Developmental abnormalities in Steel$^{17H}$ mice result from a splicing defect in the steel factor cytoplasmic tail

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The murine dominant White spotting (W) and Steel (S1) loci encode the c-kit tyrosine kinase receptor and its cognate ligand steel factor (SLF), respectively. Mutations at either locus produce deficiencies in the same three migratory cell populations—those giving rise to pigment cells, germ cells, and blood cells. The identification of the gene products of these two loci combined with the plethora of W and S1 mutations available for molecular analysis offers a unique opportunity to dissect the role of a tyrosine kinase receptor and its cognate ligand during development in a fashion not possible for most other mammalian genes. Among the most interesting S1 mutations available for study are those that induce sterility in only one sex. In studies described here, we show that one of these alleles, S1$^{17H}$, which in the homozygous condition induces sterility in males but not females, is the result of a splicing defect in the SLF cytoplasmic tail. We also characterize the nature of the germ cell defects in male and female S1$^{17H}$ mice and show that both sexes are affected equally during embryonic but not postnatal development. These studies provide new insights into the role of SLF in germ cell development and indicate that the cytoplasmic domain of SLF is important for its normal biological function.

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Mice carrying mutations at the dominant White spotting (W) and Steel (S1) loci have deficiencies in pigment cell, germ cell, and blood cell production. Although both mutations are phenotypically similar, transplantation experiments have demonstrated that the product of the W locus is cell autonomous whereas S1 mutations exert their effect in the extracellular environment (for review, see Silvers 1979). The W phenotype results from mutations in the c-kit proto-oncogene, which encodes a tyrosine kinase receptor (Chabot et al. 1988; Geissler et al. 1988). The c-kit receptor is believed to function as a homodimer, a hypothesis that is supported by the identification of c-kit dominant loss-of-function point mutations. Recently, a ligand for c-kit, designated steel factor (SLF), was identified (Anderson et al. 1990; Flanagan and Leder 1990; Huang et al. 1990; Martin et al. 1990; Williams et al. 1990; Zsebo et al. 1990a) and shown to be the product of the S1 locus (Copeland et al. 1990; Huang et al. 1990; Zsebo et al. 1990b).

Although SLF was originally purified as a soluble protein from supernatant fluids of mouse bone marrow stromal cell lines or Buffalo rat liver cells (Williams et al. 1990; Zsebo et al. 1990a), sequence analysis of SLF cDNAs suggested that SLF is synthesized as a transmembrane protein with an amino-terminal secretion signal peptide, a 189-amino-acid extracellular domain, a single 23-amino-acid transmembrane domain, and a relatively short 36-amino-acid intracellular domain (Anderson et al. 1990; Martin et al. 1990). Sequences encoding the soluble form of SLF are located in the extracellular domain and are thought to be liberated from the membrane-bound factor by proteolytic cleavage. A tissue-specific alternatively spliced form of SLF has also been identified that lacks the putative proteolytic cleavage site and gives rise preferentially to the cell-surface form (Anderson et al. 1991; Flanagan et al. 1991). The presence of both cleavable and noncleavable forms of SLF suggest that both the membrane-bound and soluble forms have important biological roles in development. The need for the membrane-bound form of SLF has been demonstrated for primordial germ cells (PGCs) in vitro (Dolci et al. 1991).

We and others have shown that SLF-coding sequences are completely deleted in homozygous lethal S1 alleles but are present in homozygous viable S1 alleles (Copeland et al. 1990; Huang et al. 1990; Zsebo et al. 1990b).
These results suggest that null alleles are lethal while viable alleles retain at least some normal SLF function. This interpretation is consistent with previous studies showing that the homozygous viable Steel-Dickie \( S^d \) mutation is the result of a discrete 4.0-kb genomic deletion that removes SLF transmembrane and cytoplasmic domains [Branum et al. 1991]. \( S^d \) mice are still capable, however, of producing soluble truncated SLF protein [Branum et al. 1991, Flanagan et al. 1991]. The fact that \( S^d \) mice are severely anemic, sterile in both sexes, and lack pigmentation in the coat provides further in vivo evidence for the importance of the membrane-bound form of SLF in mammalian development. These studies further suggest that molecular characterization of other viable SL alleles will provide additional insight into the function of SLF in mammalian development.

