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Deletion and Interallelic Complementation Analysis of *Steel* Mutant Mice

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ABSTRACT

Mutations at the *Steel* (*Sl*) locus produce pleiotropic effects on viability as well as hematopoiesis, pigmentation and fertility. Several homozygous viable *Sl* alleles have previously been shown to contain either structural alterations in mast cell growth factor (Mgf) or regulatory mutations that affect expression of the *Mgf* gene. More severe *Sl* alleles cause lethality to homozygous embryos and all lethal *Sl* alleles examined to date contain deletions that remove the entire *Mgf* coding region. As the timing of the lethality varies from early to late in gestation, it is possible that some deletions may affect other closely linked genes in addition to *Mgf*. We have analyzed the extent of deleted sequences in seven homozygous lethal *Sl* alleles. The results of this analysis suggest that late gestation lethality represents the *Sl* null phenotype and that peri-implantation lethality results from the deletion of at least one essential gene that maps proximal to *Sl*. We have also examined gene dosage effects of *Sl* by comparing the phenotypes of mice homozygous and hemizygous for each of four viable *Sl* alleles. Lastly, we show that certain combinations of the viable *Sl* alleles exhibit interallelic complementation. Possible mechanisms by which such complementation could occur are discussed.

THE pleiotropic effects on viability, hematopoiesis, pigmentation and fertility found in mice that carry *Steel* (*Sl*) mutant alleles are due to mutations in mast cell growth factor (Mgf) (Copeland *et al.* 1990; Huang *et al.* 1990; Zsebo *et al.* 1990a). Mgf is the ligand for Kit (Flanagan and Leder 1990; Huang *et al.* 1990; Williams *et al.* 1990; Zsebo *et al.* 1990a), a receptor tyrosine kinase that is encoded by the *Dominant White Spotting* (*W*) locus (Chabot *et al.* 1988; Geissler *et al.* 1988). These two gene products comprise an intercellular signaling pathway that is required for the development of mast cells, erythroid cells, neural crest-derived melanocytes and germ cells. Mgf is biologically active as both a transmembrane, cell surface protein and as a soluble factor produced by proteolytic cleavage of the membrane bound form (Anderson *et al.* 1990; Flanagan *et al.* 1991; Toksoz *et al.* 1992). Gel filtration and sedimentation analysis has suggested that the soluble form of Mgf is a noncovalently associated homodimer (Arakawa *et al.* 1991; Zsebo *et al.* 1990b); however, it is presently not known whether the membrane-bound form also functions as a dimer. Upon binding of Mgf to Kit, dimerization of the receptor is induced, leading to activation of the tyrosine kinase function with subsequent phosphorylation and binding to downstream signaling molecules (Blume-Jensen *et al.* 1991; Lev *et al.* 1991, 1992; Rottapel *et al.* 1991).

A large collection of *Sl* and *W* mutant alleles has been identified that are useful for elucidation of the *in vivo* functions of this signaling pathway. All *Sl* and *W* mutant alleles are semidominant in that they cause a mild phenotype in heterozygous mice (Silvers 1979a,b). However, the severity of the homozygous phenotype varies considerably between different alleles. In homozygous mice, the most severe *Sl* alleles cause embryonic lethality while milder alleles allow viability but have characteristic effects on anemia, pigmentation and fertility. Previously, it was demonstrated that many embryonic lethal *Sl* alleles are associated with complete deletions of *Mgf* coding sequences (Copeland *et al.* 1990; Huang *et al.* 1990; Zsebo *et al.* 1990a), suggesting that Mgf is essential for embryonic survival. However, the timing of embryonic death caused by various *Sl* mutations varies from before implantation to late in gestation (Silvers 1979a; Cattanach *et al.* 1993). While severe anemia is thought to be the cause of death in late gestation *Sl* embryos (Sarvella and Russell 1956; Silvers 1979a), lethality at earlier stages of embryogenesis is likely to result from deletion of other closely linked essential genes. In contrast to the lethal *Sl* alleles, alleles such as *Steel-Dickie* (Sl^{d}), *Steel-17H* (Sl^{17H}), *Steel-panda* (Sl^{pan}) and *Steel-contrasted* (Sl^{con}) are hypomorphic in that they retain residual Mgf activity that allows viability. The Sl^{d} allele contains an intragenic deletion that affects the transmembrane and cytoplasmic domains of Mgf such that only soluble protein is encoded (Brannan *et al.* 1991; Flanagan *et al.* 1991) while the protein encoded by Sl^{17H} mice lacks the normal cytoplasmic domain due to a splicing defect (Brannan *et al.* 1992). Last, Sl^{pan} and Sl^{con} mice were shown to have regulatory mutations that both positively and negatively affect *Mgf* mRNA

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levels but do not affect the *Mgf* coding sequences (HUANG *et al.* 1993; BEDELL *et al.* 1995). Each of these viable alleles causes characteristic defects in pigmentation, hematopoiesis and fertility in homozygous mice.

Here we describe a physical and genetic analysis of *Sl* mutants. These studies provide evidence for a second gene that lies proximal of *Sl* that affects embryonic viability and show that certain alleles of *Sl* can exhibit interallelic complementation. The implications of these studies for *Sl* gene function are discussed.

MATERIALS AND METHODS

Mice: The lethal and viable *Sl* alleles studied here are listed in Tables 1 and 3, respectively. Sl^{gb}, Sl^{10H}, Sl^{8H}, Sl^{12H}, Sl^{18H}, Sl^{pan}, Sl^{con} and Sl^{17H} mice were obtained from the MRC Radiobiology Unit (Chilton, Didcot, UK) while Sl^{J} and Sl^{d} mice and *Sl*/+ DNA were obtained from The Jackson Laboratory (Bar Harbor, ME). Some *Sl* mutant stocks have been maintained at the NCI-FCRDC by backcrossing to C57BL/6J (Sl^{d}) or C3H/HeN (other *Sl* alleles) mice. Sl^{J} was maintained by brother-sister matings of Sl^{J}/+ and +/+ segregants and was originally on a 129/Sv background. Homozygous embryos were obtained by intercrossing *Sl* heterozygotes. Mice heterozygous for different *Sl* alleles were mated to obtain F_1 progeny that were heteroallelic, *i.e.*, $Sl^{x}/+ \times Sl^{y}/+ \rightarrow Sl^{x}/Sl^{y}$. The genotype of putative heteroallelic mice was determined by either Southern blot analysis, using probes that recognize allele-specific genomic alterations, or by progeny testing.

