Gonadal expression of c-kit encoded at the W locus of the mouse

KATYA MANOVA¹,², KARL NOCKA³, PETER BESMER³ and ROSEMARY F. BACHVAROVA¹*

¹Department of Cell Biology and Anatomy, Cornell University Medical College, 1300 York Avenue, New York, NY 10021, USA
²Permanent address: Institute of Cell Biology and Morphology, Bulgarian Academy of Sciences, Sofia, Bulgaria
³Molecular Biology Program, Sloan Kettering Institute and Cornell University Graduate School of Medical Sciences, New York, NY 10021, USA

*To whom correspondence should be addressed

Summary

Recently, it has been shown that the c-kit proto-oncogene is encoded at the white spotting (W) locus in mice. Mutations of this gene cause depletion of germ cells, some hematopoietic cells and melanocytes. In order to define further the role of c-kit in gametogenesis, we have examined its expression in late fetal and postnatal ovaries and in postnatal testis. By RNA blot analysis, c-kit transcripts were not detected in late fetal ovaries but appeared at birth. The relative amount reached a maximum in ovaries of juvenile mice, and decreased in adult ovaries. c-kit transcripts were present in increasing amounts in isolated primordial, growing and full-grown oocytes, as well as in ovulated eggs. Little was detected in early 2-cell embryos and none in blastocysts. In situ hybridization revealed c-kit transcripts in a few oocytes of late fetal ovaries and in all oocytes (from primordial to full-grown) in ovaries from juvenile and adult mice. Expression was also observed in ovarian interstitial tissue from 14 days of age onward. Using indirect immunofluorescence, the c-kit protein was detected on the surface of primordial, growing and full-grown oocytes, as well as on embryos at the 1- and 2-cell stages; little remained in blastocysts.

In situ hybridization analysis of testes from mice of different ages demonstrated expression in spermatogonia from 6 days of age onward. Using information provided by determining the stage of the cycle of the seminiferous epithelium for a given tubule and by following the age dependence of labeling, it was concluded that the period of expression of c-kit extends from at least as early as type A₂ spermatogonia through type B spermatogonia and into preleptotene spermatocytes. Leydig cells were labelled at all ages examined.

The expression pattern in oocytes correlates most strongly with oocyte growth and in male germ cells with gonial proliferation.

Key words: gonadal expression, c-kit, W locus, mouse embryo, proto-oncogene, oocyte, spermatogonia.

Introduction

Mice homozygous for mutations at the white spotting (W) locus are deficient in generation of three classes of cells: germ cells, melanocytes and hematopoietic cells (specifically stem cells, erythrocytes and mast cells) (for reviews, see Russell, 1979; Silvers, 1979). A large number of alleles associated with a range of deficient phenotypes has been described (Geissler et al. 1981). Germ cells can first be identified as alkaline phosphatase-positive cells around 8 days of development when 10–100 cells are found at the base of the allantois adjoining the caudal primitive streak. In normal mice the number of germ cells increases rapidly to 2500–5500 over the next four days as they migrate from the hindgut to the gonadal ridge (see Heath, 1978, for review). In mice homozygous for more severe W alleles, the germ cells are present at 8 days but fail to increase in number thereafter (Mintz and Russell, 1957). Such mice die of macrocytic anemia around the time of birth, and lack melanocytes and germ cells. Heterozygotes are fertile, display variable degrees of white spotting, and may be mildly anemic. Transplantation experiments have shown the defect to be intrinsic to the melanoblast and the hematopoietic cells (Russell, 1979; Silvers, 1979).

Recently, it has been discovered that the W locus encodes the proto-oncogene c-kit (Chabot et al. 1988; Geissler et al. 1988; Nocka et al. 1989), the normal cellular homolog of v-kit, the oncogene of the HZ4 feline sarcoma virus. Its sequence predicts a tyrosine kinase receptor in the CSF-1 and PDGF receptor family (Besmer et al. 1986; Yarden et al. 1987; Qiu et al. 1988). Antibodies generated to the encoded protein detect glycosylated forms of 124–165×10³ Mr, and a nonglycosylated form of 106×10³ Mr consistent with the size of the predicted protein; it is a cell surface protein with autophosphorylation activity (Yarden et al. 1987; Majumder et al. 1988). Characterization of the molecu-
lar defects of several mutant alleles at the W locus has shown that they affect kinase activity and other aspects of c-kit receptor function (Tan et al. 1990; Reith et al. 1990; Nocka et al. 1990).