Among the most interesting of all homozygous viable SL alleles are those that produce sterility in only one sex. These alleles include Steel-17\(^H\) \( S^{17H} \), which produces male sterility in the homozygous condition [Peters et al. 1987], and Steel panda \( S^{pan} \), Steel contrasted \( S^{con} \), and Steel t \( S^t \), which produce female sterility in the homozygous condition [Beechey and Searle 1983, 1985; Kuroda et al. 1988]. Unlike other SL alleles [e.g., \( S^d \), \( S^{17H} \), \( S^{pan} \), and \( S^{con} \) homozygotes are only mildly anemic and retain some wild-type pigmentation in the coat [Beechey and Searle 1983, 1985; Peters et al. 1987]. The mild phenotype associated with these mutations suggests that the lesions responsible for these mutations may have a limited effect on SLF function.

In studies described here, we show that \( S^{17H} \) results from a splicing defect that causes the SLF cytoplasmic domain to be read from an alternative reading frame. We also characterize the nature of the germ cell defect in \( S^{17H} \) mice. The results of these studies provide new insight into the role of SLF/c-kit interactions in mammalian development and indicate that sequences encoded in the cytoplasmic domain of SLF are indispensable for its normal biological function.

**Results**

**SLF mRNA is expressed at wild-type levels in \( S^{17H} \) mice**

The effect of the \( S^{17H} \) mutation on steady-state SLF mRNA expression was determined by Northern analysis of total RNA isolated from brain, lung, kidney, and spleen of adult C3H/He--+/+ and C3H/He--\( S^{17H} \)/\( S^{17H} \) mice (Fig. 1). In normal adult mouse tissues, a major 5.5-kb and occasionally minor 3.7- and 2.0-kb transcripts are observed. These size differences appear to reflect alternate use of 3′ poly[A] addition sites rather than differences in protein-coding sequences [M.A. Bedell, unpubl.]. As shown in Figure 1, no differences in SLF mRNA size were observed in \( S^{17H} \) mice when compared with wild-type littermate controls. Any difference in expression levels was the result of loading [data not shown]. These Northern analysis results suggest that the \( S^{17H} \) mutation results from a subtle defect in SLF-coding sequences. The \( S^{17H} \) mutation arose in ethylnitrosourea (ENU)-treated C3H/HeH mice [Peters et al. 1987]. ENU is a potent mutagen that causes primarily point mutations and is unlikely to result in gene rearrangements [Peters et al. 1990].

**\( S^{17H} \) results from a splicing defect in the SLF cytoplasmic tail**

The sequence of the complete coding region of wild-type (\( W \)) and homozygous \( S^{17H} \) mutant (\( M \)) tissues were fractionated and probed with a 1-kb SLF cDNA probe. Arrows at left indicate approximate sizes of the SLF RNA transcripts. To control for RNA loading, the blot was stripped and reprobed with a GAPDH probe [data not shown].

**Figure 1.** Northern analysis of SLF mRNA in adult tissues. Ten megagrams of total RNA from wild-type (\( W \)) and homozygous \( S^{17H} \) mutant (\( M \)) tissues were fractionated and probed with a 1-kb SLF cDNA probe. Arrows at left indicate approximate sizes of the SLF RNA transcripts. To control for RNA loading, the blot was stripped and reprobed with a GAPDH probe [data not shown].
Figure 2. Sequence of Wt and S117H SLF cDNAs and their predicted protein products. The nucleotide sequence of the Wt SLF sequence as reported previously (Anderson et al. 1990) is shown in full, with the deduced amino acid sequence shown below. The putative transmembrane domain is boxed. The gap in the sequence of the S11zH allele is indicated above the Wt sequence by an arrow. The last 28-amino-acids encoded by the S11zH cDNA are shown above the sequence. (A) Exon boundaries. S117H DNA. A single 1.8-kb fragment was detected with both DNAs, suggesting that the mutation was not caused by an intragenic deletion. Direct sequencing of wild-type and S117H 1.8-kb fragments revealed a single nucleotide difference, a T→A transversion in the 3' splice acceptor site of intron 7 (Fig. 3). We also sequenced this region from four other strains and substrains of mice (C3H/HeJ, C57BL/6J, DBA/2J, and D138W101) and found them to be identical to C3H/HeH. We surmise that the point mutation impedes splicing; as a consequence, exon 8 is skipped and exon 7 is spliced directly to exon 9.

