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Genetics

Pluripotent hematopoietic stem cells contain high levels of mRNA for c-*kit*, GATA-2, p45 NF-E2, and c-*myb* and low levels or no mRNA for c-*fms* and the receptors for granulocyte colony-stimulating factor and interleukins 5 and 7

(growth factor receptors/transcription factors/gene expression)

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ABSTRACT **Pluripotent hematopoietic stem cells (PHSCs) were highly enriched from mouse bone marrow by counterflow centrifugal elutriation, lineage subtraction, and fluorescence-activated cell sorting based on high c-*kit* receptor expression (c-*kit*^{BR}). We used reverse transcriptase polymerase chain reaction to assay the c-*kit*^{BR} subset and the subsets expressing low (c-*kit*^{DULL}) and no (c-*kit*^{NEG}) c-*kit* receptor for expression of mRNA encoding hematopoietic growth factor receptors and transcription factors. The c-*kit*^{BR} cells had ≈3.5-fold more c-*kit* mRNA than unfractionated bone marrow cells. The c-*kit*^{DULL} cells had 47–58% of the c-*kit* mRNA found in c-*kit*^{BR} cells and the c-*kit*^{NEG} cells had 4–9% of the c-*kit* mRNA present in c-*kit*^{BR} cells. By comparing mRNA levels in c-*kit*^{BR} cells (enriched for PHSCs) with those of unfractionated bone marrow, we demonstrated that c-*kit*^{BR} cells contained low or undetectable levels of mRNA for c-*fms*, granulocyte colony-stimulating factor receptor, interleukin 5 receptor (IL-5R), and IL-7R. These same cells had moderate levels of mRNA for erythropoietin receptor, IL-3R subunits IL-3Rα (SUT-1), AIC-2A, and AIC-2B, IL-6R and its partner gp-130, and the transcription factor GATA-1 and high levels of mRNA for transcription factors GATA-2, p45 NF-E2, and c-*myb*. We conclude from these findings that PHSCs are programed to interact with stem cell factor, IL-3, and IL-6 but not with granulocyte or macrophage colony-stimulating factor. These findings also indicate that GATA-2, p45 NF-E2, and c-*myb* activities may be involved in PHSC maintenance or proliferation.**

All circulating blood cells are derived from a limited number of pluripotent hematopoietic stem cells (PHSCs). PHSCs in mouse bone marrow (BM) are a rare population of cells (estimates vary between 1/10,000 and 1/100,000) that are capable of long-term reconstitution of the entire lympho-hematopoietic system of lethally irradiated mice (1–3). The properties of PHSCs differ markedly from those of the more numerous, committed hematopoietic progenitor cells such as erythroid-burst-forming units (BFU-E) and granulocyte/macrophage colony-forming units (CFU-GM) (for review see ref. 4). The progenitor cells (≈1/200–500 BM cells) respond to late-acting growth factors and differentiate into specific cell lineages. Since their ability to self-renew is limited they can only provide short-term repopulation. The discovery of the hematopoietic growth factors that control proliferation and differentiation of BFU-E and CFU-GM has been a direct consequence of the availability of specific clonogenic *in vitro* assays (5, 6). Similar assays for the proliferation and differentiation of PHSCs would be useful for studies attempting to expand PHSCs prior to retroviral transduction and BM transplantation.

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Several papers have described procedures to separate PHSCs from whole BM populations. Spangrude *et al.* (7) obtained a subset of BM cells enriched for PHSCs and spleen colony-forming units (CFU-S) by subtracting cells that express lineage-specific antigens associated with mature cells (Lin$^-$) followed by flow cytometry based on low expression of Thy-1.1 and high expression of Sca-1 antigens. Using another fractionation protocol, Jordan *et al.* (8) enriched fetal liver PHSCs by sorting Lin$^-$ cells on the basis of AA4.1 antigen expression. Although we have not purified PHSCs to homogeneity, we (9, 10) and others (11) have enriched PHSCs from mouse BM on the basis of high expression of c-*kit* (c-*kit*^{BR}) receptor. By combining counterflow centrifugal elutriation at a flow rate of 25 ml/min (FR25) with lineage subtraction and c-*kit* selection, we were able to isolate a population of FR25 Lin$^-$ c-*kit*^{BR} cells highly enriched for PHSC activity and nearly depleted of CFU-S. As few as 100–200 cells from this population completely repopulated the lympho-hematopoietic system in the *W*BB6 F1 *W*/*W*^{v} mouse. PHSCs were also highly enriched in populations of Lin$^-$ c-*kit*^{BR} cells elutriated at 35 ml/min (FR35 Lin$^-$ c-*kit*^{BR}), but this population contained CFU-S as well. No PHSCs were found in the c-*kit*^{DULL} and c-*kit*^{NEG} subsets (10).