Phenotypes very similar to those at the W locus are caused by mutations at another genetically defined locus called steel (Sl) (Russell, 1979; Silvers, 1979). In this case, the defect has been located to the environment of the developing cells, and thus the locus could encode a putative factor that interacts with the c-kit receptor or a product affecting the activity of such a factor (Russell, 1979; Chabot et al. 1988). In fact, wild-type fibroblasts are able to produce a factor that interacts with the c-kit receptor, but Sl/Sld fibroblasts do not (Jarboe et al. 1989; Fujita et al. 1989).

Phenotypic analyses indicate that the W gene may function not only during early development of precursors of a disparate set of cell types, but also may act at different points along the developmental pathway of an individual cell type. In the haematopoietic system, W mutations affect early pluripotential precursors (see Russell, 1979), erythroid precursors (i.e. the transition from BFU-E to CFU-E (Gregory and Eaves, 1978; Iscove, 1978; Nocka et al. 1989), and the proliferation and/or differentiation of mast cells (Kitamura and Go, 1978; Fujita et al. 1988). In melanocytes, W mutations affect melanoblast development, resulting in a lack of pigmentation of the skin. For mice heterozygous for some alleles, melanocytes are produced but the coat color is diluted. This was shown in the case of Wv/+ to Wv/+ genetic background (Russell, 1979; Nishimune et al. 1980; Kuroda et al. 1989). Wv/+ mice showed a reduction in pigment granule number and size, in the differentiated melanocyte (Russell, 1949). Primordial germ cells require the gene during early proliferation as described above. For W and Sl genotypes with mild phenotypic effects, some germ cells survive, but show deficiencies in postnatal development (Coulombre and Russell, 1954; Nishimune et al. 1980; Kuroda et al. 1988; Geissler et al. 1981) (see Discussion).

Consistent with the cell autonomous character of W mutations, there is evidence for expression of c-kit in the three major cell lineages that are affected by such mutations (Nocka et al. 1989). c-kit gene products have been detected in fetal liver, bone marrow and mast cells. Expression is observed in differentiated melanocyte lines, consistent with its function at multiple stages in the development of this cell type. Finally, the mRNA is detected in both ovary and testis. In addition, c-kit is expressed in several tissues not known to be affected by W mutations: brain, placenta, lung and lymphoid tissue (Yarden et al. 1987; Qiu et al. 1988; Nocka et al. 1989).

The present work was undertaken to define the time course of expression of c-kit in germ cells, providing a basis for further studies on the function of the gene. Here we describe the appearance of c-kit mRNA and protein in oocytes during late fetal and postnatal development, and of c-kit mRNA in postnatal male germ cells. We find that the gene is expressed in the female before, during and after the major growth phase of the oocyte, and in the male primarily in spermatogonia. In addition, we observe expression in specific somatic cells: interstitial gland cells of the ovary and Leydig cells in the testis.

**Materials and methods**

**Mice**

Mice used in these studies were derived from mating of ICR females to CB6F1 males. C57BL/6 Wv/+ mice were obtained from the Jackson Laboratory.

**Preparation of RNA and Northern blots**

RNA was extracted from ovaries of mice of different ages as described (Kaplan et al. 1985). Oocytes of different stages were collected as follows. Primordial oocytes less than 20 μm in diameter were collected one day after setting up an in vitro culture of pronase-digested ovaries from 8–10 day old mice (Bachvarova et al. 1980). Growing oocytes from 8 day old mice were obtained directly from collagenase-digested ovaries (Eppig, 1976) by disrupting the follicles with a stirring cycle. Full-grown oocytes were released by pricking ovaries of 20–22 day old females stimulated 2 days previously with 4 units of PMS. Unfertilized ova were collected after superovulation of prepupal or adult mice. Two-cell embryos were obtained about 14 days after fertilization from superovulated CB6F1 females mated to CB6F1 males; for late blastocysts, 2-cell embryos were cultured for 3 days in HTF fluid plus 5 mg ml⁻¹ BSA (Quinn et al. 1988). RNA was extracted from oocytes and embryos as described (Paynton et al. 1988).

