DNA rearrangements located over 100 kb 5' of the *Steel* (*Sl*)-coding region in *Steel-panda* and *Steel-contrasted* mice deregulate *Sl* expression and cause female sterility by disrupting ovarian follicle development

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The *Steel* (*Sl*) locus is essential for the development of germ cells, hematopoietic cells, and melanocytes and encodes a growth factor (Mgf) that is the ligand for *c-kit*, a receptor tyrosine kinase encoded by the *W* locus. We have identified the molecular and germ cell defects in two mutant *Sl* alleles, *Steel-panda* (*Sl^{pan}*) and *Steel-contrasted* (*Sl^{con}*), that cause sterility only in females. Unexpectedly, both mutant alleles are shown to contain DNA rearrangements, located >100 kb 5' of Mgf-coding sequences, that lead to tissue-specific effects on Mgf mRNA expression. In *Sl^{pan}* embryos, decreased Mgf mRNA expression in the gonads causes a reduced number of primordial germ cells in both sexes. However, Mgf expression and spermatogenesis in the postnatal mutant testes is normal, and spermatogonial proliferation compensates for deficiencies in germ cell numbers. In *Sl^{pan}* and *Sl^{con}* homozygous females, decreased Mgf mRNA expression causes sterility by affecting the initiation and maintenance of ovarian follicle development. Thus, regulated expression of Mgf is required for multiple stages of embryonic and postnatal germ cell development. Surprisingly, other areas of the *Sl^{con}* female reproductive tract displayed ectopic expression of Mgf mRNA. We propose that the *Sl^{pan}* and *Sl^{con}* rearrangements alter Mgf mRNA abundance through position effects on expression that act at a distance from the *Sl* gene.

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The pleiotropic effects of mutations at the *Dominant White Spotting* (*W*) and *Steel* (*Sl*) loci are attributable to deficiencies in the development of germ cells, neural crest-derived melanocytes, and hematopoietic cells. Mice that are homozygous mutant for either locus characteristically are sterile, have a white coat with black eyes, are deficient in mast cells, and are anemic (for review, see Silvers 1979). The *W* gene product is a receptor tyrosine kinase, termed *c-kit* (Chabot et al. 1988; Geissler et al. 1988) that is expressed on the surface of the affected cell populations (Nocka et al. 1989; Manova et al. 1990; Orr-Urtreger et al. 1990). The *Sl* locus encodes mast cell growth factor [Mgf; also called *Steel* factor

(SLF); stem cell factor (SCF), or kit ligand (KL)] that is the ligand for *c-kit* (Anderson et al. 1990; Copeland et al. 1990; Flanagan and Leder 1990; Huang et al. 1990; Martin et al. 1990; Williams et al. 1990; Zsebo et al. 1990a,b). Mgf is active as both a soluble and membrane bound factor (Anderson et al. 1990) and is expressed by stromal cells that support the growth and differentiation of germ cells, melanoblasts, and hematopoietic cells (Matsui et al. 1990; Keshet et al. 1991). Upon binding of the ligand to *c-kit*, autophosphorylation of the receptor occurs and causes the activation of downstream signaling molecules. Thus, the products of the *W* and *Sl* loci are integral components of a signal transduction pathway required for intercellular communication in the development of germ cells, hematopoietic cells, and melanocytes.

For both *W* and *Sl*, a large number of independent al-

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les have been identified that result in phenotypes of varying severity (for review, see Silvers 1979). All mutant alleles are semidominant; some are lethal when homozygous and others are viable. One of the most extensively studied *Sl* alleles is *Steel-Dickie* (Sl^d). Although homozygous Sl^d mice are viable, they are severely anemic, sterile in both sexes, and have white coats (Silvers 1979). We and others have shown that the Sl^d mutation is the result of an intragenic deletion producing a biologically active form of Mgf that lacks the transmembrane and cytoplasmic domains (Brannan et al. 1991; Flanagan et al. 1991). The molecular characterization of Sl^d illustrates the specific importance of the membrane-bound form of Mgf in development. Several other viable *Sl* alleles are of interest, particularly with regard to their effects on the germ line. In mice that are homozygous for *Steel-17H* (Sl^{17H}), *Steel-panda* (Sl^{pan}), *Steel-contrasted* (Sl^{con}), or *Steel-t* (Sl^t), a sex-specific effect on fertility is observed (Beechey and Searle 1983, 1985; Beechey et al. 1986; Peters et al. 1987; Kuroda et al. 1988). All four of these alleles also cause mild effects on hematopoiesis and pigmentation. Male Sl^{17H}/Sl^{17H} mice are completely sterile, whereas the fertility of homozygous mutant females is unaffected (Peters et al. 1987). We have demonstrated that the Sl^{17H} allele contains a point mutation that affects splicing of the carboxy-terminal exon encoding the cytoplasmic tail of Mgf (Brannan et al. 1992). As the result of this mutation, the Mgf protein encoded by Sl^{17H} is predicted to lack the normal cytoplasmic domain. An analysis of germ cell development in Sl^{17H} mice indicated that Mgf has distinct roles in both embryonic and adult gonads (Brannan et al. 1992). During embryogenesis, the numbers of primordial germ cells (PGCs) were equally reduced in both male and female mutant embryos relative to wild-type littermates. In the postnatal Sl^{17H}/Sl^{17H} ovary, follicle development proceeded essentially as in wild-type animals despite a reduced number of oocytes. However, postnatal male germ cell development was defective in homozygous mutants and was characterized by a reduced number of germ cells, delayed onset, and early cessation of spermatogenesis. Thus, Mgf may have equivalent functions during embryogenesis in male and female gonads but in adults appears to perform sex-specific functions that, in the male, requires the wild-type cytoplasmic tail.

Female Sl^{pan}/Sl^{pan} and Sl^t/SI^t mice are completely sterile, whereas the fertility of males of the same genotype is unaffected (Beechey and Searle 1985; Beechey et al. 1986; Kuroda et al. 1988). The numbers of primordial follicles in ovaries of young Sl^t/SI^t females are equivalent to those found in wild-type females but these follicles are growth arrested and never mature (Kuroda et al. 1988). Like Sl^{pan} and Sl^t , the Sl^{con} mutation specifically affects female fertility. However, Sl^{con} homozygous females may occasionally bear a single litter if mated at a young age (Beechey and Searle 1983). In the same study, morphological analysis indicated that ovarian follicles of Sl^{con}/Sl^{con} mice degenerate after the first or second estrous cycle. Collectively, the phenotypes associated with Sl^{pan} , Sl^t , and Sl^{con} suggest that Mgf may be involved in

the development of the ovarian follicle; however, the molecular basis for the mutant phenotypes was not known.

We have investigated the molecular and developmental defects in the germ cell lineage that are associated with the Sl^{pan} and Sl^{con} alleles. In the studies described here, we show that both mutations contain chromosomal rearrangements that disrupt sequences located between 100 and 200 kb upstream of the Mgf-coding sequences and cause tissue-specific effects on Mgf mRNA abundance in embryonic and adult tissues. In homozygous Sl^{pan} embryos, decreased numbers of PGCs in both sexes are associated with lower levels of Mgf mRNA in the gonad. In the postnatal ovary, expression of Mgf mRNA in both Sl^{pan} and Sl^{con} homozygous mice was reduced leading to defects in ovarian follicle development. The effect was most pronounced in Sl^{pan}/Sl^{pan} females and caused the early arrest of ovarian follicles. In Sl^{con}/Sl^{con} females, an intermediate effect on Mgf mRNA expression was observed that was sufficient for ovarian follicle growth in young mice but not in older mice. Mgf mRNA is ectopically expressed at high levels in some tissues of Sl^{con} mice and causes abnormal accumulation of mast cells and melanocytes. The Sl^{pan} and Sl^{con} rearrangements appear to cause position effects on the expression of the *Sl* gene.

