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A Genetic Determinant That Specifically Regulates the Frequency of Hematopoietic Stem Cells

Sean J. Morrison,*‡† Dalong Qian,† Libuse Jerabek,§ Bonnie A. Thiel,¶ In-Kyung Park,* Preston S. Ford,† Mark J. Kiel,*‡ Nicholas J. Schork,§ Irving L. Weissman,§ and Michael F. Clarke2†

The regulation of hematopoietic stem cell (HSC) homeostasis is not well understood. We screened for genetic polymorphisms that were linked to differences between mouse strains in the numbers of long-term reconstituting HSCs or restricted progenitors in the bone marrow. AKR/J mice had significantly higher frequencies and numbers of both HSCs and restricted progenitors in their bone marrow than C57BL/Ka-Thy-1.1 mice. The C57BL/Ka-Thy-1.1 alleles were partially dominant. A locus on chromosome 17, including the H-2 complex, was significantly linked to the frequency of long-term self-renewing HSCs but showed no evidence of linkage to the frequency of restricted progenitors. Conversely, a chromosome 1 locus exhibited suggestive linkage to restricted progenitor frequencies but was not linked to HSC frequency. This demonstrates that there are distinct genetic determinants of the frequencies of HSCs and restricted progenitors in vivo. The AKR/J chromosome 17 locus was not sufficient to increase HSC frequencies when bred onto a C57BL background. This suggests that to affect HSC frequencies, the product(s) of this locus likely depend on interactions with unlinked modifying loci. The Journal of Immunology, 2002, 168: 635–642.

Hematopoietic stem cells (HSCs) have three important properties that explain the lifelong perpetuation of hematopoiesis and the remarkable regenerative capacity of the hematopoietic system: extensive self-renewal potential, pluripotency, and frequent cell cycle quiescence. The genetic regulation of these properties is poorly understood. Insights into the genetic regulation of HSCs have mainly come from the phenotypes of spontaneously occurring mutations or gene-targeted mice. For example, deficiencies in steel factor or its receptor (1), thrombopoietin receptor (2), or Ikaros (3) lead to reductions in the numbers and frequencies of HSCs and progenitors in the bone marrow. These loss of function studies identify genes that regulate the survival, proliferation, or differentiation of hematopoietic progenitors.

An alternate approach is to identify the genes that cause HSC properties to vary among different genetic backgrounds. Van Zant and colleagues (4–6) showed that the cell cycle kinetics and/or self-renewal of HSCs varies among different mouse strains and that such variations have important effects on HSC population dynamics; furthermore, the numbers of primitive hematopoietic progenitors vary considerably between mouse strains based on differences in the frequencies of long-term culture-initiating cells (LTC-IC) (7) and cobblestone area-forming cells (CAFC) (8, 9). Muller-Sieburg and Riblet (7) found that differences in mouse LTC-IC frequency were associated with polymorphisms at one or two loci on chromosome 1. de Haan and Van Zant (9, 10) found that the frequency of day 35 CAFC was significantly linked to a 4-cM interval of chromosome 18 and exhibited suggestive linkage to additional loci on chromosomes 2, 14, and X. It is important to identify the genetic determinants of HSC frequency in bone marrow because HSC frequency reflects several aspects of HSC regulation, and the ability to increase HSC numbers would greatly facilitate potential clinical applications using HSCs.

Factors that modulate the frequency of HSCs in vivo usually modulate the frequencies of restricted progenitors as well. Administration of cytokines such as steel factor and G-CSF with or without cytoreductive drugs can lead to an increase in HSC frequency (11–13); however, the same treatments concomitantly increase the frequency of a wide variety of committed progenitors (14, 15). The transcription factor GATA-2 is required in both HSCs and mast cell precursors (16–18). Mutation of the SCL/tal-1 transcription factor eliminates all hematopoiesis (19) but, in addition to regulating the formation or maintenance of HSCs, it may independently regulate restricted progenitors (20). The tyrosine kinase flk2/fl3 was thought to be a specific regulator of multipotent progenitors (21, 22) but later was found to regulate committed B lineage progenitors as well (23). In contrast, recent studies of cyclin-dependent kinase inhibitors provide the first evidence of a distinction in cell cycle regulation between HSCs and restricted progenitors: p21 deficiency increased the frequency and proliferation of HSCs (24), while p27 deficiency increased the frequency and proliferation of restricted progenitors (25).

We demonstrate that AKR/J mice have more long-term reconstituting HSCs than C57BL/Ka-Thy-1.1 mice. We systematically examined long-term self-renewing HSC frequencies in (C57BL/Ka-Thy-1.1 × AKR/J)F2 mice and genotyped them by...
PCR sequence length polymorphisms generated using primers to sequence tags distributed throughout the genome. By identifying polymorphisms that were statistically significantly linked to HSC frequencies we demonstrate that variations in HSC frequency were determined by a locus on chromosome 17. This locus specifically influenced HSC frequency but not the frequency of more restricted progenitors which were regulated by a distinct locus on chromosome 1. These data support the idea that HSCs and restricted hematopoietic progenitors are independently regulated by distinct sets of genes.