32P-labeled riboprobes were derived by transcription with SP6 polymerase of appropriately linearized pGEM7 plasmids containing c-kit inserts (Krieg and Melton, 1987). One probe was transcribed from a 3.6 kb insert including the entire coding region of the message. A second 1.1 kb probe, derived from a 1.6 kb insert, extended from the PvuII site in the region encoding the transmembrane segment to the BstHII site near the 5' end of the region encoding the extracellular domain. This second probe lacks the more conserved region encoding the kinase domain. Hybridization of the blots was carried out as described (Paynton et al. 1988) at 60°C.

In situ hybridization

Mouse ovaries and testes of various ages were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 overnight, washed, dehydrated and embedded in 56°–57°C paraaffin (TissuePrep). Sections 2–5 μm thick were mounted on poly-L-lysine-coated slides and dried. 35S-labeled antisense and sense riboprobes (4x10⁶ cts min⁻¹ μg⁻¹) were synthesized with SP6 and T7 polymerase, respectively, and hydrolysed in mild alkali to a length of 100–200 nucleotides (see Wilkinson et al. 1987a). The slides were hydrated, postfixed in paraformaldehyde followed by treatment with proteinase K and acetic anhydride (Wilkinson et al. 1987a) and prehybridization (Mutter and Wolgemuth, 1987). Slides were hybridized at 65°C overnight in a moist chamber as described (Lehnert and Akhurst, 1988) using 0.3 M NaCl and 60% formamide in the mix and a probe concentration of 0.25 ng μl⁻¹. The 1.1 kb probe described above was used in most experiments. The sense strand probe was derived from the 3.6 kb insert. Slides were washed as described (Wilkinson et al. 1987b) except that the RNase conditions were 50 μg ml⁻¹ in 0.3 M NaCl, 0.01 M Tris pH 8, 1 mM EDTA, for 30 min at 37°C. Slides were dipped in NTB2 emulsion, exposed for 1–4 wks, developed and stained with hematoxylin and eosin according to standard techniques.
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Immunofluorescence
Growing and primordial oocytes were collected 1 day after setting up cultures of pronase-digested ovaries (Bachvarova et al. 1980). Full-grown oocytes and 2-cell embryos were obtained as described above. Some samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min on ice, and washed twice in PBS containing 5% calf serum. Immune serum was derived from a rabbit immunized with vaccinia virus expressing full-length c-kit protein (Nocka et al. 1990). Preimmune and immune sera were passed through DEAE Affi-gel Blue (BioRad) to enrich for immunoglobulins. For absorption, antibody at a dilution of 1:10 was incubated with an equal volume of mast cells from either W/W mice or from +/+ mice (Nocka et al. 1990). Fresh or fixed oocytes and embryos were incubated in a 1:100 dilution of antibody for 30 min on ice. Cells were transferred through PBS containing 5% calf serum, and incubated in FITC-conjugated goat anti-rabbit antibody. Samples were washed and mounted under supported coverslips for analysis in a Nikon Microphot microscope with epi-fluorescence attachment.

Results
RNA blot analysis of c-kit expression in ovaries and oocytes
An initial survey of the expression of c-kit mRNA in ovaries was carried out by RNA blot analysis. Expression was not detected in ovaries from 17½ day fetal ovaries, but a band with a mobility corresponding to the 5.5 kb c-kit mRNA first appears at about the time of birth (Fig. 1A). Expression relative to that of actin mRNA reaches a peak in ovaries from young juvenile mice (8–17 days old), and is reduced in adult mice (Fig. 1A).

Oocytes pass through meiotic prophase in late fetal life, reaching the diplotene stage around the time of birth (Speed, 1982). Oocytes are stored in primordial follicles, which initiate growth at any time from birth to the end of fertile life. A large cohort of oocytes enters the growth phase at approximately the day of birth and reaches full size at about 15 days of age (see Bachvarova, 1985, for review). From this point the bulk of the ovarian tissue consists of multilayered and antral follicles and, after the first ovulation, corpora lutea. The time course described above is consistent with the expression of c-kit in primordial and growing oocytes.

To explore this possibility further, RNA samples prepared from primordial to full-grown oocytes, and ovulated eggs, were analyzed on blots, and found to contain c-kit mRNA (Fig. 1B,C). Using sense strand RNA as a standard in two separate experiments, the amount was estimated roughly as 15 fg per full-grown oocyte or egg and 0.5 fg per primordial oocyte (Fig. 1C). After fertilization, the amount of c-kit transcripts declines several fold by the early 2-cell stage, and is undetectable in blastocysts (Fig. 1B).