These findings were extended to other tissues by RNase protection. As expected, RNAs from tissues of wild-type mice protected a 280-bp fragment (Fig. 4). In contrast, RNAs from S117H mice protected 150- and 60-bp fragments, indicative of an exon 8 deletion. A very small but discernible amount of wild-type 280-bp fragment was also detected in mutant lanes, and mutant 150- and 60-bp fragments were detected in wild-type lanes. This suggests that the intron 7 splice acceptor is weak and that the point mutation in S11zH merely shifts the equilibrium in favor of the 3' splice acceptor of intron 8.

The RNase protection results also provide quantitative information on SLF mRNA expression levels. In most tissues, it appears that SLF mRNA is expressed at wild-type levels in S11zH mice. However, in testis, there is an increased abundance of SLF mRNA in S11zH mice relative to wild-type controls. This is not unexpected because, as discussed later, testes from S11zH/S11zH mice do not contain germ cells (which do not express SLF mRNA) and therefore have a greater contribution of somatic tissue (known to express SLF mRNA)(Keshet et al. 1991; Motto et al. 1991). Finally, lower amounts of SLF mRNA were observed in ovaries of S11zH mice when compared with wild-type controls; but owing to the small size of S11zH ovaries, it has been difficult to determine whether this difference is truly significant.

We also examined whether the alternative splice that removes the putative proteolytic cleavage site in the SLF extracellular domain proceeds normally in S11zH mice. This cleavage site is located within exon 6, and in the alternatively spliced form, exon 5 is fused directly to exon 9.
Splicing defect in Steel\textsuperscript{17H} mice

Figure 3. S\textsuperscript{17H} contains a T → A transversion in the 3' splice acceptor site of intron 7. The mutation is circled, and the arrowheads point to the altered band in the sequencing ladder. Lowercase letters indicate intronic sequences; uppercase letters indicate exon 8 sequences. The intron 7-exon 8 boundary is indicated with a colon.

exon 7. PCR amplification of the region spanning exon 6 using RNA derived from many wild-type and S\textsuperscript{17H} tissues failed to uncover any differences between wild-type and S\textsuperscript{17H} in the amount of alternative splicing (data not shown).

S\textsuperscript{17H} encodes a biologically active SLF protein

Biological activity of SLF protein encoded by S\textsuperscript{17H} mice was assayed by measuring its ability to stimulate \[^3\text{H}\]thymidine incorporation in cultured SLF-dependent murine mast cells (Williams et al. 1990). In this assay, mast cells are cocultured with irradiated CV-1 African green monkey kidney cells that had been transiently transfected with expression constructs containing the coding region of either wild-type or mutant S\textsuperscript{17H} SLF protein. After 24 hr, the cultures were pulsed for 4–5 hr with \[^3\text{H}\]thymidine and counted by scintillation spectrometry. As shown in Table 1, both wild-type and mutant SLF protein stimulated \[^3\text{H}\]thymidine uptake to a similar extent. In these experiments, the transfected CV-1 cells expressing wild-type or mutant protein were shown by Northern analysis to express SLF mRNA equally (data not shown), suggesting that the mutant growth factor contains near wild-type biological activity in this in vitro assay.

Abnormal development of PGCs in S\textsuperscript{17H} mice

We then investigated the nature of the germ cell defect in S\textsuperscript{17H} mice. In initial studies we focused on development of PGCs during embryonic development. This has only recently become possible with the identification of molecular probes that can be used to genotype the embryos for S\textsuperscript{17H}. PGCs are identified as large alkaline phosphatase-positive cells and are first observed in the extraembryonic mesoderm just proximal of the primitive streak around day 7 in development (Ginsburg et al. 1990). By day 8–8.5, PGCs are found mainly in the hind-gut endoderm and at the base of the allantois. Over the next 4 days, they migrate through the hindgut mesentery to the genital ridge. PGC proliferation occurs during the migratory period and for 1 or 2 days after colonization of the genital ridge, which, by 12.5 days postcoitum (dpc), has undergone overt sexual differentiation into the male or female gonad (for review, see Eddy et al. 1981; McLaren 1981).

