Identification of a Novel DNA Sequence Differentially Expressed Between Normal Human CD34+CD38hi and CD34+CD38lo Marrow Cells

By Lynn Graf and Beverly Torok-Storb

We have applied the recently developed differential display method to extend the molecular characterization of the less mature CD34+CD38hi bone marrow progenitors in comparison with the CD34+CD38lo cells to better understand their functional differences. Immunomagnetic enrichment of CD34+ cells followed by flow cytometry was used to isolate CD34+CD38hi and CD34+CD38lo cells from human organ donor bone marrow. A limited set of the poly A+ RNA sequences present in these two cell populations was amplified by a combination of reverse transcription with an anchored oligo dT-based primer and polymerase chain reaction with the same oligo dT primer and arbitrary decamers. A radioactive tracer allowed these sequences to be displayed as a series of bands on a denaturing polyacrylamide gel. Eight bands were chosen that appeared in multiple displays to represent gene sequences differentially expressed between CD34+CD38hi and CD34+CD38lo cells. Comparison of the sequences with public DNA sequence databases available identified one sequence as myeloperoxidase. Two other clones matched sequence fragments of unknown function, whereas the remaining five are novel sequences not present in existing databases. The relative level of expression of all of the sequences was tested by an independent reverse transcriptase-polymerase chain reaction with sequence-specific oligonucleotide primers. The lower level of myeloperoxidase mRNA in CD34+CD38lo cells was confirmed, as was the higher expression of the novel sequence 345. Sequence 345 expression is highest in CD34+CD38lo cells and decreases with increased CD38 expression. It is expressed in negligible amounts in hematopoietic cell lines and other sources of human tissue, suggesting it may have a functional role in normal hematopoiesis.

MATERIALS AND METHODS

Cell preparation. BM was obtained from organ donors through the Northwest Tissue Center (Seattle, WA). Low-density cells were prepared by Lymphoprep density gradient centrifugation (density, 1.077; from Accurate Scientific and Chemical Corp, Westbury, NY) and frozen in RPMI 1640 Medium (GIBCO, Grand Island, NY) containing 50% fetal bovine serum (HyClone Laboratories, Logan, UT) and 10% dimethyl sulfoxide at −170°C until use. Frozen cells were thawed by dilution in Iscove’s Medium (GIBCO) containing 10% fetal bovine serum and 10 μg/mL DNase I (Worthington, Freehold, NJ), washed once in the same medium, and stained with the fluorescein isothiocyanate-labeled monoclonal antibody HPCA-1 directed against the CD34 antigen. Cells were then incubated with a magnetic microbead-coupled rat-antimouse IgG1 secondary antibody. CD34+ cells were preenriched by immunomagnetic selection (MACS) on an MACS A1 column (Miltenyi Biotec GmbH, Sunnyvale, CA) and stained for CD38 with phycoerythrin-coupled Leu18 monoclonal antibody. All antibodies were from Becton Dickinson Immunocytometry Systems (San Jose, CA).

Flow cytometry. Cell sorting was performed on a FACStar Plus (Becton Dickinson) equipped with an Argon ion laser tuned at 490 nm and with a Helium laser at 633 nm. Data were acquired in list mode and analyzed with ReproMan software (FINEFacts Software, Seattle, WA). Instrument settings were standardized for forward light scatter with 8.7-μm beads and for fluorescein isothiocyanate and phycoerythrin with QC3 beads (Flow Cytometry Standards, Research Triangle Park, NC) to facilitate comparison of gating parameters in different flow cytometry separations. CD34+ cells within a low-forward and 90° light scatter gate were separated on the basis of CD38 antigen expression into CD38hi and CD38lo subpopulations.