In situ hybridization analysis of c-kit expression in ovaries: germ cells
In situ hybridization was used to confirm and extend the investigation of expression of c-kit RNA in oocytes and
to determine whether somatic cells make any contribution to expression in the ovary. The level of label over the region of tissue displaying the lowest grain density, while containing closely packed cells, was taken as the background level; a similar density of labeling was observed using the sense strand probe on slides processed in parallel (see below). The 1.1 kb probe and 3.6 kb probes gave similar results; the 1.1 kb probe corresponding to most of the extracellular domain of the c-kit protein was used for all data presented here, except as indicated in the legends.

Little expression was observed in 17½ day fetal ovaries, except in a few cases in which a few oocytes were labeled (Fig. 2A,D). These are interpreted as being the most advanced oocytes, which have probably reached the early diplotene stage. Newborn ovaries viewed at low magnification displayed numerous scattered points of high labeling closely packed in the periphery and distributed more sparsely in the central region (Fig. 2B). At high magnification abundant grains were observed over nests of diplotene oocytes in the periphery (Fig. 2E) and over oocytes isolated in primordial follicles, which tended to be located more centrally. Little expression above background could be detected in somatic cells. This was confirmed by examining the ovary of a W<sup>−/+</sup> or W<sup>−/+</sup> newborn mouse in a litter derived from a cross between W<sup>−/+</sup> heterozygotes. The number of oocytes was substantially reduced and grains were confined to the regions containing oocytes (Fig. 2C,F).

Abundant expression of c-kit RNA was seen in primordial and growing oocytes in ovaries from juvenile mice 5, 8 and 10 days old (Fig. 3A,B,E), and in oocytes of all sizes up to those in large antral follicles of ovaries from 17 day old and adult mice (Fig. 4A,C,D). Hybridization to the sense probe resulted in little label (Figs 3C, 4B). The grain density in oocytes of different sizes from 8, 10, 17 day and 4½ week old mice declined only 40% during the approximately 100-fold increase in volume from primordial to full grown oocytes.
Fig. 3. Expression of c-kit RNA in primordial and growing oocytes in ovaries of juvenile mice. (A,B,D,E) Hybridized to the c-kit antisense probe. (A) Dark-field image of 5 day ovary. (B) Dark-field image of 8 day ovary. Primordial oocytes are abundant in the periphery and growing oocytes are scattered throughout. (C) Dark-field image of 5 day ovary hybridized to the c-kit sense probe. (D) Bright-field image corresponding to B. Scale bar=200 µm for panels A–D. (E) 10 day ovary, phase-contrast optics. Oocytes in primordial (arrows) and growing follicles are labeled. Scale bar=20 µm.

Table 1. c-kit transcripts in oocytes of different sizes

<table>
<thead>
<tr>
<th>Oocyte stage</th>
<th>Number counted</th>
<th>Oocyte diameter (average in µm)</th>
<th>Grain density (arbitrary units±S.E.M.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial</td>
<td>45</td>
<td>14</td>
<td>100±5</td>
</tr>
<tr>
<td>Small growing</td>
<td>28</td>
<td>30</td>
<td>84±5</td>
</tr>
<tr>
<td>Mid-growth</td>
<td>28</td>
<td>44</td>
<td>72±6</td>
</tr>
<tr>
<td>Large growing</td>
<td>16</td>
<td>58</td>
<td>77±7</td>
</tr>
<tr>
<td>Full-grown</td>
<td>21</td>
<td>67</td>
<td>59±5</td>
</tr>
</tbody>
</table>

Samples were taken from sections of ovaries hybridized to the c-kit antisense probe. Grains were counted over the whole area of the oocyte on its largest cross section. Diameter was the average of two diameters measured at right angles. The average grain density for the groups of oocytes on a given slide were normalized to the value for primordial oocytes on that slide. No significant difference was observed for the relative values obtained from ovaries of 8 day old through adult mice, so the data were combined.

This indicates a 60-fold increase in content of transcripts per oocyte, reasonably similar to the 30-fold increase estimated from RNA blots (see above).

