gene action on gametogenesis.

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