RNA preparation. Isolated cells were washed once in Tris-HCl–
buffered saline (pH 7.5) and pelleted in microfuge tubes; at which point, they were either lyed or snap-frozen and stored at −80°C. For differential display of sequences, total nucleic acid was purified from cells lysed in RNAzol B (Biotexc Laboratories, Houston, TX), according to the manufacturer’s directions, for small amounts of tissue. For semiquantitative PCR of sorted cells, nucleic acid was prepared from cells lysed in 100 μL buffer containing 20 mmol/L Tris-HCl (pH 7.5), 100 mmol/L NaCl, 1 mmol/L EDTA, 1% sodium dodecyl sulfate, and 0.5 mg/mL Proteinase K (Sigma, St Louis, MO).23 The Proteinase K sodium dodecyl sulfate lysate was incubated for 45 minutes at 50°C to allow digestion of RNA-degrading enzymes and was extracted with buffered phenol-chloroform, and the nucleic acid was precipitated with ethanol using 10 μg glycogen (Mytilus edulus; Sigma) as a carrier. Total RNA from human tissues was obtained from Clontech (Palo Alto, CA). Total RNA from cell lines was prepared by the RNAzol B method. Any contaminating genomic DNA was removed from all samples by treating the total nucleic acid pellet for 30 minutes at 37°C with 10 U RNase-free DNase I (MessageClean Kit from GenHunter Corp, Brookline, MA) or RQ1 DNase (Promega, Madison, WI), followed by phenol-chloroform extraction and ethanol precipitation as described above.

Differential display. Differential display was performed essentially as described22 with reagents from GenHunter Kit A (Gen-hunter). Total DNA-free RNA was prepared as detailed above. Four RT reactions were performed on each RNA sample (0.2 μg RNA/reaction) in 1× transcriptions buffer; 10 mmol/L dithiothreitol; 20 μmol/L each deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), and deoxythymidine triphosphate (dTPP); and 1 μmol/L of either T7, T3, T3MC, T3MT, or T7, T7MA oligonucleotide primer (where M is degenerate for A, G, C, and T) from the kit. Samples were heated to 65°C for 5 minutes and cooled to 37°C for 10 minutes before addition of 100 U Moloney Murine Leukemia Virus RTase. After incubation at 37°C for 1 hour, the reactions were heated to 95°C for 5 minutes before storage at −20°C or PCR amplification. PCR was performed on a Perkin-Elmer Thermal Cycler (94°C for 30 seconds, 52°C for 2 minutes, 72°C for 30 seconds for 40 cycles, followed by a 5-minute extension at 72°C; Perkin Elmer-Cetus, Norwalk, CT). The 20-μL reactions contained 2 μL RT cDNA template, 50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 2.5 mmol/L MgCl2, 0.5 μmol/L T7, T7M primer, 0.2 μmol/L AP, 2 μmol/L each deoxynucleotides triphosphate (dNTP), 0.1 μg/μL dideoxy-ATP, 0.5 μL 2×-ATP, 1.200 CIU/ml from Dupont NEN (Albany, NY), and 1 U Taq DNA polymerase (Promega). Amplification products (4 μL) were desalted by heating for 3 minutes to 80°C in sequencing gel stop buffer (US Biochemical [USB], Cleveland, OH) and electrophoresed at 55 W constant power on a 6% denaturing polyacrylamide gel until the xylene cyanol was 10 cm from the bottom of the gel. γ32P-end-labeled PhiX-HincII molecular weight markers (USB) were coelectrophoresed to serve as orientation markers for comparison of gels run at different times from different templates. Gels were dried without fixation onto Whatman 3MM filter paper (VWR, Seattle, WA) and exposed for 1 to 4 days to Kodak XAR-5 film (CMX, Seattle, WA) with fluorescent ink orientation markers. All reactions were repeated with the same cDNA template as well as with a new source of RNA and cDNA template.

