Genetic Control of the Frequency of Hematopoietic Stem Cells in Mice: Mapping of a Candidate Locus to Chromosome 1

By Christa E. Müller-Sieburg and Roy Riblet

From the Medical Biology Institute, La Jolla, California 92037

Summary

The genetic elements that govern the differentiation and proliferation of hematopoietic stem cells remain to be defined. We describe here marked strain-specific differences in the frequency of long-term culture–initiating cells (LTC-IC) in the bone marrow of different strains of mice. Mice of C57Bl/6 background showed the lowest levels of stem cells in marrow, averaging 2.4 ± 0.6 LTC-IC/10^5 cells, BALB/c is intermediate ($9.1 \pm 4.2/10^5$ cells), and DBA/2 mice contained a 11-fold higher frequency of LTC-IC ($28.1 \pm 16.5/10^5$ cells) than C57Bl/6 mice. The genetic factors affecting the size of the stem cell pool were analyzed in the C57Bl/6 $\times$ DBA/2 recombinant inbred strains; LTC-IC frequencies ranged widely, indicating that stem cell frequencies are controlled by multiple genes. Quantitative trait linkage analysis suggested that two loci that have major quantitative effects are located on chromosome 1 near *Adprp* and *Acrg*, respectively. The mapping of the locus near *Adprp* was confirmed by finding an elevated stem cell frequency in B6.C-H25, a C57Bl/6 congenic strain that carries a portion of chromosome 1 derived from BALB/c mice. We have named this gene *Scfr1* (stem cell frequency regulator 1). The allelic forms of this gene may be an important predictor of stem cell number and thus would be useful for evaluating cell sources in clinical stem cell transplantation.

Hematopoietic stem cells replenish mature cells through a cascade of differentiation steps. How stem cells decide whether to differentiate and how the pool of stem cells is maintained is not well understood. The incidence of stem cells has been estimated to be about $1/10^4$ to $1/10^5$ cell in marrow both in mouse and man (1–8). Stem cell levels in mice are relatively stable in individuals of a given strain of mice (1–5; for review see reference 9). Furthermore, the size of the stem cell pool remains constant throughout the adult life of a mouse (1). This indicates that the size of the stem cell pool is tightly controlled. In the human system, the level of stem cells can vary strikingly between individual marrow samples (10). Undoubtedly, some of this variability can be explained by sampling procedures. It is also possible that the genetic diversity of the outbred human population contributes to the variability in stem cell levels. In contrast, the homogeneous genetic background of inbred mice could program a consistent level of stem cell activity.

Strain-specific differences in mice have been invaluable for analyzing the genetic mechanisms that govern hematopoiesis. For instance, the Sl and *W* mouse mutants were crucial for the identification of the cytokine receptor c-kit and its ligand (reviewed in 11). Van Zant and colleagues (12) demonstrated in an elegant allophenic system that stem cells

from C57Bl/6 and DBA/2 mice differed in the kinetics of generating peripheral hematopoietic cells. Stem cell–enriched populations from these two strains also show distinguishable kinetics of activation after cytokine or marrow-ablative treatment (13). These data suggest that stem cell behavior is governed, at least in part, by genetic principles, although the exact mechanisms remain to be established.

Progress in identifying genetic mechanisms that govern stem cells have been hampered by the low frequency of stem cells and the complexity of stem cell assays. The most stringent detection system for stem cells, the in vivo repopulation assay, tends to be influenced by a number of variables that make it difficult to compare stem cell levels in different mouse strains. Potential sources of variation include the following: (*a*) strain differences in radiosensitivity affect the conditioning of the host and thereby repopulation; (*b*) differences in antigen expression of stem cells render it difficult to standardize purification protocols; and (*c*) expression of receptors that guide homing to the marrow differ between strains (14–16). Furthermore, differences in stem cell levels or repopulation capacity could be obscured by compensatory mechanisms that can lead to a normal representation of blood cells in the periphery (12).

One approach to circumvent the variables intrinsic to re-

population systems is to enumerate stem cells in vitro. The long-term culture–initiating cell (LTC-IC)[1] assay has emerged as a system that measures a cell that is highly related, if not identical, to the marrow-repopulating stem cell (4–8, 9, 17). Currently, there are several variants of the system, all of which measure the ability of a stem cell to give rise to myeloid progeny in response to stromal layers in limiting dilution culture. However, the assays differ in the endpoint assessed 4–5 wk after initiation of the cultures. Wells that contain cobblestone-forming areas (CFA) (4-6, 17), colonies of mature myeloid cells (18), or colony-forming units culture (CFUc) (6, 8) have been measured and yield comparable results. All groups have shown that their evaluation system detects a cell that is phenotypically highly correlated to the marrow-repopulating stem cell.

We have modified the LTC-IC system, in that we used the cloned stromal cell line S17 (19) as a feeder layer. On this line, all three evaluation endpoints are highly correlated, thereby combining ease of evaluation with the perceived stringency of other methods. We have used this system to demonstrate that the size of the stem cell pool is genetically controlled. The frequency of stem cells differs ∼11-fold between DBA/2 and C57Bl/6 mice. Mapping analysis of the C57Bl/6 × DBA/2 recombinant inbred (BXD RI) strains indicated the stem cell frequency is regulated by multiple genes. Quantitative trait (QT) linkage analysis, together with analysis of congenic strains, identified a locus on chromosome 1 that has a major quantitative effect on stem cell levels. We have named this gene Scfr1 (stem cell frequency regulator 1).

Materials and Methods

Mice. BALB/c, C57Bl/6, and the Ly5-congenic C57Bl/6-Ly5.1 mice were bred at our facility. Since the C57Bl/6 and C57Bl-Ly5.1–congenic strains have identical levels of LTC-IC, they were used interchangeably in our experiments. BXD RI, B6.C-H25 and -H35, and other parental and F1 strains were purchased from The Jackson Laboratory (Bar Harbor, ME).

Stromal Cell Lines. The lines S17 (19) and FMB-D1 (17) have been described previously. The line 2018 (20, 21) was derived from fetal liver of a C57Bl/6 mouse and was the generous gift of Dr. Ihor Lemischka (Princeton University, Princeton, NJ). All lines were maintained in RPMI supplemented with 5% FCS, with weekly passages as described previously (20, 21).

