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An Allelic Series of Mutations in the *Kit ligand* Gene of Mice. II. Effects of Ethylnitrosourea-Induced *Kitl* Point Mutations on Survival and Peripheral Blood Cells of *Kitl*^{Steel} Mice

S. Rajaraman,*[,1] W. S. Davis,*[,1,2] A. Mahakali-Zama,* H. K. Evans,*[,3] L. B. Russell† and M. A. Bedell*[,4]

**Department of Genetics, University of Georgia, Athens, Georgia 30602-7223 and †Life Sciences Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37831-8077*

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ABSTRACT

The ligand for the Kit receptor tyrosine kinase is Kit ligand (Kitl; also known as mast cell growth factor, stem cell factor, and Steel factor), which is encoded at the *Steel* (*Sl*) locus of mice. Previous studies revealed that *Kitl*^{Sl} mutations have semidominant effects; mild pigmentation defects and macrocytic, hypoplastic anemia occur in heterozygous mice, and more severe pigmentation defects and anemia occur in homozygotes. Lethality also occurs in mice homozygous for severe *Kitl*^{Sl} mutations. We describe the effects of seven new *N*-ethyl-*N*-nitrosourea (ENU)-induced *Kitl*^{Sl} mutations and two previously characterized severe *Kitl*^{Sl} mutations on pigmentation, peripheral blood cells, and mouse survival. Mice heterozygous for each of the nine mutations had reduced coat pigmentation and macrocytosis of peripheral blood. In the case of some of these mutations, however, red blood cell (RBC) counts, hemoglobin concentrations, and hematocrits were normal in heterozygotes, even though homozygotes exhibited severely reduced RBC counts and lethality. In homozygous mice, the extent of anemia generally correlates with effects on viability for most *Kitl*^{Sl} mutations; *i.e.*, most mutations that cause lethality also cause a more severe anemia than that of mutations that allow viability. Interestingly, lethality and anemia were not directly correlated in the case of one *Kitl*^{Sl} mutation.

MUTATIONS at the *Steel* (*Sl*) and *Dominant White Spotting* (*W*) loci of mice identify two genes essential for the development of hematopoietic cells, germ cells, and melanocytes (reviewed by Besmer *et al.* 1993; Lev *et al.* 1994). The *W* locus encodes Kit, a type III receptor tyrosine kinase, and the *Sl* locus encodes Kitl (also known as mast cell growth factor, stem cell factor, and Steel factor), which is the only known ligand for Kit and is a member of the short-chain subgroup of helical cytokines (Jiang *et al.* 2000; Zhang *et al.* 2000). While Kit is expressed on the surface of hematopoietic cells, germ cells, and melanocytes, Kitl is expressed by various cells that support the survival, proliferation, and differentiation of the former cell types. Interestingly, recent evidence suggests that the Kitl/Kit signaling pathway may operate differently in the different cell types (Jordan *et al.* 1999; Blume-Jensen *et al.* 2000; Kissel *et al.* 2000).

A large collection of *Kitl*^{Sl} mutations exists and offers a powerful genetic resource for understanding the *in vivo* functions of the Kitl/Kit signaling pathway. Importantly, different *Kitl*^{Sl} mutations produce phenotypes that are graded with respect to severity; *i.e.*, some *Kitl*^{Sl} mutations produce very severe phenotypes while other *Kitl*^{Sl} mutations produce very mild phenotypes. While severe mutations are critical to understanding the consequences of the near or complete absence of function of a particular gene, identification and characterization of milder mutations may reveal requirements during later developmental stages (Schumacher *et al.* 1996). In *Kitl*^{Sl} mutants, homozygous null mutations cause prenatal or perinatal lethality with severe effects on numbers of hematopoietic cells, germ cells, and melanocytes. On the other hand, homozygous hypomorphic *Kitl*^{Sl} mutations allow viability but have milder effects on each cell type. Gene dosage is important to Kitl function, as all *Kitl*^{Sl} mutations (with the exception of one extinct allele) are semidominant (Mouse Genome Database 2002) and this is likely to be due to haploinsufficiency (Bedell *et al.* 1996a). The best-known hematopoietic defects in *Kitl*^{Sl} mutants are specific for stem cells, erythroid cells, mast cells, and megakaryocytes (Besmer *et al.* 1993; Lev *et al.* 1994). *Kitl*^{Sl} mutants have a macrocytic, hypoplastic anemia resulting from an increased volume of red blood cells (RBCs) and a reduced number of RBCs (Russell 1979). Recent evidence indi-

[1]These authors contributed equally to this work.

[2]*Present address:* Horizon Molecular Medicine, Norcross, GA 30071.

[3]*Present address:* University Program in Genetics, Duke University, Durham, NC 27710.

[4]*Corresponding author:* Department of Genetics, B416 Life Sciences, University of Georgia, Athens, GA 30602-7223.
E-mail: bedell@arches.uga.edu

cates that, in addition to these classically defined targets, other cell types are defective in Kit^{W} and $Kitl^{Sl}$ mutants (Huizinga *et al.* 1995; Rodewald *et al.* 1995; Motro *et al.* 1996; Laky *et al.* 1997).

In this report we describe the effects of seven new ethylnitrosourea (ENU)-induced $Kitl^{Sl}$ mutations and two previously characterized $Kitl^{Sl}$ mutations on pigmentation, peripheral blood cells, and survival of mice. We describe the molecular defects associated with the ENU-induced mutations in the accompanying article (Rajaraman *et al.* 2002, this issue). Our analysis of the effects of these mutations on survival and peripheral blood cells reveals a graded effect, with five mutations having very severe effects and two mutations having milder effects. However, with some $Kitl^{Sl}$ mutations, there is no direct relationship between severity of heterozygous and homozygous phenotypes nor is there a direct relationship between severity of effects on different blood-cell parameters.

MATERIALS AND METHODS

Mice: The generation and molecular characterization of the seven ENU-induced $Kitl^{Sl}$ mutations ($Kitl^{Sl\text{-}30R}$, $Kitl^{Sl\text{-}31R}$, $Kitl^{Sl\text{-}22R}$, $Kitl^{Sl\text{-}28R}$, $Kitl^{Sl\text{-}42R}$, $Kitl^{Sl\text{-}39R}$, and $Kitl^{Sl\text{-}36R}$) studied in this article have been described in the accompanying article (Rajaraman *et al.* 2002). Each mutant allele contains a point mutation, and the positions of the Kitl sequences affected in each are shown in Table 1. The ENU-induced $Kitl^{Sl}$ mutations were made congenic on a common strain background by backcrossing to C3H/Rl for >20 generations and subsequently to C3H/HeNCR for at least 5 generations. Two previously characterized mutations, $Kitl^{Sl\text{-}gb}$ and $Kitl^{Sl\text{-}d}$, were also used in this study (Table 1). $Kitl^{Sl\text{-}gb}$ contains an ~120-kb deletion whose proximal breakpoint is located ~60 kb upstream of the *Kitl* transcription unit and whose distal breakpoint is located within the 3′ untranslated region of *Kitl* (Bedell *et al.* 1996a). $Kitl^{Sl\text{-}gb}$ mice were originally obtained from the MRC Radiobiology Unit (Chilton, Didcot, UK) and have been maintained on a C3H/HeNCR background for >20 generations. $Kitl^{Sl\text{-}d}$ contains a 4-kb intragenic deletion that removes the transmembrane and cytoplasmic domains of Kitl (Brannan *et al.* 1991; Flanagan *et al.* 1991). $Kitl^{Sl\text{-}d}$ mice were originally obtained from the Jackson Laboratory (Bar Harbor, ME) on a C57BL/6J background and the $Kitl^{Sl\text{-}d}$ mutation was made congenic on the C3H/HeNCR strain by backcrossing for >12 generations. All strains are currently maintained in a pathogen-free colony at the University of Georgia.

Survival studies: To generate homozygous mutant mice or compound heterozygous mice, mice heterozygous for each mutant allele were intercrossed. Each about-to-deliver female and each litter were examined daily until postnatal day 18 (P18) and the numbers of pups of each genotype recorded. Homozygous $Kitl^{Sl}$ mice are readily identified at birth by their runted size and pallor due to anemia. A subset of presumed homozygous mutants for each mutant allele was subjected to molecular genotyping (see below). In every case, the genotype assigned by phenotype was confirmed.