Amplification and cloning of differential display gel bands. Bands that appeared to be consistently differential in multiple RNA isolations and cDNA preparations were excised from the dried sequencing gels with a razor blade. The gel piece was soaked for 15 minutes in 100 μL H2O2, then boiled for 15 minutes to elute the DNA. The eluate was transferred to a fresh tube. The DNA was precipitated by addition of 3 mol/L sodium acetate (pH 5.2) to a concentration of 0.3 mol/L, 10 μg glycogen as a carrier, and 3 vol of 100% ethanol, followed by 30 minutes of incubation at −80°C, and centrifugation at 13,000 rpm for 15 minutes in a microfuge. The DNA pellet was resuspended in water after an 85% ethanol wash. Reamplification by PCR was performed in 40 μL using the appropriate primers and conditions as described above except for dNTP concentrations of 20 μmol/L and no radiisotope. Four microliters of the above reaction was reamplified under the same reaction conditions for a second 40 cycles, and the products of both reactions were separated on a 2% agarose gel (Intermountain Scientific, Bountiful, UT). DNA bands were excised, eluted from the gel with a USBioclean glass bead purification kit (USB), and used directly for cloning. Vectors were pBluescript II (Novagen, Madison, WI) or pBS+ (Strategene, La Jolla, CA), which had been converted to a TA-type cloning vector after cutting with the restriction endonuclease Hinc II (USB), as described by Finney.26 For each excised band, a minimum of 5 white (recombiant) colonies were picked and tested for inserts of appropriate size by PCR with flanking primers (T3 and T7 for pBS+ or T7 and U19 for pBluescript II), as described by Zon et al.27 in a Hybaid Thermal Cycler (National Labnet, Woodbridge, NJ). Recombinant plasmid DNA was purified with a Magic Miniprep Kit (Promega), and double-stranded dideoxy sequencing was performed with dye terminators on an Applied Biosystems model 373A automated sequencer (Applied Biosystems, Foster City, CA). Synthesis of flanking primer primers and sequencing were both performed by the Fred Hutchinson Cancer Research Center (FHRC) Biotechnology Shared Resource (Seattle, WA). The inserts from a minimum of three recombinant plasmids originating from at least two different differential display gels were sequenced to establish a consensus sequence.

Sequence and database searches. Sequence homology searches26-30 were performed with the Genetics Computer Group (GCC) Software Package Version 7.3.1 provided by the FHRC Biocomputing Shared Resource under National Cancer Institute (Bethesda, MD) Grant No. CA15704. The combined EMBl (Release 37) and GenBank (Release 84) databases were searched with the MAILFASrT automated program service and BLAST server parts of the GCC software.

cDNA template preparation for semiquantitative PCR. DNA complementary to the poly A+ RNA (cDNA) present in total RNA preparations was synthesized by oligo dT (Oligo dT12-18; Pharmacia, Piscataway, NJ) primed RT with RTase (Bethesda Research Laboratories, Bethesda, MD) according to manufacturer’s directions. Starting amounts of RNA were 5 μg (cell lines and tissues) or 100,000 cell equivalents (sorted cells). The cDNA was separated from primer and unincorporated nucleotides on a 1-ML Biogel A-5M (Biorad, Richmond, CA) column in 10 mmol/L ammonium bicarbonate buffer containing 1 mmol/L Tris-HCl (pH 7.5) and 0.1 mmol/L EDTA. Pooled fractions were dried in a Speed-Vac (Savant Instruments, Farmingdale, NY), and the cDNA was resuspended in H2O at 500 cell equivalents/μL.