Results

Sl^{pan} and Sl^{con} affect abundance but not the size of Mgf mRNA

The effects of the Sl^{pan} and Sl^{con} mutations on steady-state levels of Mgf mRNA were determined by Northern blot analysis of several tissues from adult mice. Although neither mutation affects the size of the Mgf transcript, both exert tissue-specific effects on its abundance (Fig. 1). The negative effects on expression are more pronounced in Sl^{pan} tissues than in Sl^{con} tissues. Whereas the Sl^{pan} allele expresses less Mgf mRNA in all tissues examined except brain, the Sl^{con} mutation has a differential effect on Mgf mRNA abundance; in Sl^{con}/Sl^{con} brain, Mgf mRNA levels are increased four- to fivefold while other tissues have a slightly decreased level. Thus, both the Sl^{pan} and Sl^{con} mutations appear to affect either the rate of transcription initiation or stability of Mgf mRNA. Similar observations on Sl^{pan} expression have been reported by Huang et al. (1993).

Both Mgf-coding as well as -flanking sequences appear intact in Sl^{pan} and Sl^{con} mice

The Sl^{pan} and Sl^{con} mutations arose on irradiated (C3H/HeH $\times$ 101/H) F_1 males (Beechey and Searle 1983, 1985; Beechey et al. 1986). The most common types of lesions produced by radiation are large rearrangements and deletions; however, other more subtle mutations have also been produced. To determine whether any gross structural alterations were apparent in the Sl^{pan} - or Sl^{con} -coding regions, DNAs were prepared from homozygous mu-

Figure 1. Northern blot of Mgf mRNA in adult tissues of *Sl^{pan}* and *Sl^{con}* mice. Twenty micrograms of total RNA from wild-type (+), homozygous *Sl^{con}*, and homozygous *Sl^{pan}* tissues were electrophoresed, blotted to nitrocellulose, and hybridized with a Mgf cDNA probe [A]. The size of the Mgf mRNA is indicated at left. The same blot was stripped and hybridized to actin cDNA for loading control [B].

tants, wild-type siblings, C3H/HeH, and 101/H mice and subjected to Southern blot analysis after digestion with at least 10 different restriction enzymes. In each mutant, the pattern of bands hybridizing to a probe encompassing the Mgf-coding region was identical to wild-type DNA (data not shown). These results indicate that the overall organization and structure of the Mgf-coding region in *Sl^{pan}* and *Sl^{con}* are intact. These studies also showed that both mutations arose on a C3H chromosome (data not shown).

The nucleotide sequences of the Mgf-coding region in *Sl^{pan}* and *Sl^{con}* were determined following reverse transcriptase–polymerase chain reaction (RT–PCR) amplification of mutant RNAs. No differences from wild-type sequence were observed, indicating that both mutations lie outside the known coding region of Mgf (Huang et al. 1993; data not shown). To determine whether the structure of flanking sequences in mutant DNAs was normal, a number of probes that encompass the complete 3′-untranslated region of Mgf cDNA and a region extending 18 kb upstream of the Mgf-coding region (M.A. Bedell, L.S. Cleveland, T.N. O’Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins, in prep.) were used on Southern blots of wild-type and mutant DNAs. None of these flanking sequences detected any alterations in mutant DNAs (data not shown). Thus, the structural integrity of the coding sequences, the 3′-untranslated sequences, and a region extending approximately 18 kb upstream of the Mgf-coding region appears to be intact in both mutants. It is therefore likely that these mutations are either subtle mutations within Mgf-flanking sequences or that the mutations are not present in sequences immediately adjacent to the Mgf-coding region.

Long-range DNA rearrangements in *Sl^{pan}* and *Sl^{con}* DNA

To determine whether any alterations are present in *Sl^{pan}* and *Sl^{con}* DNA at a distance from the Mgf-coding region, high molecular weight DNA was prepared from C3H, 101, and homozygous mutant spleens and analyzed by pulsed field gel electrophoresis (PFGE) using the cDNA and genomic clones described above. A composite PFGE restriction map of the Mgf locus in wild-type and mutant DNAs is shown in Figure 2. No alterations were detectable with the cDNA probe in *EagI* fragments of either mutant that extend ~580 kb 3′ to the Mgf-coding region (Fig. 2). However, probing with an upstream genomic fragment (EcoB) revealed fragments in *Sl^{pan}* and *Sl^{con}* DNA that were different from wild-type DNA. For example, whereas EcoB hybridizes to a 680-kb *EagI* fragment in wild-type DNA, *EagI* fragments observed with this probe were 225 kb in *Sl^{pan}* and ~2.2 Mb in *Sl^{con}*. However, the same 90-kb fragment was seen in wild-type and both mutant DNAs upon double digestion with *EagI* and *SfiI*. This result indicates that *Sl^{pan}* DNA contains a rearrangement that breaks between 90 kb and 225 kb 5′ to the Mgf-coding sequences. Similarly, a rearrangement in *Sl^{con}* DNA was localized to a region between 90 and 2.2 Mb 5′ to the Mgf gene. Either rearrangement could potentially be an inversion, translocation, duplication, or deletion that breaks at a considerable distance upstream from the Mgf gene.

Additional probes for the region in proximity to the *Sl^{pan}* and *Sl^{con}* rearrangements were obtained by isolating end probes from two yeast artificial chromosomes (YACs), designated YLE394 and YLE3127, that were cloned from a wild-type mouse library (Imperial Cancer Research Fund) after probing with an Mgf upstream genomic fragment (M.A. Bedell, L.S. Cleveland, T.N. O’Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins, in prep.). The positions of these end probes (3127L, 394L, and 394R, designated L for left end and R for right end) on the wild-type and mutant PFGE maps are shown in Figure 2. The results of these PFGE studies are consistent with the conclusion that sequences extending 3′ to the Mgf-coding region are intact in both *Sl^{pan}* and *Sl^{con}* DNA (Fig. 2). The 5′ rearrangements were localized further using the left-end probes (394L and 3127L). In wild-type DNA, probes 394L and 3127L are located 100 and 220 kb upstream, respectively, of the Mgf-coding region and are contained on a 680-kb *BssHII* or *EagI* fragment (Fig. 2). However, these two probes hybridize to entirely different fragments in DNA from each mutant. For example, in *Sl^{pan}* DNA 394L is located on a 225-kb *Sl^{pan}* *EagI* fragment that also contains EcoB, whereas 3127L detects *EagI* fragments of 540 and 800 kb (Fig. 2). The latter two fragments are probably the result of incomplete methylation. Similarly, in *Sl^{con}* DNA, 394L and 3127L are located on *EagI* fragments of 2.2 and 1.6 Mb, respectively. These and other results (not shown) indicate that the left-end probes of the two YACs flank the rearranged sequences in both *Sl^{pan}* and *Sl^{con}*. However, the physical distance

Figure 2. Long-range restriction map of the *SI* locus in wild-type (C3H), *SI^{pan}* and *SI^{con}* DNAs. Restriction mapping of spleen DNAs was performed by PFGE blot analysis using the following probes: Mgf-coding sequence (Mgf); [EcoB] genomic fragment ~18 kb 5' to Mgf-coding sequences (M.A. Bedell, L.S. Cleveland, T.N. O'Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins, in prep.); (394L, 3127L, 394R) left (L) and right (R) end probes of two YACs (YLE394 and YLE3127; M.A. Bedell, L.S. Cleveland, T.N. O'Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins, in prep.; a unique probe for 3127R has not been obtained). The orientation of the Mgf transcription unit with respect to the centromere is indicated by the arrow (5' → 3') and is described in M.A. Bedell, L.S. Cleveland, T.N. O'Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins (in prep.). The exact location of 3127L within the mutant fragments is not known, nor is the distance between 3127L and 394L in mutant DNA. The probes 6.2/5RI and 1.2/6RI were derived from a chromosomal walk between 394L and 3127L and detect rearrangement breakpoints for *SI^{con}* and *SI^{pan}*, respectively (see text and Fig. 3). The positions of the breakpoints are shown as jagged lines on the *SI^{pan}* and *SI^{con}* restriction maps. Both mutations were induced on (C3H×101)_{F1} mice, although this analysis was done with DNA from both strains (101 results not shown), RFLPs between the two strains for all probes indicated that both mutations arose on the C3H chromosome. Hash marks indicate regions not drawn to scale and the sizes of these fragments are as follows: *SI^{pan}*, 394L, *Bss*HII, 2.3 Mb; *SI^{con}*, 3127L, *Bss*HII and *Eag*I, 1.6 Mb; *SI^{con}*, 394L, *Bss*HII and *Eag*I, 2.2 Mb; all DNAs, 394R, *Eag*I, 3 Mb. Restriction enzyme sites shown are for spleen DNA [*Bss*HII (B); *Eag*I (E); *Sfi*I (Sf)], whereas additional sites for *Sal*I, *Mlu*I, and *Sfi*I which are altered in mutants are not shown.

between the two probes in mutant DNA could not be determined from this analysis.