Long-term Culture Initiation Assay. Stromal cells (1–2 × 10³ cells/well) were seeded into 96-well cluster plates in RPMI supplemented with 5% FCS. 1 wk later, all medium was aspirated and replaced with Dexter-type medium consisting of Iscove's medium supplemented with 16% horse serum (lot #52079; Tissue Culture Biologicals, Tulare, CA) and 10⁻⁶ M hydrocortisone. Cultures were then seeded with serially diluted single-cell suspensions of femoral marrow in the same medium. Generally, we used marrow pooled from two animals and seeded at least 48 wells per

---

[1]*Abbreviations used in this paper:* BXD RI, C57Bl/6 × DBA/2 recombinant inbred; CFA, cobblestone-forming area; LTC-IC, long-term culture–initiating cells; QT, quantitative trait; QTL, quantitative trait locus; Scfr1, stem cell frequency regulator 1.

cell dilution, using three to four dilutions per sample. All experiments with RI strains, designed to map the genes that control stem cell frequencies, included marrow from the parental DBA/2 and C57Bl/6 strains as controls. Cultures were fed weekly with 100 μl medium after demidepletion. Wells that contained colonies (at least 500 cells) of small granulocytic cells (compare Fig. 1) were counted at 3, 4, 5 wk after initiation of the cultures, and the location of positive wells was recorded on forms that mirrored the 96-well plates. This permitted correlation of colony counts, CFUc and CFA for each well. Data reported are from 4 wk of cultures unless indicated differently.

CFUc Assay. Freshly explanted or cultured marrow cells were plated in methylcellulose in 1-cm dishes as described (23). Supernatant conditioned from the cell line Wehi-3 was used at 12% as a source of IL-3. Colonies were counted between 7 and 10 d later.

PCR Analysis. PCR genotyping of the congenic strains to define the differential region containing Scfr1 was performed for selected D1Mit loci as described (24–26), except that 50-μl reactions were used to yield sufficient product for detection by ethidium bromide staining. PCR primers (MapPairs) were purchased from Research Genetics (Huntsville, AL). DNA was prepared from mouse spleens.

Data Analysis. Evaluation of limiting dilution data was performed by standard graphical analysis (27) with Cricket Graph III (Computer Associates International, Islandia, NY). The level of LTC-IC/10⁵ cells seeded was calculated from the frequencies obtained in the limiting dilution analysis. Because only two to four RI strains could be analyzed at one time, the results of each experiment were normalized to the LTC-IC value of the C57Bl/6 control for that experiment. QT linkage analysis was performed with QT Manager, modified from Map Manager version 2.6.3 (28). This program uses one-way analysis of variance to find single Mendelian loci which are significantly associated with the quantitative trait phenotype. QT Manager, with its associated December 1994 data release, was provided by Kenneth Manly (Roswell Park Memorial Institute). Student's and Welch's t tests were performed with InStat software (GraphPAD Software for Science, San Diego, CA).

Results

The Assay System. All LTC-IC assays evaluate the ability of a stem cell to give rise to myeloid progeny in response to a stromal layer (4–8, 9, 18). Experience has shown that cloned stromal cell lines provide a reliable and reproducible environment for limiting dilution assays for hematopoietic precursors (29, 30) and several stromal cell lines have been used successfully to measure LTC-IC (17, 31). Previously, we reported that stromal cell lines differ significantly in their interaction with stem cells, and one stromal cell line, S17, was identified that maintained high levels of stem cells (22). We now show that this line also is useful as a source of stroma for limiting dilution analysis of LTC-IC (Table 1).

When the S17 line was used in the LTC-IC assay, colonies of small granulocytic cells (Fig. 1) developed that showed a similar kinetic as that reported for CFA (4–6, 17). Because of the ease and speed in evaluation, we chose to enumerate wells that contained these colonies of small granulocytic cells at 4–5 wk as an endpoint. A typical col-

Table 1. *Differences in LTC-IC Levels Are Not Affected by the Genotype of the Stromal Cell Line*

Source of marrow[*]	LTC-IC/10^5		
	S17	2018	FMB-D1
DBA/2	16.1	ND	20.8
C57Bl/6	1.1	ND	1.7
BALB/c	12.8	2.9	ND
DBA/2	27.7	1.9	ND
C57Bl/6	2.6	<0.8[‡]	ND

[*]Marrow cells from different strains of mice were seeded in limiting dilution onto the stromal cell lines indicated. For details see Fig. 3. The S17 line is from a BALB/c mouse, and lines 2018 and FMB-D1 are derived from C57Bl/6 (17, 19, 20).
[‡]No colonies detected.

ony is depicted in Fig. 1. If this type of colony was examined, most, if not all, colonies contained areas of CFA (compare Fig. 1). From hundreds of wells evaluated at 4–5 wk, all colonies showed CFA, and similarly all wells that contained CFA also contained colonies. Furthermore, most wells that contained colonies also contained CFUc, and vice versa (Fig. 2). Individual wells from limiting dilution cultures were harvested, and IL-3–responsive CFU were measured. In three independent experiments, only 10% of the wells were mismatched (compare Fig. 2). Thus, the limiting dilution assay on the S17 line permits us to combine all evaluation endpoints currently used. When we counted colonies consisting of small granulocytic cells, the graphs generated by plotting the percentage of negative wells against the number of cells seeded (27) were linear and intersected the origin (compare Fig. 3). Therefore, the system is dependent on a single limiting factor, namely, the LTC-IC, and it is permissible to calculate the frequency of the initiating stem cell.

The Frequency of Stem Cells Is Genetically Controlled. We used the S17-based LTC-IC assay to determine whether the frequency of stem cells in marrow is genetically controlled. Fig. 3 depicts representative examples of the limiting dilution analysis of stem cell content of different strains. We found that C57Bl/6, or the Ly5-congenic C57Bl-Ly5 strain, contained 2.4 ± 0.6 (n = 15) stem cells/10^5 cells seeded. This value is very close to that measured in competitive repopulation assays in the same strain of mice (1). However, the mean frequency (±SD) of LTC-IC in marrow of CBA mice (5.1; n = 1) and BALB/c mice (9.1 ± 4.2; n = 11) was higher than that of C57Bl/6 mice. Marrow of DBA/2 mice (28.1 ± 16.5; n = 11) contained 11-fold more LTC-IC than marrow of the C57Bl/6 strains. Stem cell levels in (DBA/2 × C57Bl/6)F1 mice (11.5 ± 1.1; n = 2) were intermediate between the parental strains (Fig. 3 *b*).