Semiquantitative RT-PCR. All PCR reactions for quantitative purposes included 50 mmol/L KCl; 40 mmol/L Tris-HCl (pH 8.3); 2.5 mmol/L MgCl2; 0.001% gelatin; 0.2 μmol/L each of dATP, dGTP, dTTP, and dCTP (Pharmacia); 25 pmol/L each of the 3’ and 5’ primers; and 1 U Taq polymerase (Promega) in 50 μL. Conditions for amplification were 4 minutes at 94°C (denaturation step) followed by 35 cycles of 93°C for 1 minute, 54°C for 2 minutes, 72°C for 2 minutes in a Hybaid Thermal Cycler. Amplification products were quanitified by inclusion of 20,000 cpm 32P-dCTP (DuPont NEN)-labeled oligonucleotide primer in the PCR reaction. PCR amplification products (20 μL) were separated on a 4% NuSieve agarose gel (SeaKem; FMC, Rockland, ME) in the presence of 10 μg/mL ethidium bromide. Gels were photographed, rinsed in water, and dried onto DE81 paper (Whatman, Maidstone, England, UK) for exposure to a PhosphorImaging screen (Molecular Dynamics, Sunnyvale, CA). Nucleotide incorporation into each band was calculated using
Image-Quant software (Molecular Dynamics), after subtraction of lane background. cDNA signal was adjusted for differences in actin signal from the same cDNA template tested in a separate PCR reaction. Oligonucleotide primers were all synthesized in the FHCRC Biotechnology Facility. They included actin sense (5’AGAGCTACGAGCTGCTGACGGCC 3’) and actin antisense (5’AGTGATCTCTTCTGATCCTGTCC 3’)19; CD38 sense (5’GACTTTGGGAAAAAGCCCTGGC 3’ at bp 1101-1124) and antisense (5’CCCTGCAAGAATATCTACAGAC 3’ at bp 889-915)19, GenBank Accession No. M3446122; myeloperoxidase sense (5’CCACGTTCCTTCAGGAGAATCTC 3’ at bp 3117 to 3141) and myeloperoxidase antisense (5’CTCTACTACATGCAAGGAC 3’ at bp 2890 to 2910)19, GenBank Accession No. J19507 and No. M17709,23; and sequence 345 sense (5’GGTGACTTGGAACAGCTGAAAA 3’) and antisense (5’GGCTACGTGACCTTAATGGGAAA 3’), GenBank Accession No. U15988, EST Database. Actin values for normalization were obtained by averaging at least three tests of duplicate samples with cDNA from 2.5 to 50 cell equivalents or 0.002 ng total RNA, depending on the cell source.

RESULTS
Selection of cell populations. CD34+ cells from previously frozen organ donor BM were preenriched by immunomagnetic selection (MACS). They were separated into the CD38lo and CD38hi subpopulations by flow cytometry, as shown in Fig 1. After MACS, the CD34+ cells comprised 30% to 50% of the cells within the low forward-side scatter gate. This variability could be attributed in part to the flow rate used for the MACS enrichment step. Gates for separation of CD38lo and CD38hi cells were standardized by calibration of the FACSTAR+ instrument for forward scatter and fluorescence with 8.7-μm and QC3 fluorescent beads. Purity of the two subpopulations was monitored after flow cytometry separation and was found to be greater than 98% (data not shown).

Display of differentially expressed sequences. Total RNA from cells isolated on the basis of CD34 and CD38 antigen expression was purified with RNAzol B and reverse-transcribed into cDNA with any of four anchored oligo dT primers (T12MC, T12MG, T12MA, and T12MT, where M refers to mixture of A, G, C, and T). Selected portions were amplified by PCR with the original cDNA primer plus the arbitrary defined 10-bp oligomers API-AP5 in the presence of [35S]-ATP, for a total of 20 reactions for each cell population. The PCR products were displayed on a 6% denaturing polyacrylamide gel, as shown on the left in Fig 2. Amplification products from CD34+CD38lo and CD34+CD38hi cells with the same primer pairs were electrophoresed together to enable identification of bands present in one lane but absent or at a lower level in another, representing sequences possibly expressed more strongly in one cell population than in the other. DNA eluted from the bands of interest was amplified by PCR with the original primers, as shown on the right in Fig 2, cloned into a TA-type plasmid vector, and sequenced. Bands a and b in Fig 2 were found to be the enzyme myeloperoxidase and a novel sequence, designated 345, respectively.

Table 1 summarizes information on the first 8 bands selected based on a difference in band intensity in CD34+CD38lo versus CD34+CD38hi cells from two different flow cytometry separations and cDNA preparations. Only bands clearly differential on at least two gels were selected. Multiple transformants were sequenced for each band, with approximately 6% (3 of 49) proving to be different from the predominant sequence found in DNA eluted from the differential display gels. The sequencing information obtained was compared with the combined GenBank and EMBL databases with the MAILFASTA program (BLAST Server), which uses algorithms developed by Pearson and Lipman24,25 to search for areas of local sequence homology. Because these sequences are from the most 3’ region of genes, as a result of the oligo dT-based cDNA primers, they are most probably noncoding sequencese. Only exact matches are listed.