Genotyping $Kitl^{Sl}$ mutants: Methods for genotyping were based on PCR amplification of genomic DNA from mouse tissues and are summarized in Table 1. A sequence polymorphism in the 5′-flanking region of *Kitl* was used for genotyping alleles that arose on non-C3H chromosomes [including $Kitl^{Sl\text{-}30R}$, $Kitl^{Sl\text{-}28R}$, $Kitl^{Sl\text{-}42R}$, and $Kitl^{Sl\text{-}39R}$ (Rajaraman *et al.* 2002) and $Kitl^{Sl\text{-}d}$, which arose on a DBA/2J chromosome (Brannan *et al.* 1991)]. This polymorphism consists of a 6-bp insertion located 259 bp 5′ to the *Kitl* transcription initiation site (Bedell *et al.* 1996b) that is present in C3H DNA but is absent from C57BL/6J, C57BL/10 and 101/Rl DNA, and DBA/2J (W. S. Davis and M. A. Bedell, unpublished results). Portions of the cloned $Kitl^{Sl\text{-}gb}$ deletion breakpoint (Bedell *et al.* 1996a) were sequenced (data not shown), and oligonucleotide primers that span the breakpoint were used for PCR amplification of genomic DNA. Three of the ENU-induced mutations ($Kitl^{Sl\text{-}31R}$, $Kitl^{Sl\text{-}22R}$, and $Kitl^{Sl\text{-}36R}$) arose on C3H chromosomes (Rajaraman *et al.* 2002), and allele-specific genotyping methods were developed (Table 1). In the $Kitl^{Sl\text{-}22R}$ allele, the mutation abolishes a *Dde*I site and restriction fragment length polymorphism (RFLP) analysis was used for genotyping. For $Kitl^{Sl\text{-}31R}$ and $Kitl^{Sl\text{-}36R}$ genotyping, allele-specific PCR amplification methods were developed.

Peripheral blood analysis: Newborn (P1) mice were anesthetized by hypothermia and euthanized, and peripheral blood was collected using heparinized capillary tubes. The blood was diluted in PBS, and RBCs were counted using a hemacytometer. At P24-P25, juvenile mice were euthanized using CO_2 asphyxiation, and peripheral blood was collected by cardiac puncture. Blood from four to eight mice of each genotype was analyzed. Complete blood cell analysis was performed using a Celldyne 3500 hematology analyzer. The parameters evaluated are RBC counts; hemoglobin concentration; mean corpuscular volume (MCV), which is the average volume of individual RBCs; mean corpuscular hemoglobin (MCH), which is the average hemoglobin concentration in RBCs and is calculated from hemoglobin and RBC values; mean corpuscular hemoglobin concentration (MCHC), which is the ratio of the hemoglobin concentration to the average RBC volume and is calculated from hemoglobin and hematocrit values; hematocrit, which is the percentage of whole blood made up of RBCs and calculated from RBC and MCV values; counts of platelets; the mean platelet volume (MPV), which is the average volume of individual platelets; and counts of white blood cells (WBC), segmented neutrophils, and lymphocytes. Note that all of these parameters, except MCH, MCHC, and hematocrit, are determined directly, while the latter three parameters are calculated values.

Statistical analysis: For each $Kitl^{Sl}$ mutation, the observed numbers of homozygous mutant and compound heterozygous mice at P1 were compared against the expected numbers of these mice using a chi-square test for significance. Survival curves for mice of different genotypes were calculated and compared using Prism software (GraphPad Software, San Diego); the Kaplan-Meier method was used to calculate fractional survival at each time point and the log-rank test with calculation of two-sided P values was used to do pairwise comparisons of survival curves. The survival curves of homozygous mutant mice were compared against the survival curves of control mice ($Kitl^{+}/Kitl^{+}$ and heterozygous mice) and of homozygous null ($Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$) mice, and the survival curves of compound heterozygous mice were compared against the survival curves of $Kitl^{Sl\text{-}d}$ hemizygous ($Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$) mice. Values for peripheral blood analysis were evaluated using unpaired, two-tailed t-tests using Prism software. For each parameter, the values for heterozygotes and homozygotes for each mutant allele were compared against that of $Kitl^{+}/Kitl^{+}$, $Kitl^{Sl\text{-}gb}/Kitl^{+}$, and $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice. In addition, comparisons were made between pairs of values for mice homozygous for viable alleles, *i.e.*, $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ *vs.* $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$, $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ *vs.* $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$, and $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$ *vs.* $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$.

RESULTS

Pigmentation of $Kitl^{Sl}$ mutant mice: All seven of the new $Kitl^{Sl}$ mutants described here arose from progeny

of mice derived from the specific locus test using ENU as mutagen (Russell *et al.* 1982). Although the $Kitl^{Sl}$ locus is not one of the loci used in the specific locus test, the new $Kitl^{Sl}$ mutants were apparent because of their mild pigmentation defects in heterozygous mice (Table 1). However, the severity of the heterozygous pigmentation defect does vary somewhat, with two of the viable mutations having less of an effect than that which is characteristic of lethal mutations, and the $Kitl^{Sl\text{-}39R}$ mutation having the mildest effect of all the mutations. In all mice homozygous for viable $Kitl^{Sl}$ mutations and in compound heterozygotes between each of the ENU-induced mutations and $Kitl^{Sl\text{-}d}$, white coats were observed (data not shown). Occasionally, small pigmented patches were seen on the heads of $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$ mice (not shown). Overall, these observations indicate that all the $Kitl^{Sl}$ mutations described here have semidominant effects on pigmentation.

Survival of homozygous mutant mice: Previous studies revealed that the majority of mice homozygous for severe $Kitl^{Sl}$ mutations die during late gestation with severe anemia (Sarvella and Russell 1956; Russell 1979). To determine the effects of the ENU-induced *Kitl* mutations on prenatal or perinatal survival, we examined the ratios of genotypes in P1 mice born to intercrosses of mice heterozygous for each mutant allele. According to Mendelian segregation, 0.25 of the total number of mice born to these intercrosses should be homozygous mutant. If the ratio of homozygous mutant mice is significantly <0.25 for a given allele, then lethality must be occurring either prior to birth or within a few hours after birth.

To establish the survival pattern for the null condition at *Kitl* and, by comparison, to determine whether any of the ENU-induced mutations might be null functionally, we examined progeny of intercrosses of mice heterozygous for the smallest complete *Kitl* deletion, $Kitl^{Sl\text{-}gb}$ (Bedell *et al.* 1996a). Since 209 wild-type and heterozygous mice were born to $Kitl^{Sl\text{-}gb}/Kitl^{+}$ intercrosses, ~70 $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice would have been expected in the absence of any lethality to the latter (Table 2). However, only 32 $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice were observed, indicating that 54% of the $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice die either before birth or immediately following birth. Furthermore, none of the observed homozygous null mice survived beyond P2 (Figure 1, solid black line in each of B–I). On embryonic day 14.5 (E14.5), however, the expected ratio of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ embryos was observed (data not shown). Thus, $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice on the C3H strain background die between E14.5 and P2, indicating that the $Kitl^{Sl}$ null phenotype on this background is pre- or perinatal lethality.

With four of the ENU-induced mutations ($Kitl^{Sl\text{-}31R}$, $Kitl^{Sl\text{-}22R}$, $Kitl^{Sl\text{-}28R}$, and $Kitl^{Sl\text{-}36R}$), only 61–71% of the expected homozygous mutant P1 mice were observed (Table 2) and these proportions are significantly ($P < 0.05$) below expectations. These results indicate that the $Kitl^{Sl\text{-}22R}$, $Kitl^{Sl\text{-}28R}$, $Kitl^{Sl\text{-}31R}$, and $Kitl^{Sl\text{-}36R}$ alleles have reduced activity for prenatal or perinatal survival. In contrast, the observed numbers of P1 mice homozygous for three of the ENU-induced mutations ($Kitl^{Sl\text{-}30R}$, $Kitl^{Sl\text{-}42R}$, and $Kitl^{Sl\text{-}39R}$) are not significantly different ($P > 0.05$) from expectation (Table 2), indicating that the effects of these mutations on prenatal and perinatal survival are milder than those of the null allele.

We examined the postnatal survival of mice homozygous for each of the $Kitl^{Sl}$ mutations until P18, the age at weaning (Table 3 and Figure 1). The survival of these mutant mice was compared to the survival of control mice (red line in Figure 1A), which consisted of nearly 1000 wild-type and heterozygous siblings segregating from all the intercrosses of each mutant allele. In crosses involving each mutant allele, the expected numbers of heterozygous mice were observed at P18 (data not shown), indicating that there was little or no postnatal lethality of heterozygous mice. While 83% of control mice survived to P18, none of the mice homozygous for $Kitl^{Sl\text{-}30R}$, $Kitl^{Sl\text{-}31R}$, $Kitl^{Sl\text{-}22R}$, $Kitl^{Sl\text{-}28R}$, or $Kitl^{Sl\text{-}42R}$ survived beyond P7 (Figure 1), and the survival curves of the homozygous mutants were highly significantly different from those of the control mice (Table 3). Thus, these five alleles are classified as homozygous lethal alleles. To determine whether these lethal alleles behave as null alleles with respect to postnatal viability, the survival curves of mice homozygous for each of the lethal alleles were compared to those of the $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice (solid lines in B–F of Figure 1). Interestingly, mice homozygous for the $Kitl^{Sl\text{-}22R}$, $Kitl^{Sl\text{-}28R}$, or $Kitl^{Sl\text{-}42R}$ mutations displayed postnatal survival curves significantly different ($P < 0.05$, Table 3) from those of the homozygous null mutants. In particular, the survival curves of $Kitl^{Sl\text{-}22R}/Kitl^{Sl\text{-}22R}$ mice are highly significantly different ($P < 0.0001$) from those of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice. These results suggest that although these three mutations cause severe effects on survival, the alleles may be mildly hypomorphic because they allow a slightly prolonged survival time compared to the null allele. In comparison, the postnatal survival curves of mice homozygous for the $Kitl^{Sl\text{-}30R}$ or $Kitl^{Sl\text{-}31R}$ mutations are not significantly different ($P > 0.05$) from those of homozygous null mutants, indicating that these mutations are likely to be null functionally.

