The Regulated Expression of B Lineage Associated Genes during B Cell Differentiation in Bone Marrow and Fetal Liver

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Summary

The expression of B lineage associated genes during early B cell differentiation stages is not firmly established. Using cell surface markers and multiparameter flow cytometry, bone marrow (BM) cells can be resolved into six fractions, representing sequential stages of development; i.e., pre-Pro-B, early Pro-B, late Pro-B/large Pre-B, small Pre-B, immature B, and mature B cells. Here we quantitate the levels of several B lineage associated genes in each of these fractions by RT-PCR, demonstrating different patterns of expression. We find that expression of terminal deoxynucleotidyl transferase (TdT), λ5, and VpreB is predominantly restricted to the Pro-B stages. Rag-1 and Rag-2 expression is also tightly regulated, and is found largely in the Pro-B through small Pre-B stages. Mb-1 is present from Pro-B throughout the pathway at high levels. Finally, Bcl-2 is expressed at high levels only at the pre-Pro-B and mature B stages, whereas it is low during all the intermediate stages. We also correlate this expression data with an analysis of the onset of Ig gene rearrangement as assessed by amplifying D-J_H, V_H-DJ_H, and V_κ-J_κ. Finally, we report differences in gene expression during B lymphopoiesis at two distinct ontogenic timings, in fetal liver and adult BM: both TdT and the precursor lymphocyte regulated myosin-like light chain are expressed at high levels in the Pro-B cell stage in bone marrow, but are absent from the corresponding fraction in fetal liver. In contrast, λ5, VpreB, Rag-1, and Rag-2 are expressed at comparable levels.

Bone marrow (BM)[1], the site of B lymphopoiesis in the adult mouse, consists of a mixture of diverse cell types at various differentiation stages. B lineage cells can be recognized by expression of the high molecular weight form of the common leukocyte antigen CD45 (termed B220), present on early developing cells before detectable surface Ig, and continuing through the mature B cell stage (1). We reported previously (2) that early B lineage BM cells ($B220^+IgM^-$) can be divided into populations of less mature and more mature cells: the "early" cells express an epitope of leukosialin (CD43) recognized by the mAb S7 ($B220^+CD43^+$), whereas the more mature cells lack this determinant ($B220^+CD43^-$). We also showed (see Fig. 1) that the early $B220^+CD43^+$ cells can be subdivided into three fractions (Fr. A–C) based on the correlated expression of the heat stable antigen (HSA) and BP-1, whereas the latter $B220^+CD43^-$ cells can be subdivided into three fractions (Fr. D–F) based on the correlated expression of IgM and IgD.

Using flow cytometry we isolated these B lineage subsets and found that the growth and proliferation of cells from the earliest fraction (Fr. A) was absolutely dependent on contact with a stromal layer, whereas cells from later $CD43^+$ fractions (Fr. B and C) could proliferate in soluble mediators (e.g., IL-7) alone. This is consistent with earlier work (3–5) that had suggested contact dependence for the earliest cells, followed by a shift to contact independent growth. Cells recovered from such short-term culture demonstrated phenotypic progression from $CD43^+$ to $CD43^-$. Furthermore, using a deletional PCR assay we found that cells in the earliest fractions showed little evidence of Ig rearrangement, those in the intermediate fractions possessed D-J_H but not V-DJ_H rearrangements, and those in latter ($CD43^-$) fractions possessed V-DJ_H and κ L chain rearrangements. From these data, we proposed an ordered B lineage cell differentiation pathway: Fr. A ($HSA^-BP\text{-}1^-$; pre-Pro-B), Fr. B ($HSA^+BP\text{-}1^-$; Pro B), Fr. C ($HSA^+BP\text{-}1^+$; Pro-B plus large Pre-B), Fr. D (IgM^-; small Pre-B), Fr. E (IgM^+IgD^-; immature B), and Fr. F (IgM^+IgD^+; mature B).