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**Fig 1.** Selection of CD34+CD38lo and CD34+CD38hi cells from organ donor BM by flow cytometry. (A) Correlated expression of CD34 and CD38 on the ungated cell population after preenrichment for CD34+ cells by MACS. (B) Gates used to select CD38lo and CD38hi subpopulations of CD34+ cells within a preestablished window for low forward and side scatter.
Two clones matched partial sequences entered in the database from large-scale sequencing of cDNA libraries. Clone no. 7-15 is 99.4% homologous over 145 bp to a tubulin intron sequence (GenBank No. X01703) from a brain cDNA library. Clone no. 12-15 matches the sequence hbc751 from a pancreatic islet library (GenBank No. T10640) with 95% homology. The former is an obvious genomic DNA contamination, whereas sequence hbc751 is of unknown function. Clone no. 2-11, expressed more highly in the more mature CD34⁺CD38⁻ cells, was identical to the enzyme myeloperoxidase (bp 2861-3210) except for two mismatched bases at the 5' end of the AP2 primer sequence.

Independent quantitation of differences in expression level of differential display sequences. To confirm the expression level difference observed in the differential display analysis, we used RT-PCR as an alternative to Northern blots because of the small amount of RNA available from the CD38⁺ and CD38⁻ cells. We designed oligonucleotide primers specific for each sequence. Primers were chosen to exclude the original differential display primer sequences and contain approximately 50% G+C. The relative sensitivity and linear range of the PCR amplification for each sequence and primer pair was determined by serial dilutions of the plasmid containing the sequence of interest. Quantitation was by incorporation of trace amounts of ³²P-labeled specific primer in the PCR reaction and PhosphorImager analysis. All bands quantitated by PhosphorImaging were also visible by ethidium-bromide staining of the PCR products in an agarose gel. The titration curves for sequence 345 and β-actin are shown in Fig 3, together with an example of an agarose gel and PhosphorImage used for quantitation of amplified product. Even below the plateau level, the template-to-product ratio is not perfectly linear, leading to an underestimate of quantitative differences.

Nonanchored oligo-dT₁₂₋₁₈-primed cDNA from three independent flow cytometry separations was tested for level of expression of seven of the identified sequences, plus actin and the CD38 antigen as controls. Pretests of cDNA template equivalents of cDNA per reaction. It was critical to eliminate any contribution of genomic DNA to the quantitation, because it was not possible to design primers to cross exon-intron boundaries. All RNA samples were treated with DNase to remove any contaminating DNA and tested negative before cDNA synthesis. Two of the seven sequences tested, myeloperoxidase and the novel sequence 345, were
confirmed to be expressed at a consistently differential level in the cells from three independent sorts, one of which is shown in Fig 4. The remaining sequences were all found to be expressed in the CD38 low and CD38 high subpopulations, but at equal levels. Table 2 summarizes the results of multiple RT-PCR tests on cells from the three separate flow cytometry separations, showing that the differences in level of clone 345 and myeloperoxidase mRNA expression are consistent and similar to that of the CD38 antigen. Results of several tests were combined by calculating the fold-difference between the CD38 low and CD38 high samples, after normalization for differences in actin level.

**Sequence 345 expression in CD34+CD38- cells.** Some consistent differences between sorts in expression level of sequence 345 and myeloperoxidase were observed, probably as a result of minor differences in the selection windows. The largest difference in the expression of sequence 345 between CD38 high and CD38 low cells was found when the gate for CD38 high was the most stringent (data not shown). To investigate this question more rigorously, we isolated a CD34+CD38 low population of cells in addition to the CD34+CD38 high and CD34+CD38 low populations and tested these cells for the expression of sequence 345. The gates selected and PCR results (Fig 5) suggest an inverse correlation between differentiation or activation, as assessed by CD38 antigen, and expression of sequence 345.