The breakpoints of the *SI^{pan}* and *SI^{con}* rearrangements were identified using probes derived from a chromosomal walk between 394L and 3127L. Unique sequences derived from phage inserts of walking clones were used as probes on both conventional Southern and PFGE blots of *SI^{pan}* and *SI^{con}* DNA. Two walking probes, 1.2/6RI and 6.2/5RI (see Fig. 2), were identified that detected altered fragments in *SI^{pan}* and *SI^{con}* DNAs, respectively, on conventional Southern blots with a number of restriction enzymes (Fig. 3). For example, probe 1.2/6RI hybridized to a *Hind*III fragment of 6 kb in wild type that corresponds to an 8-kb band in *SI^{pan}* DNA (Fig. 3A, lanes 1 and 2), whereas probe 6.2/5RI hybridized to *Bgl*II fragments of 5 and 20 kb in wild-type and 1.8 and 20 kb in *SI^{con}* DNA (Fig. 3B, lanes 1 and 2). These results indicate that breakpoint fragments for each rearrangement had been identified. The rearrangement breakpoints are located 115 and 195 kb upstream of the Mgf-coding sequences, respectively, for *SI^{pan}* and *SI^{con}* (see Fig. 2). None of the YAC end-probes or walking probes tested displayed any difference in hybridization intensity of mutant versus wild-type fragments (Fig. 3; data not shown). This sug-

gests that neither of the mutations involves deletions or duplicated sequences in this region, a supposition further supported by the absence of lethality in *SI^{pan}* or *SI^{con}* homozygotes.

The *SI^{pan}* mutation results from a large chromosomal inversion

A genomic fragment that contains the *SI^{pan}* breakpoint was cloned from homozygous *SI^{pan}* DNA by hybridization to probe 1.2/6RI. Restriction mapping of the mutant fragment versus the corresponding wild-type fragment revealed the approximate position of the *SI^{pan}* breakpoint. A unique probe from the proximal side of the *SI^{pan}* breakpoint (pan2RP) hybridized to the same mutant fragments as did probe 1.2/6RI in PFGE blots (not shown) and in Southern blots (Fig. 3A; cf. lane 2 vs. 4 and 6 vs. 8) but different wild-type fragments (Fig. 3A, cf. lane 1 vs. 3 and 5 vs. 7), confirming the authenticity of this breakpoint probe. The nature of the *SI^{pan}* rearrangement was then investigated by mapping the chromosomal location of this upstream breakpoint probe in an interspecific backcross mapping panel (Copeland and Jenkins 1991) and by cytogenetic analysis of *SI^{pan}* chromosomes. The

Figure 3. Southern blots of altered DNA bands at the *Sl^{pan}* (A) and *Sl^{con}* (B) rearrangement breakpoints. (A) C3H (lanes 1,3,5,7) and *Sl^{pan}/Sl^{pan}* (lanes 2,4,6,8) DNAs were digested with *Hind*III (lanes 1–4) or *Xba*I (lanes 5–8). The blot was first probed with 1.2/6RI [a walking probe derived from wild-type DNA (see Fig. 2, lanes 1,2,5,6)] and then stripped and probed with pan2RP [a probe derived from the mutant portion of the *Sl^{pan}* breakpoint fragment (see text) lanes 3,4,7,8]. (B) C3H (lanes 1,3,5,7) and *Sl^{con}/Sl^{con}* (lanes 2,4,6,8) DNAs were digested with *Bgl*II (lanes 1–4) or *Hind*III (lanes 5–8). The blot was first probed with 6.2/5RI [a walking probe derived from wild-type DNA (see Fig. 2) lanes 1,2,5,6] and then stripped and probed with con0.4RI [a probe derived from the *Sl^{con}* breakpoint fragment (see text); lanes 3,4,7,8]. Asterisks indicate the positions of cross-hybridizing bands in B. Arrows in A and B indicate the mutant bands, and the sizes (in kb) are indicated at left. 101 DNA was also used as a control (not shown); however RFLPs between this strain and C3H indicated that both mutants arose on the latter chromosome.

segregation of a polymorphic *Bgl*II fragment hybridizing to pan2RP (C57BL/6J, 4 kb; *Mus spretus*, 12 kb) was determined in 169 backcross progeny and the locus detected by this probe, designated *D10Fcr2*, was mapped to a position 43.2 ± 3.8 cM proximal of *Mgf* (Fig. 4A). The mapping data predict that the *Sl^{pan}* mutation is an inversion with the proximal breakpoint just distal to *Myb* and the distal breakpoint 115 kb upstream from *Mgf*-coding sequences (Fig. 4A). Confirmation of a large paracentric inversion was obtained by karyotypic analysis of

Sl^{pan} chromosomes (Fig. 4B). Comparison of the G-banding pattern of wild-type and *Sl^{pan}* chromosomes indicates that an intercalary portion of the mutant chromosome has been inverted; the proximal break in *Sl^{pan}* is at the junction of bands 10B1/10B2 and the distal break is at the junction of bands 10C2/10C3. The positions of the cytological breakpoints exactly correspond to the positions of the breakpoints predicted from the mapping data and are further consistent with a ~ 40 cM inversion in the *Sl^{pan}* chromosome that is cytologically $\sim 40\%$ of the physical length of chromosome 10.

A 7-kb *Sl^{con}* breakpoint fragment has been cloned, and a portion of this (con0.4RI) was shown to detect the same altered bands as probe 6.2/5RI on Southern blot analysis of *Sl^{con}/Sl^{con}* DNA (Fig. 3B, cf. lane 2 vs. 4 and 6 vs. 8). However, further analysis of the *Sl^{con}* breakpoint has so far been limited by the repetitive nature of the probes obtained from the rearranged side of the *Sl^{con}* breakpoint. Although con0.4RI spans the breakpoint and detects the altered bands in C3H DNA, this probe does not sufficiently overlap the rearrangement to follow segregation of the corresponding sequences in the *M. spretus* allele of the backcross panel. Karyotypic analysis of *Sl^{con}* chromosomes has not revealed any obvious abnormalities (Davisson and Lewis 1990; data not shown) indicating that the *Sl^{con}* rearrangement is relatively subtle. It is still possible that the *Sl^{con}* rearrangement could also be an inversion that is too small to be detected by karyotypic analysis.

Analysis of primordial germ cell development in *Sl^{pan}* mice

Germ cell development was first examined in *Sl^{pan}* mice during embryogenesis. PGCs were quantitated in mutant and wild-type embryos as described previously (Brannan et al. 1992). Three to five mutant embryos of each genotype and sex were analyzed at embryonic day 11.5 (E11.5), and the fraction of PGCs relative to wild-type littermates was as follows: female *Sl^{pan}/+*, 0.56 ± 0.11 ; male *Sl^{pan}/+*, 0.45 ± 0.15 ; female *Sl^{pan}/Sl^{pan}*, 0.25 ± 0.15 ; male *Sl^{pan}/Sl^{pan}*, 0.23 ± 0.12 . These values were reduced further at E12.5 with homozygous mutants of each sex having $\sim 10\%$ the number of wild-type PGCs. The effect of the *Sl^{pan}* mutation on PGC numbers is quantitatively similar to that observed previously with *Sl^{17H}*, a male-sterile allele (Brannan et al. 1992). Thus, two *Sl* alleles that cause sterility in opposite sexes have equivalent effects on male and female PGC development.