Southern blot analysis and pulsed field gel electrophoresis (PFGE): All DNAs analyzed were from mouse tissues except *Sl*/*Sl* DNA that had been prepared from a fetal liver stromal cell line (ZSEBO *et al.* 1990a) and was kindly provided by D. A. WILLIAMS. The isolation and analysis of genomic DNA by conventional Southern blot analysis was performed as previously described (JENKINS *et al.* 1982) except that either Zetabind (Cuno, Inc., Meriden, CT) or Hybond N+ (Amersham, Arlington Heights, IL) nylon membranes were used and ^{32}P-labeled DNA probes were prepared using a Prime-It II kit (Stratagene, La Jolla, CA). The probes used are described below and were hybridized and washed under high stringency. The blots were first analyzed by autoradiography and then hybridization intensity was quantitated using a Phosphor Imager SF (Molecular Dynamics, Sunnyvale, CA). High molecular weight spleen DNA was prepared, digested and subjected to PFGE using methods described by KINGSLEY *et al.* (1992) and the blots were analyzed by autoradiography.

Probes that contain the 5′ flanking region and the full-length *Mgf* cDNA are described in the companion paper (BEDELL *et al.* 1996). A YAC library of mouse DNA (ICRF reference library, LEHRACH *et al.* 1990) was screened with an upstream genomic probe (*Eco*RI fragment B, Figure 4 of BEDELL *et al.* 1996). Probes from the ends of the YAC inserts were prepared either by bubble PCR (RILEY *et al.* 1990) or from cloned fragments isolated following hybridization of size-fractionated YAC fragments to vector sequences. Two probes from the proximal portion of chromosome *10* were used to control for DNA loading on Southern blots, *Myb* (JUSTICE *et al.* 1990) and *D10Fcr2* (BEDELL *et al.* 1995). The Sl^{gb} deletion breakpoint fragment was cloned by size-fractionating *Eco*RI fragments of DNA from a Sl^{gb} homozygous embryo, ligating the fragments into λZAP vector followed by conventional screening, isolation and subcloning. Sequencing was performed using a dideoxy method (United States Biochemical, Cleveland, OH).

Interspecific backcross (IB) mapping: The chromosomal position of DNA probes was determined using a [(C57BL/6J × *Mus spretus*) × C57BL/6J] backcross mapping panel as described previously (COPELAND and JENKINS 1991). The segregation pattern of probes polymorphic for the two species was compared with the segregation of 2000 other probes that have been typed in the same panel.

RESULTS

Deletion analysis of homozygous lethal *Sl* alleles: The extent of deleted sequences was determined in seven *Sl* alleles that cause lethality to homozygous embryos during various stages of development (see Table 1). All seven alleles were previously shown to be associated with deletions that remove the *Mgf* coding region (COPELAND *et al.* 1990), a region that corresponds to ~40 kb of genomic DNA. To determine whether these deletions extend into sequences immediately flanking the *Mgf* coding region, Southern blot analyses of DNA from mice heterozygous for each of the lethal *Sl* alleles was performed with probes derived from the full-length *Mgf* transcription unit and ~14 kb of 5′ flanking region. These clones span ~64 kb of genomic DNA and are described in the companion paper (BEDELL *et al.* 1996). In all heterozygous DNAs except for Sl^{gb}/+, the intensity of hybridization of each probe was approximately half that seen in wild-type DNA (data not shown). Hybridization of control probes, *Myb* (JUSTICE *et al.* 1990) and *D10Fcr2* (BEDELL *et al.* 1995), to the same blots demonstrated that equivalent amounts of DNA had been loaded in each lane. These results indicate that, with the exception of Sl^{gb}, the sequences deleted in all *Sl* alleles examined includes the 5′ and 3′ flanking regions of *Mgf* and are therefore a minimum of 64 kb in size.

A probe from the 3′ untranslated region (UTR) of *Mgf* (see BEDELL *et al.* 1996) detected numerous RFLPs in Sl^{gb} DNA, suggesting the presence of a deletion breakpoint. For example, a 5.5-kb *Eco*RI fragment was observed in Sl^{gb}/Sl^{gb} DNA while two *Eco*RI fragments of 5 and 2.7 kb are present in wild-type DNA upon hybridization with 3′ *Mgf* (Figure 1A, left). A subgenomic library of *Eco*RI-digested Sl^{gb}/Sl^{gb} DNA was prepared and the 5.5-kb mutant breakpoint fragment isolated. The approximate position of the Sl^{gb} breakpoint was determined to be in the distal region of the 3′ UTR of *Mgf* by restriction site mapping and hybridization analysis. Sequencing of the distal portion of the Sl^{gb} fragment revealed that the deletion breakpoint is at position 5287 of the 5.4-kb *Mgf* mRNA (BEDELL *et al.* 1996). As the 3′ *Mgf* probe contains only ~100 bp that overlaps the sequence of Sl^{gb} genomic fragments, the hybridization intensity of this probe to mutant DNA is less intense than in wild-type DNA (Figure 1A, left). Hybridization of the gbB/H probe, derived from the Sl^{gb} breakpoint fragment, to Sl^{gb}/+ DNAs confirmed that the authentic breakpoint fragment had been cloned (Figure 1A, right). Analysis of pulsed field gel

TABLE 1

Lethal *Steel* alleles

Gene symbol[a]	Gene name	Size of deletion (kb)	Phenotype[b]		References
			Heterozygous mice	Homozygous mice	
Sl^{gb}	*Steel-Grizzle Belly*	120	Light-colored belly, dilution of coat, head spots	Anemia; embryos die after E15	SCHAIBLE (1961, 1963); M. A. BEDELL, unpublished data
Sl^{J}	*Steel-J*	650	Dilution of color on the belly; generally, a white tip on tail	Prenatal lethal; embryos die after E15	STEVENS (1979); P. J. DONOVAN, personal communication
Sl^{10H}	*Steel-10H*	680	Lighter coat and feet than wild-type mice; occasional spotting	Anemia; embryos die after E15	BEECHEY and SEARLE 1985; M. A. BEDELL, unpublished data
Sl	*Steel*	>810	Slight dilution of coat color; occasional spotting	Anemia, prenatal lethal; embryos die after E15	SARVELLA and RUSSELL 1956
Sl^{8H}	*Steel-8H*	>810	Lighter coat and feet than wild-type mice; occasional spotting	Prenatal lethal (timing not reported)	BEECHEY and SEARLE 1985
Sl^{12H}	*Steel-12H*	>810	Light coat; runting; reduced viability	Prenatal lethal: embyros die before implantation	CATTANACH and RASBERRY 1988
Sl^{18H}	*Steel-18H*	>810	Greyish coat; pale ears and tail; head spotting; occasional nose and belly spotting; reduced viability; anemia	Prenatal lethal: embryos die shortly after implantation	CATTANACH *et al.* 1988