In situ hybridization analysis of c-kit expression in ovaries: somatic cells

Significant somatic cell expression could first be seen in ovaries of 14 day old mice, and became more pronounced at 17 days, located primarily over interstitial tissue (Fig. 4A). This pattern was maintained in adult ovaries, where accumulating interstitial tissue makes a substantial contribution to the overall expression (Fig. 4C,D,H). A few labeled thecal cells surrounding follicles judged to be healthy or only in early stages of atresia were consistently present (Fig. 4F,G). These correspond to the distribution of lipid-containing cells in the theca interna as viewed in plastic sections. In addition, cells of the corpus luteum in cycling adults showed a moderate level of labeling lower than that in interstitial tissue (Fig. 4D,E), as well as corporal lutea at 10 days of pregnancy (not shown). Labeling over follicular cells was at background level at all stages. Hybridization to the sense probe resulted in a background level of labeling (Fig. 4B).

Expression of c-kit protein in oocytes

To determine whether c-kit was expressed at the protein level, live oocytes and early embryos were examined by
Fig. 4. Expression of c-kit RNA in oocytes and somatic ovarian cells of 17 day and older mice. All panels except B hybridized to c-kit antisense probes. The 3.6 kb probe was used for panels A and F. (A) Dark-field image of 17 day ovary. Oocytes and interstitial tissue surrounding follicles are labeled. (B) Dark-field image of ovary from a 4½ wk old mouse hybridized to the sense probe. (C) Dark-field image of a ovary from a 4½ wk old mouse. Arrows indicate two follicles in advanced state of atresia, surrounded by highly labeled interstitial tissue. (D) Dark-field image of adult ovary. A full-grown oocyte in an antral follicle is indicated by the arrow. (E) Bright-field image corresponding to D. cl: corpora lutea. Scale bar=200 µm for panels A-E. (F) Bright-field image of a 17 day ovary. A follicle with several layers has a few labeled cells in the theca interna (arrows). The grains over the oocyte are out of focus. (G) Bright-field image of ovary from a 4½ wk old mouse. A follicle with several layers has a few labeled cells in the theca interna (arrows). (H) Bright-field image of ovary from a 4½ wk old mouse. Interstitial cells are highly labeled, as well as oocytes in a small growing follicle (lower right) and in a primordial follicle (arrow). Scale bar=20 µm for panels F-H.

indirect immunofluorescence, using a rabbit immune serum raised against the c-kit protein (Nocka et al. 1990). Oocytes of all stages showed strong surface fluorescence with immune serum (Fig. 5A,D), but not with preimmune (Fig. 5B,C). The fluorescence on primordial oocytes is patchy and/or less intense compared to growing oocytes (Fig. 5A). Fluorescence is maintained in ovulated eggs and in 1- and 2-cell embryos (Fig. 6A,C). Absorption with +/+ mast cells decreased the fluorescence to near background levels (Figs 5E,F, 6B), while absorption with W/W mast cells, which lack the c-kit receptor (Nocka et al. 1990), did not (Figs 5D, 6A,C). This result confirms the specificity of the anti-c-kit antiserum. In 8-cell embryos, the fluor-
escence is reduced and in blastocysts, in which only the outer surface is exposed to the antibody, it is near the level seen in controls (Fig. 6A,C). Fixed oocytes and embryos displayed a distinct but lower level of fluorescence (not shown).

In summary, c-kit transcripts appear approximately at the time that oocytes enter the diplotene stage of meiosis in late fetal life, and is maintained in primordial, growing, full-grown, and maturing oocytes. The protein is present on primordial oocytes (the earliest stage analysed), and persists during growth, ovulation, and through the 2-cell stage of embryogenesis, disappearing thereafter.

In situ hybridization analysis of c-kit RNA in postnatal testis

To test the generality of c-kit expression in postnatal developing germ cells, in situ hybridization was carried out on testes from newborn to adult mice. At birth, testicular cords contain quiescent nonproliferating gonocytes (T1 spermatogonia) located centrally, and a continuous layer of Sertoli cells located basally. At this stage, no significant label above background was observed (not shown). Further development and establishment of the cycle of the seminiferous epithelium have been described (Clermont and Peret, 1957; McKinney and Desjardins, 1973; Bellve et al. 1977; Sutchiffe and Burgoyne, 1989). At 3–4 days of age, a few spermatogonia were labeled, presumably the most advanced type A, and a few interstitial cells (not shown). At 6 days of age, the progeny of spermatogonia have become the definitive spermatogonia that will be maintained throughout life, including proliferating undifferentiated type A, type A1–4, and a few type B spermatogonia. These have migrated basally and make up about 16% of the cells in tubular cross sections (Bellve et al. 1977).