Materials and Methods

Mouse strains

C57BL/6J (Ly5.1), C57BL/Ka-Thy-1.1 (Ly 5.2), AKR/J, AKR/cumberland, C57BL/FlaEg.Thy-1.1, and (C57BL/6J (Ly5.1), C57BL/Ka-Thy1.1 (Ly 5.2), AKR/J, AKR/cumberland, AKR/Cu) using an x-ray machine operated at 200 kV, delivering 85 rad/ min. The radiation was delivered in two doses, with 3 h between doses. This locus specifically influenced HSC frequency but not the frequency of more restricted progenitors which were regulated by a distinct locus on chromosome 1. These data support the idea that HSCs and restricted hematopoietic progenitors are independently regulated by distinct sets of genes.

Materials and Methods

Mouse strains

C57BL/6J (Ly5.1), C57BL/Ka-Thy-1.1 (Ly 5.2), AKR/J, AKR/cumberland, C57BL/FlaEg.Thy-1.1, and (C57BL/6J (Ly5.1), C57BL/Ka-Thy1.1 (Ly 5.2), AKR/J, AKR/cumberland, AKR/Cu) using an x-ray machine operated at 200 kV, delivering 85 rad/ min. The radiation was delivered in two doses, with 3 h between doses. This locus specifically influenced HSC frequency but not the frequency of more restricted progenitors which were regulated by a distinct locus on chromosome 1. These data support the idea that HSCs and restricted hematopoietic progenitors are independently regulated by distinct sets of genes.

Bone marrow preparation and staining

Marrow was flushed from the femurs and tibias of donor mice. Single cell suspensions were prepared by drawing the bone marrow through a 25-gauge needle, then expelling them back through a nylon mesh screen. Cells were suspended in HBSS (without phenol red; Applied Scientific, San Francisco, CA) containing 2% calf serum (Irvine Scientific, Santa Ana, CA), pH 7.2. Marrow samples from individual mice were always processed separately, not pooled.

For HSC purification or analysis, cells were incubated with a mixture of unlabeled lineage marker Abs including those specific for CD3 (KT3.1), CD4 (GK1.5), CD5 (53-7.3), CD8 (53-6.7), Gr-1 (8C5), Ly-76 (Ter119), B220 (6B2), and Mac-1 (M170). All Ab incubations were 20 min on ice.

After incubation, cells were diluted and centrifuged through a calf serum cushion. The cells were incubated with a PE-conjugated anti-rat IgG Ab to stain lineage markers. The cells were then incubated with directly conjugated Abs against Thy-1.1 (19-12-2F11), Sca-1 (E13 bio), and c-kit (2B8 APC). The cells were resuspended in 0.5 µg/ml propidium iodide before analysis. Analyses and cell sorts were performed on a modified dual laser FACS (BD Biosciences, Mountain View, CA). For functional assays, progenitors were purified by sorting and sorting to obtain precise numbers of cells that were homogeneous for the indicated surface marker phenotype.

Functional analyses

Recipient mice were lethally irradiated (920 rad for C57BL. 800 rad for AKR/Cu) using an x-ray machine operated at 200 kV, delivering 85 rad/ min. The radiation was delivered in two doses, with ~3 h between doses. After irradiation, mice were maintained on antibiotic water containing 1.1 g/L neomycin sulfate and 10 U/L polymixin B sulfate. For reconstitution assays, double-sorted progenitor populations were injected into the retro-orbital venous sinus of irradiated recipient mice along with recipient type whole bone marrow (WBM) cells (200,000 for C57BL and 40,000–200,000 for AKR due to the higher frequency of multipotent progenitors) to protect recipient mice from radiation-induced hematopoietic failure. Reconstituted mice were periodically bled via the tail vein to monitor reconstitution by donor marked progenitors. RBCs were depleted by sedimentation and the remaining RBCs were lysed by a 5-min incubation on ice in 0.15 M ammonium chloride plus 0.01 M potassium bicarbonate. Cells were stained with three color combinations of Abs against a donor marker (Ly5.2 for C57BL and Thy-1.1 for AKR) and lineage markers and analyzed by flow cytometry.

Stromal cell cultures were prepared by plating AC-6 cells in 24-well plates. After the AC-6 cells became confluent, the cultures were irradiated and single Thy-1lowSca-1 Lin c-kit+ cells were sorted into each well. The medium (RPMI 1640, 10% FBS, 50 µM 2-ME, penicillin/streptomycin, and 1 µM sodium pyruvate) was changed weekly. Colony formation was monitored using an inverted microscope from 1 to 6 wk after cloning HSCs into the cultures. All cultures were incubated at 37°C in a humidified chamber flushed with 5% CO2. ACFC were also assayed on AC-6 stromal feeder cells in 12-well plates.