Enriched populations of PHSCs provide an opportunity to study some of the molecular events that occur in these most primitive of all hematopoietic cells. Jordan *et al.* (8) generated a cDNA library from RNA extracted from Lin$^-$ AA4.1$^+$ fetal liver cells and identified the cDNA for a hematopoietic-specific tyrosine kinase receptor, *flk*-2. Using adult BM, we were able to detect *flk*-2 mRNA in the total cellular RNA extracted from FR35 Lin$^-$ c-*kit*^{BR} cells but not in the PHSC-enriched FR25 Lin$^-$ c-*kit*^{BR} cells (10). This suggested that there may be differences in gene expression between PHSCs in fetal liver and adult BM. It also suggested that the phenotypically different PHSCs in the FR25 Lin$^-$ c-*kit*^{BR} and FR35 Lin$^-$ c-*kit*^{BR} fractions may express differences in mRNA levels for receptors for early- and late-acting growth factors and transcription factors.

To test the hypothesis that specific hematopoietic growth factor receptors and transcription factors are differentially expressed in heterogeneous populations of mouse PHSCs, we used reverse transcriptase polymerase chain reaction (RT-PCR)

Abbreviations: PHSC, pluripotent hematopoietic stem cell; CFU-S, colony-forming unit(s), spleen; FR25 and FR35, flow rates of 25 and 35 ml/min; Lin$^-$, lineage negative; IL, interleukin; ILR, IL receptor; BM, bone marrow; RT-PCR, reverse transcriptase polymerase chain reaction; β_2m, β_2-microglobulin; FACS, fluorescence-activated cell sorting; G-CSFR, granulocyte colony-stimulating factor receptor; EpoR, erythropoietin receptor; ES, embryonic stem; EB, embryoid bodies.

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to analyze mRNA levels in PHSC-enriched and PHSC-depleted BM fractions. The PHSC-enriched FR25 Lin$^-$ c-*kit*^{BR} fraction expressed high levels of mRNAs encoding the c-*kit* receptor, as predicted from our flow cytometry data, and high levels of mRNA for the transcription factors GATA-2, p45 NF-E2, and c-*myb*. This same fraction contained low levels or no mRNA for c-*fms*, granulocyte colony-stimulating factor receptor (G-CSFR), interleukin 5 receptor (IL-5R), and IL-7R.

METHODS

Mice. Adult, female C57BL/6 mice 3–5 weeks old were used in this study.

Fractionation of BM Cells. Single cell suspensions were fractionated by counterflow centrifugal elutriation followed by subtraction of lineage-positive cells as described (10, 12). The FR25 Lin$^-$ and FR35 Lin$^-$ cell populations were fractionated by fluorescence-activated cell sorting (FACS) into subsets expressing high (c-*kit*^{BR}), low (c-*kit*^{DULL}), and no (c-*kit*^{NEG}) c-*kit* receptor.

RT-PCR. Total RNA from unfractionated BM and the FR25 Lin$^-$ c-*kit*^{BR}, c-*kit*^{DULL}, and c-*kit*^{NEG} and FR35 Lin$^-$ c-*kit*^{BR}, c-*kit*^{DULL}, and c-*kit*^{NEG} fractions obtained by FACS was prepared using RNAzol B (Tel-Test, Friendswood, TX) and first strand cDNA was prepared according to the manufacturer's instructions (Perkin–Elmer/Cetus). The cDNA was amplified by PCR at 94°C for 30 sec, 58°C for 1 min, and 72°C for 2 min using 33 cycles for β_2-microglobulin (β_2m) cDNA and 36 cycles for all other cDNAs.

Quantitation of the mRNA Populations Amplified by RT-PCR. To obtain an estimate of the concentration of RNA in each sample (13), limiting dilution aliquots of RNA were assayed by RT-PCR to identify the linear and plateau portions of the β_2m cDNA amplification curve.

The mRNA levels obtained for β_2m were used as internal standards in our estimates of the mRNA levels for receptor and transcription factor genes. The ratio of mRNA for these genes to β_2m mRNA was determined for each sample of fractionated BM. These ratios were compared with the ratio obtained for unfractionated BM in order to quantify relative gene expression in each fraction of BM cells (see Fig. 2).

RESULTS

Purification of Cells Expressing c-*kit*. The results of four BM fractionations are seen in Table 1. The FR25 Lin$^-$ and FR35 Lin$^-$ subsets contained an average of 3% and 4% c-*kit*^{BR} cells, 7% and 16% c-*kit*^{DULL} cells, and 90% and 80% c-*kit*^{NEG} cells, respectively. Approximately 1×10^5 and 2×10^5 cells were obtained for the FR25 Lin$^-$ c-*kit*^{BR} and FR35 Lin$^-$ c-*kit*^{BR} subsets and these had an average c-*kit*^{BR} purity of 88% and 91% by FACS analysis.

Morphologic Analysis of Purified Cell Populations. The cells in FR25 and FR35 Lin$^-$ c-*kit*^{BR} and c-*kit*^{DULL} fractions were mononuclear cells with a few contaminating erythroid and granulocytic cells when viewed in cytocentrifuge preparations. In contrast, numerous immature erythroblasts and myelocytes were present in the FR25 Lin$^-$ c-*kit*^{NEG} and FR35 Lin$^-$ c-*kit*^{NEG} subsets. This information was used to interpret our data regarding c-*fms*, erythropoietin receptor (EpoR), and transcription factors.