In contrast to mice homozygous for lethal mutations, the majority of $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$ and $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice survived beyond P7, with 73 and 63% survival to P18, respectively, compared to 83% survival of control mice (Figure 1A). Thus, both of these alleles are classified as homozygous viable alleles. However, the survival of $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice is significantly less than that of control mice ($P = 0.024$), while the survival of $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$ mice is not significantly different ($P = 0.126$) from that of control mice (Table 3). For $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice, the decreased viability is restricted to the period before P7 (see Figure 1A). This early lethality in $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice is consistent with observations made on P1 mice (see above and Table 2), where the ratio of

TABLE 1

Summary of *Kitl*[Sl] mutant alleles used in this study

Kitl allele	*Kitl* sequence alteration[a]	Effect on Kitl[b]	Genotyping method[c]	Coat of heterozygous mice	Viability of homozygous mice[d]
Sl-gb	Deletion of coding region	Null mutant	PCR across breakpoint	Head spot, diluted ventrum	Perinatally lethal
Sl-30R	Point mutation (T325G)	Missense mutant (L18R)	Strain polymorphism	Head spot, diluted ventrum	Perinatally lethal
Sl-31R	Point mutation (C340T)	Missense mutant (P23L)	Allele-specific PCR	Head spot, diluted ventrum	Perinatally lethal
Sl-22R	Point mutation (T433C)	Missense mutant (L54P)	RFLP analysis	Head spot, diluted ventrum	Perinatally lethal
Sl-28R	Point mutation (T626A)	Missense mutant (I118N)	Strain polymorphism	Head spot, diluted ventrum	Perinatally lethal
Sl-42R	Point mutation (T → C, 5′ splice site)	Truncated S-Kitl (96 aa + 2 aa)	Strain polymorphism	Head spot, diluted ventrum	Perinatally lethal
Sl-39R	Point mutation (C637T)	Missense mutant (S122F)	Strain polymorphism	Faint head spot, diluted ventrum	Viable
Sl-36R	Point mutation (G711T) and exon skipping	2 truncated S-Kitl (147 aa; 96 aa + 25 aa)	Allele-specific PCR	Faint head spot, diluted ventrum	Viable
Sl-d	Intragenic deletion	Truncated S-Kitl (180 aa + 3 aa)	Strain polymorphism	Head spot, diluted ventrum	Viable

[a] The nucleotide numbering for *Kitl* is from Bedell *et al.* (1996b; GenBank accession no. U44725). Molecular genetic analysis of the mutations is described in Rajaraman *et al.* (2002), except for *Kitl*[Sl-gb] and *Kitl*[Sl-d], which are described in Bedell *et al.* (1996a) and Brannan *et al.* (1991), respectively. All mutations are congenic on a C3H background.

[b] The amino acid numbering is for the processed form of Kitl that lacks the 25-aa signal sequence. Note that two biologically active isoforms of wild-type Kitl are produced by alternative mRNA splicing and post-translational processing (Flanagan *et al.* 1991; Huang *et al.* 1992), a membrane-bound isoform (MB-Kitl) and soluble isoform (S-Kitl), respectively. Each of the missense mutants shown here would affect both S-Kitl and MB-Kitl isoforms. However, premature termination occurs in the cytoplasmic domain of the *Kitl*[Sl-39R], *Kitl*[Sl-36R], and *Kitl*[Sl-d] mutants such that no MB isoform would be expected.

[c] The methods used in this study for genotyping are described briefly in materials and methods. Detailed methods are available on request.

[d] The effects of the mutants on mouse viability are summarized from this study.

TABLE 2

Ratios of P1 homozygous and compound heterozygous mice

Kitl allele[a]	$Kitl^{+}/Kitl^{+}$, $Kitl^{Sl}/Kitl^{+}$ observed	Total expected[b]	$Kitl^{Sl}/Kitl^{Sl}$ observed	$Kitl^{Sl}/Kitl^{Sl}$ expected[c]	$Kitl^{Sl}/Kitl^{Sl}$ observed/expected[d]	*P* value[e]
			Homozygous mice			
Sl-gb	209	279	32	70	0.46	0.0000
Sl-30R	169	225	47	56	0.83	0.2137
Sl-31R	215	287	44	72	0.61	0.0011
Sl-22R	158	211	34	53	0.65	0.0101
Sl-28R	182	243	43	61	0.71	0.0233
Sl-42R	137	183	38	46	0.83	0.2566
Sl-39R	115	153	48	38	1.25	0.1185
Sl-36R	135	180	30	45	0.67	0.0253
Sl-d	103	137	32	34	0.93	0.6905
			Compound heterozygous mice (each allele *in trans* with $Kitl^{Sl\text{-}d}$)			
Sl-gb	142	232	31	47	0.65	0.0176
Sl-30R	174	213	43	58	0.74	0.0489
Sl-31R	160	123	36	53	0.68	0.0176
Sl-22R	123	164	25	41	0.61	0.0125
Sl-28R	86	115	20	29	0.70	0.1055
Sl-42R	139	185	29	46	0.63	0.0109
Sl-39R	95	127	25	32	0.79	0.2361
Sl-36R	125	167	36	42	0.86	0.3800

[a] Homozygous mice were produced by intercrossing mice heterozygous for each allele while compound heterozygous mice were generated by crossing $Kitl^{Sl\text{-}d}/Kitl^{+}$ mice with mice heterozygous for each of the indicated alleles.

[b] The total number of expected mice of all genotypes ($Kitl^{+}/Kitl^{+}$, $Kitl^{Sl}/Kitl^{+}$, and $Kitl^{Sl}/Kitl^{Sl}$) was calculated by dividing the number of $Kitl^{+}/Kitl^{-}$ mice observed for each cross by 0.75.

[c] The number of homozygous or compound heterozygous mice that were expected to be born to each cross was calculated by multiplying the total mice expected by 0.25.

[d] If there were no prenatal or perinatal loss of homozygous or compound heterozygous mice, then the observed-to-expected ratio should be 1.

[e] *P* value calculated from chi-square analysis (with 1 d.f.).

$Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice was less than expected. Thus, the $Kitl^{Sl\text{-}36R}$ mutation affects perinatal and juvenile viability in some homozygous mice, but has less of an effect on homozygous mice surviving longer than 1 week.

Additive effect of some $Kitl^{Sl}$ mutations on survival of compound heterozygous mice: During the course of our studies with the ENU-induced $Kitl^{Sl}$ mutations, experiments with $Kitl^{Sl\text{-}d}$ mice revealed that the latter mutation exerts gene dosage effects on mouse survival (Figure 1I). This gene dosage effect was observed when survival of $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice (pink line in Figure 1I), which carry two copies of the $Kitl^{Sl\text{-}d}$ allele, was compared with survival of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ mice (dashed black line in Figure 1I), which carry only one copy of the $Kitl^{Sl\text{-}d}$ allele. In $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice, the expected numbers of P1 mice were observed (Table 2), and 82% of them survived to P18 (Figure 1I). However, we observed only 65% of the expected number of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ P1 mice (Table 2) and the postnatal survival curve of these mice is intermediate between that of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice and $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice (Figure 1I). Thus, hemizygosity for $Kitl^{Sl\text{-}d}$ causes an intermediate phenotype for postnatal survival.