In situ hybridization analysis of *Sl^{pan}* embryos was performed to determine whether the observed PGC deficiencies are attributable to decreased *Mgf* expression. Several *+/+*, *Sl^{pan}/+*, and *Sl^{pan}/Sl^{pan}* embryos of each sex were examined at E12.5 after hybridization to a *Mgf* antisense probe. The pattern of expression observed in *+/+* embryos was identical to that previously described (Matsui et al. 1990; Keshet et al. 1991). However, some tissues of homozygous *Sl^{pan}* embryos displayed reduced levels of *Mgf* mRNA (Fig. 5). In contrast to the high ex-

Figure 4. The *SI^{pan}* chromosome contains a large inversion. (A) The chromosomal position of a probe derived from the novel portion of the *SI^{pan}* breakpoint fragment (pan2RP; see text) was determined using an interspecific backcross panel (Copeland and Jenkins 1991). Only selected loci that have been mapped previously for wild-type chromosome 10 are shown. The locus detected by pan2RP is *D10Fcr2* and maps 43.2 ± 3.8 cM proximal to the *Mgf* cDNA probe and 4.35 ± 1.61 cM distal to *Myb*. The most likely explanation for these mapping results is that the *SI^{pan}* chromosome (right) contains a very large inversion such that *D10Fcr2* is juxtaposed to only 115 kb from the *Mgf* gene. (B) An ideogram of wild-type chromosome 10 is shown at left; the *SI* gene has been localized previously to band D1. Comparison of the banding patterns of wild-type and *SI^{pan}* chromosomes confirms that the latter contains a large inversion whose breakpoints correspond to those predicted from the mapping data (A). The positions of the inversion breakpoints are shown as dotted lines on the ideogram.

pression in the gonads of wild-type embryos of both sexes, (Fig. 5B,F), no detectable expression was seen in the homozygous mutant ovary (Fig. 5H) and only minimal expression was observed in homozygous mutant testes (Fig. 5D). Overall expression in the body of the mutant embryos, as well as the mutant dermis, was also reduced but nonetheless was still detectable. In the neural tube of homozygous mutants, expression of *Mgf* mRNA in the floor plate and motor neurons was moderately reduced compared to wild-type, whereas some tissues (kidney and liver) displayed no detectable difference from wild-type expression (Fig. 5; data not shown). Thus, the *SI^{pan}* mutation displays a tissue-specific effect on embryonic as well as adult mRNA expression. Although decreased expression of *Mgf* mRNA during embryogenesis is likely the cause of the PGC deficiencies, these effects occur in both sexes, indicating that the basis for the specific effect of the *SI^{pan}* allele on female fertility must be occurring later in development.

Postnatal development of germ cells in *SI^{pan}* and *SI^{con}* mice

The postnatal development of normal ovarian follicles is summarized in Figure 7C (below) and involves an intricate pattern of bidirectional communication between so-

matic cells and oocytes and is hormonally regulated (for review, see Freeman 1988; Buccione et al. 1990; Eppig 1994). In newborn wild-type female mice, each oocyte is enclosed in a primordial follicle consisting of a single layer of flattened somatic cells that are precursors to the follicle (or granulosa) cells. As the oocyte expands in size, the follicle cells differentiate to form primary follicles with a single layer of cuboidal cells. Secondary follicles with multiple layers of follicle cells first appear at ~2 weeks after birth, and the presence of a fluid-filled cavity (antrum) marks the appearance of tertiary follicles. The development of both tertiary and Graafian follicles and the release of mature eggs are controlled by pituitary gonadotrophins, follicle-stimulating hormone, and luteinizing hormone. At 4–5 weeks after birth, sexual maturity is reached and the first eggs are ovulated in response to a surge in luteinizing hormone. The remnants of the follicle then differentiate into the progesterone-secreting corpus luteum. If pregnancy does not ensue, the corpus luteum degenerates within 12–14 days and a new pool of follicles is recruited every 4–5 days in concert with the estrous cycle.

Because the results described above showed that the *SI^{pan}* mutation affects the steady-state levels of *Mgf* mRNA in many adult tissues as well as embryonic gonads, it was possible that expression in the postnatal

Figure 5. Expression of *Mgf* mRNA in wild-type and *Sl^{pan}* embryos. Bright-field (top) and dark-field (bottom) photomicrographs of sections from E12.5 mice after in situ hybridization to *Mgf* antisense cDNA probe. Males (A–D) and females (E–H) of the indicated genotypes are littermates. (NT) Neural tube; (Dm) dermis; (Mn) metanephros; (Te) testes; (Ov) ovary; (Li) liver; (St) stomach; (G) gut. Arrows indicate the gonads in the dark-field photomicrographs. All were photographed using a 10× objective. The section shown in C and D only has one testis. Note that although some tissues in homozygotes of both sexes express similar levels of *Mgf* mRNA relative to wild-type, expression in the gonads of *Sl^{pan}/Sl^{pan}* embryos is greatly reduced (males) or not detectable (females).

ovary was also affected. *Mgf* has been shown previously to be expressed in follicle cells, whereas *c-kit* is expressed by oocytes and thecal cells (Manova et al. 1990, 1993; Keshet et al. 1991; Yoshinaga et al. 1991; Motro and Bernstein 1993). In situ hybridization analysis of wild-type ovaries revealed that *Mgf* mRNA expression is up-regulated during early follicle growth such that secondary follicles express much higher levels than primordial or primary follicles (Fig. 6B; see also Manova et al. 1993). However, we were unable to detect expression of *Mgf* mRNA in *Sl^{pan}/Sl^{pan}* ovaries, and only a few oocytes were present (Fig. 6C,D), consistent with the previous observations made in embryonic mutant ovaries. Huang et al. (1993) also showed that *Mgf* expression and oocyte numbers are severely reduced in the homozygous *Sl^{pan}* ovary. Although primordial follicles are initiated around the few oocytes remaining in homozygous *Sl^{pan}* ovaries at postnatal day 12 (P12), development of the ovarian follicles is arrested at the primary follicle stage (Fig. 6C; Huang et al. 1993). Thus, the sterility of *Sl^{pan}/Sl^{pan}* females is attributable to decreased numbers of oocytes with early arrest of ovarian follicle development that is probably caused by the virtual absence of *Mgf* mRNA.

Beechey and Searle (1983) have reported that although ovaries of young homozygous *Sl^{con}* mice display normal follicle development, albeit with reduced numbers of oocytes and growing follicles, ovaries of older homozygous mice contain no follicles. This suggests that the developmental defect(s) causing sterility in *Sl^{con}* females may be occurring postnatally but at a different stage than in *Sl^{pan}* females. Ovarian follicle development in *Sl^{con}* mice was examined in more detail by histological analysis of *Sl^{con}* ovaries at ages ranging from 2 to 10 weeks after birth (Fig. 7). At all ages examined, the heterozygous (not shown) and homozygous (Fig. 7) *Sl^{con}* ovaries had fewer oocytes than wild-type controls. In 2-week-old *Sl^{con}/Sl^{con}* ovaries, secondary follicles were present; however, their development appeared to be delayed and the follicle structures were not as orderly as in wild type (Fig. 7A,B). Apparently normal follicles, including all stages up to tertiary follicles, were seen in 4-week-old *Sl^{con}/Sl^{con}* ovaries (Fig. 7D,E), but after 5 weeks, many of the follicles were undergoing atresia (Fig. 7F). At 8 weeks, only an occasional atretic follicle could be seen (Fig. 7G), and by 10 weeks, the entire mutant ovary was comprised of corpus luteum (Fig. 7H). Thus, young *Sl^{con}*