Results

AKR/J Thy-1lowSca-1 Lin c-kit+ cells are highly enriched for long-term reconstituting HSC activity

Long-term reconstituting HSCs can be isolated as Thy-1lowSca-1 Lin c-kit+ cells (30–32) from mouse strains with the Thy-1.1, Ly-6th genotype (33, 34). Both C57BL/Ka-Thy-1.1 and AKR/J mice are Thy-1.1, Ly-6th. Thy-1lowSca-1 Lin c-kit+ cells from C57BL/Ka are highly enriched for long-term multilineage reconstituting activity. Based on limit dilution analyses, lethally irradiated C57BL-Ly5.2 mice were competitively reconstituted on average by a single long-term self-renewing multipotent progenitor when injected i.v. with 13 Thy-1lowSca-1 Lin c-kit+ cells from C57BL/Ka-Thy-1.1 mice (31, 35, 36). The Thy-1lowSca-1 Lin c-kit+ population from C57BL/Ka-Thy-1.1 mice is thought to be a nearly pure population of long-term reconstituting HSCs because nearly 80% of limit dilution reconstituted mice were long-term multilineage reconstituted (31), ~90% of single cells formed primitive colonies in methylcellulose, and 49% of single cells formed long-term colonies on AC-6 stroma (13, 32, 37, 38).

Thy-1lowSca-1 Lin c-kit+ cells purified from AKR/J were phenotypically indistinguishable from the long-term reconstituting HSC population in C57BL/Ka-Thy-1.1 (Fig. 1). As expected based on their genotype, AKR/J Thy-1lowSca-1 Lin c-kit+ cells were also highly enriched for long-term reconstituting HSCs. Male AKR/J Thy-1lowSca-1 Lin c-kit+ cells were competed against 40,000–200,000 female AKR/cumberland WBM cells and assayed for reconstituting activity in lethally irradiated female AKR/Cu mice. AKR/Cu are Thy-1.2, thus reconstitution by AKR/J donor HSCs was detected by the presence of Thy-1.1+ T cells and by Y chromosome-containing myeloid and B cells. In many experiments with Thy-1lowSca-1 Lin c-kit+ cells from C57BL/Ka-Thy-1.1 mice, recipients that were reconstituted by donor T cells were always multilineage reconstituted. Thus AKR/Cu mice were assayed for reconstitution by screening for donor T cells by FACS between 3 and 6 mo after reconstitution. In two experiments, 11 of 16 recipients of 10 donor Thy-1lowSca-1 Lin c-kit+ cells had donor T cells at 13–14 wk after reconstitution. Two of three recipients injected with 15 Thy-1lowSca-1 Lin c-kit+ cells were reconstituted by donor T cells at 6 mo and eight of nine recipients injected with 50 cells were reconstituted by donor T cells at 14 wk after reconstitution. It was confirmed that recipients of 10 or 50 donor Thy-1lowSca-1 Lin c-kit+ cells that became reconstituted by donor T cells were multilineage reconstituted. Myeloid and B cells were purified by flow cytometry from the blood of recipients and tested for Y chromosome by PCR. As shown in Fig. 2, every...
recipient that was reconstituted by donor T cells was also reconstituted by donor myeloid and B cells. By limit dilution analysis, recipients were reconstituted by an average of one long-term HSC when injected with 11 cells (Table I). Thus AKR/J Thy-1 low Sca-1 cells were similarly highly enriched for long-term multilineage reconstituting activity as the corresponding population from C57BL/Ka mice.

At least some AKR/J Thy-1 low Sca-1 cells had day 12 CFU-spleen (CFU-S) activity (Table I), as would be expected for a long-term HSC population under our assay conditions (31, 39, 40). While AKR/J Thy-1 low Sca-1 cells appeared to have somewhat more CFU-S activity than the corresponding C57BL/Ka population, the significance of this is hard to interpret because the CFU-S activity of HSCs probably varies by cell cycle status and mouse strain. Fifty-six percent of single AKR/J Thy-1 low Sca-1 cells formed colonies of hundreds of thousands of hematopoietic cells that persisted for >6 wk on AC-6 stromal cells, as compared with 49% of cells from the same C57BL/Ka population.

**AKR/J mice have more HSCs than C57BL/Ka-Thy-1.1 mice**

We compared the frequencies of bone marrow HSCs between AKR/J and C57BL/Ka-Thy-1.1 mice. In addition to determining the frequencies of Thy-1 low Sca-1 cells, which contain mainly long-term reconstituting HSCs, we compared the frequencies of Thy-1 low Sca-1 cells, which contain mainly transiently reconstituting multipotent progenitors (31). There was no difference in the total number of bone marrow cells that could be recovered from the femurs and tibia of AKR/J and C57BL/Ka-Thy-1.1 mice. In addition to determining the frequencies of Thy-1 low Sca-1 cells, which contain mainly long-term reconstituting HSCs, we compared the frequencies of Thy-1 low Sca-1 cells, which contain mainly transiently reconstituting multipotent progenitors (31). There was no difference in the total number of bone marrow cells that could be recovered from the femurs and tibia of AKR/J and C57BL/Ka-Thy-1.1 mice.