The gene dosage effects observed with the $Kitl^{Sl\text{-}d}$ allele provided the basis for a second test for activity of the ENU-induced $Kitl^{Sl}$ gene products, namely, whether a given allele could exert an additive effect when *in trans* with $Kitl^{Sl\text{-}d}$. To accomplish this, mice heterozygous for each ENU-induced mutation were crossed with $Kitl^{Sl\text{-}d}/Kitl^{+}$ mice to generate compound heterozygous mice (*i.e.*, $Kitl^{Sl\text{-}X}/Kitl^{Sl\text{-}d}$, where X stands for any ENU-induced mutation). The survival of the compound heterozygotes was then determined and compared to that of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ mice (see Table 3 and Figure 1). If the test mutation is null functionally, then the survival curve of the compound heterozygotes should be identical to that of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ mice. If the test mutation is hypomorphic, then it should exert an additive effect with $Kitl^{Sl\text{-}d}$ and the survival curve of the compound heterozygote would be shifted to the right of the $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ survival curve. To validate this test, we first determined whether the two homozygous viable alleles ($Kitl^{Sl\text{-}39R}$ and $Kitl^{Sl\text{-}36R}$) could exert an additive effect with the $Kitl^{Sl\text{-}d}$ allele. As expected, the survival curves of $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}d}$ and $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}d}$ mice shifted to the right (dashed, colored lines in Figure 1, G and H, respectively) and are highly significantly different ($P < 0.0001$, Table 3) from the

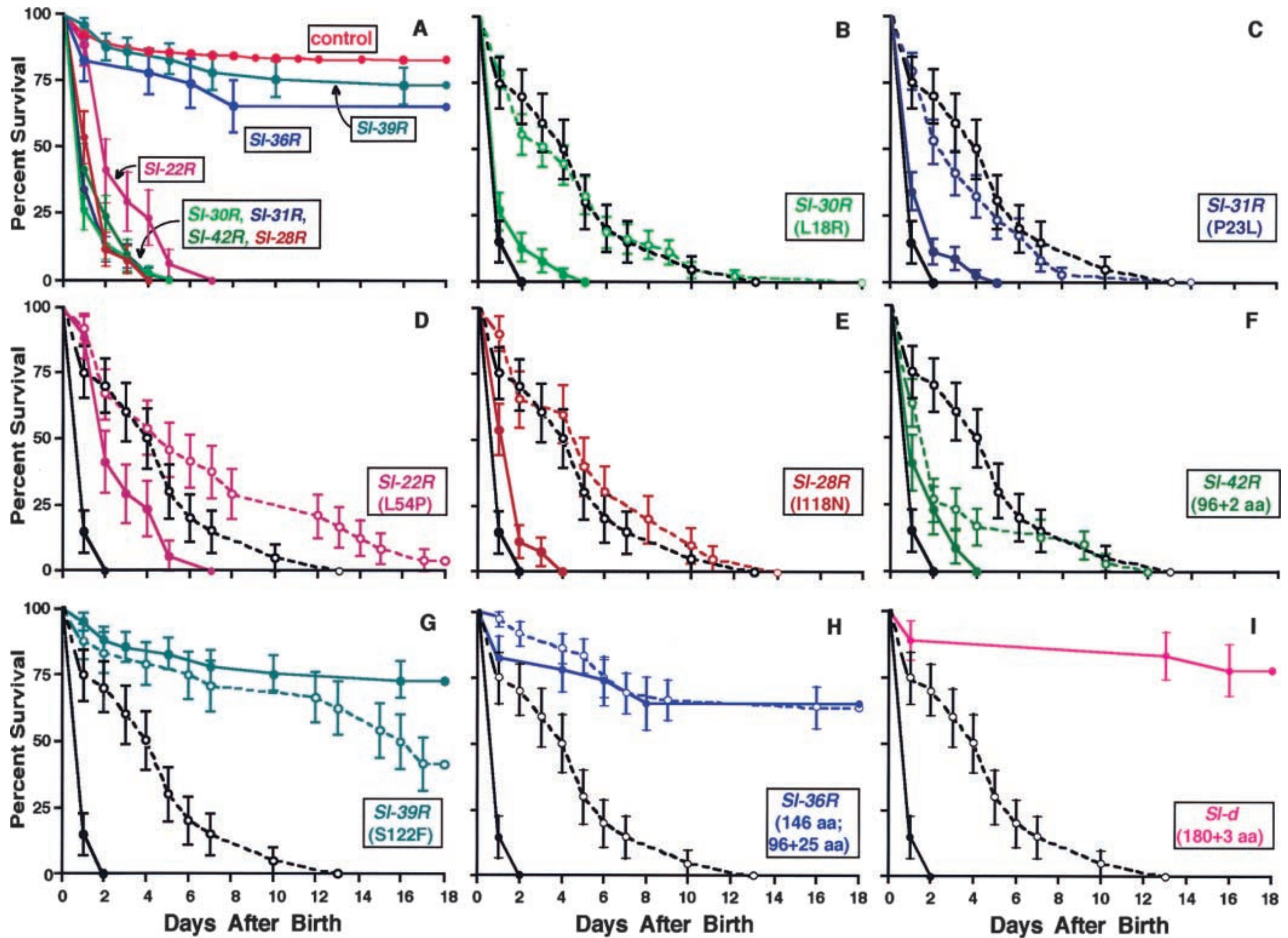

FIGURE 1.—Survival curves of homozygous mutant and compound heterozygous mice carrying $Kitl^{Sl}$ mutations. For each allele, heterozygous mice were mated and the resulting progeny observed every day after birth until P18. The values at P0 represent the sum of the numbers of dead and living homozygous mutant or compound heterozygous mice observed at P1. For subsequent ages, the fractional survival and SEM at each age were calculated using the Kaplan-Meier method. (A) The red line is the survival curve for 942 wild-type mice and heterozygotes combined from all intercrosses and the other survival curves are for mice homozygous for each of the $Kitl^{Sl}$ mutant alleles. (B–H) The survival curves for mice carrying each of the ENU-induced $Kitl^{Sl}$ mutant alleles. (I) The survival curve for $Kitl^{Sl\text{-}d}$. The lines and symbols used in the graphs are as follows: solid lines with solid circles, homozygous mice; dashed lines with open circles, compound heterozygous mice ($Kitl^{Sl\text{-}X}/Kitl^{Sl\text{-}d}$, where X represents any of the ENU alleles); solid black lines, $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$; dashed black lines with open symbols, $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$).

$Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ survival curve. In contrast, none of the lethal alleles ($Kitl^{Sl\text{-}30R}$, $Kitl^{Sl\text{-}31R}$, $Kitl^{Sl\text{-}22R}$, $Kitl^{Sl\text{-}42R}$, and $Kitl^{Sl\text{-}28R}$) exhibited a significant additive effect with $Kitl^{Sl\text{-}d}$ (Table 3 and dashed colored lines in Figure 1, B–F). Thus these alleles are likely to be null, or nearly null, for activity required for prenatal and postnatal survival. Although the survival curve for $Kitl^{Sl\text{-}22R}/Kitl^{Sl\text{-}d}$ mice was not statistically different from that of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ mice ($P = 0.0719$, Table 3), examination of these curves (dashed colored line in Figure 1D) suggests a trend toward increased survival in the former that is consistent with the enhanced survival of some $Kitl^{Sl\text{-}22R}/Kitl^{Sl\text{-}22R}$ mice. Thus, from the analysis of homozygous mutant and compound heterozygous mice, $Kitl^{Sl\text{-}22R}$ mutation appears to be mildly hypomorphic for postnatal survival.

Effects of $Kitl^{Sl}$ mutations on RBCs of newborn mice: Previous studies revealed that $Kitl^{Sl}$ mutations cause a macrocytic, hypoplastic anemia that is apparent during embryogenesis and continues into adulthood (RUSSELL 1979). Like the pigmentation phenotype, the anemia phenotype in $Kitl^{Sl}$ mutants is semidominant; *i.e.*, the number of RBCs is mildly reduced in heterozygous mice but markedly reduced in homozygous mice. Because mice homozygous for severe $Kitl^{Sl}$ mutations die either pre- or perinatally with severe anemia, it is likely that the anemia is the cause of the lethality. If so, then mice homozygous for each lethal $Kitl^{Sl}$ mutation should exhibit effects on RBCs that are more severe than those of mice homozygous for each viable mutation. Furthermore, the effect on heterozygous mice would be ex-

TABLE 3

Postnatal survival of $Kitl^{Sl}/Kitl^{Sl}$ mice

	Homozygous mice[a]			Compound heterozygous mice[b]	
Kitl allele	No. of mice $Kitl^{Sl\text{-}X}/Kitl^{Sl\text{-}X}$	*vs.* control	*vs.* $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$	No. of mice $Kitl^{Sl\text{-}X}/Kitl^{Sl\text{-}d}$	*vs.* $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$
Sl-gb	20	<0.0001	—	20	—
Sl-30R	40	<0.0001	0.1222	43	0.8619
Sl-31R	40	<0.0001	0.0590	34	0.4071
Sl-22R	17	<0.0001	<0.0001	24	0.0719
Sl-28R	21	<0.0001	0.0176	18	0.1069
Sl-42R	22	<0.0001	0.0050	15	0.3969
Sl-39R	41	0.1257	<0.0001	14	<0.0001
Sl-36R	24	0.0241	<0.0001	36	<0.0001
Sl-d	17	0.5890	<0.0001	17	<0.0001

[a] The survival of mice homozygous for each of the indicated alleles was monitored for 18 days after birth. The numbers of homozygous mutants listed here are not the same as in Table 2 because postnatal survival was not monitored for all litters. For each allele ($Kitl^{Sl\text{-}X}$, where X indicates gb, d, or one of the ENU-induced alleles) fractional survival was calculated using the Kaplan-Meier method. The resulting survival curves for homozygous mice were compared against survival curves of control mice (pooled $Kitl^{+}/Kitl^{+}$ and heterozygous mice) and against $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice (homozygous null mice) using the log-rank test. The two-sided P values are shown for each comparison.