As S\textsuperscript{17H} induces sterility only in males and as day 12.5 is the earliest embryonic stage at which overt sexual differentiation can be observed, we first examined PGC development in day 12.5 embryos. In male and female S\textsuperscript{17H}/+ embryos, PGC numbers were reduced to ~6% the levels of wild-type controls (Table 2; Fig. 5). Surprisingly, PGC development was equally affected in both sexes. In two heterozygous S\textsuperscript{17H}+/ female embryos, PGC numbers were reduced to ~46% the levels of wild-type controls (Table 2). This suggests that S\textsuperscript{17H} exerts a semidominant effect on germ cell development.

To determine whether the PGC defect can be observed before overt sexual differentiation occurs, we also examined four litters of day 11.5 embryos (Table 2). Even at this early stage, a semidominant effect of S\textsuperscript{17H} on PGC development was observed (Table 2). Again, both sexes were equally affected. PGC numbers were reduced to
This conclusion is consistent with recent reports [Dolci et al. 1991; Godin et al. 1991; Matsui et al. 1991] showing that SLF is required for PGC survival in culture and, under certain circumstances, can also act as a PGC mitogen.

**Effect of Sl17H on postnatal germ cell development**

Our results with Sl17H embryos showed that PGC development is affected equally in both sexes. To define the effects of Sl17H on postnatal germ cell development, we examined the ovaries and testes of Sl17H/Sl17H mice during postnatal development.

**Female development**

In the female mouse, germ cells pass through all oogonal divisions and reach the oocyte stage while still in the embryo. By 3 days after birth they are the static dictyate state, which is maintained until a few hours before ovulation. At each estrus cycle (4.5–5 days in mouse), only a small number of the available oocytes are recruited to mature. It has been estimated that during the total reproductive life of a female mouse no more than a few percent of oocytes will ever be recruited [for review, see Rugh 1990].

The effect of the Sl17H mutation on oocyte growth and development was first examined in three 12-day-old Sl17H/Sl17H female mice. At this stage, the first round of oocyte growth can easily be scored. For all 12-day-old homozygous Sl17H females screened, the ovaries were noticeably smaller than their wild-type littermate controls (Fig. 6A, B). In addition, the mutants contained fewer oocytes: 270 ± 70 (mean ± s.e.m.) vs. 2108 ± 277 in controls (P = 0.03, t-test). Growing oocytes were seen in both the mutant and control ovaries, but fewer were present in the mutant ovaries: 71 ± 29 vs. 393 ± 60 (P = 0.02). This level of reduction in oocyte numbers could be explained solely by the observed effects of Sl17H on embryonic germ cell development. Other than a reduction in oocyte numbers, no other germ cell defects were observed, suggesting that Sl17H exerts little, if any, effect on female postnatal germ cell development. Although we would predict that Sl17H females are subfertile, they can be bred to produce multiple litters; we have bred eight such females and found them to have an average of 3.9 litters containing 5.0 pups each.

The histology of 7-month-old Sl17H/Sl17H female ovaries was also assessed. No follicular tissue or oocytes were observed, although some remnants of corpora lutea were present [Fig. 6C]. Large invaginations of the ovarian surface epithelium were seen, as well as an extensive network of complex tubular adenoma and fat infiltration. This tumor phenotype is identical to that reported by Murphy [1972] for germ cell-deficient Wx/wv mice. Murphy proposed that the lack of germ cells in Wx/wv mice leads to overproduction of pituitary gonadotrophic hormone with excess stimulation of the gonad. A similar mechanism could operate in Sl17H/Sl17H mice, which have reduced germ cell numbers.