Figure 6. Expression of *Mgf* mRNA in P12 ovaries of wild-type (A,B) and *Sl^{pan}/Sl^{pan}* (C,D) mice. Bright-field (A,C) and dark-field (B,D) photomicrographs after in situ hybridization to *Mgf* antisense cDNA probe. (Pr) Primordial follicles; (1°) primary follicles; (2°) secondary follicles. In wild-type ovaries, *Mgf* mRNA expression is up-regulated during follicle growth. *Sl^{pan}/Sl^{pan}* ovaries express no detectable *Mgf* mRNA, and growth of the few remaining follicles has initiated but is arrested at the primary follicle stage. Arrows indicate arrested follicles in the mutant ovaries (C,D).

homozygous females have a reduced number of growing follicles that are delayed in their initial development. Subsequently, these follicles develop normally for a short period but then fail to be maintained after ~5 weeks of age, about the time of the first estrous cycle. These results are consistent with previous reports that young homozygous *Sl^{con}* females may occasionally have a single litter but that older homozygous females are completely sterile (Beechey and Searle 1983).

To determine whether the delayed follicle development and the lack of maintenance of follicles in *Sl^{con}* mice results from abnormal *Mgf* mRNA expression in the postnatal ovary, in situ hybridization analysis was performed on ovaries from wild-type and *Sl^{con}* homozygous mice at 2, 4, and 5 weeks after birth (Fig. 8). At 2 weeks of age, *Sl^{con}/Sl^{con}* ovaries expressed low but de-

tectable amounts of *Mgf* mRNA (Fig. 8B), and the signal was more diffuse than the restricted expression seen in growing follicles of wild-type ovaries at the same age (Fig. 8A). This reduced and diffuse expression is likely the reason for the delay in *Sl^{con}* follicle development described above. Although overall expression in the 4-week-old *Sl^{con}/Sl^{con}* ovaries was reduced relative to wild-type ovaries of the same age, about the same amount of *Mgf* mRNA was observed in tertiary follicles of both genotypes (Fig. 8C,D). By 5 weeks of age, *Mgf* mRNA expression was not detectable in *Sl^{con}/Sl^{con}* ovaries (Fig. 8F). At all ages, *Mgf* mRNA was expressed at lower levels in *Sl^{con}/Sl^{con}* ovaries than in wild-type ovaries but at higher levels than in *Sl^{pan}/Sl^{pan}* ovaries. This intermediate effect of the *Sl^{con}* mutation relative to the *Sl^{pan}* mutation on ovarian expression is consistent with

Figure 7. Morphology of *Sl^{con}* ovaries in young and older mice. Photomicrographs of hematoxylin/eosin-stained sections of ovaries from wild-type (A,D) and *Sl^{con}/Sl^{con}* (B,E,F,G,H) at 2 weeks (A,B), 4 weeks (D,E), 5 weeks (F), 8 weeks (G), and 10 weeks (H) after birth. All panels were photographed using a 10× objective. A schematic of stages of ovarian follicle development is shown in C (reprinted, with permission, from Erickson et al. 1985). (Pr) Primordial follicles; (1°) primary follicles; (2°) secondary follicles; (3°) tertiary follicles; (At) atretic follicles; (CL) corpus luteum. At all ages, *Sl^{con}* homozygous ovaries have decreased numbers of oocytes and follicles. At 2 weeks (B), follicle development in the mutant has initiated but is delayed, and the overall follicle structure is disorderly. By 4 weeks (E), some follicles in *Sl^{con}/Sl^{con}* ovaries have developed to the normal stage. At 5 weeks (F), many of the remaining follicles in *Sl^{con}/Sl^{con}* ovaries are undergoing atresia. At 8 weeks (G) only an occasional atretic follicle is seen with most of the ovary comprised of CL, whereas at 10 weeks (H) no growing follicles were seen and the entire ovary is CL.

Figure 7. (See facing page for legend.)

Figure 8. Expression of *Mgf* mRNA in postnatal ovaries of *Sl^{con}* mice. Dark-field photomicrographs of ovaries from wild-type (A,C,E) and *Sl^{con}/Sl^{con}* (B,D,F) ovaries of various ages of mice after in situ hybridization to an *Mgf* antisense cDNA probe. The ages chosen represent the period of early follicle growth (2 weeks; A,B), apparently normal (4 weeks; C,D), and degeneration (5 weeks; E,F) in *Sl^{con}/Sl^{con}* ovaries. (1°) Primary follicles; (2°) secondary follicles; (3°) tertiary follicles; (ovd) oviducts; (ut) uterus. (A–D) Photographed using a 10× objective; (E,F) photographed at 5×. At 2 weeks of age, *Mgf* mRNA expression is significantly reduced in *Sl^{con}/Sl^{con}* ovaries but is detected at a low level and is more diffuse than the localized expression in growing follicles of wild-type (B compared with A). In 4-week *Sl^{con}/Sl^{con}* ovaries, a low level of expression is seen in the mural granulosa cells of 3° follicles. However, by 5 weeks of age there is no detectable expression in *Sl^{con}/Sl^{con}* ovaries (F), whereas expression is apparent in mural granulosa cells of wild-type ovaries (E). Note also the abundant expression of *Mgf* mRNA in the *Sl^{con}/Sl^{con}* oviducts (B,F) compared with little or no expression in oviducts of wild type (A,F).

the steady-state levels of *Mgf* mRNA observed in other adult tissues by Northern blot analysis (see Fig. 1).

In contrast to the defects in postnatal ovarian follicle development described above for *Sl^{pan}* and *Sl^{con}* females, no major defects were found in the postnatal testes of mice homozygous for either mutation (not shown). Unlike the decreased expression seen in embryonic testes and postnatal ovary, the postnatal testes of homozygous *Sl^{pan}* mice expressed essentially normal levels of *Mgf* mRNA as revealed by in situ hybridization of neonatal, juvenile, and adult testes. Morphological analysis of the neonatal testes revealed that males homozygous for either *Sl^{pan}* or *Sl^{con}* had reduced numbers of germ cells compared with wild type. In both mutants, the initial germ cell deficiencies in males were compensated for by mitotic proliferation of spermatogonia such that nearly normal numbers of differentiating germ cells were seen in older males and all subsequent stages of spermatogenesis occurred normally.

Ectopic expression of Mgf mRNA in the reproductive tract of Sl^{con} mice

Although *Mgf* mRNA expression in the ovaries of *Sl^{con}* mice is lower than in wild-type ovaries, the oviducts and uterus of *Sl^{con}* mice express abnormally high levels of

this gene. This effect was observed in *Sl^{con}* homozygous mice (e.g., Fig. 8, B and F; Fig. 9, B and D), with heterozygous mice displaying an intermediate level of expression (not shown). In the uterus of immature wild-type mice (2 weeks old), low expression of *Mgf* mRNA was detected in the stromal cells of the endometrium (Fig. 9A). Similar localization of *Mgf* mRNA has been reported in the porcine uterus (Zhang and Anthony 1994). In 2-week-old *Sl^{con}/Sl^{con}* mice, expression in the endometrium was increased slightly compared with wild type, whereas extremely high expression was seen in the myometrium, specifically the inner circular layer of muscle cells (Fig. 9B). In the oviduct of *Sl^{con}* mice (Fig. 9D), ectopic expression of *Mgf* mRNA was restricted to certain regions with maximal expression observed in the isthmus and ampulla but no detectable expression was seen in the mutant infundibulum or any portion of the wild-type oviduct (Fig. 9C). As the ectopic expression occurs in muscle cells, these regional differences in expression appear to be related to the thickness of the muscular layer in different portions of the oviduct.