[b] Survival curves of mice that were compound heterozygotes of $Kitl^{Sl\text{-}d}$ and $Kitl^{Sl\text{-}gb}$ and of $Kitl^{Sl\text{-}d}$ and of each of the ENU-induced alleles were calculated and compared as described for homozygous mice. In this case, if the ENU-induced allele behaves as a null, then additive effects would not be observed and the survival curve of the compound heterozygote would not be significantly different from that of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ mice. If the ENU-induced allele is hypomorphic, then additive effects would be observed and the survival curve of the compound heterozygote would be significantly different from that of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$ mice.

pected to parallel the effect on homozygous mice; *i.e.*, mutations that cause lethality when homozygous would be expected to have a heterozygous phenotype that is more severe than that of mutations that allow viability when homozygous. We tested this by examining RBC counts in P1 mice that were heterozygous or homozygous for each of the nine $Kitl^{Sl}$ mutations and by studying peripheral blood parameters in P24-P25 mice that were heterozygous for each of the nine $Kitl^{Sl}$ mutations and in mice that were homozygous for each of the three viable $Kitl^{Sl}$ mutations.

In general, the mean RBC counts were reduced in P1 $Kitl^{Sl}$ mice when the mutations were heterozygous or homozygous (Figure 2). In comparison to mean RBC counts of $4.1 \pm 0.1 \times 10^9$ cells/ml in P1 $Kitl^{+}/Kitl^{+}$ mice, the corresponding values for heterozygous $Kitl^{Sl}$ mutants ranged from $2.7 \pm 0.2 \times 10^9$ cells/ml (66% of wild type) in P1 $Kitl^{Sl\text{-}39R}/Kitl^{+}$ mice to $3.4 \pm 0.2 \times 10^9$ cells/ml (83% of wild type) in P1 $Kitl^{Sl\text{-}22R}/Kitl^{+}$ mice. With the notable exception of $Kitl^{Sl\text{-}22R}$, each lethal and viable $Kitl^{Sl}$ mutation resulted in RBC counts in heterozygous P1 mice that are significantly different ($P < 0.02$) from wild-type values. Given that $Kitl^{Sl\text{-}22R}$ is a lethal mutation when homozygous, it is surprising that the mean RBC counts for P1 $Kitl^{Sl\text{-}22R}/Kitl^{+}$ mice are not significantly different ($P = 0.08$) from the P1 wild-type mean. The mean RBC counts for P1 mice heterozygous for viable mutations ($Kitl^{Sl\text{-}39R}$, $Kitl^{Sl\text{-}36R}$, and $Kitl^{Sl\text{-}d}$) are not significantly different from the $2.9 \pm 0.1 \times 10^9$ cells/ml (71% of wild type) observed in P1 $Kitl^{Sl\text{-}gb}/Kitl^{+}$ mice ($P = 0.50$, 0.26, and 0.08, respectively), and they are not significantly different from each other ($Kitl^{Sl\text{-}39R}/+$ *vs.* $Kitl^{Sl\text{-}36R}/+$, $P = 0.13$; $Kitl^{Sl\text{-}39R}/+$ *vs.* $Kitl^{Sl\text{-}d}/+$, $P = 0.03$; $Kitl^{Sl\text{-}36R}/+$ *vs.* $Kitl^{Sl\text{-}d}/+$, $P = 0.82$). Thus, the severity of the anemia phenotype in P1 heterozygous mice does not correlate directly with severity of the survival phenotype in homozygous mice (see Table 4). Furthermore, the heterozygous anemia phenotype does not correlate with the pigmentation defects in older heterozygous mice (see Table 4), as juvenile $Kitl^{Sl\text{-}22R}/Kitl^{+}$ mice have a pigmentation defect that is more severe than that of $Kitl^{Sl\text{-}39R}/Kitl^{+}$ mice (Table 1) even though the former mice are less anemic at birth than the latter (Figure 2).

With P1 homozygous mice, RBC counts in all $Kitl^{Sl}$ mutants are significantly reduced ($P < 0.0001$) relative to wild-type values (Figure 2). Moreover, unlike the situation in P1 heterozygous mice, there is a direct correlation between lethality and RBC counts in P1 mice homozygous for each of the nine mutations. While mean RBC counts in P1 $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice were $0.71 \pm 0.04 \times 10^9$ cells/ml (17% of wild type), mean RBC counts in P1 mice homozygous for the other lethal mutations ranged from $0.71 \pm 0.07 \times 10^9$ cells/ml (17% of wild type) in $Kitl^{Sl\text{-}31R}/Kitl^{Sl\text{-}31R}$ mice to $0.82 \pm 0.06 \times 10^9$ cells/ml (20% of wild type) in $Kitl^{Sl\text{-}22R}/Kitl^{Sl\text{-}22R}$ mice. Importantly, none

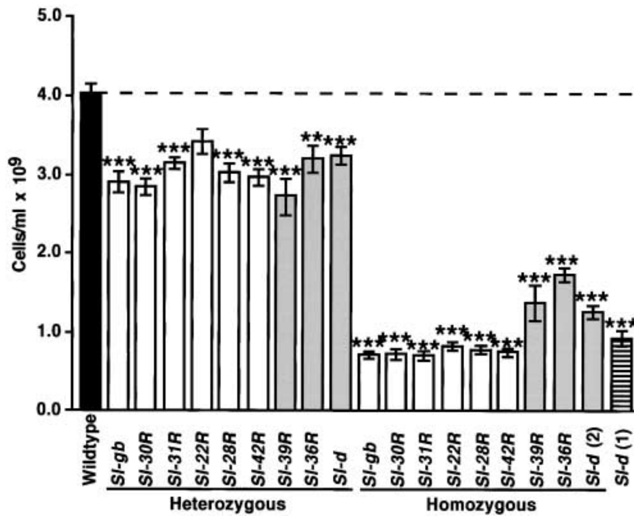

FIGURE 2.—Peripheral RBC counts in newborn $Kitl^{Sl}$ mutant mice. Blood was collected from euthanized P1 mice and RBCs counted using a hemacytometer. The mean and SEM are shown for each genotype. Each set of values for heterozygous or homozygous mutants was compared against the values of wild-type mice using unpaired *t*-test: (**) $P = 0.002$–0.02; (***) $P < 0.002$. Solid bar, wild type; open bars, alleles that cause lethality to homozygous mice; shaded bars, alleles that allow viability to homozygous mice; bar with lines, hemizygous $Kitl^{Sl\text{-}d}$ mice ($Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}d}$). The dashed horizontal line is the mean value for wild-type mice.

of the values for homozygous lethal $Kitl^{Sl}$ mutations are significantly different ($P > 0.2$) from that of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice. In comparison, mean RBC counts in $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$, $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$, and $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice were $1.4 \pm 0.2 \times 10^9$ cells/ml (34% of wild type), $1.7 \pm 0.1 \times 10^9$ cells/ml (41% of wild type), and $1.3 \pm 0.1 \times 10^9$ cells/ml (32% of wild type), respectively. Each of these values for homozygous viable mutations is significantly different ($P < 0.02$) from that of P1 $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice. Although the mean in P1 $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice is not significantly different from that of $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$ mice ($P = 0.18$), it is significantly different from that of $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice ($P = 0.003$). Thus, the RBC counts found in P1 mice homozygous for viable mutations are significantly higher than those found in mice homozygous for lethal mutations, and the $Kitl^{Sl\text{-}36R}$ mutation has the mildest effect of all mutants on RBC counts when in the homozygous condition. In conclusion, in P1 homozygous mice, the lethal mutations behave as null alleles with respect to RBC counts while the viable mutations are hypomorphic with respect to RBC counts.

The relationship between extent of anemia and survival is extended further by examination of the gene dosage effects of the $Kitl^{Sl\text{-}d}$ mutation. The mean P1 RBC counts in $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}gb}$ mice were $0.9 \pm 0.1 \times 10^9$ cells/ml (22% of wild type), which is significantly different from that of $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice ($P < 0.02$) but is not significantly different from that of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ mice ($P = 0.12$). This intermediate effect on RBC counts is consistent with the intermediate effect seen in the survival of $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}gb}$ mice (see above and Figure 1I). However, the relationship between viability and extent of anemia does not extend to all $Kitl^{Sl}$ mutations. Although $Kitl^{Sl\text{-}36R}$ exerts the mildest effect on RBC counts of the three viable mutations (Figure 2), it is the only viable mutation that causes significant lethality to homozygous mice prior to and during the first week after birth (see Figure 1, G–I, and above). If perinatal or juvenile lethality occurs in some $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice because of severe anemia, then a range of RBC counts would have been observed in individual mice of this genotype. However, all six $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mutants sampled had very similar RBC counts (not shown), none of which were below the values seen in $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$ and $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mutants. Thus, the cause of lethality in some $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice during the first week after birth does not appear to be due to severe anemia.