**Table 1. The properties of Thy-1 low Sca-1 cells from C57BL/Ka-Thy-1.1 and AKR/J mice**

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Limit Dilution Dose for LTMR</th>
<th>CFU-S</th>
<th>Clonogenic on Stroma (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL</td>
<td>1 in 13</td>
<td>1 in 59 ± 24</td>
<td>49 ± 10</td>
</tr>
<tr>
<td>AKR/J</td>
<td>1 in 11 ± 3</td>
<td>1 in 18</td>
<td>56</td>
</tr>
</tbody>
</table>

a Data are presented as mean ± SD. AKR statistics lacking SDs are the means of two experiments.

b The number of cells that must be i.v. injected into lethally irradiated recipients in order for an average of one cell to engraft and give long-term multilineage reconstitution (based on limit dilution analyses of competitively reconstituted mice).

c The percentage of single cells that can proliferate on AC-6 stroma to form colonies of hundreds of thousands of cells that persist for more than 6 wk.
In side-by-side pairwise comparisons, AKR/J averaged four times as many Thy-1lowSca-1" Lin"c-kit" cells and nearly three times as many Thy-1lowSca-1"Lin"c-kit" cells as C57BL/Ka-Thy-1.1 (Table II). Both differences were highly statistically significant ($p < 0.001$). Because Thy-1lowSca-1" Lin"c-kit" cells are similarly enriched for long-term reconstituting HSCs in AKR/J and C57BL/Ka-Thy-1.1 mice, we conclude that AKR/J mice have four times as many long-term reconstituting HSCs and significantly more transiently reconstituting multipotent progenitors as well.

Analysis of HSC frequency in (C57BL/Ka-Thy-1.1 × AKR/J)F1 progeny

AKR/J mice were bred with C57BL/Ka-Thy-1.1 mice and the frequency of Thy-1lowSca-1" Lin"c-kit" HSCs was assayed in the bone marrow of the femurs and tibias of the F1 mice (Table III). Of 12 F1 mice examined, four mice had HSC frequencies that fell around the high end of the C57BL/Ka-Thy-1.1 range (0.014–0.016). All remaining mice were intermediate between the C57BL/Ka-Thy-1.1 and AKR/J ranges. No F1 mice had HSC frequencies within or near the AKR/J range. The F1 data demonstrate that the C57BL/Ka-Thy-1.1 alleles of polymorphic gene(s) that influence HSC frequency are partially dominant.

Analysis of HSC frequency in F2 progeny

The frequencies of Thy-1lowSca-1" Lin"c-kit" cells were assayed in 290 (C57BL/Ka-Thy-1.1 × AKR/J)F2 mice (Table IV). Within each analysis, bone marrow cells from C57BL/Ka and AKR/J mice were analyzed for comparison. Of 32 C57BL/Ka-Thy-1.1 controls analyzed, 29 fell within the normal low range, while three had HSC frequencies intermediate to those normally seen in C57BL/Ka-Thy-1.1 and AKR/J. Of 28 AKR/J mice examined, 26 had HSC frequencies that fell within their normal high range while two mice had intermediate HSC frequencies. Of the F2 mice examined, 32% had low HSC frequencies that were equal to or less than normal C57BL/Ka-Thy-1.1 controls, 12% had high HSC frequencies that were equal to or greater than normal AKR/J controls, and 56% had intermediate HSC frequencies.

Interval mapping of F2 progeny

The genome was scanned to identify chromosome regions that contain genes that regulate HSC frequency. Seventy-six markers spanning the genome (including the X chromosome but not the Y chromosome) were assessed for linkage to HSC frequency in a screen that used F2 mice with low or high HSC frequencies. The genome scan was initially done using DNA isolated from 10 F2 mice with high and 10 F2 mice with low Thy-1lowSca-1" Lin"c-kit" cell frequencies (indistinguishable from the AKR/J or C57BL/Ka-Thy-1.1 parental mice, respectively). Twenty-six F2 mice with high HSC frequencies and 42 F2 mice with low HSC frequencies were then genotyped using a number of markers near several loci that showed evidence of linkage in the initial screen. Ultimately, only one locus showed suggestive or significant linkage to HSC frequency: a region of chromosome 17, around the marker D17Mit65 (Table V). Assuming a partial dominance model, this association was highly significant ($p < 0.001$). Neighboring markers D17Mit65 and D17Mit66 exhibited LOD scores of 4.85 and 4.64, respectively, based on a likelihood ratio test of association for contingency tables (28). Both markers exhibited significant linkage based on criteria for intercross analysis (29).

The influence of the chromosome 17 locus is specific to HSCs

We sought to test whether the locus linked to D17Mit66 influenced progenitor numbers generally or if its effect was specific to HSCs. The frequency of restricted progenitors can be quantified in CAFC assays by counting the number of progenitors that form cobblestone areas at early time points from 7 to 10 days (41, 42). The frequencies of day 8 CAFC were 16.1 ± 6.3/10⁴ WBM cells in C57BL/Ka-Thy-1.1 (n = 14) and 26.5 ± 15.0/10⁴ WBM cells in AKR/J (n = 15). This difference was statistically significant ($p = 0.02$). The day 8 CAFC frequencies we observed were consistent with a previous report (41). Unfortunately, the surface marker phenotypes of restricted progenitors that form day 8 cobblestone areas are not characterized, so this difference in day 8 CAFC cannot be confirmed by assaying for a corresponding difference in the frequency of a phenotypically defined restricted progenitor population. Nonetheless, we assayed for differences in the frequencies of pro-B cells (B220+CD43hiIgM+ (43)), common myeloid progenitors (Lin"IL-7Rc-kitSca-1FcRlow" (44)) and common lymphoid progenitors (Lin"IL-7Rc-kiflowSca-1low" (45)). We did not observe a difference in the frequency of these progenitor populations between AKR/J and C57BL/Ka-Thy-1.1 (data not shown). This suggests that not all progenitor frequencies are regulated differently between these two mouse strains, and provides further evidence that polymorphisms that influence HSC frequencies do not have global effects on all progenitors.