Quantitative Analysis of mRNA Using RT-PCR. The primer pairs were designed to span one or more intron/exon boundary. Each primer was 17–27 nt in length (Table 2). We used a limiting dilution RT-PCR for unfractionated BM and for each BM fraction to obtain an estimate of the mRNA level for the constitutively expressed β_2m gene (14). By selecting a point on the linear phase of the reaction curve we were able to equalize the quantity of RNA used in each subsequent RT-PCR (Fig. 1).

RT-PCR Analysis of Receptor mRNA. To confirm the flow cytometry data, we quantified the level of c-*kit* mRNA in FR25 Lin$^-$ c-*kit*^{BR}, c-*kit*^{DULL}, and c-*kit*^{NEG} and in FR35 Lin$^-$ c-*kit*^{BR}, c-*kit*^{DULL}, and c-*kit*^{NEG} cells. Primers were used that amplified two cDNA transcripts, 95 and 107 bp in length, referred to as c-*kit* and c-*kit*A, respectively (19). Data for the fractionated cells are presented as the ratio of mRNA for c-*kit* (c-*kit* plus c-*kit*A) to the mRNA for β_2m divided by the ratio obtained for unfractionated BM (Fig. 2).

Table 1. Results of flow cytometry based on c-*kit* expression

Sample*	% of total†	Average no. of cells‡	% purity§
FR25 Lin$^-$ c-*kit*^{BR}	3	99,000	88
FR25 Lin$^-$ c-*kit*^{DULL}	7	243,000	87
FR25 Lin$^-$ c-*kit*^{NEG}	90	982,000	99
FR35 Lin$^-$ c-*kit*^{BR}	4	189,000	91
FR35 Lin$^-$ c-*kit*^{DULL}	16	549,000	91
FR35 Lin$^-$ c-*kit*^{NEG}	80	920,000	94

*Samples were obtained by counterflow centrifugal elutriation, lineage subtraction, and staining with anti c-*kit* monoclonal antibody.
†Estimates of the percent of FR25 Lin$^-$ and FR35 Lin$^-$ fractions sorted as c-*kit*^{BR}, c-*kit*^{DULL}, and c-*kit*^{NEG} cells.
‡The c-*kit*^{BR} cells were collected throughout the entire sorting procedure, whereas the second stream was alternated between the c-*kit*^{DULL} and c-*kit*^{NEG} subsets.
§The purity of each c-*kit* fraction was determined by FACS reanalysis.

Both c-*kit*^{BR} fractions had ≈3.5-fold more c-*kit* mRNA than the cells in unfractionated BM. The c-*kit*^{DULL} subsets had 48–58% of the c-*kit* mRNA found in the c-*kit*^{BR} subsets and the c-*kit*^{NEG} subsets had 4–9% of the c-*kit* transcripts found in their respective c-*kit*^{BR} subsets. These results were consistent with our FACS analysis of these fractions and demonstrated that the RT-PCR assay was sufficiently accurate to quantify cytokine receptor transcript levels in different subsets of hematopoietic cells. Unfractionated BM and all fractionated populations contained both c-*kit* and c-*kit*A transcripts. In each sample the expression of c-*kit*A was lower than that of c-*kit* with the ratio of c-*kit*A:c-*kit* being highest (1:2) in the two c-*kit*^{BR} subsets and lowest (1:12 and 1:6) in the two c-*kit*^{NEG} subsets (Table 3). These differences in c-*kit*A expression in the c-*kit*^{BR} and c-*kit*^{NEG} subsets were significant ($P < 0.003$).

The results of our survey of cytokine receptor mRNAs are shown in Table 4. The mRNA levels for G-CSFR, IL-5R, and IL-7R were low or undetectable in the PHSC-enriched, CFU-S-depleted FR25 Lin$^-$ c-*kit*^{BR} fraction and in the FR35 Lin$^-$ c-*kit*^{BR} fraction, which contained both PHSCs and CFU-S. In contrast, the FR25 Lin$^-$ c-*kit*^{BR} cells contained significantly less mRNA for c-*fms* and EpoR compared with the FR35 Lin$^-$ c-*kit*^{BR} subset. The levels of mRNA for c-*fms* and G-CSFR in the FR35 Lin$^-$ c-*kit*^{NEG} fraction, which contained the myelocytes not removed by lineage subtraction, were 57-fold and 66-fold higher than the levels of these mRNAs in the myelocyte-depleted FR25 Lin$^-$ c-*kit*^{BR} fraction.

The difference between the RNA levels in FR25 Lin$^-$ c-*kit*^{BR} and FR35 Lin$^-$ c-*kit*^{BR} cells did not differ significantly for 10 of the 12 receptor mRNAs assayed. However, the mean mRNA levels in the FR25 Lin$^-$ c-*kit*^{BR}, c-*kit*^{DULL}, and c-*kit*^{NEG} cells were generally lower than those observed in the corresponding FR35 Lin$^-$ c-*kit*^{BR}, c-*kit*^{DULL}, and c-*kit*^{NEG} cells. We also observed that mRNA levels in the FR25 Lin$^-$ c-*kit*^{BR} cells were generally lower than the levels present in the FR25 Lin$^-$ c-*kit*^{DULL} and FR25 Lin$^-$ c-*kit*^{NEG} cells.