We asked whether the genetic background of the stromal cell line influences the results. Since the S17 line is de-
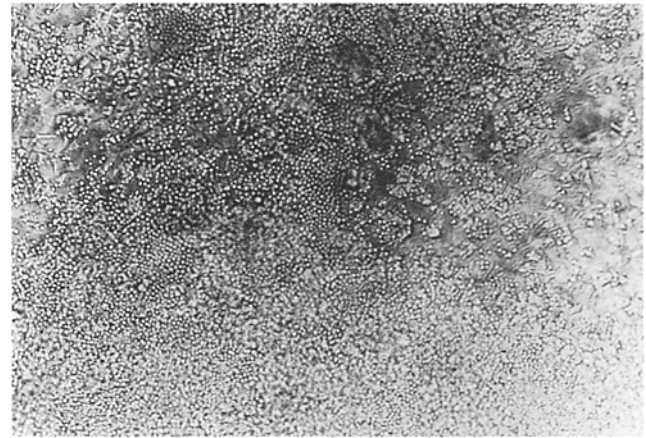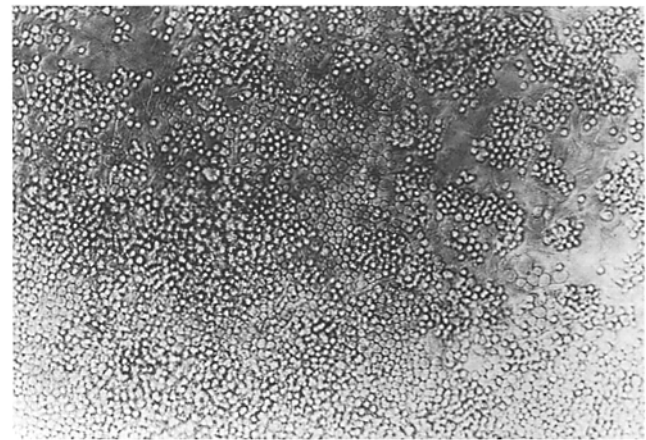
Figure 1. Colonies of small, granulocytic cells at 4 wk contain cobblestone areas. A typical colony of small, granulocytic cells that forms in response to the stromal cell line S17 in a 4-wk culture in limiting dilution is depicted here. Several areas of CFA are found in the center of the colony. The colony is from a culture seeded with 1.5 × 10^3 C57Bl-Ly5.1 marrow cells. ×10 (*A*); ×40 (*B*).

rived from a BALB/c mouse, we used the lines 2018 (20, 21) and FMB-D1 (17) as controls, both of which are derived from the C57Bl/6 background. All stromal cell lines tested supported limiting dilution analysis of LTC-IC, although the lines differed in how well they revealed colonies. The difference between stem cell levels in DBA/2 and C57Bl/6 marrow persisted on all lines (Table 1). Thus, the genetic background of the stromal cell lines did not play a role in this system, indicating that the differences in stem cell frequency reflect stem cell properties rather than stromal cell influences.

Genetic Analysis and Mapping. In our limited strain survey, the C57Bl/6 and DBA/2 strains exhibited the largest difference in stem cell frequency. This allowed us to use the large and extensively characterized panel of BXD RI
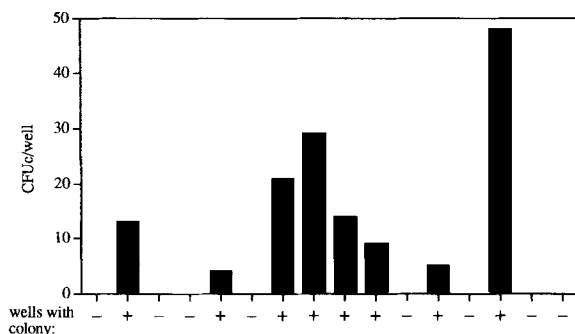
Figure 2. Good agreement in evaluating granulocytic colonies with CFUc content of the wells. DBA/2 marrow cells were seeded at 1.25 × 10³ cells/well. Four wk later, wells that contained granulocytic colonies were counted. Wells that contained (+) or lacked (−) colonies are indicated on the x axis. Thereafter, individual wells were harvested, and the content of CFUc was assessed in each well. The bars represent the number of CFUc in individual wells. Data are representative of three independent experiments.

strains (32) to analyze the genetic factors controlling this difference. The currently available 26 BXD strains were obtained and their bone marrow stem cell frequencies were evaluated in the LTC-IC assay. These results are shown in Fig. 4. It is apparent that there is wide variation in the stem cell levels of these strains, and that there is quantitative rather than qualitative variation. Some of the RI lines, like B6D2F1 mice, exhibit high levels of LTC-IC, but none of the RI strains showed frequencies of LTC-IC as high as the DBA/2 progenitor. Some strains have LTC-IC frequencies as low as C57Bl/6, and some are intermediate. This picture indicates that there are multiple genes that interact to regulate the stem cell level. If the entire difference of LTC-IC levels between C57Bl/6 and DBA/2 was determined by as few as three genes, it is likely that several of the RI lines would have displayed stem cell levels equal to DBA/2. The absence of the parental DBA phenotype in the RI panel suggests that the control is more complex.

The data shown in Fig. 4 were examined with QT Manager to identify, in the RI strains, any loci whose allele patterns are significantly associated with the quantitative variation of stem cell frequency. At a 99% confidence limit three loci were identified, adjacent to the acetylcholine receptor γ gene (*Acrg*) and the poly (ADP-ribose) polymerase gene (*Adprp*) on chromosome 1 and the histone 3 protein gene (*Hist3*) on chromosome 11. Paradoxically, the attribute that gives the RI lines so much power genetically, i.e., the hundreds of typed loci throughout the genome, creates a likelihood of false-positive findings in this quantitative analysis. This problem has been addressed by Lander and Schork (33) with the result that for genome-wide assessments like the RI analysis, a confidence level of 95% requires a *P* value of 0.00002. The *P* values for the three loci identified here fall short of this threshold, being 0.0007 for *Acrg*, 0.005 for *Adprp*, and 0.002 for *Hist3*. Thus, the RI data do not identify with high confidence a candidate locus for a regulator of stem cell frequency. Nevertheless, the co-
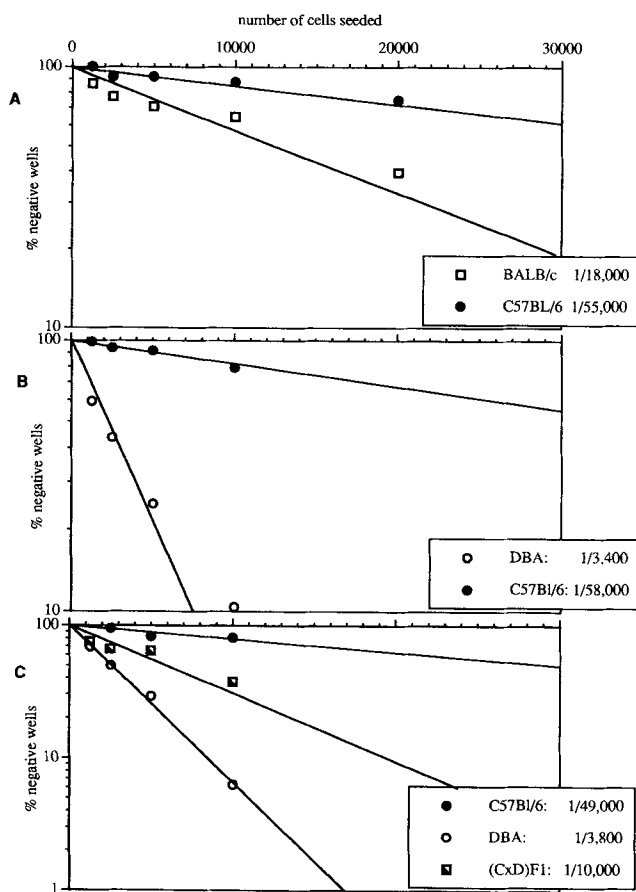