[a] Sl^{J}, Sl^{gb} and *Sl* all arose spontaneously whereas Sl^{8H}, Sl^{10H}, Sl^{12H} and Sl^{18H} were each induced by X-irradiation. Mutant stocks were maintained on a C3H/HeN background except Sl^{J} that was maintained by brother-sister mating and was originally on a 129/Sv background. *Sl*/*Sl* DNA was obtained from a stromal cell lined derived from homozygous *Sl* embryos (ZSEBO *et al.* 1990a).

[b] Unless otherwise noted, the phenotypes are as described in the original published reports for each allele. Differences in the phenotypes of both heterozygous and homozygous mice may be due to strain background effects. Sl^{gb}/Sl^{gb} and Sl^{10H}/Sl^{10H} were originally reported to be neonatal lethals. However, no liveborn homozygous mice were observed (M. A. BEDELL, unpublished data) while live homozygous embroys have been observed at E12.5 and E15.

electrophoresis (PFGE) blots of YAC, wild-type, and $Sl^{gb}/+$ mouse DNAs placed the proximal end of the Sl^{gb} deletion ~60 kb 5′ of the *Mgf* coding region and the size of the Sl^{gb} deletion was estimated to be ~120 kb (Figure 2). The gbB/H probe was deleted in all other homozygous lethal *Sl* alleles (Table 2).

Additional probes were obtained from YACs that were isolated from the ICRF Reference Library (LEHRACH *et al.* 1990) after screening with EcoB (Figure 2), a genomic fragment from the 5′ flanking region of *Mgf* (see BEDELL *et al.* 1996). Two YAC clones were obtained from a C3H library; clone ICRFy902G03127 (YLE3127), which is 400 kb and clone ICRFy902A0394 (YLE394), which is 610 kb. End-probes from each YAC were prepared and three of these (394R, 394L and 3127L) hybridized to unique sequences on Southern blots of mouse DNA while the fourth (3127R) detected highly repetitive sequences. The two YACs were aligned with respect to each other and the *Mgf* coding region by partial and complete restriction digest analysis of YAC DNA, hybridization of *Mgf* cDNA, genomic clones or YAC end probes to YAC DNA, and hybridization of YAC end probes to PFGE blots of mouse genomic DNA. These results revealed that 394L is ~100 kb upstream of the *Mgf* coding region, that 3127L is an additional 120 kb upstream from 394L and that 394R maps ~590 kb 3′ to *Mgf* (see Figure 2). The total genomic region spanned by 3127L and 394R is estimated to be 810 kb. The chromosomal positions of 394R, 394L and 3127L were determined by interspecific backcross mapping (COPELAND and JENKINS 1991). This analysis confirmed that 394L and 3127L are very closely linked to *Mgf*; they