The mRNA for *Etk2*, a candidate growth factor receptor (20), is expressed in embryonic stem (ES) cells, in 4- to 20-day embryoid bodies (EB) containing mature hematopoietic cells and progenitors, and in adult brain. However, we were not able to detect mRNA for *Etk2* in unfractionated adult BM or in any of the fractions obtained by flow cytometry (data not shown).

RT-PCR Analysis of Transcription Factor mRNA. We assayed for the expression of several transcription factors involved in erythropoiesis (Table 4). To determine whether contaminating erythroid progenitors or erythroblasts were present in the frac-

Table 2. Primer pairs used for RT-PCR analysis of mRNA levels for cytokine receptors and transcription factors

Gene		Oligonucleotide primers	nt*	Ref.
β_2m	s	5′-TGCTATCCAGAAAACCCCTC-3′	258	14
	a	3′-GGACGTCTCAATTCGTACTG-5′		
c-*kit*†	s	5′-GGGCAAGAGTTCCGCCTTCTT-3′	95 and 105	NA
	a	3′-CCGAAACACCAGCGTCG-5′		
c-*fms*	s	5′-CTGAGTCAGAAGCCCTTCGACAAAG-3′	449	15‡
	a	3′-CGATGTCGGAAACCAGACCCGTTTC-5′		
EpoR	s	5′-AGGCCGCACTGAGTGTGTTC-3′	356	39
	a	3′-ACCAACAGACACCACCTCGG-5′		
IL-6R	s	5′-GTTCTACAGAAGCAACGAGTGTCCTC-3′	336	15‡
	a	3′-GGAATACTGTTGTCGTTGTCTCTGA-5′		
gp-130	s	5′-CTCACCTGCAACATCCTGTCCT-3′	249	16
	a	3′-ACCGTGAAGTACATACCAGTCG-5′		
IL-3Rα	s	5′-GAACAGATTCCACCATGGCCTCCTTG-3′	228	15‡
	a	3′-GTCTCCACTACGGACACTTCTGTC-5′		
AIC-2A	s	5′-CAGCCTTCGCGACTAAGAAC-3′	242	15‡
	a	3′-TTAAGGAAGATCGGCTGGGTCC-5′		
AIC-2B	s	5′-AAGATGGCTTACTCATTCATT-3′	654	15‡
	a	3′-GTTGAGGATTGTCTGGATCC-5′		
G-CSFR	s	5′-CGTCACCCTAAACATCTCCCTCCAT-3′	309	15‡
	a	3′-CAACGGGTGGTAGTACTGTCTCCTT-5′		
IL-5Rα	s	5′-GCCCTTTGATCAGCTGTTCAGTCCAC-3′	451	15‡
	a	3′-CTGGTCCAACAAAGGTGGCCAAGGC-5′		
IL-7R	s	5′-CAAAGTCCGATCCATTCCCCATAAC-3′	237	15‡
	a	3′-GGATCAGAGGGGCTAGTATTCTTTTG-5′		
β-Globin	s	5′-GAAGTTGGGTGCTTGGAGAC-3′	401	17
	a	3′-GCTAAGATCCCTCGTCAGAG-5′		
GATA-1	s	5′-GGAGCCCTCTCAGCTCAGC-3′	470	18
	a	3′-GACTTCCTGGTCGACCACCG-5′		
GATA-2§	s	5′-GGCGTCAAGTACCAAGTGTCAC-3′	258	NA
	a	3′-GAGGACGAGTCTTCCGGCCCTC-5′		
p45 NF-E2§	s	5′-GGGGCCAACCGTGCTCCGCGCCCA-3′	210	NA
	a	3′-TTAGGAAACACGAACACCTCTGGA-5′		
c-*myb*	s	5′-GAGCTTGTCCAGAAATATGGTCCGAAG-3′	523	15‡
	a	3′-CAGGGAGTCGGCCGACGCCGTCGG-5′		
Etk2	s	5′-ACCAAATTGCCCAATCCCAG-3′	593	NA
	a	3′-GACCAGGTCCTCAGTCCGTAAA-5′		

s, Sense; a, antisense; NA, not applicable.
*Number of nucleotides (nt) in the amplified fragments.
†Provided by Xiao Piao and Alan Bernstein (Mount Sinai Hospital, Toronto).
‡See ref. 15 for original references for these primer pairs.
§Provided by Nancy C. Andrews and Stuart H. Orkin (the Children's Hospital, Boston).

tionated BM subsets we tested for the presence of β-globin mRNA. The levels of mRNA for β-globin in the FR25 Lin$^-$ c-*kit*^{BR} and FR35 Lin$^-$ c-*kit*^{BR} subsets were 16% and 4%, respectively, of unfractionated BM, indicating that some erythroid cells were present in both PHSC-enriched cell populations.