Figure 3. The frequency of stem cells in marrow differs among inbred strains of mice. Shown are representative examples of limiting dilution data of LTC-IC in the marrow of inbred strains of mice. Colonies of small granulocytic cells were counted at 4 wk of culture, and the percentage of negative wells was plotted against the number of cells seeded per well. The intersection of the graphs with 37% negative wells on the *y* axis yields the frequency of LTC-IC as the corresponding value on the *x* axis. The frequency of LTC-IC for each strain is indicated in the figures. (*A*) BALB/c vs. C57Bl/6; (*B*) DBA vs. C57Bl/6; (*C*) DBA vs. C57Bl/6 vs (DBA/2 × C57Bl/6)F1.

incidence of two selected loci on chromosome 1 was sufficiently intriguing to suggest a confirmatory study of congenic strains.

The congenic strains B6.C-H35 and B6.C-H25 have a C57Bl/6 background and carry parts of chromosome 1 derived from BALB/c mice (34). LTC-IC levels in the marrow of H-35 mice were similar to that of C57Bl/6 mice (data not shown). However, H-25 mice contained 4.2 ± 1.6 LTC-IC/10⁵ cells ($n = 5$), significantly higher ($p = 0.022$) than C57Bl/6 mice (1.8 ± 1, $n = 5$, in this series of experiments) and significantly lower than BALB/c (9.5 ± 2.9, $n = 5$, $p = 0.0071$). Thus, the level of LTC-IC in H-25 mice is intermediate between C57Bl/6 and BALB/c mice (Fig. 5). These results indicate that a substantial portion of the quantitative variation can be ascribed to a gene mapping on chromosome 1 in the region contributed by BALB/c to the B6.C-H25–congenic strain. We have named this gene *Scfr1*.
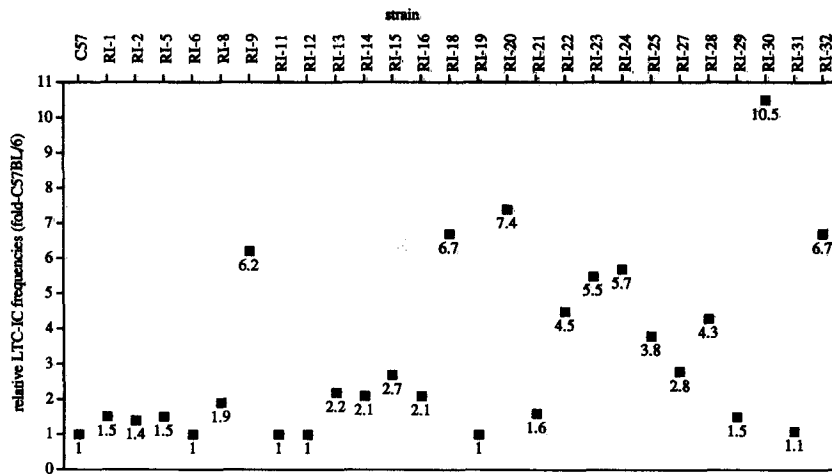
Figure 4. Distribution of relative stem cell frequencies in BXD RI strains. Limiting dilution analysis was performed on the stromal cell line S17, and colonies were enumerated at 4 wk. In each experiment, the parental strains DBA/2 and C57Bl/6 were included as controls. Frequencies were determined as outlined in Fig. 3. To facilitate comparison between experiments, data are expressed as relative frequencies calculated as LTC-IC per 10^5 cells seeded in each RI strain/LTC/IC per 10^5 in C57Bl/6 mice obtained in the same experiment.

The extent of the donor, or differential, region that contains *Scfr1* has not been carefully determined. Previous studies have shown that the B6.C-H25 strain carries BALB/c alleles of the *Apoa2*, *Ly9*, and *Sap* genes along with *H25* (35–37). We have typed the congenic strains at a series of simple sequence-length polymorphism loci (24–26) covering the distal half of chromosome 1 to more accurately define the differential region. Beginning near the midpoint of the chromosome and extending to the terminal known marker, we typed B6.C-H25 and -H35 at D1Mit215, -365, -26, -218, -33, -452, -113, -166, -17, and -155 (Fig. 6). B6.C-H35 carried the C57Bl/6 allele at all loci tested; in contrast, B6.C-H25 typed like BALB/c at D1Mit113, -166, and -17, and like C57Bl/6 at the other loci. Thus, the differential region donated by BALB/c extends at least from D1Mit113 to D1Mit17, a segment 10–15 centiMorgans long that contains *Apoa2*, *Ly9*, *Sap*, *H25*, the *Scfr1*, and a variety of other genes as shown in Fig. 7.