Effects of $Kitl^{Sl}$ mutations on peripheral blood of juvenile mice: We examined the effects on peripheral blood in P24-P25 mice, each of which was heterozygous for one of the nine $Kitl^{Sl}$ mutations or homozygous for one of the three viable $Kitl^{Sl}$ mutations. Significant differences between wild-type and mutant mice were observed for RBC counts, hemoglobin, MCV, MCH, MCHC, and hematocrit (Figure 3). However, no differences were observed between any mutant and wild-type mice for MPV, WBC counts, platelet counts, or lymphocytes (data not shown). Counts of segmented neutrophils (data not shown) were marginally reduced in $Kitl^{Sl\text{-}31R}/Kitl^{+}$, $Kitl^{Sl\text{-}39R}/Kitl^{+}$, and $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$ mice; however, the values are not significantly different from wild-type values ($P = 0.032$, $P = 0.021$, and $P = 0.041$, respectively). While neutrophil counts in $Kitl^{+}/Kitl^{+}$ mice were $0.9 \pm 0.1 \times 10^6$ cells/ml, these values in $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice were $0.5 \pm 0.1 \times 10^6$ cells/ml. Although the neutrophil counts for $Kitl^{+}/Kitl^{+}$ and $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice are significantly different ($P = 0.01$), there are no previous reports of neutrophil defects in $Kitl^{Sl}$ mutants. Because the effect on neutrophils was marginal and restricted to a specific allele, the relevance of these data is uncertain.

Consistent with the semidominant pigmentation defects (Table 1), every heterozygous $Kitl^{Sl}$ mutation caused a significant effect on at least one peripheral blood parameter in P24-P25 mice (Figure 3). Interestingly, the severity of the heterozygous effect on peripheral blood did not relate directly to the severity of the heterozygous pigmentation phenotype or to the severity of the homozygous survival phenotype (Table 4). In comparison to RBC counts of $6.9 \pm 0.1 \times 10^9$ cells/ml in wild-type mice, RBC counts in $Kitl^{Sl\text{-}30R}/Kitl^{+}$, $Kitl^{Sl\text{-}28R}/Kitl^{+}$, and $Kitl^{Sl\text{-}42R}/Kitl^{+}$ mice are significantly reduced (Figure 3A), ranging from $5.5 \pm 0.1 \times 10^9$ to $6.0 \pm 0.1 \times 10^9$ cells/ml (80–87% of wild type). None of these values is significantly different from RBC counts of $5.8 \pm 0.1 \times 10^9$ cells/ml (84% of wild type) in $Kitl^{Sl\text{-}gb}/Kitl^{+}$ mice.

TABLE 4

Relative effects of Kit^{Sl} mutations on different aspects of the mutant phenotype

		$Kitl^{Sl}/Kitl^{+}$ mice[a]							$Kitl^{Sl}/Kitl^{Sl}$ mice[a]	
Kitl allele	Kitl defect	P1 RBC	P24 RBC	P24 HGB	P24 MCV	P24 MCH	P24 MCHC	P24 coat pigment	P1 RBC	Postnatal viability
Sl-30R	Missense (L18R)	Null	Null	Null	Null	Null	No effect	Null	Null	Null
Sl-31R	Missense (P23L)	Null	No effect	No effect	Null	Null	No effect	Null	Null	Null
Sl-22R	Missense (L54P)	Mildly hypomorphic[b]	No effect	No effect	Hypomorphic	Hypomorphic	No effect	Null	Null	Mildly hypomorphic
Sl-28R	Missense (I118N)	Null	Null	No effect	Null	Null	No effect	Null	Null	Null
Sl-42R	Truncated S-Kitl (96 + 2 aa)	Null	Null	Null	Null	Null	No effect	Null	Null	Null
Sl-39R	Missense (S122F)	Null	Hypomorphic	No effect	Hypomorphic	Hypomorphic	No effect	Hypomorphic	Hypomorphic	Hypomorphic
Sl-36R	2 truncated S-Kitl (147 aa; 96 + 25 aa)	Null[c]	Hypomorphic	Null	Hypomorphic	Hypomorphic	No effect	Hypomorphic	Hypomorphic	Hypomorphic
Sl-d	Truncated S-Kitl (180 + 25 aa)	Null[c]	Null	No effect	Hypomorphic	Hypomorphic	No effect	Null	Hypomorphic	Hypomorphic

[a] For each $Kitl^{Sl}$ mutation and for each aspect of the heterozygous or homozygous phenotype, "No effect" indicates that the effect was similar to that of wild-type animals, "Null" indicates that the effect was similar to that of $Kitl^{Sl\text{-}gb}/Kitl^{+}$ for heterozygous mutations or of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ for homozygous mutations, and "Hypomorphic" indicates that the effect was less than that of $Kitl^{Sl\text{-}gb}/Kitl^{+}$ for heterozygous mutations or of $Kitl^{Sl\text{-}gb}/Kitl^{Sl\text{-}gb}$ for homozygous mutations.

[b] Although the mean is higher than that for any other heterozygous mutant, the values are not significantly different from those for either wild-type or $Kitl^{Sl\text{-}gb}/Kitl^{+}$ mice ($P > 0.05$).

[c] Although the values are higher than those for $Kitl^{Sl\text{-}gb}/Kitl^{+}$ mice, they are not significantly different ($P > 0.05$).