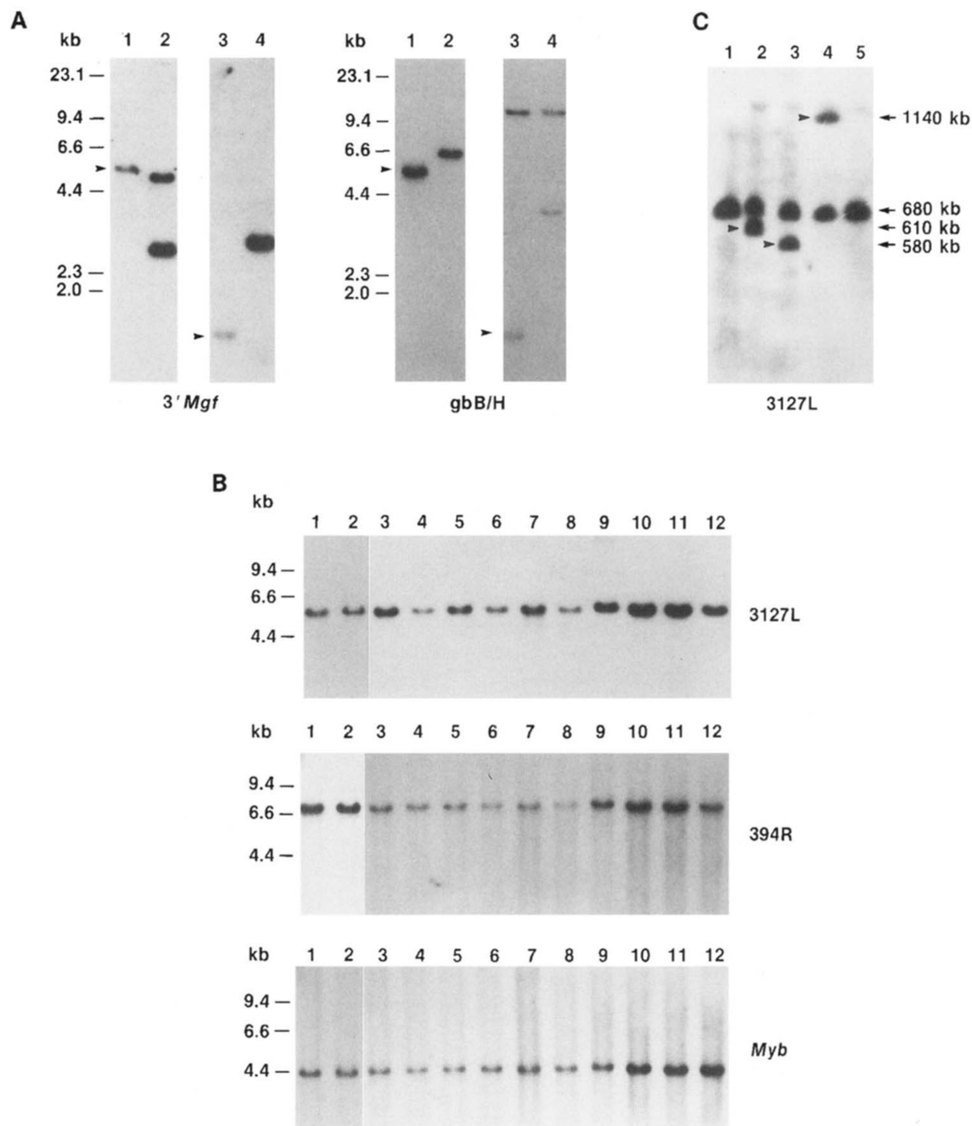

FIGURE 1.—Representative Southern blots of DNA from *Sl* mutant mice. (A) DNAs from Sl^{gb}/Sl^{gb} embryos (lanes 1 and 3) and +/+ mice (lanes 2 and 4) were digested with *Eco*RI (lanes 1 and 2) or *Hind*II (lanes 3 and 4) and the blot was sequentially probed with a portion of the 3′ UTR region of *Mgf* cDNA (3′ *Mgf*) (see BEDELL *et al.* 1996) and with gbB/H, a probe derived from the Sl^{gb} deletion breakpoint fragment (see text). Note that the two probes detect the same band in Sl^{gb}/Sl^{gb} DNA but different bands in +/+ DNA. Hybridization with a control probe, *Myb* (not shown), revealed that each lane contained equal amounts of DNA. (B) DNAs from +/+ (lanes 1, 3, 5, 7, 9 and 11), $Sl^{10H}/+$ (lane 2), $Sl^{18H}/+$ (lane 4), $Sl^{8H}/+$ (lane 6), $Sl^{12H}/+$ (lane 8), $Sl^{J}/+$ (lane 10) and $Sl/+$ (lane 12) mice were digested with *Eco*RI and the blot was sequentially probed with 3127L (the most proximal probe, top), 394R (the most distal probe, middle) and *Myb* (a control probe, bottom). Quantitation of the hybridization intensity with each of these probes, as well as other probes and other blots, are summarized in Table 2. In A and B, λ*Hind*III size markers are shown to the left of the panels. (C) CHEF blot of spleen DNAs from +/+ (129/Sv and C3H/HeN, lanes 1 and 5, respectively), $Sl^{J}/+$ (lane 2), $Sl^{10H}/+$ (lane 3) and $Sl^{gb}/+$ (lane 4) were digested with *Eag*I and the blot probed with 3127L, the most proximal probe. The estimated sizes of the different fragments are indicated to the right of the panel. In panels A and C, arrowheads point to the mutant fragments.

did not recombine with each other or with the *Mgf* cDNA probe in 174–183 N_2 animals typed in common. In contrast, 394R mapped 0.5 +/− 0.5 cM distal to *Mgf* cDNA after typing of 188 N_2 animals. Because 394R was determined to be 3′ to the *Mgf* cDNA (see above), the genetic mapping results allowed the direction of *Mgf* transcription to be oriented with respect to the centromere (Figure 2).

Further analyses of the various *Sl* deletions were done using the YAC end probes, 3127L, 394L and 394R (Figure 1, B and C, Table 2). As expected, all three probes were present at normal intensity in Sl^{gb} DNA (Table 2). Sequences hybridizing to 3127L and 394R were also present at normal intensity in DNA from $Sl^{J}/+$ and $Sl^{10H}/+$ mice (Table 2, Figure 1B) indicating that the proximal and distal limits of these deletions are within the region encompassed by the two YACs. However, hybridization of 394L to both $Sl^{J}/+$ and $Sl^{10H}/+$ DNA

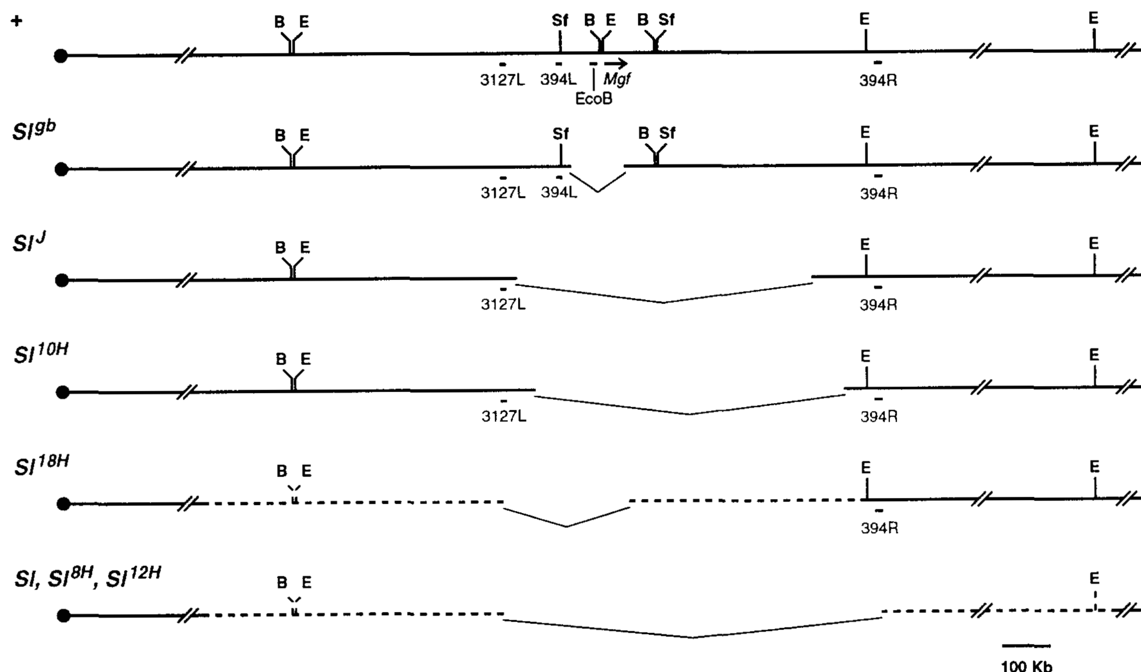

FIGURE 2.—The extent of deleted sequences in lethal *Sl* alleles. Schematics of chromosome *10* of wild-type C3H(+) and seven homozygous lethal *Sl* alleles are shown with the centromere (●) and telomere oriented left to right, respectively. Deleted regions are shown as gaps connected by offset thin lines. Hash marks represent portions not drawn to scale, the solid lines indicate intact DNA sequences and the dashed lines indicate sequences that were not analyzed so deletions are of unknown size. The probes shown are as follows: 3127L, left end probe of YAC YLE3127; 394L and 394R, left and right end probes, respectively, of YAC YLE394; EcoB, *Eco*RI fragment B of 5′ flanking region of *Mgf* (see BEDELL *et al.* 1996); *Mgf*, transcriptional unit of Mgf, oriented 5′ to 3′ with respect to the centromere (see text). Restriction enzyme sites: *Bss*HII (B), *Eag*I (E), *Sfi*I (Sf). Other *Sfi*I sites are not shown. See Table 1 for the estimated sizes of the deletions.