Discussion

We demonstrate here that the size of the stem cell pool differs markedly in the marrow of inbred strains. Analysis of
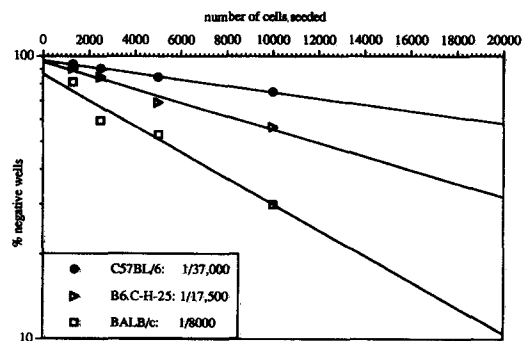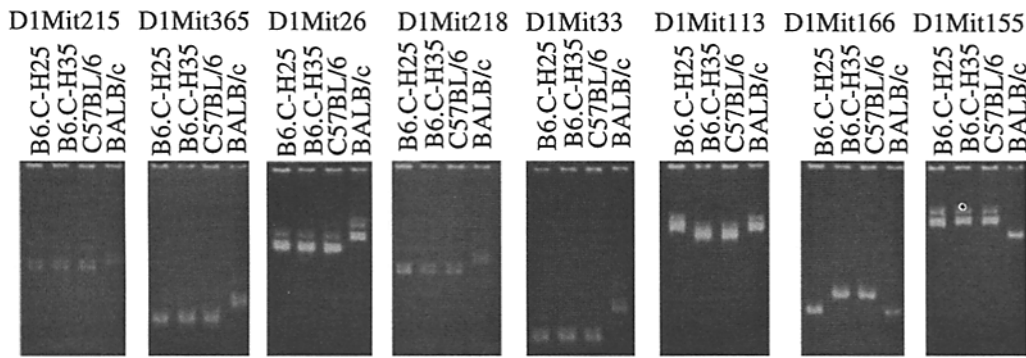

Figure 5. The congenic H-25 strain confirms the existence of a stem cell frequency controlling gene, *Scfr1*. Shown are limiting dilution data demonstrating the levels of LTC-IC in the marrow of BALB/c, C57Bl/6, and B6.C-H25 mice. Data are representative of five independent experiments. For details see legend to Fig. 3.

the large set of BXD RI strains and confirmatory studies of congenic strains identified a small region on distal chromosome 1 that accounts for a portion of the difference in stem cell level. Our mapping studies open avenues to identify the regulatory genes, among them *Scfr1*, by examining candidate genes in this region or by positional cloning.

A very efficient approach to analyzing the genetic control of a trait or character in mice and to map the responsible gene(s) is to analyze a panel of RI strains. Such strains, "cousins" derived from the F2 generation of an intercross between two inbred strains, comprise a fixed and stable genetic segregation experiment that can be analyzed repeatedly in many laboratories over indefinite periods of time. The typing results are cumulative; each typing increases the information and the density of markers on the genomic map of the mouse. We are fortunate in this study to have identified C57Bl/6 and DBA/2 as strains that differ greatly in stem cell level, because the panel of BXD RI lines, derived from these progenitor strains, is one of the largest available and is by far the most densely mapped.

Historically, RI strain mapping has been extensively used for mapping discrete, or qualitatively varying, characters such as differences in DNA sequence, protein electrophoretic mobility, or antigenic structure (32). Recently, RI strains have been used to analyze quantitatively varying characters such as alcohol and drug preferences in an efficient approach to quantitative trait locus (QTL) analysis (38). When a character is determined by multiple genes with varying degrees of effect, analysis of RI lines can readily identify those genes that contribute most strongly (38).

In the analysis presented here, the strain distribution pattern of stem cell frequency in 26 BXD RI lines reveals multiple genetic differences between the parental strains that affect this frequency. None of the strains have a level as high as DBA/2, indicating that the number of such genes probably exceeds three; otherwise we should have observed a DBA genotype among the 26 strains. QT analysis suggests that genes, located near *Acrg* and *Adprp* on chromosome 1 and *Hist3* on chromosome 11, have the largest effects on stem cell frequency. Although these locations

D1Mit215   D1Mit365   D1Mit26   D1Mit218   D1Mit33   D1Mit113   D1Mit166   D1Mit155

B6.C-H25
B6.C-H35
C57BL/6
BALB/c
(repeated lane labels above each gel)

D1Mit marker	215	365	26	218	33	452	113	166	17	155
map position	49	60	67	72	82	85	90	99	110	116

```
                                                        c         c       c
B6.C-H25 -//-----------------------------------------X===========X-------
            b     b     b    b     b    b                                  b

B6.C-H35 -//-----------------------------------------------------------------
            b     b     b    b     b    b     b    b     b    b
```
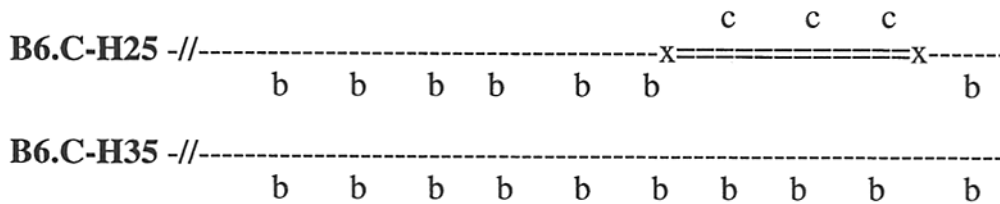
Figure 6. Genotyping the H25- and H35-congenic strains at D1Mit loci to map the differential regions. PCR products for each D1Mit marker were electrophoresed on polyacrylamide minigels and stained with ethidium bromide to visualize the simple sequence length polymorphism. B6.C-H35 has the C57Bl/6 ("b") allele at all typed loci, and B6.C-H25 is "b" at most loci but has the BALB/c ("a") allele at three markers near the distal end of chromosome 1, as represented below the gel images. Data for D1Mit452 and -17 are not shown.

were not defined with high confidence, they offered guides for additional studies of congenic strains. We found no congenic strains constructed from C57Bl/6 and DBA/2, the inbred strains with the largest stem cell level differences. But the stem cell level in BALB/c is nearly as high as in DBA/2, and two lines carrying chromosome 1 minor histocompatibility loci from BALB/c bred onto the C57Bl/6 background were available. The H35 locus is likely to be on the proximal part of chromosome 1, and H25 is near the distal end. Since congenic strains carry a segment of donor chromosome that contains the selected locus, we could assess two portions of chromosome 1 for their effect on stem cell levels. The position of H35 is not well defined, and if it is on chromosome 1, then the differential segment in B6.C-H35 must be proximal to the midpoint marker, D1Mit215, that is just proximal to the Acrg locus. This agrees well with the phenotype of this stain, which showed stem cell levels indistinguishable from that of C57Bl/6. Thus, our most significantly associated map location was not tested by this strain and awaits confirmation by F2 and backcross analysis. The location of H25 is more felicitous; the differential region contains the Adprp locus, identified by the QTL analysis as a site of a stem cell–regulating gene. This allows a direct test and confirmation of the effect of this region on stem cell regulation. The B6.C-H25 strain had stem cell levels intermediate to the parental C57Bl/6 and BALB/c strains, demonstrating that at least one of the

genetic factors that affect stem cell level is present in the small donor chromosome segment. This region contains a variety of genes that are expressed in cells of the hematopoietic lineages that could be examined for potential roles in stem cell regulation. These include genes of known regulatory function, like the Pbx and Hlx homeobox genes, the Otf1 octamer-binding transcription factor 1 gene, and the Tgfb2 cytokine gene; genes of presumptive regulatory function, like Tal2, a T cell leukemia gene, and Ril3, a radiation-induced leukemia sensitivity locus; and a wide variety of cell surface receptor genes including Rxrg, a retinoic acid receptor–related gene, various Fcε and Fcγ receptors, selectins, and the CD34 antigen.