In situ preparations of testes from 6-day-old mice demonstrated moderate to high labeling of some basal tubular cells. The labeled cells could often be seen to have round nuclei and a halo of clear cytoplasm, presumably type A spermatogonia (Fig. 7A). Many of the unlabeled cells could be identified as Sertoli cells. Interstitial cells, apparently Leydig cells, were also labeled (Fig. 7A). Sections hybridized to the sense strand showed background levels of grains (not shown).

At 9 days of age, type A spermatogonia are still the predominant germ cell. In some cases the labeling defined a distinct basal layer of spermatogonia (not shown).

By 13 days of age, the progeny of type B spermatogonia have entered the next interphase, and some have reached the pachytene stage of meiotic prophase. The 12 stages of the cycle of the seminiferous epithelium (Oakberg, 1956; Clermont, 1972) can be recognized in tubules by the meiotic and postmeiotic cell types present and their arrangement in the layers of the epithelium. This information in turn permits identification of the gonial types. By 13 days of age, four subdivisions of the cycle can be recognized based on the meiotic stages present and their arrangement in the layers of the epithelium (see McKinney and Desjardins, 1973 for a description of 10–21 day old mice and Clermont and Peret, 1957, for the 15 day old rat). Patterns of labeling were characterized as discrete (label over only the basal layer of cells) or diffuse (label over more than one cell layer of the epithelium (Fig. 7B)). The discrete pattern was seen to correspond...
Fig. 6. Expression of c-kit protein on the surface of full-grown oocytes and embryos. Fluorescence images of embryos obtained 1½, 2½, or 3½ days after fertilization. (A) Embryos incubated with c-kit immune serum absorbed with W/W mast cells. 1c: 1-cell fertilized embryo. 8c-mor: compact 8-cell embryos or morulae. blast: early blastocyst. (B) Two-cell embryos incubated with immune serum absorbed with +/+ mast cells. (C) A full-grown oocyte (fgo) and 2-cell to 8-cell embryos incubated with c-kit immune serum absorbed with W/W mast cells. (D) Two-cell embryos incubated with second antibody only. Scale bar=50 µm for all panels.

to tubules with early pachytene spermatocytes and labeled type A spermatogonia (Fig. 8A) (the stage of the cycle of the epithelium is indicated in the legends). It was also seen in tubules with a few pachytene spermatocytes and labeled intermediate or type B spermatogonia (Fig. 8B). The type A spermatogonia appear to have the highest grain density. The diffuse pattern was seen in tubules containing primarily moderately labeled preleptotene spermatocytes (Fig. 8C). Preleptotene cells were identified by their small size and presence in both the basal and inner layers. Sertoli cells were not labeled (e.g. Fig. 8B). No significant label was observed with the sense strand probe (Fig. 7E).

By 19 days of age, all stages of spermatocytes are present. Labeled basal cells (Fig. 7C) were identified as spermatogonia of types A4 to B (Fig. 8D,E) and types A2-3 (Fig. 8F) (see Huckins, 1971, for placement of types A1-4 in the cycle). Preleptotene spermatocytes, now located basally, were also labeled (Fig. 8E). Leydig cells continue to show abundant grains (Fig. 8E).

In adults, the cross-sectional area of the tubules has expanded. A large fraction of the epithelium is occupied by developing spermatids, and the spermatogonia are more sparsely distributed. Identification of the approximate stage of the cycle of the seminiferous epithelium was facilitated by the use of such parameters as the number of layers of spermatids, their position in the epithelium and their nuclear shape (Roosen-Runge and Giesel, 1950; Leblond and Clermont, 1952). At low magnification, two patterns of labeling were most obvious: isolated basal cells with variable numbers of grains and a quite continuous band of basal cells with a low density of grains (Fig. 9A). From the stage of the cycle, it was determined that the former consist of type A4, intermediate and type B spermatogonia (Fig. 9E,F). The latter were preleptotene spermatocytes, seen most typically in tubules at stage VII (Fig. 9G); their identity was confirmed by their small size and high number. Rare type A1-3 spermatogonia could be found labeled in tubules with unlabeled leptotene/zygotene spermatocytes (Fig. 9H). Pachytene spermatocytes at all stages were not labeled above background. Leydig cells were highly labeled (Fig. 9A,D) and unlabeled cells could often be ident-
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Fig. 7. Expression of c-kit RNA in juvenile testis. All panels except E hybridized to the c-kit antisense probe. (A) Bright-field image of 6 day testis. Arrows indicate labeled spermatogonia. L: a small cluster of labeled Leydig cells. Scale bar=20 μm. (B) Dark-field image of 13 day testis. Arrows indicate diffusely labeled tubules. (C) Dark-field image of 19 day testis. (D) Bright-field image corresponding to B. (E) Dark-field image of 13 day testis hybridized to the sense strand probe. Scale bar=200 μm for panels B–E.