### Table II. The frequencies of multipotent progenitors in C57BL/Ka-Thy-1.1 and AKR/J mice

<table>
<thead>
<tr>
<th>Subpopulation</th>
<th>C57BL/Ka</th>
<th>AKR/J</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lineage&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.013 ± 0.004</td>
<td>0.052 ± 0.023</td>
</tr>
<tr>
<td>Lineage&lt;sup&gt;−&lt;/sup&gt;</td>
<td>0.059 ± 0.014</td>
<td>0.166 ± 0.050</td>
</tr>
</tbody>
</table>

The frequencies of Lineage<sup>+</sup> and Lineage<sup>−</sup> cells were based on paired analyses of 22–29 mice of each strain. The differences in the frequencies of both multipotent progenitor populations were highly statistically significant ($p < 0.001$).

### Table III. The frequency of Thy-1<sup>−</sup>Sca-1" Lin"c-kit" cells in individual C57BL/Ka-Thy-1.1, AKR/J, and (C57BL/Ka-Thy-1.1 × AKR/J)F2 mice

<table>
<thead>
<tr>
<th>Percentage of WBM Cells</th>
<th>C57BL/Ka-Thy-1.1</th>
<th>AKR/J</th>
<th>(C57BL/Ka × AKR/J)F2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.014</td>
<td>0.071</td>
<td>0.034</td>
<td></td>
</tr>
<tr>
<td>0.009</td>
<td>0.065</td>
<td>0.028</td>
<td></td>
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<td></td>
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<td>0.028</td>
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<tr>
<td></td>
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<td>0.024</td>
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<td></td>
<td></td>
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<td></td>
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<td></td>
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<td>0.015</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.014</td>
<td></td>
</tr>
</tbody>
</table>

### Table IV. Distribution of (C57BL/Ka-Thy-1.1 × AKR/J)F2 mice between low, intermediate, and high frequencies of HSC

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Frequency of Thy-1lowSca-1&quot; Lin&quot;c-kit&quot; Cells</th>
<th>Low</th>
<th>Intermediate</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/Ka-Thy-1.1</td>
<td>29</td>
<td>3</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>AKR/J</td>
<td>0</td>
<td>2</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>(C57BL × AKR)F2</td>
<td>94</td>
<td>161</td>
<td>35</td>
<td></td>
</tr>
</tbody>
</table>
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Table V. Significant linkage of HSC frequency to genotype at chromosome 17 loci based on an analysis of (C57BL/Ka-Thy-1.1 × AKR/J)F₂ mice with parental (C57BL or AKR) HSC frequencies

<table>
<thead>
<tr>
<th>Genotype</th>
<th>D17 Mit65 F₂ HSC Frequency</th>
<th>D17 Mit66 F₂ HSC Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
<td>High</td>
</tr>
<tr>
<td>C57BL/C57BL</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>AKR/C57BL</td>
<td>16</td>
<td>10</td>
</tr>
<tr>
<td>AKR/AKR</td>
<td>8</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>26</td>
</tr>
</tbody>
</table>

* F₂ mice were characterized as low or high if they fell within the C57BL/Ka-Thy-1.1 or AKR/J parental HSC frequency ranges, respectively. Values of \( p \) represent probability of obtaining a \( \chi^2 \) statistic by chance at the locus examined that is as great as the one observed. The LOD score represents association of the HSC phenotype with genotype at the given marker. The LOD scores for both markers indicate significant linkage.

Table VI. No evidence for linkage of day 8 CAFC frequency to genotype at D17 Mit66 based on an analysis of (C57BL/Ka-Thy-1.1 × AKR/J)F₂ mice with parental levels of day 8 CAFC

<table>
<thead>
<tr>
<th>Genotype</th>
<th>F₂ Day 8 CAFC Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Low</td>
</tr>
<tr>
<td>C57BL/C57BL</td>
<td>5</td>
</tr>
<tr>
<td>AKR/C57BL</td>
<td>11</td>
</tr>
<tr>
<td>AKR/AKR</td>
<td>4</td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
</tr>
</tbody>
</table>

* F₂ mice were characterized as low or high if their day 8 CAFC frequency fell within the C57BL/Ka-Thy-1.1 (<10/10,000 WBM cells) or AKR/J (>25/10,000 WBM cells) ranges respectively. Likelihood ratio \( \chi^2 \), 0.611. Probability of obtaining a \( \chi^2 \) statistic by chance at the locus examined that is as large as the one observed, \( p \) = 0.737. The LOD score for association of the day 8 CAFC phenotype with genotype at D17 Mit66 was 0.133.