That the size of the stem cell pool differs between DBA/2 and C57Bl/6 mice has been previously suggested. Philips and colleagues (13) demonstrated that C57Bl/6 mice have more Sca-1+ rhodamine^{lo} marrow cells than DBA/2 mice, and concluded that the stem cell pool in C57Bl/6 is larger than in DBA/2 mice. Experience has shown that identification of stem cells on the sole basis of phenotypic markers is risky, particularly when different strains are involved (16). We used a functional assay, the LTC-IC assay, to demonstrate that the frequency of stem cells in DBA/2 mice is considerably higher than in C57Bl/6 mice. As with all in vitro assays, it is not absolutely certain that this assay detects the most primitive stem cells. However, it is well documented that the cell measured in this assay is highly
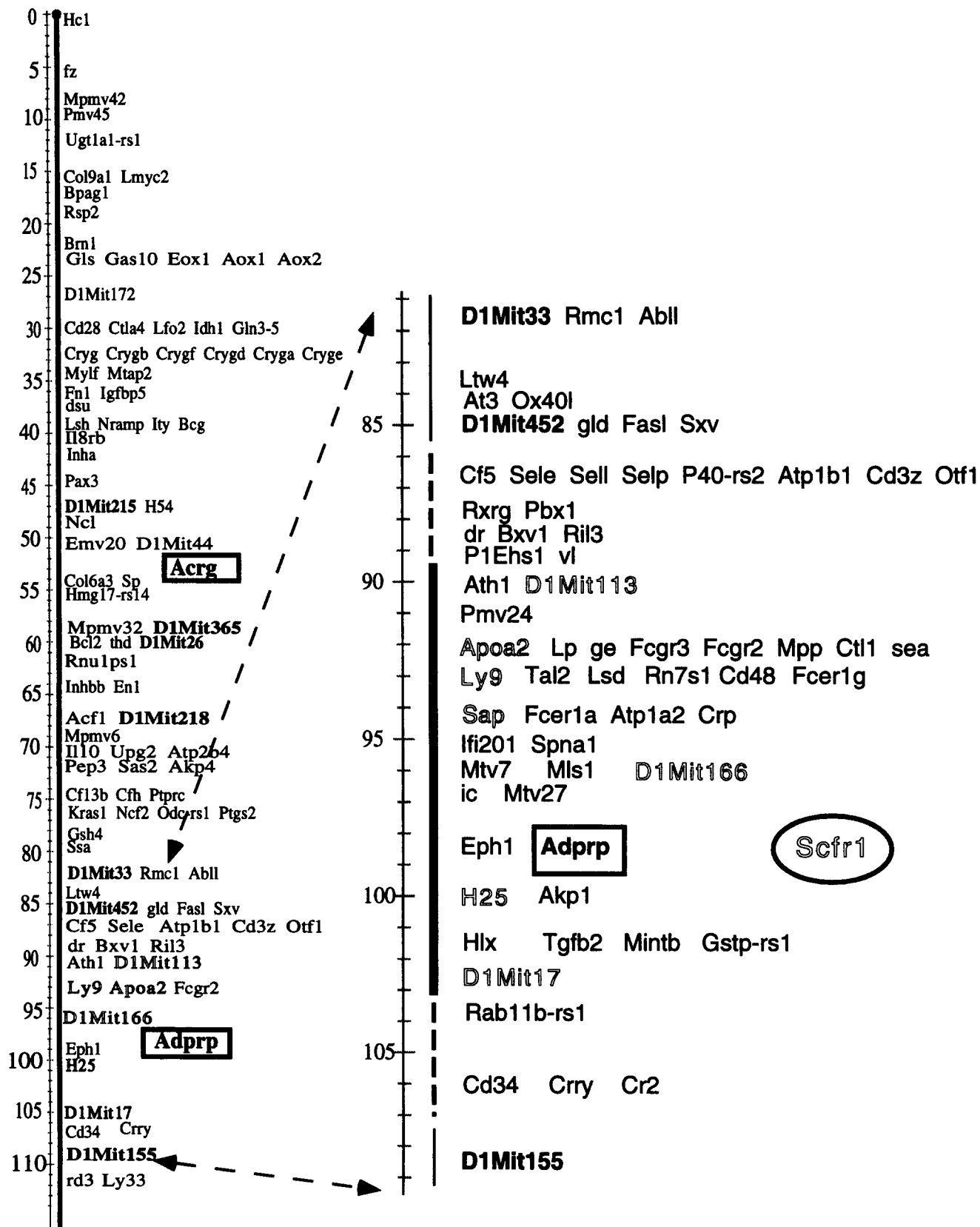
Figure 7. The B6.C-H25 differential region and location of *Scfr1* on chromosome 1 of the mouse. This partial map is adapted from the 1994 report of the Mouse Chromosome 1 Committee as presented in the Encyclopedia of the Mouse Genome (39, 40). Units are in centiMorgans. The B6.C-H25 differential region containing the stem cell frequency controlling gene *Scfr1* is expanded on the right side. Genes shown in bold have the C57Bl/6 allele in the B6.C-H25 strain; those shown in outline font have the BALB/c allele. The differential region is indicated by a heavier chromosome bar. The two loci strongly associated with stem frequency in the QTL analysis are boxed. *Scfr1* is not precisely located, but is within the differential region.

correlated to repopulating stem cells (4–8, 9, 17). This seems to be true both in C57Bl/6 and DBA/2 mice, since in both strains the progeny of the primitive rhodamine^{lo} subset of stem cells permanently displaced the progeny of the less primitive rhodamine^{hi} stem cells as early as 2 wk of culture (13). The LTC-IC assay that we used differs from previously described assays only in that the stromal cell line S17 allowed us to combine all three endpoints used in other variants of this assay. These three endpoints, colonies of small cells, CFA, and CFU, showed a good correlation and yielded similar results in this system. Ploughmacher et al. (4, 5) demonstrated good correlation of CFA and CFU but little correlation with colonies formed on the primary stroma. Primary stroma is a heterogeneous mixture of cells, both inhibitory and stimulatory, for hematopoietic differentiation. It is likely that the use of a monoclonal stromal cell line provided a more standardized environment in which CFA and colonies are correlated. Alternatively, it is possible that the S17 line provides a better environment for the proliferation of primitive cells. In fact, we selected this line because we knew that S17 supports high levels of stem cells with in vivo repopulation capacity (21).