**Expression of sequence 345 in human tissues and hematopoietic cell lines.** As a first step in cloning and identifying the function of sequence 345, we have assayed its expression by RT-PCR, as described above, in a series of normal human tissues as well as a selection of human hematopoietic cell lines. The results are shown in Fig 6. The highest level of expression in normal tissues is in brain, followed closely by kidney. All tissues and cell lines show much lower expres-

**Fig 4.** RT-PCR quantitation of mRNA levels of differentially expressed gene sequences. Bands represent PCR amplification products from duplicate reactions from cells isolated by flow cytometry. cDNA template from RT reactions with oligo dT priming was amplified with sequence-specific primers in the presence of tracer amounts of one 32P-labeled primer. PCR products were separated on a 4% GTG agarose gel that was dried and exposed to a Phosphorimage screen.

**Fig 3.** Determination of quantitative limits of PCR amplification. Each set of oligonucleotide primers to be used for cDNA quantitation was tested under the standard PCR conditions (94°C for 1 minute, 54°C for 2 minutes, and 72°C for 3 minutes for 35 cycles) with a threefold plasmid dilution series containing the appropriate insert. One radiolabeled primer was incorporated. PCR products were separated on a 4% GTG agarose gel that was dried and exposed to a Phosphorimage screen, allowing quantitation of amplified product. The idealized exponential curve for each graph is indicated by a dotted line. Each experimental line is from a separate test with duplicate or triplicate samples. An example of the agarose gel and Phosphorimage file for one experimental line from duplicate samples of a β-actin plasmid dilution series is shown below the graphs. The input plasmid template in femtograms, calculated from A0, is plotted against amplified product, after normalization to the value for 10 fg.
expression, when normalized to β-actin, than do CD34+CD38hi cells. Differences in source and preparation of total RNA render it impossible to compare the levels on a per-cell or per-nanogram-of-nucleic-acid basis.

**DISCUSSION**

The characterization of hematopoietic stem cells has focused on cell surface antigen selection criteria that yield cells with the greatest long-term repopulating ability, as assessed by a variety of in vitro assays. Currently, the selection criteria for the most immature cells are predominantly negative, because these cells are characterized by their lack or low expression of markers such as HLA-DR, CD71, CD45RA, or CD38. The molecular characterization of the pluripotent human hematopoietic stem cell, as distinguished from a still immature but lineage-committed progenitor cell, has been hampered by the inability to obtain such cells in sufficient numbers to make high-quality cDNA libraries as well as by the problems inherent in the commonly used subtractive hybridization techniques. In this report, the use of organ donor BM, together with immunomagnetic preenrichment of CD34+ cells on a MACS column, made it possible to obtain adequate numbers of the most primitive cells. The decision to use CD38 as the selection criteria for the most primitive cells was based on the work by Terstappen et al.25 as well as on our own results,46,49 which have shown that the CD34+CD38hi population contains the multipotent cells with the largest proliferative capacity.

Our study shows that the differential display technique can be used to identify genes differentially expressed between CD34+CD38hi and CD34+CD38lo cells from human BM. This cDNA cloning technique offers several advantages over subtractive hybridization-based screening.41 First, it requires only a relatively small amount of total (not polyA+) RNA and no library construction. Second, uniquely expressed RNAs enriched or underexpressed in two or more different tissues or cell types can be identified simultaneously, in contrast to subtractive hybridization, which selects for those overexpressed in a single tissue. Also, it eliminates the danger of removal of novel genes sharing stretches of sequence with abundant members of gene families. It has been used successfully to study processes as diverse as breast cancer,21,42,43 cardiac rejection,44 and the development of the preimplantation mouse embryo.45

Using 20 primer combinations (all 4 oligo-dT-anchored primers and a set of 5 arbitrary decamers), we identified eight bands that appeared to be reproducibly differentially expressed between CD34+CD38hi and CD34+CD38lo cells isolated by flow cytometry at two different time points. Of these eight, only two could be confirmed by an independent, although also PCR-based, technique to be expressed at different levels in the two cell populations. This is comparable with the experience of other investigators46,49 attempting to confirm differential bands with Northern blots of relevant tissue. To our knowledge, no other reports have used RT-PCR. The sequence homology of clone 2-11 to myeloperoxidase was unequivocal. Only two bases at the 5′ end of the AP2 primer were mismatched in a stretch of 480 bp that ended at bp 3209 of a known splice variant of the human myeloperoxidase gene.25,33,34 A detailed investigation of the properties of the 10mers noted that 1 to 4 mismatched bases at the 5′ end are tolerated.44 Because it is an early marker