was half as intense as in wild-type (Table 2), suggesting that the proximal breakpoint of each deletion is located between 3127L and 394L, a distance of ~120 kb. To identify the exact location of the Sl^{J} and Sl^{10H} breakpoints, a chromosomal walk was initiated from these two probes. Overlapping phage clones of wild-type DNA were isolated and the direction of the walk determined by hybridization to DNA from YLE3127 and YLE394. Unique sequences derived from the walking clones were used as probes on conventional Southern blots of Sl^{J} and Sl^{10H} DNAs to identify probes that are located near the two breakpoints. The results of these analyses indicate that the Sl^{J} deletion breakpoint lies ~38 kb proximal to the *Sl10H* breakpoint (Figure 2). Although the distal extent of these two deletions has not been precisely determined, the sizes of mutant fragments on PFGE blots were used to estimate the total amount of deleted DNA. In DNA from wild-type mice, 3127L hybridizes to an *Eag*I fragment of 680 kb while this probe detects mutant *Eag*I fragments of 610 and 580 kb, respectively, in $Sl^{J}/+$ and $Sl^{10H}/+$ DNA (Figure 1C). Wild-type DNA also contains a second *Eag*I fragment of 580 kb, that contains the *Mgf* coding region (see Figure 2). However, an *Eag*I site near the coding region is removed by the deletions in Sl^{J} and Sl^{10H} DNA, so that only one *Eag*I fragment is present in each mutant allele. Therefore, the extent of deleted sequences was estimated by subtracting the sizes of the mutant *Eag*I fragments from the sum of the two wild-type *Eag*I fragments (1260 kb). The results indicate that the Sl^{J} and Sl^{10H} alleles contain deletions of 650 and 680 kb, respectively (see Figure 2). Similar analysis of $Sl^{gb}/+$ DNA revealed a 1140 kb mutant *Eag*I fragment (Figure

TABLE 2

Quantitation of Southern blot hybridization results

	Probe			
Mutant	3127L	394L	gbB/H	394R
$Sl^{gb}/+$	1.0	0.92	RFLPs	1.1
$Sl^{J}/+$	0.84	0.51	0.48	0.90
$Sl^{10H}/+$	1.1	0.55	0.53	1.4
$Sl^{18H}/+$	0.56	0.39	0.44	0.99
$Sl/+$	0.51	0.51	0.48	0.52
$Sl^{8H}/+$	0.64	0.56	0.49	0.56
$Sl^{12H}/+$	0.60	0.55	0.49	0.68

Southern blots of $Sl/+$ and $+/+$ DNAs were sequentially hybridized to each probe; 3127L is 220 kb 5′ to *Mgf*, 394L is 100 kb 5′ to *Mgf*, (see text and Figure 1), gbB/H is derived from Sl^{gb} deletion breakpoint and is 60 kb 5′ to *Mgf* (see text), and 394R is 590 kb 3′ to *Mgf* (see text and Figure 1). The intensity of hybridization of each expermiental probe was normalized to that of control probes, *Myb* and *D10Fcr2* (see text). For each heterozygous DNA, the data are expressed as the ratio of hybridization intensity relative to $+/+$ DNA and are the average of two separate samples. RFLPs, restriction fragment length polymorphisms.

1C) and confirmed that the Sl^{gb} allele contains a deletion of 120 kb.

In DNA from $Sl/+$, $Sl^{8H}/+$ and $Sl^{12H}/+$ mice, hybridization of 3127L, 394L and 394R was half as intense as in wild-type DNA (Figure 1B and Table 2). This indicates that the proximal and distal ends of these three deletions lie outside the region spanned by YLE3127 and YLE394 and are therefore ≥810 kb in size (Figure 2). Last, hybridization of 3127L and 394L, but not 394R, to $Sl^{18H}/+$ DNA was half as intense as to wild-type DNA (Figure 1B and Table 2). This suggests that the distal breakpoint of the Sl^{18H} deletion must be located between the *Mgf* transcription unit and 394R (Figure 2). The results of the analysis of deleted sequences in all seven lethal *Sl* alleles are shown in Figure 2.

Genetic interactions of lethal and viable *Sl* alleles: The information gained through the analysis of *Sl* deletions was used to conduct additional tests of the *in vivo* function of Mgf. While the entire *Mgf* coding region is deleted in Sl^{gb} DNA, this allele contains the smallest deletion of the lethal alleles examined and is least likely to be affecting any other genes required for embryonic viability. By creating mice that are heteroallelic for Sl^{gb} and various viable *Sl* alleles, the phenotype of mice hemizygous for each hypomorphic allele can be determined. This analysis was done with four homozygous viable *Sl* alleles (Sl^{d}, Sl^{17H}, Sl^{pan} and Sl^{con}) for which each of the molecular defects has been previously reported (Table 3). Hemizygous mice were produced by intercrossing $Sl^{gb}/+$ mice with mice heterozygous for each viable *Sl* allele. One-quarter of the F_1 progeny from each cross displayed pigmentation defects consistent with their containing two mutant *Sl* alleles. These results are summarized in Table 3 with representative mice shown in Figure 3. Mice with the genotype Sl^{d}/Sl^{gb}, Sl^{pan}/Sl^{gb} and Sl^{con}/Sl^{gb} were viable and had normal lifespans. While both Sl^{d}/Sl^{gb} and Sl^{pan}/Sl^{gb} mice were completely white, Sl^{con}/Sl^{gb} mice had slightly less pigmentation than Sl^{con}/Sl^{con} mice (see Figure 3A). In sharp contrast, only a few Sl^{17H}/Sl^{gb} mice survived the first few weeks of birth. These hemizygous mice were easily recognizable as being runted, pale and severely anemic. The period of survival observed with neonatal Sl^{17H}/Sl^{gb} mice is only a few weeks longer than that observed for homozygous Sl^{gb} mice.