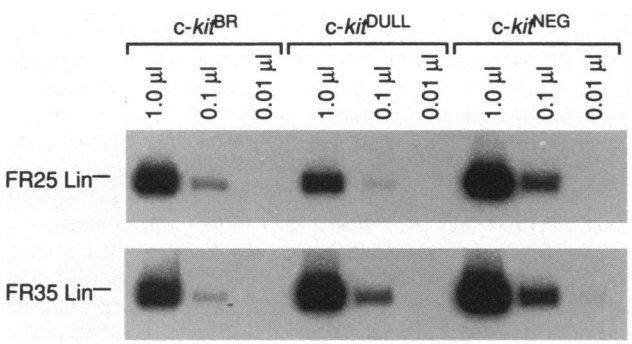

FIG. 1. RT-PCR analysis of the concentration of β_2m mRNA in each of six subsets of BM cells obtained by flow cytometry. This limiting dilution assay enabled us to determine the linear and plateau phases of the PCR. The RNA in each BM fraction was adjusted by selecting a point on the linear phase of the reaction and this volume of RNA was then used in all subsequent assays.

All three FR25 Lin$^-$ c-*kit* fractions contained moderate levels of GATA-1 mRNA including the c-*kit*^{DULL} and c-*kit*^{NEG} fractions, which had higher levels of β-globin mRNA. Moderate to low levels of GATA-1 were found in the FR35 Lin$^-$ c-*kit*^{BR}, c-*kit*^{DULL}, and c-*kit*^{NEG} fractions. In contrast, the expression of GATA-2 and p45 NF-E2 was extremely high in both PHSC-enriched c-*kit*^{BR} fractions. This pattern was reversed in the two PHSC and CFU-S-depleted c-*kit*^{NEG} fractions, which showed significantly lower quantities of both GATA-2 and p45 NF-E2 mRNA. The level of c-*myb* mRNA followed a pattern similar to that of GATA-2 and p45 NF-E2 with the highest levels in the two c-*kit*^{BR} fractions and the lowest levels in the two c-*kit*^{NEG} fractions.

DISCUSSION

In this study we have analyzed mRNA levels in highly enriched populations of PHSCs that have been separated from most progenitors and maturing blood cells on the basis of c-*kit* expression. Several methods currently in use for quantitative PCR include the addition of cloned genomic templates (21) or deleted versions of sample cDNA templates (22). This allows use of the same primer set for the standard and sample template; however, amplification efficiency may still differ due to

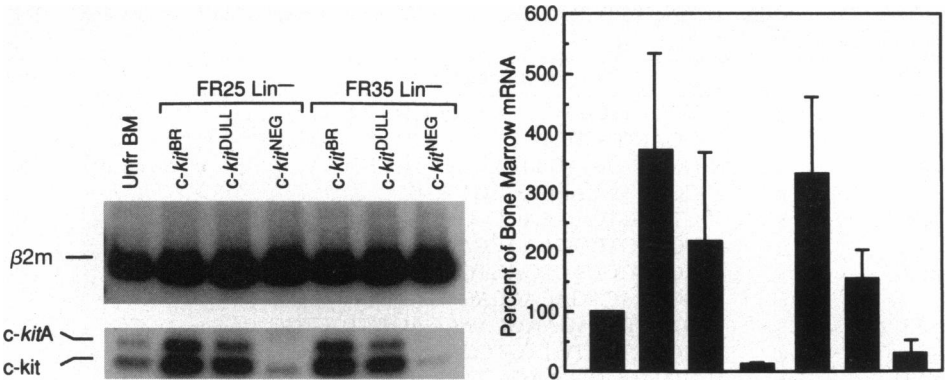

FIG. 2. RT-PCR assay of β_2m, c-*kit*, and c-*kit*A mRNA in unfractionated (Unfr) BM and fractionated (Fr) BM (FR25 and FR35). The quantity of RNA in each sample was approximately equal as seen by the β_2m bands. The c-*kit*A (107 bp) band is evident in the c-kit^{BR} and c-kit^{DULL} cells but is difficult to resolve in the two c-kit^{NEG} subsets. The c-*kit* (95 bp) band is seen in all fractions. The c-*kit* and c-*kit*A isoforms in each fraction were quantified as a combined single value. Each mean ± SD is based on four separate experiments. These values and those reported in Table 3 were obtained using the following equation:

$$\left(\frac{\text{Test gene mRNA in FrBM}}{\beta_2\text{m gene mRNA in FrBM}} \Big/ \frac{\text{test gene mRNA in Unfr BM}}{\beta_2\text{m gene mRNA in Unfr BM}}\right) \times 100 = \%\text{Unfr BM mRNA}$$

$$\left[\frac{\text{c-}kit\text{ mRNA in FR25 Lin}^- \text{ c-}kit^{BR}\ (126{,}406)}{\beta_2\text{m mRNA in FR25 Lin}^- \text{ c-}kit^{BR}\ (272{,}682)} \Big/ \frac{\text{c-}kit\text{ mRNA in Unfr BM}\ (19{,}064)}{\beta_2\text{m mRNA in Unfr BM}\ (143{,}141)}\right] = 353\%.$$

differences in template size. We chose to use an endogenous internal standard, β_2m, based on our earlier finding that β_2m and β-actin primer sets had a very similar efficiency of amplification and either could be used to quantify MDR-1 and c-*kit* cDNA (13).