Expression of *Mgf* mRNA in the ovary is differentially regulated at various stages of the estrous cycle (Motro and Bernstein 1993). However, the ectopic expression that we have noted in *Sl^{con}* uteri and oviducts occurs independently of the estrous cycle. First, high expression

Figure 9. The *Sl^{con}* mutation causes ectopic expression of *Mgf* mRNA and accumulation of mast cells in uterus and oviducts. Dark-field (A,B) and bright-field (C–F) photomicrographs of sections from wild-type (A,C) and *Sl^{con}/Sl^{con}* (B,D–F) after in situ hybridization to *Mgf* (A–E) or *c-kit* (F) antisense cDNA probes. The sections shown in C–F are stained with toluidine blue. Two-week uterus (A,B), 2-week oviducts (C,D), and 4-week oviducts (E,F). (En) Endometrium; (My) myometrium; (M) muscle fibers; (S) serosal membranes. Arrows in D–F point to mast cells. (A,B) 5× objective; (C,D) 10× objective plus 1.2× magnifier; (E,F) 40× objective plus 1.6× magnifier. In wild-type uterus, a low level of *Mgf* mRNA expression was detected in the endometrium of immature mice (A). Whereas expression of *Mgf* mRNA in endometrium of *Sl^{con}/Sl^{con}* mice was increased moderately compared with control, massive overexpression in the circular layer of the mutant myometrium was observed (B). This ectopic expression of *Mgf* mRNA in the mutant is associated with greatly increased numbers of mast cells in serosal tissues as indicated by their metachromatic staining with toluidine blue (D–F, compared with wild type in C) and the expression of *c-kit* (F). In G, the reproductive tract of a 4-week-old *Sl^{con}* homozygous female is shown with arrows indicating areas of abnormal pigmentation.

was seen in the uteri and oviducts of sexually immature *Sl^{con}* mice (Fig. 9B,D), before the onset of the first estrous cycle. Second, the tissues from wild-type and mutant mice at 5 weeks of age (Fig. 8E,F) are both from mice at the same stage of the estrous cycle as indicated by the thick cornified epithelium of the vagina (not shown).

One consequence of the ectopic expression of *Mgf* in the reproductive tract of *Sl^{con}* mice is the abnormal accumulation of mast cells (Fig. 9C–F). These cells were identified on the basis of their metachromatic, granular appearance upon staining with toluidine blue (Fig. 9C–F) or Giemsa (not shown). In addition, many of the metachromatic cells expressed *c-kit* (Figure 9F). The number of mast cells was moderately increased throughout the female reproductive tract of *Sl^{con}* mice, with the highest accumulation observed in the serosal membranes, adjacent to the muscular layer expressing high amounts of *Mgf* mRNA (Fig. 9C–F). Areas of abnormal pigmentation also have been observed in histological sections of the *Sl^{con}* reproductive tract (not shown) and on occasion are grossly apparent in the oviducts (Fig. 9G), indicative of the abnormal accumulation of melanocytes. Thus, both mast cells and melanocytes appear to accumulate in high numbers in response to ectopic expression of *Mgf* mRNA in *Sl^{con}* mice.

The Sl^{pan} and Sl^{con} phenotypes are not complemented by other Sl mutations

Although we have shown that both of the *Sl^{pan}* and *Sl^{con}* rearrangements affect expression of *Mgf* mRNA, it was formally possible that the rearranged sequences could be affecting another gene. To test this, the ability of other *Sl* mutant alleles to complement the *Sl^{pan}* and *Sl^{con}* phenotypes was examined. The mutants chosen for the complementation test were *Grizzle-belly* (*Sl^{gb}*) and *Sl^d*; both of these alleles contain deletions that affect the *Mgf*-coding sequences but leave intact the region corresponding to the *Sl^{pan}* and *Sl^{con}* rearrangements. The *Sl^d* allele contains a small intragenic deletion of *Mgf*-coding sequences and is sterile in both sexes (Brannan et al. 1991; Flanagan et al. 1991). *Sl^{gb}* is a lethal allele that contains a 90-kb deletion encompassing the entire *Mgf*-coding region with the proximal deletion breakpoint located ~55 and 135 kb distal to the *Sl^{pan}* and *Sl^{con}* rearrangement breakpoints, respectively (M.A. Bedell, L.S. Cleveland, T.N. O'Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins, in prep.). If *Sl^{gb}* or *Sl^d* expressed a gene required for ovarian follicle development that was defective in either rearrangement, the phenotypes of compound heterozygotes would be expected to be less severe than that

of the corresponding homozygous mutants. Sl^{pan}/Sl^{gb} and Sl^{pan}/Sl^d mice were produced by intercrossing $Sl^{pan}/+$ mice with mice heterozygous for either Sl^{gb} or Sl^d , respectively. One-quarter of the mice produced from these crosses had white coats and were shown to contain both mutant alleles (see Materials and methods). Male compound heterozygous mice were fertile indicating that one copy of the Sl^{pan} allele is sufficient for male germ cell development. However, female compound heterozygotes were consistently sterile because of defects in ovarian follicle development (not shown). In Sl^{pan}/Sl^{gb} females, the effect on the ovaries was similar to that of Sl^{pan}/Sl^{pan} mice with follicle development arrested at the primary follicle stage. In Sl^{pan}/Sl^d ovaries, a single mature follicle was occasionally seen; however, the vast majority of follicles arrested at the primary follicle stage. In both Sl^{gb}/Sl^{pan} and Sl^{pan}/Sl^d , reduced numbers of oocytes were seen in the postnatal ovary that were similar to or less than those observed in Sl^{pan}/Sl^{pan} mice. Thus, the ovarian follicle defects of Sl^{pan} mice were not complemented by either of the Sl^{gb} or Sl^d alleles. Although our studies focused on ovarian follicle development, the compound heterozygotes had white coats indicating that pigmentation defects were also not complemented. Similarly, the effects of the Sl^{con} allele on female fertility and pigmentation were not complemented by either Sl^{gb} or Sl^d (Beechey and Searle 1972, 1983; M.A. Bedell, unpubl.).

Discussion

The evidence described here demonstrates that the Sl^{pan} and Sl^{con} alleles contain DNA rearrangements that are located 115 and 195 kb, respectively, upstream of the *Mgf*-coding region and affect expression of the *Mgf* gene. Although it was conceivable that another gene had been disrupted by these rearrangements, genetic evidence suggests that this is unlikely. The phenotypes of Sl^{pan} and Sl^{con} mutant mice were not complemented by either of two *Sl* mutant alleles, Sl^d and Sl^{gb} (this study; Beechey and Searle 1972, 1983), whose mutated sequences affect only the *Mgf*-coding sequences and leave intact the sequences corresponding to the rearrangements (Brannan et al. 1991; Flanagan et al. 1991; M.A. Bedell, L.S. Cleveland, T.N. O'Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins, in prep.). The lack of complementation therefore indicates that *Sl* is the only gene affected by the Sl^{pan} and Sl^{con} rearrangements that contributes to ovarian follicle development.