Heteroallelic mice were produced by intercrossing mice heterozygous for each of the four viable *Sl* alleles. The pigmentation of approximately one-quarter of the F_1 progeny was not that expected of either heterozygous or wild-type mice. All six types of heteroallelic mice produced (Sl^{d}/Sl^{17H}, Sl^{d}/Sl^{pan}, Sl^{d}/Sl^{con}, Sl^{17H}/Sl^{pan}, Sl^{17H}/Sl^{con} and Sl^{con}/Sl^{pan}) were viable and displayed varying extents of pigmentation (Table 3 and Figure 3). While mice homozygous for each of the alleles displayed significant deficiencies in pigmentation that range from completely white to grey, some heteroallelic mice displayed more pigmentation than the corresponding homozygotes. For example, although Sl^{17H}/Sl^{17H} mice are completely white and the only pigmented areas of Sl^{pan}/Sl^{pan} mice are the ears, Sl^{17H}/Sl^{pan} mice are grey and white (see Figure 3B). There is some variability in the extent of pigmentation in different Sl^{17H}/Sl^{pan} mice, with one-third to two-thirds of the dorsal surface being pigmented. However, the ventrum of Sl^{17H}/Sl^{pan} mice was invariably white. In Sl^{17H}/Sl^{con} mice, much more pigment was observed than even the grey of Sl^{con}/Sl^{con} mice, with nearly normal pigmentation in most of the coat with the exception of a prominent white head spot (see Figure 3C). Thus, both Sl^{pan} and Sl^{con} partially complement the coat color deficiencies produced by Sl^{17H}. In comparison, the pigmentation defects caused by Sl^{17H}, Sl^{pan} and Sl^{con} were not complemented by Sl^{d} as Sl^{17H}/Sl^{d} and Sl^{pan}/Sl^{d} mice are completely white (see Table 3) and Sl^{con}/Sl^{d} mice have about the same pigmentation as Sl^{con}/Sl^{con} mice (Figure 3A). In addition, Sl^{con}/Sl^{pan} mice had slightly less coat pigment than Sl^{con}/Sl^{con} mice (Figure 3D). Comparison of the entire series of Sl^{con} heteroallelic mice results in the following classification on the basis of pigmentation (from least to most pigment); $Sl^{gb}/Sl^{con} = Sl^{pan}/Sl^{con} < Sl^{d}/Sl^{con} = Sl^{con}/Sl^{con} < Sl^{17H}/Sl^{con} <$ wild type.

DISCUSSION

Deletion analysis of various lethal *Sl* alleles has provided information regarding sequences that contribute to the lethal phenotype. The smallest deletion we have identified in the homozygous lethal class of *Sl* mutants is ~120 kb in the Sl^{gb} allele, while the next smallest deletions are 650 and 680 kb, respectively, in Sl^{J} and Sl^{10H}. The relatively small size of the Sl^{gb} deletion suggests that it may not affect any genes other than *Mgf* that are essential for viability. Neonatal lethality has previously been reported to occur in both Sl^{gb}/Sl^{gb} and Sl^{10H}/Sl^{10H} mice (Schaible 1961, 1963; Beechey and Searle 1985). However, no live-born homozygous offspring were observed when $Sl^{gb}/+$ or $Sl^{10H}/+$ mice were intercrossed (M. A. Bedell, unpublished results), suggesting that strain background may affect the viability of homozygous mice. Because our analysis indicates that the deletions in Sl^{gb}, Sl^{J} and Sl^{10H} are considerably smaller than that of the original *Sl* allele (Figure 2) and analysis of Sl/Sl and Sl^{J}/Sl^{J} mice demonstrated that lethality occurred during embryogenesis after E15 (Sarvella and Russell 1956; P. J. Donovan, personal communication), all four alleles would be expected to cause homozygous lethality at the same stage of development when on a uniform genetic background. Late gestation lethality therefore appears to be the true null phenotype of *Sl* mutations. However, detailed comparison of the development of Sl^{gb}/Sl^{gb} and Sl/Sl embryos will be required to determine if the larger deletion affects additional genes essential for embryonic survival. In comparison to deletions that remove all *Mgf* coding se-

TABLE 3

Genetic interactions of *Sl* alleles

Allele[a]	Mutation	Consequence of mutation	Phenotype of heteroallelic *Sl* mice				
			Sl^{gb}	Sl^{d}	Sl^{17H}	Sl^{pan}	Sl^{con}
+	—	—	Light belly, dilution of coat, head spots	Light belly, dilution of coat, head spots	Light belly, slight dilution of coat, head spots	Light belly	Dark genitalia
Sl^{gb}	120 kb deletion[b]	Absence of Mgf	Prenatal lethal	Viable, white coat	Neonatal lethal	Viable, white coat	Viable, light grey coat, dark genitalia
Sl^{d}	Intragenic deletion[c]	Lack of membrane bound Mgf		White coat	White coat	White coat	Grey coat, dark genitalia
Sl^{17H}	Point mutation in splice acceptor[d]	Absence of normal Mgf cytoplasmic domain			White coat	Grey and white coat	Nearly normal pigmentation, head spot, dark genitalia
Sl^{pan}	Distant rearrangement[e]	Altered *Mgf* mRNA abundance				White coat, black ears	Light grey coat, dark genitalia
Sl^{con}	Distant rearrangement[e]	Altered *Mgf* mRNA abundance					Grey coat, dark genitalia

[a] All mutants were maintained on a C3H/HeN background except Sl^{d} that was maintained on C57BL/6J.
[b] Present study.
[c] Intragenic deletion of 4 kb that causes the absence of transmembrane and cytoplasmic domains (BRANNAN *et al.* 1991; FLANAGAN *et al.* 1991).
[d] Point mutation in 3′ splice site such that normal cytoplasmic domain is removed by splicing, leaving an out-of-frame cytoplsmic domain (BRANNAN *et al.* 1992).
[e] Chromosomal rearrangements in Sl^{pan} and Sl^{con} that break 115 and 195 kb, respectively, upstream of *Mgf* coding sequences and have tissue specific effects on *Mgf* mRNA abundance (BEDELL *et al.* 1995).

quences, the Sl^{d} mutation is an intragenic deletion that affects only the membrane-bound form of the growth factor and is able to produce soluble factor (BRANNAN *et al.* 1991; FLANAGAN *et al.* 1991). Sl^{d} homozygous mice are viable on some backgrounds even though they are severely anemic. The difference in homozygous phenotypes between the lethal deletions and Sl^{d} indicates that although soluble Mgf alone is sufficient for viability, the complete absence of both soluble and membrane-bound Mgf results in lethality caused by severe anemia (SARVELLA and RUSSELL 1956; SILVERS 1979a). It will be of interest to determine the phenotypic consequences of expressing only membrane-bound Mgf, in the complete absence of soluble factor.