Even though our analysis revealed marked differences in stem cell levels, all of the strains tested apparently have normal hematopoiesis. It is clear that the number of stem cells in C57Bl/6 is entirely sufficient to provide blood cells for the lifespan of the animal. Therefore, DBA/2 could have an excess of stem cells that could remain silent and thus would not be evident in the periphery. Alternatively, DBA/2 mice might require higher levels of stem cells to compensate for a defective hematopoietic compartment. Van Zant and colleagues (12) noted that CFU spleen and CFUc in the marrow of DBA/2 mice have a higher incidence of cycling cells than the corresponding cells in C57Bl/6 mice. It is possible that mature cells in DBA/2 mice have a higher turnover than C57Bl/6 mice, placing an increased demand on precursors and stem cells in DBA/2 mice. The decay of DBA/2-derived peripheral hematopoi-

etic cells observed in allophenic mice (12) supports this interpretation. Van Zant and colleagues (12) postulated the existence of a genetic locus, called Stk, that would regulate stem cell kinetics in these mice. Whether our system measures a gene(s) related to the Stk locus remains to be established. In either case, it is likely that the identification and eventual cloning of the genes that control stem cell frequencies will be valuable for understanding the biology of stem cells.

Sakakeeny and Greenberger (41) showed that granulopoiesis persisted longer if cultures were initiated with DBA/2 cells than with C57Bl/6 cells. In their culture system, differences between strains were noted between 20 and 60 wk, and it seems unlikely that our 4–5-wk cultures are affected by these differences. To our knowledge, the underlying mechanism for differences in longevity of the bone marrow cultures has not been determined. Thus, it is tempting to speculate that longevity of granulopoiesis is actually a function of the number of stem cells originally seeded. Cultures initiated with DBA/2 cells contain higher levels of stem cells that by sequential activation (42, 43) would sustain granulopoiesis in these cultures.

Stem cell transplantation is increasingly applied to the treatment of a variety of diseases in humans. One problem encountered in this procedure is the difficulty in accurately assessing the number of stem cells and thereby the reconstituting ability of the graft. The level of stem cells in human marrow tend to be more variable than in murine marrow. Some of these differences can be accounted for by the medical history of the patients or variations in sampling efficiency. However, even in samples obtained from healthy donors, the level of stem cells varies widely (10). It is tempting to speculate that some of the variation seen in human stem cell levels could be a reflection of allelic differences in the human homologues of Scfr1 or other Scfr genes. If so, these genes may well provide a desperately needed, rapid screening test for repopulation capacity.

Address correspondence to Christa E. Müller-Sieburg, Medical Biology Institute, 11077 North Torrey Pines Road, La Jolla, CA 92037.

References

1. Harrison, D.E., C.M. Astle, and M. Stone. 1989. Number and function of transplantable primitive immunohematopoietic stem cells. Effects of age. J. Immunol. 142:3833–3840.
2. Harrison, D.E., C.T. Jordan, R.K. Zhong, and C.A. Astle.

1993. Primitive hemopoietic stem cells: direct assay of most productive populations by competitive repopulation with simple binomial, correlation and covariance calculation. Exp. Hematol. 21:206–219.

3. Nakano, T., N. Waki, H. Asai, and Y. Kitamura. 1989. Effect of 5-fluorouracil on "primitive" hematopoietic stem cells that reconstitute whole erythopoiesis in genetically anemic W/W^v mice. Blood. 73:425–430.

4. Ploughmacher, R.E., J.P. van der Sluijs, J.S.A. Voerman, and N.H.C. Brons. 1989. An in vitro limiting-dilution assay of long-term repopulating hematopoietic stem cells in the mouse. Blood. 74:2755–2763.

5. Ploughmacher, R.E., J.P. van der Sluijs, C.A.J. van Beurden, M.R.M. Baert, and P.L. Chan. 1991. Use of limiting-dilution type long-term marrow cultures in frequency analysis of marrow repopulating and spleen colony-forming hematopoietic stem cells in the mouse. Blood. 78:2527–2533.

6. Pettengell, R., T. Luft, R. Henschler, J.M. Hows, T.M. Dexter, D. Ryder, and N.G. Testa. 1994. Direct comparison by limiting dilution analysis of long-term-culture-initiating cells in human bone marrow, umbilical cord blood, and blood stem cells. Blood. 84:3653–3659.

7. Sutherland, H.J., C.J. Eaves, A. Eaves, W. Dragowska, and P.M. Lansdorp. 1989. Characterization and partial purification of human marrow cells capable of initiating long-term hematopoiesis in vitro. Blood. 74:1563–1570.

8. Sutherland, H.J., P.M. Lansdorp, D.H. Henkelman, A.C. Eaves, and C.J. Eaves. 1990. Functional characterization of individual human hematopoietic stem cell cultured at limiting dilution on supportive marrow stromal layers. Proc. Natl. Acad. Sci. U.S.A. 87:3584–3588.

9. Müller-Sieburg, C.E., B. Torok-Storb, J. Visser, and R. Storb, editors. 1991. Hematopoietic stem cells. Curr. Top. Microbiol. Immunol. 177:1–251.

10. Lansdorp, P.M., and W. Dragowka. 1993. Maintenance of hematopoiesis in serum-free bone marrow cultures involved sequential recruitment of quiescent progenitors. Exp. Hematol. 21:1321–1327.

11. Witte, O.N. 1990. Steel locus defines new multipotent growth factor. Cell. 63:5–6.

12. Van Zant, G., P.W. Eldridge, R.R. Behringer, and M.J. Dewey. 1983. Genetic control of hematopoietic kinetics revealed by analyses of allophenic mice and stem cell suicide. Cell. 35:639–645.

13. Phillips, R.L., A.J. Reinhart, and G. Van Zant. 1992. Genetic control of murine hematopoietic stem cell pool sizes and cycling kinetics. Proc. Natl. Acad. Sci. USA. 89:11607–11611.