~64–66% of wild-type levels in Sl17H/+ embryos and 18–24% in Sl17H/Sl17H embryos. Its interesting to note that in wild-type embryos PGC numbers increased from 1952 PGCs at day 11.5 to 3584 PGCs at day 12.5 while PGC numbers actually decreased during this period [351–468 PGCs at day 11.5 to 215 PGCs at day 12.5] in Sl17H/Sl17H embryos. This suggests that both PGC survival and/or proliferation may be impaired in Sl17H mice.
Table 1. Proliferation of the SLF-dependent mast cell line MC-6 on transfected CV-1 cells

| Construct | transfected | [3H]Thymidine incorporation (cpm) |
|-----------|-------------|----------------------------------|
| Wt        |             | 25,374 ± 1,806                   |
| S117H     |             | 21,017 ± 1,598                   |
| Vector    |             | 2,112 ± 1,459                    |

Male development

Spermatogenesis in the mouse proceeds through a series of well-defined stages beginning with the spermatogonial stage (includes type-A, intermediate, and type-B spermatogonia) and proceeding to the primary spermatocyte, secondary spermatocyte and, finally, the spermatid stage. At 12 days of age, 50% of tubule cross sections from SI17H/S117H littermates had only Sertoli cells, 27% had Sertoli cells as well as a few spermatogonia, and only 23% showed cells advanced to spermatocyte stages (Fig. 7B). In control mice at this age, all tubules contained spermatogonia and Sertoli cells, and 90% of the tubules showed all germ cell stages up to meiotic prophase (Fig. 7A). By 4 weeks of age, all tubule cross sections from control males showed all stages of germ cell differentiation up to either round spermatids (56% of the tubule cross sections) or elongating and maturing spermatids (44% of the tubule cross sections). In contrast, 28% of the tubule cross sections from SI17H/S117H littermates had only Sertoli cells, none had germ cells that had progressed to the elongating or mature spermatid stages, and only 32% had germ cells that had progressed to the round spermatid stage. By 5 weeks of age (Fig. 7C,D), 52% of the tubule cross sections of SI17H/S117H males had germ cells that had progressed to the elongating or mature spermatid stage; however, most of these were virtually devoid of developing spermatocytes representing the next wave of spermatogenesis. Hyperplasia of the interstitial cells was also apparent. Cessation of subsequent germ cell differentiation was even more apparent by 8 weeks of age, when most of the tubules of mutant mice contained Sertoli cells and only a few spermatogonia, although some still showed a few late-stage spermatids with no spermatocytes from the next wave of spermatogenesis. In summary, germ cell development in the testes of SI17H/S117H males can be characterized by delayed onset, reduction in numbers of germ cells, and early cessation of development leading to virtual depletion of differentiating germ cells by 8 weeks of age.

Discussion

We have shown that the developmental abnormalities in SI17H mice result from a splicing defect in the SLF cytoplasmic tail. This splicing defect results from a T → A transversion within the polypyrrimidine tract that lies
Table 2. Abnormal development of primordial germ cells in S117H mice

| Genotype* | +/+ | S117H/+ | S117H/S117H |
|-----------|-----|---------|-------------|
| Age       | Litter | PGC no. | male        | female      | male       | female      |
| 12.5      | 1     | 3584 ± 256 [3] | —           | 0.46 ± 0.07 [2] | 0.06 (1)   | 0.06 ± 0.03 [2] |
| 11.5      | 1     | 1496 [1]     | 0.68 ± 0.01 [2] | 0.87 ± 0.16 [4] | 0.15 [1]   | —           |
| 11.5      | 2     | 1676 ± 201 [3] | 0.65 [1]     | 0.63 ± 0.05 [4] | —           | 0.23 ± 0.03 [3] |
| 11.5      | 3     | 2274 ± 258 [3] | 0.75 [1]     | 0.58 ± 0.10 [4] | —           | 0.28 ± 0.06 [3] |
| 11.5      | 4     | 2277 [1]     | 0.61 ± 0.09 [3] | 0.69 [1]     | 0.20 [1]   | 0.13 [1]    |

*Alkaline phosphatase-positive PGCs were quantitated following serial section of +/+, S117H/+, or S117H/S117H embryos at 11.5 and 12.5 days in development. Embryos were produced, sexed, and genotyped as described in Materials and methods. Numbers listed for wild-type embryos represent the average total number of PGCs per animal (male and female numbers did not differ significantly and were averaged) ± the s.d.; the numbers in parentheses represent the total number of embryos of a given genotype analyzed. Numbers listed for S117H/+ and S117H/S117H embryos represent the percentage of wild-type littermate controls ± the s.d.