The phenotypes of Sl^{pan} and Sl^{con} mice appear to be attributable to long-range effects of these upstream rearrangements on *Mgf* mRNA expression. However, we do not at present know the mechanism for these effects. One possibility is that *cis*-acting sequences required for normal *Mgf* transcription, such as promoters or enhancers, are located in the region affected by the rearrangements. Alternatively, it is possible that there are previously unidentified *Mgf* exons that are disrupted by these rearrangements. Even though these potential regulatory

sequences or upstream exons for *Mgf* would be >100 kb upstream from the known coding sequences, there are examples of similarly large distances involved in transcription of other genes (Boyce et al. 1991; Duhl et al. 1994). However, we consider both of these possibilities unlikely for the following reasons: (1) The breakpoints of the Sl^{pan} and Sl^{con} rearrangements are 80 kb apart, implying that multiple regulatory sequences or upstream exons that span at least 80 kb would be required; (2) the Sl^{con} rearrangement causes both positive and negative effects on *Mgf* mRNA abundance, arguing against disruption of a single regulatory sequence; and (3) a major site for transcription initiation in a number of tissues has been mapped just upstream of the coding sequences (M.A. Bedell, unpubl.) with no current evidence for the transcription of far-upstream sequences. Although the final proof of the mechanism involved in the transcriptional effects of the rearrangements awaits further analysis, a mechanism that we currently favor is that the Sl^{pan} and Sl^{con} rearrangements cause position effects on expression. This situation could arise through juxtaposition of exogenous regulatory sequences, removal of regulatory sequences required for *Mgf* expression, or alteration of the chromatin structure of the *Mgf* locus. Any of these alterations, either singly or in combination, could disrupt the normal regulation of *Mgf* expression in a tissue-specific fashion. Position effect mutations are well documented in *Drosophila* (for review, see Eissenberg 1989) and yeast (see, e.g., Brand et al. 1985; Gottschling et al. 1990). However, the best evidence for position effects in the mouse comes from the variable expression of transgenes integrated at different chromosomal locations (Townes and Behringer 1990; Wilson et al. 1990). In addition, expression of the *Myc* proto-oncogene has been shown to be activated by chromosomal translocations and retroviral insertions located up to 270 kb from the gene (Lazo et al. 1990; Shaughnessy et al. 1994). In Sl^{pan} mice, *Mgf* expression is either not affected or is reduced in some tissues, indicating negative effects on transcription. However, both positive and negative effects on expression are seen in tissues of Sl^{con} mice. This suggests that the rearrangement has either removed a tissue-specific negative regulator or brought a tissue-specific positive regulator into proximity with the gene. Regulated expression of *Mgf* mRNA therefore seems to be complex and particularly sensitive to long-range effects.

The identification of mutations that act over considerable distances to affect expression of a gene has important practical, as well as biological, implications. Strategies for the positional cloning of genes often utilize rearrangement or deletion breakpoints as starting points to search for a gene responsible for a mutant phenotype. In addition, the cloning of genes affected by transgene insertions relies on the assumption that a disrupted gene lies near the integration site. The latter situation is complicated by the fact that transgene insertion sites are often complex and may involve very large deletions and other rearrangements. As is evident from the effects of the Sl^{pan} and Sl^{con} mutations on *Mgf* expression, the relevant gene could lie much farther from a rearrange-

ment than suspected previously. It is also possible that position effect mutations could affect multiple genes or be specific for only certain transcription units within a set of closely linked genes. For these reasons, the final proof that a gene identified by positional cloning is responsible for a mutant phenotype should be obtained through either analysis of multiple mutant alleles, gene knockout, or mutant rescue.

The defects that we observed in germ cell development in *SI^{pan}* and *SI^{con}* mice are likely to be the result of negative effects of the rearrangements on *Mgf* mRNA expression. During embryogenesis, *Mgf* is thought to be required for the migration and survival of PGCs (McCoshen and McCallion 1975; Dolci et al. 1991; Matsui et al. 1991). In *SI^{pan}* mice, decreased *Mgf* expression occurs early in the embryonic development of the gonads of both sexes leading to a reduced number of PGCs in both male and female mutant embryos. This suggests that a critical amount of *Mgf* is required for PGC survival or migration in both sexes. Although we have not determined the effect of the *SI^{con}* mutation on PGC development, the germ cell deficiencies in neonatal *SI^{con}* mice of both sexes suggest that the *SI^{con}* mutation may also affect early germ cell development. It will be of interest to determine whether there are temporal or quantitative differences in the effects of these two mutations on PGCs. Reduced expression of *Mgf* mRNA was not, however, apparent in the postnatal mutant testes, and spermatogenesis proceeds essentially normally in both *SI^{pan}* and *SI^{con}* homozygous males. The differential effects of the *SI^{pan}* mutation on *Mgf* expression in embryonic versus postnatal testes suggests that *Mgf* transcription may be alternatively regulated at different stages of testes development.

Our observations on the postnatal mutant ovaries are in agreement with Huang et al. (1993) and indicate that the sterility of *SI^{pan}/SI^{pan}* females is attributable to early arrest of ovarian follicle development with concomitant arrest of oocyte maturation. Although reduced expression of *Mgf* was observed in the homozygous *SI^{pan}* ovary and is probably the cause of arrested follicle growth, the mutant follicles arrest at the primary follicle stage when *Mgf* is not normally expressed at high levels. Because oocytes secrete factors that affect granulosa cell proliferation (for review, see Eppig 1994), it is possible that the decreased *Mgf* expression in *SI^{pan}* ovaries may cause the arrested growth of oocytes, which is in turn responsible for the arrested development of the somatic cells. Thus, *Mgf* expression may indirectly influence the development of granulosa cells. Clearly, *Mgf* is required at this stage of ovarian follicle development as two other *Sl* alleles, *SI^t* and *SI^d*, are sterile in homozygous females and also cause growth arrest at the primary follicle stage (Kuroda et al. 1988; M.A. Bedell, unpubl.). The *SI^d* allele produces only soluble factor (Brannan et al. 1991; Flanagan et al. 1991), suggesting that a critical amount of membrane-bound *Mgf* may be required for mediating communication between oocytes and follicle cells. Huang et al. (1993) have proposed that between 20% and 50% of the normal amount of *Mgf* is required for con-

tinued follicle and oocyte development. The expression of *Mgf* in young *SI^{con}* ovaries may be just above that threshold as we have demonstrated that *Mgf* mRNA levels were intermediate between that of *SI^{pan}* and wild type and caused an initial delay, followed by normal development, of some follicles. The lack of maintenance of oocytes and follicles in older *SI^{con}* females may be attributable to a requirement for a higher threshold of *Mgf* in ovaries of older mice, to changes in the follicular dynamics that result from the reduced number of oocytes and follicles, or to the combined effects of both. Further analysis of ovarian follicle development in *SI* mutant mice should provide additional information on the role of *Mgf* in the development and function of the ovary. Although our current analysis has focused on the germ line, the decreased coat pigmentation and mild anemia of *SI^{pan}/SI^{pan}* and *SI^{con}/SI^{con}* mice indicates that deregulated *Mgf* expression may also affect development of melanocytes and hematopoietic cells.

In addition to the negative effects on *Mgf* expression observed in *SI^{pan}* and *SI^{con}* tissues, the latter mutation also causes positive effects on *Mgf* mRNA abundance in some tissues. In the *SI^{con}* female reproductive tract, ectopic expression occurs in smooth muscle and causes the abnormal accumulation of high numbers of mast cells and melanocytes. A similar effect has been observed in patients with cutaneous mastocytosis; these lesions often display areas of hyperpigmentation and have been shown to have aberrant distribution of soluble *Mgf* protein (Longley et al. 1993). Presently, it is not known how increased *Mgf* expression in the *SI^{con}* reproductive tract affects the numbers of mast cells and melanocytes. *Mgf* was purified on the basis of growth-stimulating activity for mast cells (Anderson et al. 1990; Huang et al. 1990; Williams et al. 1990; Zsebo et al. 1990a); it has also been shown to be a chemoattractant for mast cells (Meininger et al. 1992), to induce the maturation of mast cells (Tsai et al. 1991), and to be a survival factor for melanoblasts (Steel et al. 1992). The abnormal levels of *Mgf* in the *SI^{con}* reproductive tract could thus affect virtually all aspects of mast cell and melanocyte functions.