[1] *Abbreviations used in this paper:* APC, allophycocyanin; BI, biotin; BM, bone marrow; FL, fluorescein; Fr., fraction; HSA, heat stable antigen; PLRLC, precursor lymphocyte regulated myosin-like light chain; TdT, terminal deoxynucleotidyl transferase; TR, Texas red.

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To understand more about the events occurring in these early B cell differentiation stages, we have quantitated the expression of a number of B lineage associated genes using a PCR assay to amplify cDNA generated from RNA isolated from small numbers of sorted cells of each fraction. We achieve quantitation by simultaneously determining the signal obtained from amplification of a housekeeping gene, β-actin, using this value to normalize the results with other genes. The genes examined include those involved in rearrangement, such as terminal deoxynucleotidyl transferase (TdT; 6, 7), Rag-1 and Rag-2 (8, 9), genes that associate with Ig H chain, i.e., Mb-1 (10–12), λ5, and VpreB (13), and a gene that can block programmed cell death known as Bcl-2 (14–17). Additionally, we have investigated the status of Ig genes in these cells by assessing the degree of D-J_H, V_H-DJ_H, and V_κ-J_κ rearrangements. Finally, as recent work suggests differences in B lymphopoiesis during fetal and adult timings (18, 19), we have compared gene expression in corresponding fractions isolated from fetal liver with that in adult BM.

We find that the expression of most of these genes is tightly controlled during the early B lineage pathway in BM suggesting that, in many cases, precise timing of expression is critical to their functional progression. For example, TdT is found during stages where H chain rearrangement predominates, but is absent from the next stage where most L chain rearrangement occurs. Furthermore, our comparison of Pro-B cells from fetal liver and adult BM reveals a striking difference in the expression of two genes: TdT and the precursor lymphocyte regulated myosin-like L chain (PLRLC; 20), both found at high levels in Pro-B cells of BM, are absent from Pro-B cell fractions in fetal liver, whereas other genes such as λ5, VpreB, Rag-1, and Rag-2 are expressed at comparable levels. This supports the hypothesis that there are differences in the B cell differentiation pathway at distinct ontogenic timings.

Materials and Methods

Animals and Cell Preparation. BM was obtained from 2–4-mo-old female BALB/cAnN mice bred in our animal facility. A single cell suspension of BM (femur and tibia) was prepared by injecting medium (staining medium; deficient RPMI, containing 10 mM Hepes, 3% FCS, and 0.1% NaN_3; Irvine Scientific, Santa Ana, CA) into the bone to flush out cells, followed by gentle mixing with a 1-ml syringe. Fetal liver was obtained from timed matings of BALB/cAnN mice. A single cell suspension of fetal liver was prepared by dissociation between frosted glass slides. Cells were treated with 0.165 M NH_4Cl to eliminate erythrocytes.

Staining, Flow Cytometry, and Cell Sorting. Cells were incubated with appropriate reagents in staining medium on ice for 15 min, washed three times with staining medium, then incubated a further 15 min with Texas red (TR)–avidin to reveal the biotin reagent, and finally washed twice with staining medium. Flow cytometry analysis and sorting was carried out using a dual laser/dye laser flow cytometer (FACStar Plus®, Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with appropriate filters for four color immunofluorescence. Samples were held on ice during sorting. Reanalysis of sorted fractions consistently showed purities in excess of 95%. Selected populations were sorted directly into microcentrifuge tubes (for RNA and DNA preparation). Preparation of labeled reagents has been described previously (21). The BP-1 hybridoma was generously provided by Dr. M. D. Cooper (University of Alabama, Birmingham, AL).