Although a number of splicing mutations in the AG dinucleotide have been identified, few have been reported in the polypyrimidine tract. Given that polypyrimidine tracts are somewhat degenerate and often punctuated with purine bases, we were surprised to find that a subtle T → A transversion could have such a dramatic effect on splicing. Smith et al. (1989) have demonstrated that the branchpoint is specified by its immediate sequence context and its proximity to an adjacent polypyrimidine tract. The 3' splice site itself is only recognized in the second step of the splicing reaction and is determined with reference to the branchpoint sequence by a distance-independent scanning process for the first AG dinucleotide downstream of the branchpoint and polypyrimidine tract (Smith et al. 1989). For the 3' acceptor splice site of SLF intron 7, there are 16 nucleotides between the presumed branchpoint and the AG dinucleotide at the 3' splice junction [ctctttttgtttttcag: AA-GAA], 15 of which are pyrimidines. The T → A transversion in S117H reduces the number of pyrimidines by only one but creates a second AG dinucleotide in the middle of the polypyrimidine tract [ctctttttgtttttcag: AAGAA]. Neither AG dinucleotide is now preceded by a true polypyrimidine tract, and splicing at either AG dinucleotide would be inefficient.

In the absence of exon 8, the SLF cytoplasmic tail is read from an alternative frame. Instead of being 36 amino acids long, sequencing data predict that the S117H cytoplasmic tail is 28 residues long with only the first amino acid (lysine) read in the correct frame. A number of hypotheses could explain the effect of this mutation on SLF function.

SLF encoded by S117H mice may be unstable or produced at decreased levels. We consider this unlikely, however, because we have shown that CV-1 cells transfected with expression constructs containing either wild-type or S117H SLF-coding sequences are able to stimulate incorporation of [3H]thymidine by cultured mast cells to approximately the same degree.

Alternatively, the mutant SLF may not be well an-
chored in the cell membrane. Positively charged amino acids within cytoplasmic domains located immediately adjacent to transmembrane regions have been shown to be important for anchoring transmembrane proteins in the membrane (Cutler and Garoff 1986). Mutations that reduce the net positive charge in this region can lead to membrane instability (Cutler et al. 1986). In the case of SLF, the first 3 amino acids of the cytoplasmic domain are Lys-Lys-Lys [+]3. In S117H these amino acids are Lys-Tyr-Ala [+]1, resulting in a net +2 reduction in positive charge. This reduction in positive charge could significantly reduce the stability of SLF within the cell membrane. Antibodies to mouse SLF will allow this model to be tested.

The SLF cytoplasmic tail may have an important undiscovered function that is impaired in S117H mice. This function could involve a feedback or signaling mechanism normally transmitted through the SLF cytoplasmic tail upon interaction with c-kit-expressing cells. Another possible role of the cytoplasmic tail may be to dimerize SLF. The c-kit receptor is thought to function as a homodimer, and it is possible that SLF also acts as a dimer. Dimerization of SLF may be mediated by the cytoplasmic tail while still embedded in the membrane.

One of the unusual developmental effects of S117H is its ability to induce sterility only in males. By examining PGC development in 11.5 and 12.5 dpc embryos, we have shown that S117H leads to abnormal germ cell development before overt sexual differentiation of the embryo at 12.5 dpc. Surprisingly, both male and female mutant embryos are affected equally, with both sexes containing ~6% the normal number of germ cells at 12.5 days in development.

In 12-day-old S117H/S117H female mice we detected a six- to eightfold reduction in the number of primordial and growing oocytes compared with wild-type littermate controls, indicating that S117H/S117H females are actually subfertile. As discussed previously, this reduction most likely results from the effects observed of S117H on embryonic germ cell development rather than an effect of S117H on postnatal germ cell development. Our studies with S117H mice do not help clarify the role of SLF in postnatal female germ cell development. For example, SLF may be required, but sufficient SLF activity could still be present in the S117H ovaries to allow for normal development of the remaining oocytes. Alternatively, SLF or at least its cytoplasmic tail, may not be required for postnatal female germ cell development.