Interestingly, the skin appears to display regional differences in the effects of the *SI^{con}* mutation on *Mgf* function. Whereas the overall pigmentation of *SI^{con}* homozygotes is reduced, the genital papillae are hyperpigmented (Beechey and Searle 1983). This suggests that *Mgf* expression in most of the *SI^{con}* dermis may be reduced at some crucial point in melanocyte development but overexpressed in a restricted area of the genital papillae, leading to melanocyte accumulation. An effect opposite to this has been observed for a regulatory mutation at the *W* locus, *W-sash* (*W^{sh}*), which causes ectopic expression of *c-kit* in some tissues (Duttlinger et al. 1993). These investigators proposed that in *W^{sh}* embryos, abnormal expression of *c-kit* in the somitic dermatome, a site that normally expresses *Mgf*, leads to a reduction in *Mgf* protein available for migrating melanoblasts. This, in turn, could decrease the survival and/or proliferation of the melanoblasts, resulting in the dominant pigmentation defects apparent in *W^{sh}* mice. In principle, a similar ef-

fect could occur in Sl^{con} mice; ectopic expression of Mgf in cells that normally express *c-kit* could bind to the *c-kit* receptor in an autocrine fashion, causing either constitutive activation of the receptor or inhibition of migration of cells to the normal sites of Mgf expression. More detailed analysis of Mgf mRNA expression in Sl^{con} embryos and adults is in progress.

In future studies it will be important to determine whether the abnormal accumulation of Mgf as seen in the reproductive tract of Sl^{con} mice has an adverse effect on reproductive functions. A number of cytokines and their receptors have been identified in the placenta and uterus and have been implicated in the establishment of pregnancy as well as preimplantation embryonic development (Hunt 1989; Pollard 1991). Although mast cells are present at low numbers in the normal female reproductive tract and their appearance is regulated with the estrous cycle, a role for these cells in reproduction is presently unclear. Clearly, mast cells are not essential for pregnancy, as live offspring have been produced in mast cell deficient, W/W^v mice after transplantation of wild-type donor blastocysts (Wordinger et al. 1986). However, it is possible that abnormal accumulations of mast cells in the female reproductive tract could affect the maternal recognition of pregnancy or embryonic development. Further analysis of reproductive functions in Sl^{con} and wild-type mice containing ovary transplants may help clarify these issues.

Materials and methods

Animals

The Sl^{pan} and Sl^{con} mutations were induced by radiation treatment of (C3H/HeH $\times$ 101/H) F_1 males (Beechey and Searle 1983; Beechey et al. 1986). Each stock was maintained at the NCI-FCRDC animal facility on a C3H/HeN or C3H/HeJ background. Mice that are heterozygous for either allele are fertile in both sexes. After birth, mice that were heterozygous or homozygous for the mutations were identified by coat color: $Sl^{pan}/+$ mice have a slight dilution of pigmentation; Sl^{pan}/Sl^{pan} mice have white coats with black ears; $Sl^{con}/+$ mice have normal coat pigmentation but hyperpigmented genital papillae, and Sl^{con}/Sl^{con} mice have a uniformly gray coat and hyperpigmentation in the genitals. To distinguish embryos, F_2 animals were derived from outcrossing C3H- Sl^{pan} to C57BL/6J so that restriction fragment length polymorphisms (RFLPs) within the Mgf coding sequences of the two strains could be used for genotyping by Southern blot analysis. Some of the F_2 animals have been examined postnatally for ovarian follicle development, and no significant differences have been observed between mutants on various genetic backgrounds.

The Sl^d stock was maintained on the C57BL6/J strain, whereas Sl^{gb} was carried on either C3H/HeN or C3H/HeJ background. Mice heterozygous for either allele are fertile in both sexes and have diluted coats and/or head spots, Sl^d/Sl^d mice have white coats and Sl^{gb}/Sl^{gb} mice die during late gestation (Silvers 1979; M.A. Bedell, unpubl.). Compound heterozygous mice were produced by mating $Sl^{pan}/+$ or $Sl^{con}/+$ mice with mice heterozygous for either Sl^{gb} or Sl^d ; one-quarter of the progeny of these crosses had less coat pigmentation than Sl^{pan}/Sl^{pan} or Sl^{con}/Sl^{con} mice and were shown to contain both mutant alleles by either Southern blot analysis or by progeny testing of the males (not shown).

Northern blot and sequencing analysis

Total RNA from tissues of wild-type or homozygous mutant mice was prepared using RNazol (Tel-Test, Inc.). Northern blot analysis was performed as described previously (Brannan et al. 1992) except that 20 μ g of RNA was used and transferred to Hybond N (Amersham). The Sl^{pan} and Sl^{con} coding sequences were determined by direct sequencing of RT-PCR products as described previously (Brannan et al. 1992). In brief, 2 μ g of total RNA from homozygous mutant tissues was used as template to synthesize cDNA followed by asymmetric PCR and dideoxy sequencing of single-strand PCR products.

Southern blot analysis and PFGE

For conventional Southern blot analysis, genomic DNA was isolated, electrophoresed, and blotted as described previously (Jenkins et al. 1982) except that either Zetabind (Cuono) or Hybond N+ (Amersham) nylon membranes were used. The blots were hybridized with 32 P-labeled DNA probes prepared using a Prime-It II kit (Stratagene) and washed at high stringency. For long-range mapping, high molecular weight DNA was prepared, digested, and electrophoresed using PFGE as described by Kingsley et al. (1992). The probes used for both Southern blot and PFGE analysis are as follows: cDNAs that contain the Mgf coding region (Anderson et al. 1990), 3'-untranslated cDNAs and upstream genomic probes (M.A. Bedell, L.S. Cleveland, T.N. O'Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins, in prep.), and end probes of *Sl* YACs (M.A. Bedell, L.S. Cleveland, T.N. O'Sullivan, P.J. Donovan, N.G. Copeland, and N.A. Jenkins, in prep.). The latter were derived from two overlapping YAC clones of 610 and 400 kb, and their relationship to Mgf coding sequences is shown in Figure 2. The probes, 6.2/5RI and 1.2/6RI [see Fig. 2], are unique fragments derived from the inserts of two λ clones of a chromosomal walk between 3127L and 394L using a genomic library of 129 mouse DNA (Stratagene). Mutant breakpoint fragments were obtained from size-fractionated libraries of Sl^{con}/Sl^{con} and Sl^{pan}/Sl^{pan} DNA by hybridization to 6.2/5RI and 1.2/6RI, respectively, and cloned into the λ ZAP vector (Stratagene). Phage libraries were screened, and the clones were purified and their inserts cloned into plasmid using conventional techniques.

Interspecific backcross mapping and cytogenetic analysis

The chromosomal positions of DNA probes were determined using a [(C57BL/6J) $\times$ *M. spretus*] $\times$ C57BL/6J backcross panel as described previously (Copeland and Jenkins 1991). The segregation pattern of a probe polymorphic for the two strains was compared against the segregation of >1700 other probes in the same panel (Copeland and Jenkins 1991). Chromosome preparations were obtained directly from bone marrow, G-banded, and analyzed as described previously (Evans 1987).

In situ hybridization analysis and alkaline phosphatase histochemistry

Sl^{pan} embryos were obtained by intercrossing B6C3F1 $Sl^{pan}/+$ mice, with the presence of a vaginal plug considered E0.5. Embryos were dissected at E12.5, and the caudal part of each embryo was processed and sectioned while heads of the embryos were used for genotyping. For in situ hybridization, tissues from adult mice or embryos were fixed in paraformaldehyde, embedded in paraffin, and sectioned at 5 μ m. Tissue sections were hybridized to 35 S-labeled cDNA probes for Mgf or *c-kit* and washed as described previously (Keshet et al. 1991), dipped in

NTB-2 emulsion, and exposed for 6–22 days. After photographic developing, slides were counterstained with toluidine blue. PGCs were identified as described previously (Brannan et al. 1992) by alkaline phosphatase histochemistry. Embryos were fixed in paraformaldehyde, infiltrated with 0.5 M sucrose, and embedded in gelatin. Frozen sections were cut at 15 μ m, and the sections were stained with Fast Red TR and Naphthol AS-MX phosphate (Sigma). PGCs were visualized by their intense red staining and counted using light microscopy.

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DNA rearrangements located over 100 kb 5' of the Steel (Sl)-coding region in Steel-panda and Steel-contrasted mice deregulate Sl expression and cause female sterility by disrupting ovarian follicle development.

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