We interpret these results as follows. c-kit mRNA is present in spermatogonial stages from about type A through type B, and in the earliest preleptotene spermatocytes. There was no detectable label in later spermatocytes or spermatids. We have not attempted to characterize the labeling of earlier undifferentiated type A spermatogonia directly. Sertoli cells do not contain detectable c-kit RNA, while Leydig cells are labeled from 6 days of age through adult life.

Discussion

The major finding presented here is that c-kit is expressed in type A and B spermatogonia and highly expressed in oocytes. These results imply that the c-kit gene may have a second period of function in germ cells of postnatal gonads, in addition to the well-known requirement for W in primordial germ cells.

In oocytes, c-kit transcripts first appear at the diplotene stage close to the time of birth. They are maintained in primordial oocytes, accumulate during oocyte growth, and persist through oocyte maturation. The c-kit protein is present on the oocyte surface at all stages tested from primordial oocytes through full-grown oocytes, and is still abundant on 2-cell embryos. In male germ cells, expression correlates with the period of spermatogonial proliferation. Transcripts are absent in gonocytes of newborn mice, but are present in proliferating type A and B spermatogonia from their first appearance in juvenile mice, and are retained in the earliest preleptotene spermatocytes. No expression was seen in later meiotic or postmeiotic stages. In addition, c-kit is expressed in some somatic cells of the gonads.

The phenotypes of mice carrying W alleles provide some evidence for a function of c-kit in postnatal female and male germ cells. In addition to a large decrease in the number of germ cells found in juvenile W/Wv and Wv/Wv mice, it was found that the rate of development of both oocytes and spermatogenic cells was slower than in normals in most cases (Coulombre and Russell, 1954). In addition, in some W genotypes, female and male germ cell development varies independently (Geissler et al. 1981), suggesting a function after the primordial germ cell stage.

The close correspondence of phenotypes seen in W and Sl mutant mice indicates that they affect the same cellular target. We can therefore infer some aspects of c-kit receptor function by analyzing Sl phenotypes. Histological examination of juvenile ovaries of infertile Sl/Sl females reveals numerous primordial follicles, but no follicles with growing oocytes (Kuroda et al. 1988), suggesting a function in initiation and possibly maintenance of oocyte growth. Growth can begin at any time from days to months after reaching the primordial oocyte stage. Thus, if the c-kit receptor is involved in oocyte growth, it appears well before it carries out this function. In Sl/Sl+:/+ chimeras, oocyte growth can be initiated and maintained even in follicles comprised entirely of Sl/Sl' cells; thus, the Sl product may be produced outside the follicle (Kuroda et al. 1988). Nevertheless, the principal source of the Sl product could normally be intrafollicular, since only a small contribution of wild-type factor may be sufficient to reinforce the mildly defective factor released by
Fig. 8. Expression of c-kit RNA in individual cells of seminiferous tubules of 13 and 19 day old mice, shown in high-magnification bright-field images of sections hybridized to the c-kit antisense probe. (A–C) 13 day old mice; (D–E) 19 day old mice. (A) Tubule with zygotene or early pachytene spermatocytes, and large labeled basal type A spermatogonia. Approximately stage I of the cycle of the seminiferous epithelium. (B) Tubule with a few pachytene spermatocytes, and basal moderately labeled intermediate or type B spermatogonia. Stage III–V of the cycle. Two unlabeled Sertoli cells are indicated at S. (C) Diffusely labeled tubule with labeled preleptotene spermatocytes in both basal and more apical layers at this age. Approximately stage VII of the cycle. (D) Tubule with early pachytene spermatocytes and labeled type A4 spermatogonia. Approximately stage I of the cycle. (E) Below: tubule with pachytene spermatocytes and moderately labeled intermediate or type B spermatogonia. Stage III–V of the cycle. Above: tubule with pachytene spermatocytes and labeled preleptotene spermatocytes. Approximately stage VII of the cycle. A small cluster of labeled Leydig cells is indicated at L. (F) Tubule with leptotene/zygotene and pachytene spermatocytes, and a few large labeled type A2–3 spermatogonia (arrows). Stage IX–XI of the cycle identified by two layers of meiotic cells. Scale bar=20 μm for all panels.