Our studies with S117H mice do, however, suggest an important role for SLF in male postnatal germ cell development. In S117H/S117H mice, male germ cell development was initially characterized by a reduction in germ cell numbers, which again is most likely the result of the reduction in PGC numbers observed at 11.5 and 12.5 dpc. Later, after the first wave of spermatogenesis was complete, a near cessation of sperm development was observed leading to a depletion of differentiating germ cells by 8 weeks of age. S117H therefore appears to affect male postnatal germ cell development in addition to embryonic germ cell development. The antibody-blocking studies of Yoshinaga et al. (1991) also support a role for c-kit/SLF in postnatal male germ cell development. However, in the antibody-blocking studies, only differentiating type-A spermatogonia were affected, whereas primitive [undifferentiated] type-A spermatogonia and spermatogenic stem cells were not affected by antibody treatment. These results contrast with our observation that virtually all stages of spermatogenesis were affected in S117H mice.

It is possible that some of the effects we observed of S117H on postnatal male germ cell development may be indirect. In the adult male, each generation of germ cells is comprised of a group of cells at approximately the same stage of development, which evolve synchronously throughout the spermatogenic process. There is also a Sertoli cell cycle corresponding to the development of...
the sperm that is thought to reflect the role of Sertoli cells as nurse cells to the developing sperm. This role of the Sertoli cell is thought to commence several weeks after birth: In the immature rat, the development of complete spermatogenesis requires follicle stimulating hormone (FSH), but the maintenance of this process in the adult rat does not (Chemes et al. 1979a, b; Dym et al. 1979). It has been postulated that this reflects a fundamental change in the regulation of spermatogenesis from a process that is regulated by FSH to a process that is primarily regulated by cell–cell interactions of the Sertoli cells and the seminiferous epithelium (Zabludoff et al. 1990). In SlzH mice, where germ cell numbers are reduced at birth, these interactions may not be properly established, which could indirectly induce some of the effects on male germ cell development found in SlzH mice.

A spontaneous mouse mutation, juvenile spermatoghnal depletion (isd), which produces a phenotype very similar to that observed in male SlzH testes, has been reported (Beamer et al. 1988). isd is a recessive mutation that maps on chromosome 1, ~6 cm proximal to isocitrate dehydrogenase-1 (Idh-1). Homozygous isd males have small testes, normal serum testosterone, elevated serum FSH, and azoospermia. By 3–4 weeks of age, a reduction in the number of spermatogenic cells is observed. By 8–10 weeks of age most tubules are devoid of germ cells, leaving only Sertoli cells. It has been postulated that Sertoli cells in isd males are deficient in their role as nurse cells for the developing sperm. It will be interesting to determine whether similar signaling pathways are deficient in isd and SlzH mice.

Materials and methods

Northern analysis

Total cellular RNA was isolated from tissues by the guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi 1987). Ten micrograms of RNA from each tissue was size-fractionated on a 1% agarose/formaldehyde gel, transferred to Zetabind membrane, hybridized, and washed according to Church and Gilbert (1984).

Sequence analysis

The SlzH cDNA sequence was determined by direct dideoxy sequencing of PCR-derived brain and lung cDNAs. Two micrograms of total RNA was used as a template to synthesize single-stranded cDNA with the Copy Kit (Invitrogen, San Diego, CA). cDNA was used as a template in two rounds of PCR. The first PCR involved 30 cycles under the following conditions: 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min using 400 ng each of the oligodeoxynucleotide primers, 5'-CGGAGTCGCCAACCAGGCGTC-3' and 5'-GTCACTTGGACAGAATGGGG-3'. The reaction product was gel purified, kinnased, filled in with T4 polymerase, and ligated into the HindII site of Bluescript KS + (Stratagene). One clone was sequenced to verify authenticity and directionality of the expected 278-bp fragment. The PvuII-digested plasmid was used to generate an [α-32P]CTP-labeled 600-bp T7 polymerase transcript, which was purified on an 8 m urea/5% acrylamide gel. RNase protection analyses were performed using the RPA kit (Ambion). Briefly, 20,000 cpm of the above probe was hybridized in solution to 10 μg of total RNA at 50°C for 16 hr followed by digestion with RNases A and T1. The RNA fragments protected from digestion were fractionated on a urea/acrylamide gel, which was used to expose film by autoradiography.