The high level of c-*kit* mRNA in the FR25 and FR35 Lin⁻ c-kit^{BR} fractions suggested that c-*kit* receptor has a rapid turnover rate as has been demonstrated for other receptors (23). Both isoforms of c-*kit* mRNA were present in these fractions. Although the functional importance of these isoforms has not been established, it has been suggested that the ratio of c-*kit*A to c-*kit* has an effect on dimerization of c-*kit* receptor subunits, thereby affecting the ability of c-*kit* receptor to bind its ligand, stem cell factor (19).

The PHSC-enriched c-kit^{BR} cells contain moderate levels of mRNA for IL-6R, gp-130, IL-3Rα, AIC-2A, and AIC-2B. This supports other findings (24–27) that indicate PHSC and CFU-S proliferation *in vitro* is enhanced by a combination of stem cell factor, IL-3, and IL-6 but not by any one of these cytokines alone. The expression of mRNA for IL-3R and IL-6R subunits in all BM fractions supports a large body of data showing that IL-3 and IL-6 have pleiotropic effects in hematopoiesis.

Of the 12 cytokine receptor mRNAs assayed, only c-*fms* and EpoR mRNA levels were significantly lower in the PHSC-enriched FR25 Lin⁻ c-kit^{BR} fraction, which contains few CFU-S, compared with the FR35 Lin⁻ c-kit^{BR} fraction, which contains a large number of CFU-S and progenitor cells. The CFU-S or the many BFU-E in the FR35 fraction (28) may contribute the higher level of EpoR mRNA (29) present in the FR35 Lin⁻ c-kit^{BR} cells. Low levels of EpoR mRNA may be present in the PHSCs of both c-kit^{BR} fractions without conferring a capacity to respond to Epo. Migliaccio *et al.* (30) have suggested that mRNA expression is not correlated with EpoR formation since the translocation of the receptor to the cell membrane may be erythroid-specific. Similarly, the FR25 Lin⁻ c-kit^{BR} cells contained significantly less c-*fms* mRNA compared with the progenitor-enriched FR35 Lin⁻ c-kit^{BR} cells and the FR35 Lin⁻ c-kit^{NEG} population, which contained many myelocytes (12). We believe that these findings support the concept that PHSCs lack receptors for late-acting cytokines such as Epo and M-CSF.

Table 3. RT-PCR analysis of the ratio of c-*kit*A to c-*kit* isoforms

Sample	Ratio*	n†	P value
FR25 Lin⁻ c-kit^{BR}	0.46 ± 0.05	5	0.001‡
FR25 Lin⁻ c-kit^{DULL}	0.44 ± 0.06	3	0.018‡
FR25 Lin⁻ c-kit^{NEG}	0.08 ± 0.14	3	—
FR35 Lin⁻ c-kit^{BR}	0.46 ± 0.03	5	0.003§
FR35 Lin⁻ c-kit^{DULL}	0.36 ± 0.11	3	0.122
FR35 Lin⁻ c-kit^{NEG}	0.16 ± 0.14	3	—

*Mean ± SD.
†Number of experiments.
‡Significantly different from FR25 Lin⁻ c-kit^{NEG}.
§Significantly different from FR35 Lin⁻ c-kit^{NEG}.

G-CSFR, IL-5R, and IL-7R were expressed at very low or undetectable levels in both PHSC-enriched subsets. Although these subsets were not purified to homogeneity, our inability to detect mRNA for G-CSFR, IL-5R, and IL-7R indicates that the PHSCs in these subsets do not express these receptors. The absence of mRNA for G-CSFR was an unexpected finding since earlier studies demonstrated that G-CSF can mobilize PHSCs from BM into the circulation (31) and together with stem cell factor can induce PHSC proliferation (32). Our results suggest that G-CSF may exert an indirect effect on PHSCs or, if its action is direct, may only interact with a small population of PHSCs.

The role of GATA-1, GATA-2, p45 NF-E2, and c-*myb* in hematopoiesis is still largely unknown but there are indications that these transcription factors are expressed in early cells of several hematopoietic lineages (33–37). The transcription factors GATA-1 and GATA-2 bind to a concensus element found in the enhancers and promotors of globin loci, whereas the erythroid-specific nuclear factor, p45 NF-E2, binds to the locus control region of the globin genes. We found the highest levels of mRNA for GATA-2 and p45 NF-E2 in the primitive c-kit^{BR} cell populations and lower levels in more mature cell subsets. Thus GATA-2 and p45 NF-E2 may induce differentiation or proliferation in primitive PHSCs and CFU-S. Mice with disrupted GATA-2 genes fail to develop hematopoietic activity (40). Similarly, down-regulation of c-*myb* is a prerequisite for Epo-induced erythroid differentiation (37). Our data support the concept that these transcription factors act on the most primitive BM cells in FR25 Lin⁻ c-kit^{BR} and FR35 Lin⁻ c-kit^{BR} fractions.