Three *Sl* alleles examined, *Sl*, Sl^{8H} and Sl^{12H} are deleted for all probes tested and therefore contain deletions of 810 kb. While the timing of Sl^{8H}/Sl^{8H} lethality has not been reported, homozygous *Sl* embryos die at late gestation (SARVELLA and RUSSELL 1956). The deleted sequences in the *Sl* allele therefore do not appear to affect an essential gene(s) that is required earlier in development. The physical characterization of the breakpoints in the *Sl* allele may be used as starting points to identify other developmentally important genes that are proximal and distal to the *Sl* locus. Direct evidence for the existence of essential gene(s) in the vicinity of the *Sl* gene has been reported by CATTANACH *et al.* (1993): two *Sl* alleles that have large deletions and cause homozygous pre-implantation lethality (Sl^{22H} and Sl^{23H}) were each complemented by another deletion allele that causes homozygous postimplantation lethality (Sl^{24H}). The Sl^{18H} deletion is cytogenetically apparent and was estimated to have 2.5% of the chromosome removed (CATTANACH *et al.* 1993). Our analysis indicates that the distal end of the Sl^{18H} breakpoint is less than 590 kb distal to the *Mgf* coding region. As this allele causes lethality around the time of implantation, the identification of the distal extent of the deletion indicates that gene(s) responsible for this phenotype must be located proximal to the *Mgf* gene. The *Sl* allele that is lethal at the earliest stage of development is Sl^{12H}. Death in Sl^{12H}/Sl^{12H} embryos occurs before implantation and the extent of this deletion has been estimated from cytogenetic analysis to be ~10% of chromosome *10* (CATTANACH *et al.* 1993). It is possible that this deletion encompasses many genes, any of which could contribute to the early lethality. Further analysis of these and other *Sl* deletions should allow the identification and eventual characterization of genes essential to various stages of embryonic development.