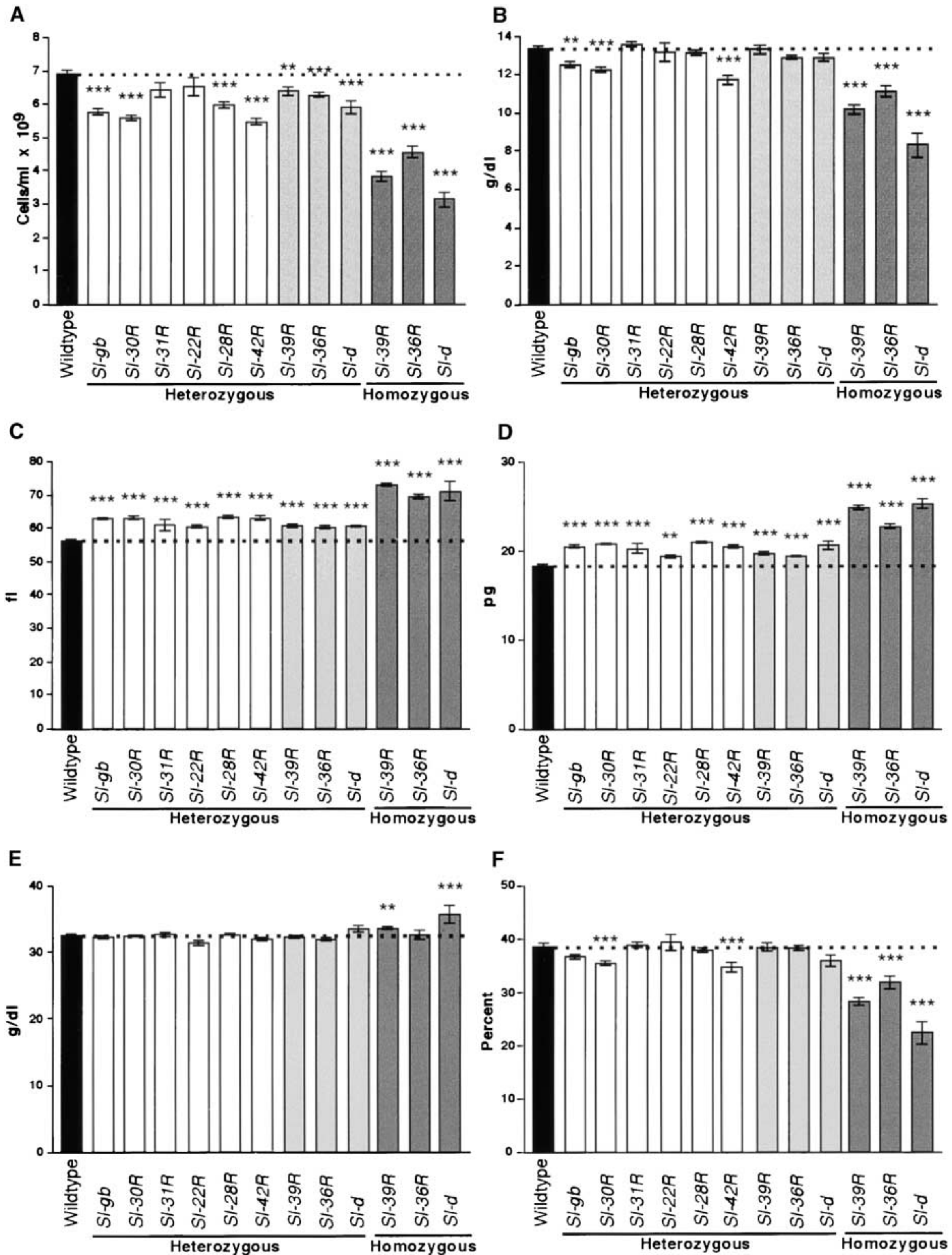

FIGURE 3.—Peripheral blood cell analysis in P24-P25 *Kitl*^{Sl} mutant mice. Blood was collected from euthanized mice and analyzed using a Celldyne 3500 hematology analyzer. The mean and SEM are shown for each genotype. (A) RBCs. (B) Hemoglobin. (C) MCV. (D) MCH. (E) MCHC. (F) Hematocrit. Each set of values for heterozygous or homozygous mutants was compared against the values of wild-type mice using unpaired *t*-test: (**) $P = 0.002$–0.02; (***) $P < 0.002$. Solid bar, wild type; open bars, alleles that cause lethality to homozygous mice; shaded bars, alleles that allow viability to homozygous mice. The dashed horizontal line is the mean value for wild-type mice.

However, in $Kitl^{Sl\text{-}31R}/Kitl^{+}$ and $Kitl^{Sl\text{-}22R}/Kitl^{+}$ mice, RBC counts are not significantly different from wild type ($6.4 \pm 0.2 \times 10^9$ and $6.5 \pm 0.3 \times 10^9$ cells/ml, respectively; Figure 3A). The lack of effect on RBC counts in $Kitl^{Sl\text{-}31R}/Kitl^{+}$ and $Kitl^{Sl\text{-}22R}/Kitl^{+}$ mice is unexpected, given the clear pigmentation defects in these mice (see Table 1) and the severe effects on survival and P1 RBC counts of $Kitl^{Sl\text{-}31R}/Kitl^{Sl\text{-}31R}$ and $Kitl^{Sl\text{-}22R}/Kitl^{Sl\text{-}22R}$ mice (Figures 1 and 2). Furthermore, neither the hemoglobin concentrations (Figure 3B) nor the hematocrit (Figure 3F) was affected in P24-P25 $Kitl^{Sl\text{-}31R}/Kitl^{+}$ and $Kitl^{Sl\text{-}22R}/Kitl^{+}$ mice. Similarly, neither hemoglobin concentrations nor hematocrits of $Kitl^{Sl\text{-}28R}/Kitl^{+}$, $Kitl^{Sl\text{-}39R}/Kitl^{+}$, $Kitl^{Sl\text{-}36R}/Kitl^{+}$, and $Kitl^{Sl\text{-}d}/Kitl^{+}$ mice were affected even though these mice had significantly reduced RBC counts.

The only parameters for which all nine P24-P25 heterozygous mutants had significant defects are MCV (Figure 3C) and MCH (Figure 3D). While the mean MCV of P24-P25 wild-type mice is 56.2 ± 0.4 fl, the mean MCV of P24-P25 heterozygous mutants ranged from 60.6 ± 0.3 fl (108%) in $Kitl^{Sl\text{-}36R}/Kitl^{+}$ mice to 63.6 ± 0.6 fl (113%) in $Kitl^{Sl\text{-}42R}/Kitl^{+}$ mice. For MCH, the mean of wild-type mice is 18.3 ± 0.2 pg, and the mean of heterozygous mutants ranged from 19.4 ± 0.2 pg (106%) in $Kitl^{Sl\text{-}22R}/Kitl^{+}$ mice to 21.0 ± 0.1 pg (115%) in $Kitl^{Sl\text{-}28R}/Kitl^{+}$ mice. Notably, the mean MCV (Figure 3C) and MCH (Figure 3D) of P24-P25 $Kitl^{Sl\text{-}31R}/Kitl^{+}$ and $Kitl^{Sl\text{-}22R}/Kitl^{+}$ mice are significantly increased relative to P24-P25 wild-type mice even though the RBC counts of these mice are not significantly different from that of wild-type mice (Figure 3A). However, it should be noted that the MCH is calculated by dividing the hemoglobin concentration by RBC counts. Since the MCV is increased in all mutants, the elevated MCH simply means that the hemoglobin concentration is higher because the cells are larger. Consistent with this, most of the MCHC values (Figure 3E) in mutant mice are not significantly different from those of wild-type mice (32.7 ± 0.3 g/dl). MCHC is calculated from hemoglobin concentrations, RBC counts, and MCV and therefore takes the volume of the cells into consideration. Thus, in mice heterozygous for each of the $Kitl^{Sl}$ mutations, the increased hemoglobin concentration is proportional to the increased MCV, and the anemia is classified as macrocytic and normochromic.

As expected, the homozygous effects of each of the viable mutations ($Kitl^{Sl\text{-}39R}$, $Kitl^{Sl\text{-}36R}$, and $Kitl^{Sl\text{-}d}$) on RBC counts, hemoglobin, MCV, MCH, MCHC, and hematocrit are significantly greater than the corresponding heterozygous effects of these mutations (Figure 3). While RBC counts in wild-type P24-P25 mice were $6.9 \pm 0.1 \times 10^9$ cells/ml, RBC counts in $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$, $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$, and $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice were $4.6 \pm 0.2 \times 10^9$ cells/ml (67% of wild type), $3.9 \pm 0.1 \times 10^9$ cells/ml (56% of wild type), and $3.1 \pm 0.1 \times 10^9$ cells/ml (45% of wild type), respectively (Figure 3A). The effects on hemoglobin concentration (Figure 3B) and hematocrit (Figure 3F) in each homozygous viable mutant were of approximately the same magnitude as for RBC counts. For RBC counts, hematocrit, and hemoglobin, pairwise comparisons between the three homozygous mutants revealed that the $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mutants have values for each of these three parameters significantly lower ($P < 0.01$ in each case) than those of the other two viable mutants. Moreover, the RBC count in $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice is significantly higher than that in $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$ and $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice ($P = 0.004$ and $P < 0.0001$, respectively) but neither the hematocrit nor the hemoglobin concentration in the former mice is significantly different from that in either of the latter mice. Nonetheless, with respect to homozygous viable mutations, the trend for RBC counts, hematocrit, and hemoglobin concentration in P24-P25 mice is the same as for RBC counts in P1 mice; *i.e.*, $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mutants have the mildest effect, and $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mutants have the most severe effect. Surprisingly, this trend was not observed with MCV. While the MCV of wild-type P24-P25 mice was 56.2 ± 0.4 fl, MCV of $Kitl^{Sl\text{-}39R}/Kitl^{Sl\text{-}39R}$, $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$, and $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice was 73.0 ± 0.5 fl (130%), 69.7 ± 0.7 fl (124%), and 71.4 ± 2.8 fl (127%), respectively. In particular, there is no difference ($P = 0.6$) in the MCV of $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice and $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice, even though the latter have only ~67% of the RBC counts of the former ($4.6 \pm 0.2 \times 10^9$ *vs.* $3.1 \pm 0.2 \times 10^9$, $P < 0.0001$). Interestingly, mice homozygous for either $Kitl^{Sl\text{-}39R}$ or $Kitl^{Sl\text{-}d}$ mutations had significantly increased MCHC (34.0 ± 0.2 and 36.0 ± 1.3 g/dl, respectively, compared to 32.7 ± 0.3 g/dl in wild-type mice, $P < 0.01$ for each pair). This suggests that hemoglobin concentration in these mutant mice is not proportional to the increased volume of RBCs, and the anemia could be classified as macrocytic and hyperchromic.

Relative effects of $Kitl^{Sl}$ mutations: The heterozygous and homozygous phenotypes observed for the seven ENU-induced $Kitl^{Sl}$ mutations and $Kitl^{Sl\text{-}d}$ are summarized in Table 4. The alleles were considered functionally null if their phenotype was similar to that of $Kitl^{Sl\text{-}gb}$. Four of the alleles ($Kitl^{Sl\text{-}30R}$, $Kitl^{Sl\text{-}31R}$, $Kitl^{Sl\text{-}28R}$, and $Kitl^{Sl\text{-}42R}$) clearly behave as null alleles, while one mutation ($Kitl^{Sl\text{-}22R}$) appears to be a strong hypomorph and three mutations ($Kitl^{Sl\text{-}39R}$, $Kitl^{Sl\text{-}36R}$, and $Kitl^{Sl\text{-}d}$) are clearly hypomorphic. While most of the $Kitl^{Sl}$ mutations cause comparable effects on different aspects of the phenotype, the $Kitl^{Sl\text{-}31R}$ and $Kitl^{Sl\text{-}22R}$ mutations are unusual in that they do not cause any detectable effects on RBC counts in heterozygous mice.