Plasmid construction and transfection

The brain-derived wild-type And SlzH cDNAs made above were used as a template in PCR for 30 cycles under the same conditions described above using 400 ng each of the following primers: 5'-CGGAGTCGCCAACCAGGCGTC-3' and 5'-GTCACTTGGACAGAATGGGG-3'. Each reaction product was gel purified, kinnased, filled in with T4 polymerase, and ligated into Bluescript (Stratagene). Each clone was sequenced to verify authenticity, Wt, 956 bp, and SlzH, 888 bp. Both were then subcloned into an expression vector under control of the SV40 early promoter using the pSG5 vector (Stratagene). CV-1 cells on 10-cm dishes grown in Dulbecco's modified Eagle medium (DMEM) (GIBCO) supplemented with 2 mM L-glutamine, 1 mM Na-pyruvate, and 10% FBS were transfected with 20 μg of expression vector by the calcium phosphate technique (Graham and van der Eb 1973). The cells were shocked with 15% glycerol, 18 hr after the addition of precipitate.

Embryo analysis

The embryos were generated by crossing homozygous SlzH/SlzH females with wild-type C57BL/6 males. The heterozygous F1 animals were then used to set up timed matings, where the morning of the plug was considered as 0.5 dpc. At either 11.5 or 12.5 days of gestation, females were sacrificed, and the embryos were dissected, heads saved for genotyping, and organs removed.
from hind sections to reveal the gonads. The sex of the 12.5 dpc embryos was determined by visual inspection of the gonads. The cleaned hind sections were fixed for 2–4 hr in 4% paraformaldehyde in PBS, overnight in 5% sucrose in PBS, and 2–4 hr in 15% sucrose in PBS. The embryos were embedded in 10% gelatin/15% sucrose in PBS and frozen for sectioning with a cryostat. Serial sections (15 μm) were stained for PGs by alkaline phosphatase histochemistry (Mintz and Russell 1957). The alkaline phosphatase-positive cells were counted in each section under a microscope.

Embryos were genotyped for Sl using a restriction fragment length polymorphism in the coding region of SL between C57BL/6 and C3H/He strains of mice. High-molecular-weight DNA was extracted from the embryo heads, digested with BglII, and fractionated on a 0.8% agarose gel. A Southern blot was probed with a 1-kb Sl cDNA probe, which detects three bands for the wild-type C57BL/6 Sl allele (29, 16.5, and 6.1 kb) and two bands for the C3H/HeH Sl allele (32 and 5.9 kb). The sex of the 11.5 dpc embryos was determined, and the sex of the 12.5 dpc embryos was confirmed by rehybridizing the Southern blot with a Y chromosome-specific probe.

Histological analysis of postnatal gonads

Ovaries were fixed in Bouins’ solution, embedded in paraffin, and serially sectioned at 7 μm. Sections were stained with periodic acid Schiff’s (PAS) reagent so that growing or fully grown oocytes could be identified easily by the PAS-positive staining of the zona pellucida. The number of oocytes in each ovary was estimated by counting the number of primordial and growing oocytes exhibiting a germinal vesicle in every third section throughout the ovary.

Testes were removed from mice and fixed in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer, rinsed in buffer, dehydrated through ethanol, and embedded in methacrylate resin. Sections (5 μm) cut through the mid-region of the testis were stained with PAS reagent for visualization of spermatid acrosomes and cell staging. Twenty-five tubule cross sections per testis were scored for stages of germ cells present. Testes were scored from mice at the ages of 12 days, 3, 4, and 5 weeks and qualitatively assessed for germ cell depletion at 6 and 8 weeks of age.

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