(C57BL/Ka-Thy-1.1 × AKR/J)F₂ mice were assayed for day 8 CAFC frequencies. F₂ mice were then categorized as low (day 8 CAFC <10/10⁴ WBM cells), intermediate, or high (day 8 CAFC >25/10⁴ WBM cells). These mice were then genotyped at selected markers to determine whether loci that were potentially linked to HSC frequency were also linked to restricted progenitor frequency. Table 6 shows that D17 Mit66 was not linked to day 8 CAFC frequency. Thus, while loci linked to D17 Mit66 influenced HSC frequencies, there was no evidence that they influenced the frequencies of more restricted progenitors. D17 Mit65, which is only 0.1 cM away from D17 Mit66, was not genotyped in these mice.

Restricted hematopoietic progenitors are regulated by a distinct locus

Genotyping of F₂ mice with high and low day 8 CAFC frequencies led to the identification of a chromosome 1 locus that appears to influence restricted progenitor frequencies but not HSC frequencies. D1 Mit330 is a marker located around 35 cM on chromosome 1. Table 7 shows that when the D1 Mit330 marker was tested for linkage to day 8 CAFC, a LOD score of 2.8 was generated based on a likelihood ratio test of association. This satisfies the criterion for suggestive linkage in an intercross analysis (29). D1 Mit330 showed no evidence of linkage to HSC frequency (Table 7).

Mapping of the chromosome 17 locus that influences HSC frequency

Additional markers at 5-cM intervals from 9.8 to 49.2 cM on chromosome 17 were genotyped in (C57BL/Ka-Thy-1.1 × AKR/J)F₂ mice that had high or low HSC frequencies. The first six markers (D17 Mit 31, 51, 65, 66, 139, and 87) all exhibited significant linkage to HSC frequency (LOD ≥ 4.3). The next two markers, D17 Mit153 (at 35 cM) and D17 Mit186 (at 39 cM), exhibited suggestive linkage, while the final marker, D17 Mit155 (49 cM), was not linked to HSC frequency. The resulting linkage map is shown in Fig. 3. It demonstrates that at least 20 cM of chromosome 17 proximal to the 30 cM point are significantly linked to HSC frequency. Note that marker positions are based on the Massachussetts Institute of Technology Center for Genome Research website (www.genome.wi.mit.edu). The Jackson Laboratory (Bar Harbor, ME) mouse genome database (www.informatics.jax.org) reports somewhat different positions but is based on analyses of a more distant genetic background.

The AKR/J chromosome 17 locus is not sufficient to increase HSC frequency in a C57BL background

To test whether the AKR/J chromosome 17 locus is sufficient to increase HSC frequencies, the AKR/J chromosome 17 locus between markers D17 Mit31 and D17 Mit87 was bred onto a C57BL background.
background. To do this, C57BL-FlaEg.Thy-1.1 mice were obtained by mating B6.AKR-H2k/FlaEg mice (The Jackson Laboratory) to C57BL/Ka-Thy-1.1 mice and selecting offspring that retained both the Thy.1 marker and the AKR/J-derived H-2 region from B6.AKR-H2k/FlaEg. B6.AKR-H2k/FlaEg mice are a congenic strain in which the H-2 region from AKR/J has been introduced onto a C57BL background. The AKR/J H-2 region for which the B6.AKR-H2k/FlaEg mice are congenic appears to closely correspond to the region exhibiting significant linkage in Fig. 3. That is, these mice were genotyped at all markers from D17 Mit31 to D17 Mit155 and had the AKR allele at all loci that exhibited linkage (D17 Mit31 to D17 Mit87) but had the C57BL allele at the loci that did not exhibit significant linkage: D17 Mit153, 186, and 155 (see Fig. 3). Thus, the C57BL-FlaEg.Thy-1.1 mice represent a C57BL background equivalent to C57BL/Ka-Thy-1.1, except that they contain the AKR/J H-2 region that exhibited linkage to increased HSC frequencies.

The frequencies of Thy-1^low/Sca-1^Lin^c-kit^ transiently reconstituting HSCs and Thy-1^low/Sca-1^Lin^c-kit^ transiently reconstituting multipotent progenitors were significantly higher in AKR/J mice than in C57BL/Ka-Thy-1.1 or in C57BL-FlaEg.Thy-1.1 mice (Table 8). The frequencies of these two populations did not significantly differ between C57BL/Ka-Thy-1.1 and C57BL-FlaEg.Thy-1.1 mice. This suggests that the AKR/J H-2 region is not sufficient to increase HSC frequencies in a C57BL background. The most likely explanation for this is that the influence of linked gene(s) in this region depends on interactions with nonlinked modifying loci. Such loci were not detected in the initial screen, but because only 27 polymorphic markers throughout the genome were analyzed it is likely that additional loci that influence HSC frequencies were not detected. An alternative possibility is that the AKR/J chromosome 17 locus requires an AKR/J allele between D17 Mit87 (which exhibited significant linkage and had AKR alleles in C57BL-FlaEg.Thy-1.1 mice) and D17 Mit153 (which exhibited suggestive linkage and had C57BL alleles in C57BL-FlaEg.Thy-1.1 mice). That is, the linked region may have included an important gene distal to D17 Mit87 that was not included in the congenic region in the C57BL-FlaEg.Thy-1.1 mice. We were unable to generate a higher resolution map in this region because no informative markers were available between D17 Mit87 and D17 Mit153. Thus, it is possible that an AKR/J allele that was not present in the C57BL-FlaEg.Thy-1.1 congenic mice is required in this region to elevate HSC frequencies.