Others have studied the expression of hematopoiesis-related genes in ES cells and in the EB they produce in culture. Erythroid precursors appear in EB at day 4 followed by macrophages and myelocytes. Although their quantitative

Table 4. RT-PCR analysis of mRNA contained in fractionated BM cells

Gene	FR25 Lin$^-$ c-*kit*^{BR}	FR25 Lin$^-$ c-*kit*^{DULL}	FR25 Lin$^-$ c-*kit*^{NEG}	FR35 Lin$^-$ c-*kit*^{BR}	FR35 Lin$^-$ c-*kit*^{DULL}	FR35 Lin$^-$ c-*kit*^{NEG}
c-*kit*	371 ± 163	217 ± 150	13 ± 1*	332 ± 128	155 ± 48*	30 ± 22*
c-*fms*	14 ± 6	76 ± 45	260 ± 138*	188 ± 79*	83 ± 62	803 ± 131*†
EpoR	33 ± 4	11 ± 12*	75 ± 66	127 ± 55*	40 ± 27	42 ± 49
IL-6R	55 ± 63	100 ± 92	61 ± 41	94 ± 103	312 ± 134*†	102 ± 69
gp-130	93 ± 79	291 ± 143	47 ± 33	339 ± 339	193 ± 97	105 ± 90
IL-3Rα	130 ± 57	136 ± 48	95 ± 18	342 ± 217	228 ± 61	326 ± 50*†
AIC-2A	55 ± 22	138 ± 72	40 ± 19	65 ± 39	249 ± 104*	70 ± 40
AIC-2B	59 ± 46	71 ± 42	35 ± 9	89 ± 71	166 ± 44*†	73 ± 33
G-CSFR	1 ± 3	8 ± 12	16 ± 10*	3 ± 3	223 ± 185*†	66 ± 24*
IL-5R	0 ± 0	ND	151 ± 173	ND	ND	ND
IL-7R	0 ± 0	237 ± 257	37 ± 26	1 ± 3	4 ± 5	6 ± 6
β-Globin	16 ± 12	123 ± 90*	87 ± 10*	4 ± 2	ND	26 ± 24†
GATA-1	73 ± 33	62 ± 53	46 ± 40	129 ± 55	35 ± 15	2 ± 2*
GATA-2	749 ± 269	228 ± 100*	141 ± 46*	618 ± 106	205 ± 167*	312 ± 166
p45 NF-E2	811 ± 441	210 ± 208*	214 ± 258	709 ± 786	320 ± 297	188 ± 37†
c-*myb*	157 ± 101	70 ± 32	27 ± 30	162 ± 93	32 ± 19*	5 ± 3*

All values are given as percent mean ± SD relative to unfractionated BM (mean of three or four experiments). ND, not determined.
*Significantly different from FR25 Lin$^-$ c-*kit*^{BR} ($P < 0.05$).
†Significantly different from its corresponding FR25 Lin$^-$ c-*kit* fraction ($P < 0.05$).

estimates differed, the studies of Keller *et al.* (38) and McClanahan *et al.* (15) detected c-*kit* mRNA in ES cells and early EB. Also, both demonstrated a later appearance of mRNA for the cytokine receptors c-*fms* and AIC-2A. McClanahan *et al.* (15) further observed that in comparison to the high-level expression of c-*kit* mRNA in ES cells and early EB, the mRNA for G-CSFR, IL-5R, and IL-7R occurred at only trace levels until later stages of EB and the onset of hematopoietic development. Although it is unclear whether any cells in the EB correspond to the PHSCs of adult BM, this pattern of receptor development is similar to what we find in PHSC-enriched FR25 Lin$^-$ c-*kit*^{BR} cells.

The ES cell and EB system can be used as a model for embryonic hematopoiesis; however, the EB include cells of many developing systems. Identification of c-*kit* mRNA in EB may not necessarily indicate hematopoietic activity since c-*kit* is also involved early in gonadogenesis and neural crest cell migration. This organ specificity may explain why it was possible to detect *Etk2* mRNA in ES cells and EB and in adult brain of mice (ref. 20; unpublished data) but not in adult unfractionated or fractionated BM.

Our findings provide an opportunity to correlate the mRNA levels of specific genes in enriched populations of adult BM PHSCs with what is observed in *in vitro* models such as the ES cell and EB systems. We predict that further analysis of both models will focus attention on early events in hematopoiesis.