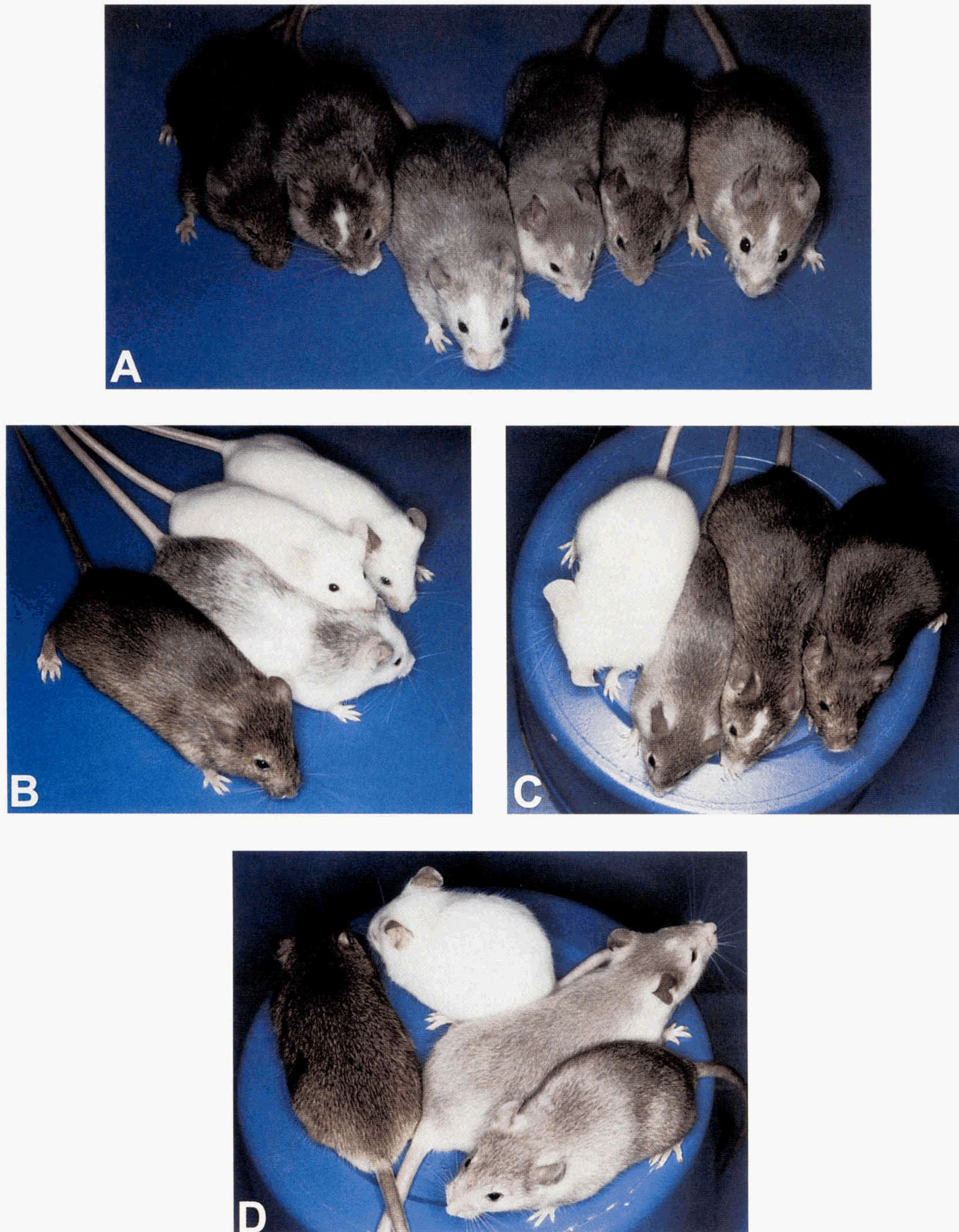

FIGURE 3.—Representative photographs of mice heteroallelic for different *Sl* mutant alleles. Listed below are the genotypes of the mice in each panel from left to right. (A) The Sl^{con} series. +/+, Sl^{17H}/Sl^{con}, Sl^{gb}/Sl^{con}, Sl^{pan}/Sl^{con}, Sl^{con}/Sl^{con}, Sl^{d}/Sl^{con}. Note that the Sl^{con} pigmentation defect is almost fully complemented by Sl^{17H} but not by Sl^{pan} or Sl^{d} and that the Sl^{con} hemizygous mouse (Sl^{gb}/Sl^{con}) has less pigmentation than the Sl^{con} homozygous mouse. (B) Complementation of the Sl^{17H} and Sl^{pan} pigmentation defects. +/+, Sl^{17H}/Sl^{pan}, Sl^{17H}/Sl^{17H}, Sl^{pan}/Sl^{pan}. (C) Complementation of the Sl^{17H} and Sl^{con} pigmentation defects. Sl^{17H}/Sl^{17H}, Sl^{con}/Sl^{con}, Sl^{17H}/Sl^{con}, +/+. In panels B and C, note that the heteroallelic mice (Sl^{17H}/Sl^{pan} and Sl^{17H}/Sl^{con}) have more pigmentation than any of the corresponding homozygous mice and that while the former is only partially complemented, most of the coat in the latter has nearly normal pigmentation. (D) Sl^{pan} and Sl^{con} do not complement each other. +/+, Sl^{pan}/Sl^{pan}, Sl^{pan}/Sl^{con}, Sl^{con}/Sl^{con}. Note that the heteroallelic mouse (Sl^{pan}/Sl^{con}) has less pigmentation than the Sl^{con}/Sl^{con} mouse. All mutants were maintained on a C3H/HeN background except Sl^{d} that was maintained on C57BL/6J.

Gene dosage appears to be a critical feature of the function of both wild-type and mutant *Sl* proteins for pigmentation and viability. All known *Sl* alleles exhibit haploinsufficiency in that heterozygous mice display mild pigmentation defects (see Tables 1 and 3) (SILVERS 1979a; CATTANACH *et al.* 1993). Because this occurs in mice heterozygous for *Sl* deletions, this semidominant effect must result from reduced gene dosage

rather than from gain-of-function mutations in the protein. The profound effects of only a single copy of Sl^{17H} on viability are somewhat surprising. It is not presently known how the absence of the normal cytoplasmic domain in Sl^{17H} disrupts Mgf function but it may cause either decreased protein stability, lack of proper presentation in the membrane, absence of proteolytic cleavage or faulty signaling (BRANNAN *et al.* 1992). Although the lethality observed in Sl^{17H}/Sl^{gb} could result from a gain-of-function mutation in the Sl^{17H}-encoded protein, this is unlikely because these hemizygous mice live longer than Sl^{gb}/Sl^{gb} mice. It is more likely that the amount of mutant protein expressed by only a single copy of the Sl^{17H} allele causes severe impairment of Mgf function. A threshold of functional Mgf activity may therefore be required for viability, as has been proposed for the activity of this growth factor during ovarian follicle development (HUANG *et al.* 1993; BEDELL *et al.* 1995). In sharp contrast to the lethality of Sl^{17H}/Sl^{gb} mice, mice that are hemizygous for Sl^{pan}, Sl^{con} or Sl^{d} express sufficient functional Mgf for viability. One caveat to direct comparisons between these alleles is that the Sl^{d}/Sl^{gb} mice were produced on a mixed (B6C3F1) background while all others were on a pure inbred background (C3H/HeN). Background effects could therefore be contributing to the observed differences in viability produced by these hypomorphic alleles.

Complementation of the pigmentation phenotype was observed with certain combinations of viable *Sl* alleles, *i.e.*, more pigmentation was observed in Sl^{17H}/Sl^{pan} and Sl^{17H}/Sl^{con} mice than in the respective homozygous mice. This complementation is even more striking when compared with the phenotypes of mice hemizygous for each of these alleles (see above). The nature of the mutations present in each of the complementing alleles is known. Sl^{17H}-encoded Mgf lacks the normal cytoplasmic domain as the result of a splice mutation (BRANNAN *et al.* 1992), and both Sl^{pan} and Sl^{con} contain intact coding sequences but have distant rearrangements that affect the expression of the gene (BEDELL *et al.* 1995). Several possible mechanisms for this complementation have been considered. Because the Sl^{pan} and Sl^{con} rearrangements are more than 100 kb from *Mgf* (BEDELL *et al.* 1995), they could affect another gene, in addition to *Mgf*, that is required for pigmentation. If this were the case, the pigmentation of Sl^{17H}/Sl^{pan} and Sl^{17H}/Sl^{con} mice would result from the mild effects of one mutant copy each of *Mgf* and the other gene. However, the Sl^{pan} and Sl^{con} phenotypes are not complemented by Sl^{gb} and Sl^{d} (BEDELL *et al.* 1995; present study), providing strong evidence that *Mgf* is the only gene required for pigmentation that is affected by the rearrangements. Therefore, the Sl^{17H}/Sl^{pan} and Sl^{17H}/Sl^{con} phenotypes represent true interallelic complementation. Two mechanisms by which interallelic complementation may be produced are transvection, whereby regulatory elements of one allele affect expression of a second allele on the homologous chromosome, and dimer formation between protein monomers expressed by two different alleles. Although it is not clear whether transvection occurs in mammals, the phenomenon is well characterized at a few loci in Drosophila and often involves complementation between a regulatory mutant and a structural mutant (for example, see reviews by MULLER and SCHAFFNER 1990; PIRROTTA 1990). If dimerization occurs, the low level of Mgf expressed from the Sl^{pan} and Sl^{con} alleles may form heterodimers with Sl^{17H}-encoded Mgf that are either more stable or provide some function that is missing from Sl^{17H} homodimers. In both transvection and heterodimer models, it is not presently clear why complementation was observed only between the regulatory mutants and the Sl^{17H} allele and not the Sl^{d} allele. Presumably, this is a reflection of a more severe effect on the protein in the latter allele, where membrane-bound Mgf is completely absent (BRANNAN *et al.* 1991; FLANAGAN *et al.* 1991). While biochemical analysis has indicated that soluble Mgf is in fact a dimer (ZSEBO *et al.* 1990b; ARAKAWA *et al.* 1991), the Sl^{d}-encoded Mgf may not dimerize. Further genetic analysis of *Sl* mutants and biochemical assays of mutant and wild-type forms of Mgf should reveal the mechanism of interallelic complementation and provide further information on the *in vivo* functions of this growth factor.

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