### Table VIII. The AKR/J chromosome 17 locus that is significantly linked to high HSC frequencies is not sufficient to significantly increase HSC numbers when crossed onto a C57BL/Ka-Thy-1.1 background

<table>
<thead>
<tr>
<th>Subpopulation (% of WBM)</th>
<th>Lineage~</th>
<th>Lineage~low</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/Ka-Thy-1.1</td>
<td>0.015 ± 0.002</td>
<td>0.071 ± 0.038</td>
</tr>
<tr>
<td>AKR/J</td>
<td>0.085 ± 0.014</td>
<td>0.334 ± 0.176</td>
</tr>
<tr>
<td>C57BL-FlaEg.Thy-1.1</td>
<td>0.024 ± 0.006</td>
<td>0.106 ± 0.046</td>
</tr>
</tbody>
</table>

* The C57BL-FlaEg.Thy-1.1 mice represent a C57BL background equivalent to C57BL/Ka-Thy-1.1 except that they contain the AKR/J H-2 region that exhibited linkage to increased HSC frequencies. These mice were genotyped at all markers from D17 Mit31 to D17 Mit155 (shown in Fig. 3), and had the AKR allele at all loci that exhibited significant linkage (D17 Mit31 to D17 Mit87) but had the C57BL allele at the other loci: D17 Mit153, 186, and 155. The AKR/J mice had significantly higher frequencies of Lineage~ and Lineage~low multipotent progenitor populations than both of the C57BL strains (p < 0.01), but the two C57BL strains did not significantly differ from each other (p = 0.08–0.17).

### Discussion

AKR/J mice have significantly greater numbers of long-term reconstituting HSCs and day 8 CAFC (restricted progenitors) than C57BL/Ka-Thy-1.1 mice. By screening for linkages between genomic markers and the frequencies of HSCs and restricted progenitors (C57BL/Ka-Thy-1.1 × AKR/J)F2 mice, we found that differences in HSC number were significantly linked to a series of markers within a 20-cM region of chromosome 17; however, this locus was not linked to differences in day 8 CAFC frequencies. Thus, this locus influences the frequency of bone marrow HSCs but not the frequency of more restricted progenitors. The only other evidence of a regulator of HSC frequency but not restricted progenitor frequency came from recent studies of cyclin-dependent kinase inhibitors. p21 deficiency increased the frequency and proliferation of HSCs but not restricted progenitors (24), while p27 deficiency increased the frequency and proliferation of restricted progenitors (25). In support of the idea that HSCs and restricted progenitors are regulated by distinct genetic determinants we further demonstrated that a chromosome 1 locus influenced the frequency of restricted progenitors but not HSCs.

Polymorphisms in p21 do not appear to explain the difference in HSC frequency between AKR and C57BL mice. p21 has been sequenced in several inbred mouse strains, including AKR and C57BL, and the coding sequence was not observed to contain any polymorphisms that affected the amino acid sequence (46). To address whether there is any polymorphism in the promoter or regulatory elements that might affect the p21 expression level in AKR/J and C57BL/Ka, we compared p21 expression level in bone marrow by Western blot and found similar levels of expression (data not shown). To determine whether p21 is similarly up-regulated after irradiation in the two strains, p21 levels were examined by Western blot at 1, 3, and 4 h after irradiation of acutely isolated bone marrow suspensions. p21 was up-regulated normally in both mouse strains and continued to be expressed at similar levels (data not shown). These observations suggest that there are no polymorphisms in regulatory elements that cause detectable differences in the expression level of p21 in AKR/J and C57BL/Ka mice. Thus, while it remains possible that there are differences in p21 expression that are specific to HSCs, it seems unlikely that polymorphisms in p21 could account for the difference in HSC frequencies between AKR/J and C57BL/Ka mice.

The proportions of F2 mice with high and low HSC frequencies suggest that a single locus on chromosome 17 cannot explain all of the variation in HSC number between C57BL/Ka-Thy-1.1 and AKR/J mice. If a single locus with Mendelian inheritance patterns determined the differences in HSC frequency between AKR/J and C57BL/Ka-Thy-1.1, then F2 mice would be expected to be distributed 1:2:1 between low, intermediate, and high HSC frequencies. Instead we observed an ~3:6:1 ratio among F2 mice. It would appear that additional loci that were not detected in our screen also influence HSC frequencies. The influence of such loci could explain why the AKR chromosome 17 locus is not sufficient in a C57BL background to increase HSC frequencies.