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**Table 2. Differences in Expression Level of Selected Gene Sequences**

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Direction of Difference</th>
<th>Fold-Difference (±SE)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>345</td>
<td>CD38hi &gt; CD38lo</td>
<td>2.47 (±0.38)</td>
<td>7</td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>CD38hi &lt; CD38lo</td>
<td>3.53 (±0.54)</td>
<td>9</td>
</tr>
<tr>
<td>CD38</td>
<td>CD38hi &lt; CD38lo</td>
<td>3.03 (±0.64)</td>
<td>3</td>
</tr>
</tbody>
</table>

Radioactivity incorporated in the PCR amplification products (bands), as shown in Fig 3, was quantified using a Phosphorimager. All samples were tested in duplicate. The mean of duplicate samples was calculated and adjusted for the differences in actin level. Actin correction factors were 1.07, 1.20, and 1.15 for sorts no. 1, 2, and 3, respectively. Fold-difference between the higher expressor and the lower expressor population was calculated for each RT-PCR test, and the results of varying numbers (n) of tests for each sort combined to calculate the mean ± standard error (SE).

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**Fig 5.** Relative expression of sequence 345 in CD38hi versus CD38lo subpopulations of CD34+ cells. Gating for the CD38lo population was based on isotype control antibody staining. PCR amplification products from cDNA from the three isolated populations shown in (A) were quantitated as described previously. Values are the mean ± SE of three tests with triplicate samples.

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of myeloid differentiation, the higher expression of myeloperoxidase in CD34+CD38+ cells is appropriate with significant amounts already accumulated in the azurophilic granules of promyelocytes. The higher expression of myeloperoxidase in CD34+CD38+ cells by Phosphorimage analysis of RT-PCR amplification products. Results are from duplicate samples in a minimum of two experiments. All values were normalized to actin to allow comparison of signals from RNA obtained by different preparation methods, as described in Materials and Methods.

The actin signal was also used to combine experiments starting from different RNA sources and preparation methods. This was of particular importance for the tissue RNAs obtained from a commercial source that varied greatly in actin signal in spite of equal A260 readings.

All of the original sequences were found to be expressed in the CD34+ cells and were detectable in the cDNA from the equivalent of 1,000 cells or less. However, there was a consistent difference in the expression level between the CD38+ and CD38− cells for only the myeloperoxidase and clone 345 sequences. Differences in expression level detected by the PCR quantitation were not large. On average, a 3.5-fold difference was observed for myeloperoxidase, and a 2.5-fold difference for sequence 345. However, this difference increased to 4.7-fold when more rigorous flow cytometry gating was used to select the small CD38−, not CD38+, subset of CD34+ cells. Although these differences may seem small, they were consistent. In addition, comparable levels of differential expression were also observed for GATA-1 (data not shown) and CD38 sequences, both of which have maturation-stage-specific expression.

The difference in CD38 sequence expression between the CD38+ and CD38− cells was only threefold, which is relatively low compared with differences observed in surface expression of the antigen, as detected by flow cytometry. However, it should be noted that the cells selected were CD38+, not CD38−, as compared with a negative control; therefore, there are cells within this subpopulation that express CD38 on their surface, albeit at a low level. In addition, CD38 is known as a classical "activation antigen" that may be regulated at other than the transcriptional level. This could also account for discrepancies between the level of expressed CD38 and the level of detectable RNA.

In summary, we have identified an apparently novel sequence that is expressed at a twofold higher level in immature hematopoietic progenitors, compared with more mature progenitors, and at more than a fivefold higher level than in normal nonhematopoietic tissue. Currently, the sequence is being monitored in immature cells after cytokine exposure. However, defining the function of the gene requires a full-length sequence. Efforts are underway to achieve this goal.

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