14. Storer, J.B. 1975. Acute responses to ionizing radiation. In Biology of the Laboratory Mouse. E.L. Green, editor. Dover Publications, Inc., New York. 427–446.

15. Lesley, J., and I.S. Trowbridge. 1982. Genetic characterization of a polymorphic murine cell-surface glycoprotein. Immunogenetics. 15:313–320.

16. Spangrude, G.J., and D.M. Brooks. 1992. Phenotypic analysis of mouse hematopoietic stem cells shows a Thy-1-negative subset. Blood. 80:1957–1964.

17. Weilbaecher, K., I. Weissman, K. Blume, and S. Heimfeld. 1991. Culture of phenotypically defined hematopoietic stem cells and other progenitors at limiting dilution on Dexter monolayers. Blood. 78:945–952.

18. Neben, S., P. Anklesaria, J. Greenberger, and P. Mauch. 1993. Quantitation of murine hematopoietic stem cells in vitro by limiting dilution analysis of cobblestone area formation on a clonal stromal cell line. Exp. Hematol. 21:438–443.

19. Collins, L.S., and K. Dorshkind. 1987. A stromal cell line from myeloid long-term bone marrow cultures can support myelopoiesis and B lymphopoiesis. J. Immunol. 138:1082–

20. Deryugina, E.I., B.I. Ratnikov, M.A. Bourdon, and C.E., Müller-Sieburg. 1994. Clonal analysis of primary marrow stroma: functional homogeneity in support of lymphoid and myeloid cell lines and identification of positive and negative regulators. Exp. Hematol. 22:910–918.

21. Wineman, J.P., K. Moore, I.A. Lemischka, and C.E. Müller-Sieburg. 1996. Functional heterogeneity of the hematopoietic microenvironment: rare stromal elements maintain long-term repopulating stem cells. Blood. In press.

22. Wineman, J.P., S.-I. Nishikawa, and C.E. Müller-Sieburg. 1993. Maintenance of high levels of pluripotent hematopoietic stem cells in vitro: effect of stromal cells and c-kit. Blood. 81:356–372.

23. Müller-Sieburg, C.E., K. Townsend, I.L. Weissman, and D. Rennick. 1988. Proliferation and differentiation of highly enriched mouse hematopoietic stem cells and progenitor cells in response to defined growth factors. J. Exp. Med. 167:1825–1840.

24. Dietrich, W.F., J.C. Miller, R.G. Steen, M. Merchant, D. Damron, R. Nahf, A. Gross, D.C. Joyce, M. Wessel, R.D. Dredge, et al. 1994. A genetic map of the mouse with 4,006 simple sequence length polymorphisms. Nature Genetics. 7: 220–245.

25. Copeland, N.G., D.J. Gilbert, N.A. Jenkins, J.H. Nadeau, J.T. Eppig, L.J. Maltais, J.C. Miller, W.F. Dietrich, R.G. Steen, S.E. Lincoln, et al. 1993. Genome maps IV. Science (Wash. DC). 262:67–82.

26. Whitehead Institute/MIT Center for Genome Research, Genetic Map of the Mouse. Database Release 10. April 28, 1995.

27. Lefkovits, I., and H. Waldmann. 1984. Limiting dilution analysis of cells of the immune system. I. The clonal basis of the immune response. Immunol. Today. 5:265–268.

28. Manly, K.F. 1993. A Macintosh program for storage and analysis of experimental genetic mapping data. Mammalian Genome. 4:303–313.

29. Müller-Sieburg, C.E., C.A. Whitlock, and I.L. Weissman. 1986. Isolation of two early B lymphocyte progenitors from mouse marrow: a committed pre-pre-B cell and a clonogenic Thy-1^{lo} hematopoietic stem cell. Cell. 4:653–662.

30. Whitlock, C.A., G.F. Tidmarsh, C. Müller-Sieburg, and I.L. Weissman. 1987. Bone marrow stromal cell lines with lymphopoietic activity express high levels of a pre-B neoplasia-associated molecule. Cell. 48:1009–1021.

31. Verfaillie, C.M., and J.S. Miller. 1994. CD34$^+$/CD33$^-$ cells reselected from macrophage inflammatory protein 1a + interleukin-3-supplemented "stroma-noncontact" cultures are highly enriched for long-term bone marrow culture initiating cells. Blood. 84:1442–1449.

32. Taylor, B.A., and P.C. Reifsnyder. 1993. Typing recombinant inbred mouse strains for microsatellite markers. Mamm. Genome. 4:239–242.

33. Lander, E.S., and N.J. Schork. 1994. Genetic dissection of complex traits. Science (Wash. DC). 265:2037–2048.

34. Bailey, D.W. 1975. Genetics of histocompatibility in mice. I. New loci and congenic lines. Immunogenetics. 2:249–256.

35. Lusis, A.J., B.A. Taylor, R.W. Wangenstein, and R.C. Le Boeuf. 1983. Genetic control of lipid transport in mice. II. Genes controlling structure of high density lipoproteins. J. Biol. Chem. 258:5071–5078.

36. Durda, P.J., S.C. Boos, and P.D. Gottlieb. 1979. T100: a new murine cell surface glycoprotein detected by anti-Lyt-2.1 serum. J. Immunol. 12:1407–1412.

37. Mortensen, R.F., P.T. Le, and B.A. Taylor. 1985. Mouse serum amyloid P-component (SAP) levels controlled by a locus on chromosome 1. *Immunogenetics*. 22:367–375.

38. Gora-Maslak, G., G.E. McClearn, J.C. Crabbe, T.J. Phillips, J.K. Belknap, and R. Plomin. 1991. Use of recombinant inbred strains to identify quantitative trait loci in psychopharmacology. *Psychopharmacology (Berl.)*. 104:413–424.

39. Encyclopedia of the Mouse Genome. Release 3. 1994. Mouse Genome Informatics Project, The Jackson Laboratory, Bar Harbor, ME 04609.

40. Seldin, M. 1994. Mouse Chromosome 1 Committee Report.

1994. *Mamm. Genome*. 5:S1–S21.

41. Sakakeeny, M.A., and J.S. Greenberger. 1982. Granulopoiesis longevity in continuous bone marrow cultures and factor-dependent cell line generation: significant variation among 28 inbred mouse strains and outbred stocks. *J. Natl. Cancer Inst.* 68:305–317.

42. Kay, H.E.M. 1965. How many cell-generations? *Lancet.* 2: 418–419.

43. Micklem, H.S., J.D. Ansell, J.E. Wayman, and L. Forrester. 1983. The clonal organization of hematopoiesis in the mouse. *Progress in Immunology*. 5:633–644.