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1. Boggs, D. R., Boggs, S. S., Saxe, D. F., Gress, L. A. & Canfield, D. R. (1982) *J. Clin. Invest.* **70,** 242–253.
2. Harrison, D. E. & Astle, C. M. (1991) *Exp. Hematol.* **19,** 347–377.
3. Visser, J. W. M., Bauman, J. G. J., Mulder, A. H., Eliason, J. F. & de Leeuw, A. M. (1984) *J. Exp. Med.* **59,** 1576–1590.
4. Orlic, D. & Bodine, D. M. (1994) *Blood* **84,** 3991–3994.
5. Pluznik, D. H. & Sachs, L. (1966) *Exp. Cell Res.* **43,** 553–563.
6. Metcalf, D. (1984) *The Hemopoietic Colony-Stimulating Factors* (Elsevier, Amsterdam).
7. Spangrude, G. L., Heimfeld, S. & Weissman, I. L. (1988) *Science* **241,** 58–62.
8. Jordan, C. T., McKearn, J. P. & Lemischka, I. R. (1990) *Cell* **61,** 953–963.
9. Orlic, D. & Bodine, D. M. (1992) *Exp. Hematol.* **20,** 291–295.
10. Orlic, D., Fischer, R., Nishikawa, S. I., Nienhuis, A. W. & Bodine, D. M. (1993) *Blood* **82,** 762–770.
11. Okuda, S., Nakauchi, H., Nagayoshi, K., Nishikawa, S., Nishikawa, S. I., Miura, Y. & Suda, T. (1991) *Blood* **78,** 1706–1712.
12. Orlic, D., Anderson, S. & Bodine, D. M. (1994) *Blood Cells* **20,** 107–120.
13. Sorrentino, B. P., McDonagh, K. T., Woods, D. & Orlic, D. (1995) *Blood,* in press.
14. Sykes, M., Sachs, D. H., Nienhuis, A. W., Pearson, D. A., Moulton, A. D. & Bodine, D. M. (1992) *Transplantation* **55,** 197–202.
15. McClanahan, T., Dalrymple, S., Barkett, M. & Lee, F. (1993) *Blood* **81,** 2903–2915.
16. Hibi, M., Murakami, M., Saito, M., Hirano, T., Taga, T. & Kishimoto, T. (1990) *Cell* **63,** 1149–1157.
17. Hansen, J. N., Konkel, D. A. & Leder, P. (1982) *J. Biol. Chem.* **257,** 1048–1052.
18. Tsai, S.-F., Martin, D. I. K., Zon, L. I., D'Andrea, A. D., Wong, G. G. & Orkin, S. H. (1989) *Nature (London)* **339,** 446–451.
19. Reith, A. D., Ellis, C., Lyman, S. D., Anderson, D. M., Williams, D. E., Bernstein, A. & Pawson, T. (1991) *EMBO J.* **10,** 2451–2459.
20. Biesecker, L. G., Gottschalk, L. R. & Emerson, S. G. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 7044–7048.
21. Gilliland, G., Perrin, S. & Bunn, H. F. (1990) *in PCR Protocols,* eds. Innis, D. H., Gelfand, D. H., Sninsky, J. J. & White, T. J. (Academic, New York), p. 60.
22. Singer-Sam, J., LeBon, J. M., Tanguay, R. L. & Riggs, A. D. (1990) *Nucleic Acids Res.* **18,** 687.
23. Wiley, H. S. (1992) *Membrane Dynamics and Signaling* (JAI, Greenwich, CT), p. 11313.
24. de Vries, P., Brasel, K. A., Eisenman, J. R., Alpert, A. R. & Williams, D. E. (1991) *J. Exp. Med.* **173,** 1205–1211.
25. Bodine, D. M., Karlson, S. & Nienhuis, A. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 8897–8901.
26. Bodine, D. M., McDonagh, K. T., Seidel, N. E. & Nienhuis, A. W. (1991) *Exp. Hematol.* **19,** 206–212.
27. Luskey, B. D., Rosenblatt, M., Zsebo, K. M. & Williams, D. A. (1992) *Blood* **80,** 396–402.
28. Jones, R. J., Wagner, J. E., Celano, P., Zicha, M. S. & Sharkis, S. J. (1990) *Nature (London)* **347,** 188–189.
29. Krantz, S. B. (1991) *Blood* **77,** 419–434.
30. Migliaccio, A. R., Migliaccio, G., D'Andrea, A., Baiocchi, M., Crotta, S., Nicolis, S., Ottolenghi, S. & Adamson, J. W. (1991) *Proc. Natl. Acad. Sci. USA* **88,** 11086–11090.
31. Bodine, D. M., Seidel, N. E., Zsebo, K. M. & Orlic, D. (1993) *Blood* **82,** 445–455.
32. Bodine, D. M., Seidel, N. E., Gale, M. S., Nienhuis, A. W. & Orlic, D. (1994) *Blood* **84,** 1482–1491.
33. Romeo, P. H., Prandini, M. H., Joulin, V., Mignotte, V., Prenant, M., Vainchenker, W., Marguerie, G. & Uzan, G. (1990) *Nature (London)* **344,** 447–449.
34. Martin, D. I. K., Zon, L. I., Mutter, G. & Orkin, S. H. (1990) *Nature (London)* **344,** 444–447.
35. Andrews, N. C., Erdjument-Bromage, H., Davidson, M. B., Tempst, P. & Orkin, S. H. (1993) *Nature (London)* **362,** 722–728.
36. Pevny, L., Simon, M. C., Robertson, E., Klein, W. H., Tsai, S. F., D'Agati, V., Orkin, S. H. & Costantini, F. (1991) *Nature (London)* **349,** 257–260.
37. Todokoro, K., Watson, R. J., Higo, H., Amanuma, H., Kuramochi, S., Yanagisawa, H. & Ikawa, Y. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 8900–8904.
38. Keller, G., Kennedy, M., Papayannopoulou, T. & Wiles, M. V. (1993) *Mol. Cell. Biol.* **13,** 473–486.
39. Kuramochi, S., Ikawa, Y. & Todokoro, K. (1990) *J. Mol. Biol.* **216,** 567–575.
40. Tsai, F. Y., Keller, G., Kuo, F. C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F. W. & Orkin, S. H. (1994) *Nature (London)* **371,** 221–226.