Previous studies reported associations between genetic markers and the frequency of primitive hematopoietic progenitors. de Haan and Van Zant (9) looked for linkages between day 35 CAFC frequency and genetic markers in recombinant inbred strains of C57BL/6 and DBA/2. They reported that a 4-cM region of chromosome 18 was linked to CAFC frequency. We genotyped F2 mice at D18 Mit182, which is within 6 cM of the region identified by de Haan and Van Zant (9), but it showed no evidence of linkage to HSC frequency. Either D18 Mit182 was too far from the locus identified by de Haan and Van Zant (9) or the alleles in this region
that influence HSC frequency are polymorphic between C57BL and DBA/2 but not AKR/J. Muller-Sieburg and Riblet (7) also examined C57BL × DBA/2 recombinant inbred strains for loci that influence primitive progenitor numbers, but they quantified primitive progenitors using the LTC-IC assay. They reported an association with two chromosome 1 loci, linked to Aegr at ~53 cM and Adprp at ~98 cM. An important question is whether the assays used in the three studies detect the same progenitors. Day 35 CAFC and LTC-IC cannot both accurately estimate HSC numbers, because Muller-Sieburg and Riblet (7) estimated based on LTC-IC that BALB/c had five times more HSCs than C57BL/6, while de Haan and Van Zant (8) estimated based on day 35 CAFC that BALB/c had fewer HSCs than C57BL/6. Trends in HSC frequencies based on day 35 CAFC measurements by de Haan and Van Zant (8) have been similar to trends that we have reported based on direct determination of the numbers of Thy-1low Sca-1+ Lin-c-kit+ cells in this and previous studies (compare Refs. 8 and 38).

Mapping of the chromosome 17 locus that influences the number of long-term self-renewing HSCs indicates that markers within an ~20-cM region were significantly linked to HSC frequency. The large size of the linked region suggests that HSC frequency may be regulated by multiple genes in the region. Unfortunately, developing an accurate consensus map of markers and genes within this region of chromosome 17 is particularly difficult because it contains inversions which differentially suppress recombination between different species of mice. This means that observed map distances vary considerably (by around 5 cM) between different genetic backgrounds. The markers have not been mapped on an AKR × C57BL background, but approximate locations were assigned based on the Massachusetts Institute of Technology database (www.genome.wi.mit.edu). Note that somewhat different genetic distances are reported in the Mouse Genome Database (The Jackson Laboratory; www.informatics.jax.org) but these distances were based primarily on crosses between different species of mice (Mus spretus and Mus musculus). Thus only approximate locations can be assigned to the markers and genes in the AKR × C57BL background.

Although precise positions of genes in this region cannot be determined without specifically mapping them in an AKR × C57BL background, several interesting candidate genes do fall within the linked region. The most striking aspect of the region is that it includes the H-2 complex, which contains most histocompatibility genes and many genes that regulate immune responsiveness. These include class I MHC alleles that are targets of NK cells and that act as barriers to the allotransplantation of HSCs (47, 48). NK cells inhibit HSC engraftment without killing (49) but perhaps by the secretion of factors such as TNF. The lymphoxygen/TNF complex is also contained within the linked region. Lymphoxygenin A and B and TNF are widely expressed by hematopoietic cells and are known to be polymorphic between C57BL and AKR (50–52). TNF inhibits HSC proliferation (53), and TNFR-deficient mice have elevated numbers of primitive hematopoietic progenitors (54). Further study will be required to determine whether these effects are specific to HSCs.

In addition to the H-2 complex, Notch3 and Notch4 (55) are also in the linked region. Notch receptors are intriguing because they regulate stem cell numbers in the nervous system by influencing whether the stem cells self-renew or differentiate (56–58). Furthermore, Notch activation promotes the survival and self-renewal of human HSCs in culture (59). Another factor contained within the linked region is vascular endothelial growth factor (VEGF), which binds to receptors Flk-1 and Flt-1. Flk-1-deficient mice have greatly reduced numbers of hematopoietic progenitors and fail to form yolk sac blood islands (60). VEGF receptors are expressed by human HSCs (61), and VEGF protected human progenitors from radiation-induced cell death (62). Mechanisms that might account for the partially dominant role of the C57BL allele in reducing HSC numbers would be relatively complex, unless there is a clear dose response relationship of HSC to Notch or Flk-1 activation.

It is tempting to speculate that the alleles that account for the increased frequency of HSCs are also involved in the greatly increased incidence and early onset of lymphoma in AKR mice (63). The linked region contains two ubiquitously expressed cell cycle regulators, p21 (cyclin-dependent kinase inhibitor 1A, a tumor suppressor gene) and cyclin F (64, 65). Protooncogenes in the region include Pim1 (66), Pim2 (67), and vav (68). Each is known to be expressed in hematopoietic cells, and at least Pim1 is polymorphic between AKR and C57BL.

Although the AKR chromosome 17 region was not sufficient to increase HSC numbers in a C57BL background, perhaps it would be sufficient to increase HSC frequencies in other genetic backgrounds that are associated with low HSC frequencies. If so, it may be possible to positionally clone the polymorphic genes that affect HSC frequency on chromosome 17 by breeding this locus onto other genetic backgrounds.

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