DISCUSSION

In this report we describe the effects of seven ENU-induced $Kitl^{Sl}$ mutations on survival and peripheral blood cells of mice and compare the severity of these effects to those caused by two previously characterized

Kitl^{Sl} mutations (*Kitl*$^{Sl\text{-}gb}$ and *Kitl*$^{Sl\text{-}d}$). Careful analysis of the survival curves and extent of anemia of homozygous mutant mice has revealed that five of the seven new mutants are functionally null (or nearly so) and two of the seven new mutants are hypomorphic. There does not seem to be a relationship between phenotypic severity and type of mutation, as four of the *Kitl*^{Sl} missense mutants are null while one is hypomorphic and one of the *Kitl*^{Sl} truncation mutants is null while the other is hypomorphic. Although we do not know how each of the *Kitl*^{Sl} mutations described here affects Kitl function, it is likely that the mutations affect some aspect of Kitl conformation, processing, localization, or binding to Kit because none of the mutations affect steady-state levels of *Kitl* mRNA (Rajaraman *et al.* 2002). In this regard, the *Kitl*$^{Sl\text{-}22R}$ mutation is particularly interesting. Several aspects of the phenotypic analysis suggest that the *Kitl*$^{Sl\text{-}22R}$ mutant is hypomorphic, yet the L54P substitution, which is in the second α-helical domain of Kitl, would be expected to have very severe effects on Kitl structure. Further structural studies of all of the mutants should provide useful information about structural requirements for Kitl function.

Three mutant alleles used in this study (*Kitl*$^{Sl\text{-}42R}$, *Kitl*$^{Sl\text{-}36R}$, and *Kitl*$^{Sl\text{-}d}$) are expected to produce truncated S-Kitl proteins with varying extents of C-terminal deletions (see Table 1 and Rajaraman *et al.* 2002). The *Kitl*$^{Sl\text{-}36R}$ mutant potentially encodes two isoforms: Kitl$^{Sl\text{-}36R\text{-}A}$, which is 147 aa, and Kitl$^{Sl\text{-}36R\text{-}B}$, which is 96 aa with an additional 25 aa out of frame (Rajaraman *et al.* 2002). Since the Kitl$^{Sl\text{-}36R\text{-}B}$ isoform contains the identical 96 N-terminal aa as Kitl$^{Sl\text{-}42R}$ (Rajaraman *et al.* 2002), and the present studies indicate that the latter is null functionally, it is likely that the former is also null functionally. This is consistent with *in vitro* studies indicating that Kitl activity is abolished by deletions that remove more than the N-terminal 142 aa (Nishikawa *et al.* 1992). However, we cannot exclude the possibility that the Kitl$^{Sl\text{-}36R\text{-}B}$ isoform is a gain-of-function mutant because of the abnormal C-terminal sequences. Regardless of which Kitl$^{Sl\text{-}36R}$ isoform is biologically active, both are deleted for more C-terminal sequences than Kitl$^{Sl\text{-}d}$. It might therefore be expected that the latter mutation would have more severe effects on function. However, comparison of *Kitl*$^{Sl\text{-}d}$ and *Kitl*$^{Sl\text{-}36R}$ mutant mice reveals that all aspects of the heterozygous and homozygous RBC phenotype are less severe in *Kitl*$^{Sl\text{-}36R}$ mutants than in *Kitl*$^{Sl\text{-}d}$ mutants. Further studies of the mutant proteins encoded by each of these alleles will be necessary to understand these phenotypic observations.

The ENU-induced *Kitl*^{Sl} mutations described here cause mild pigmentation defects when present in the heterozygous condition and severe pigmentation defects in the homozygous or compound heterozygous condition. In addition, none of the heterozygous *Kitl*^{Sl} mutations caused a more severe effect on any aspect of the mutant phenotype described here than that of *Kitl*$^{Sl\text{-}gb}$. This indicates that none of these *Kitl*^{Sl} mutations acts in a dominant-negative manner, despite the facts that Kitl is known to function as a dimer (Jiang *et al.* 2000; Zhang *et al.* 2000) and that some *Kit*^{W} mutants act in a dominant-negative fashion (Nocka *et al.* 1990). Together, these observations conform to the well-known semidominant effects of *Kitl*^{Sl} mutations due to haploinsufficiency (Bedell *et al.* 1996a). Moreover, all of the *Kitl*^{Sl} mutations described here cause an increase in MCV and MCH when in the heterozygous condition, whereas other aspects of peripheral blood, such as RBC counts, hematocrit, and hemoglobin concentration, are normal in some heterozygous mutants (see Figure 3). Thus, increased MCV, MCH, and pigmentation are the most sensitive aspects of the *Kitl*^{Sl} semidominant phenotype. Whether the differences in effects on RBC counts and pigmentation reflect true differences between Kitl signaling in erythroid progenitors and melanoblasts or different thresholds for Kitl activity in the two cell types remains to be determined. Although the basis for the increased MCV is not known, it would be expected to relate directly to effects on RBC numbers. This is not always the case; for example, *Kitl*$^{Sl\text{-}d}$/*Kitl*$^{Sl\text{-}d}$ mice have fewer RBCs than *Kitl*$^{Sl\text{-}36R}$/*Kitl*$^{Sl\text{-}36R}$ mice, but the MCV in both mutants is the same (Figure 3, A and D).

If lethality to *Kitl*^{Sl} mice was always caused by anemia, then each of the lethal *Kitl*^{Sl} mutations would be expected to cause a more severe anemia than that of each of the viable *Kitl*^{Sl} mutations. The results described in this report are in basic agreement with this hypothesis. For example, P1 mice homozygous for each of the six lethal mutations (*Kitl*$^{Sl\text{-}gb}$, *Kitl*$^{Sl\text{-}30R}$, *Kitl*$^{Sl\text{-}31R}$, *Kitl*$^{Sl\text{-}22R}$, *Kitl*$^{Sl\text{-}42R}$, and *Kitl*$^{Sl\text{-}28R}$) have nearly identical RBC counts that are all significantly lower than the RBC counts in P1 mice homozygous for each of the viable mutations (*Kitl*$^{Sl\text{-}39R}$, *Kitl*$^{Sl\text{-}36R}$, and *Kitl*$^{Sl\text{-}d}$; Figure 2). However, in P1 mice, RBC counts in homozygous lethal *Kitl*^{Sl} mutants are only slightly less than that in hemizygous *Kitl*$^{Sl\text{-}d}$ mice (see Figure 2). Although none of the latter mice survive until weaning, their survival time is significantly greater than that of mice homozygous for other lethal *Kitl*^{Sl} mutations (Figure 1I). In contrast, homozygous *Kitl*$^{Sl\text{-}d}$ mice have a nearly normal survival curve (Figure 1I), and their RBC counts are only slightly higher than that in hemizygous *Kitl*$^{Sl\text{-}d}$ mice. Thus, if *Kitl*^{Sl}-induced lethality is caused by severe RBC hypoplasia, these results suggest that the threshold for P1 RBC counts that allows survival to weaning may be between the mean values seen for hemizygous *Kitl*$^{Sl\text{-}d}$ mice (0.9×10^9 cells/ml, 22% of wild type) and homozygous *Kitl*$^{Sl\text{-}d}$ mice (1.3×10^9 cells/ml, 32% of wild type).

Although the *Kitl*$^{Sl\text{-}36R}$ mutation allows survival in the majority of homozygous mice, a significant number of *Kitl*$^{Sl\text{-}36R}$/*Kitl*$^{Sl\text{-}36R}$ mice die either perinatally or within the first week of birth. This could be explained if P1 *Kitl*$^{Sl\text{-}36R}$/*Kitl*$^{Sl\text{-}36R}$ mice had large variations in RBC counts, with death occurring to mice with low RBC counts and sur-

vival occurring in mice with higher RBC counts. However, we did not observe such variation; in fact, RBC counts in all $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ P1 mice tested were very similar and are significantly higher than those seen in $Kitl^{Sl\text{-}d}/Kitl^{Sl\text{-}d}$ mice. Therefore the perinatal and juvenile lethality of $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice does not seem to be reflected in the severity of the anemia. It is possible that some aspect of RBC function, rather than RBC numbers, is more drastically affected in a subset of $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice. Alternatively, development or function of some other cell type may be contributing to the lethality of $Kitl^{Sl\text{-}36R}/Kitl^{Sl\text{-}36R}$ mice. In this regard, it is interesting to note that the absence of Kitl/Kit signaling in the enteric nervous system causes a lethal condition in mice (Maeda *et al.* 1992; Huizinga *et al.* 1995). Whether such intestinal defects contribute to the lethality of the present $Kitl^{Sl}$ mutants remains to be determined.

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