SI/SI1 cells (Kohrogi et al. 1983). Although oocyte growth can be maintained in vitro, it has been difficult to assess the role of extracellular factors in this process, since oocytes are highly dependent on communication through gap junctions with follicle cells for growth (see Schultz, 1986, for a review; see Buccione et al. 1987).

The c-kit receptor is still present on full-grown oocytes, raising the possibility of a function in the resumption of the cell cycle at the onset of meiotic maturation. The presence of the c-kit receptor through the 2-cell stage of embryogenesis can be accounted for by continued synthesis of the protein on maternal message present in eggs and stability of the protein into the 2-cell stage. It is difficult to speculate on its role during this period.

In agreement with our in situ hybridization results on male germ cells, c-kit RNA expression has been detected in purified spermatogonial cell populations by RNA blot analysis (as well as in Leydig cells) (Sorrentino, V., personal communication). Transcription may be limited to premeiotic cells and the mRNA detected in preleptotene spermatocytes retained from the preceding interphase of type B spermatogonia. In SI/+ mice the transition from type A to type B
Fig. 9. Expression of c-kit RNA in adult testis. All panels except C hybridized to the c-kit antisense probe. (A and B) Dark- and corresponding bright-field images of adult testis (6 mon old). A tubule with an almost continuous band of label in the basal layer is marked c, and two with isolated labeled basal cells are labeled i (upper left). (C) Dark-field image of adult testis hybridized to the sense strand probe. (D) High-magnification bright-field image of a cluster of labeled Leydig cells. (E–H) High-magnification bright-field images of seminiferous tubules. (E) Tubule with labeled type A4 spermatogonia (arrows) at approximately stage I of the cycle of the seminiferous epithelium. (F) Tubule with labeled intermediate or type B spermatogonia (arrows) at stage III–V of the cycle. s: an unlabeled Sertoli cell. (G) Tubule with labeled preleptotene spermatocytes. Stage VII of the cycle of the epithelium identified by the presence of whirls of sperm tails in the lumen (not visible in the photomicrograph). (H) Two tubules with labeled A2-3 spermatogonia (arrows). Stage IX–XII of the cycle identified by the presence of only one layer of spermatids. Scale bar=20 \( \mu \)m for all panels.

spermatogonia is defective (Nishimune et al. 1980). In testicular tubules of \( SL/SL^{al};+/+ \) chimeras, normal regions alternate with undifferentiated regions, presumably corresponding to clones of wild-type and mutant cells (Nakayama et al. 1988). Also, transplantation has shown that the environmental effect of \( SL \) is endogenous to the tubules (Kuroda et al. 1989). These results implicate Sertoli cells as the site of the defect, and thus the source of the \( SL \) gene product.

Developmental transitions of late type A spermatogonia are sensitive to temperature as in cryptorchidism (Haneji et al. 1984), as well as genetic lesions (Sutchiffe and Burgoyne, 1989). The expression of a set of protooncogenes is increased in type B compared to type A spermatogonia (Wolfes et al. 1989), suggesting a new developmental program. If the c-kit receptor is involved in mediating a specific transition at this point, it is present before it carries out this function and availability of its ligand could control the process.

Expression of the c-kit receptor correlates with proliferation of spermatogonia and potential for growth of oocytes. Activation of tyrosine kinase receptors often results in intracellular signals that promote cell proliferation (including growth) (Rosen, 1987; Yarden and Ullrich, 1988; Ullrich and Schlessinger, 1990). The most economical hypothesis is that the same signal can promote the cell cycle when presented to spermatogonia and can promote growth when present to oocytes in meiotic prophase. An additional or alternate possibility is a role in cell survival, analogous to low
level stimulation of macrophages by CSF-1 (Tushinski et al. 1982).

The role if any of c-kit in ovarian interstitial tissue and Leydig cells of testis is difficult to assess. The phenotypes of W and S/J mutant mice have not provided evidence of effects in somatic cells of the gonads (see Kuroda et al. 1989; Terada et al. 1986).

In summary, we have found expression of c-kit transcripts in male and female germ cells starting at or shortly after birth, and maintained to the beginning of meiosis in males and through meiotic maturation in females. Genetic data indicate a functional role for the W and S/J pair of genes at least for a brief period during germ cell development in postnatal gonads.

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