RT-PCR Assay. Total RNA was extracted from 10^5 sorted cells using a modification of the guanidinium thiocyanate method (22) with addition of 40 μg of carrier ribosomal RNA. The cell lysate was then layered over 5.7 M CsCl and centrifuged at 80,000 rpm for 150 min in a tabletop ultracentrifuge (model TL-100; Beckman Instruments, Inc., Palo Alto, CA). After ethanol precipitation, the RNA pellet was dissolved in 20 μl of water and stored at −70°C. 4 μl of this sample was used for first strand cDNA synthesis in a total volume of 20 μl. Briefly, 4 μl of RNA and 4.5 μl of water were mixed with 2 μl of 100 mM MeHgOH and incubated at room temperature for 5 min. After addition of 2.5 μl of 0.7 M 2-ME and 1 μl of 10 U/ml random hexamers (Pharmacia, Piscataway, NJ), the reaction was incubated at 65°C for 2 min and placed on ice. The following reagents were then added: 1 μl of 20 U/μl RNase inhibitor (Promega, Madison, WI); 2 μl of 10× PCR buffer (500 mM KCl, 200 mM Tris-HCl, pH 8.3, 25 mM $MgCl_2$, and 1 mg/ml BSA); 2 μl of a 1-mM mixture of all four deoxynucleotide triphosphates and 1 μl of M-MLV reverse transcriptase at 200 U/μl (GIBCO BRL, Gaithersburg, MD). The reaction was incubated at 37°C for 60 min and heated to 95°C for 5 min, then quickly chilled on ice.

cDNA was amplified by PCR using the different primers depicted in Table 1. PCR reactions were performed in 50-μl volume containing 4 μl of cDNA sample, 1× PCR buffer, 100 μM of each of four deoxynucleotide triphosphates, 2 μM of each sense and antisense primers, and 5 U of Taq polymerase (Promega). After an initial 10-min incubation at 80°C, the PCR reactions were carried out using the following conditions: denaturation at 95°C for 30 s, annealing at 62°C (for the first five cycles) or 58°C (for further cycles) for 30 s, and polymerization at 72°C for 45 s. Aliquots were withdrawn at 22 and 26 cycles for separate analysis to ensure that amplification was within the linear range. To verify that equal amounts of RNA were added in each PCR reaction, the "housekeeping gene" β-actin was also amplified. 15 μl of the PCR samples were then separated by 1.5% agarose gel electrophoresis and blotted onto Hybond N membrane (Amersham Corp., Arlington Heights, IL). Filters were UV crosslinked, prehybridized for 1–3 h, and then hybridized overnight (at 42°C) with riboprobes prepared from the PCR products (see below). Membranes were washed (twice for 30 min in 2× SSC and twice in 0.2× SSC at 65°C) and imaged on x-ray film (1–4-h exposure) and quantitated using a two-dimensional proportional scintillation detector (Ambis, San Diego, CA). Radioactivity in individual bands representing each PCR product was measured and normalized using the β-actin signal for each sample. The maximum signal was then set to 100% and all other values were expressed accordingly. Several independently sorted samples of each cell phenotype were analyzed.

PCR products were cloned in order to generate riboprobes as this gave a very high signal with low background in hybridization. Individual PCR products were amplified using cDNA from expressing cell fractions for 30 cycles and the appropriate size ethidium bromide–stained band identified on a 1.5% agarose gel. The agarose containing the band was excised, the DNA eluted, purified, and cloned into the pCR™ vector using the TA Cloning System kit (Invitrogen, San Diego, CA). After purification and linearization of the plasmids with restriction endonuclease HindIII, riboprobes were made using T7 polymerase according to the manufacturer's procedure (RNA Transcription kit; Stratagene, La Jolla, CA). Approximately one sixth of a labeling was used per blot.

Table 1. *Oligonucleotides Used to Amplify B Lineage Associated Genes from cDNA*

Gene	5′ oligo	3′ oligo	Size
β-actin	CCTAAGGCCAACCGTGAAAAG	TCTTCATGGTGCTAGGAGCCA	623
TdT	GAAGATGGGAACAACTCGAAGAG	CAGGTGCTGGAACATTCTGGGAG	313
λ5	CTTGAGGGTCAATGAAGCTCAGAAGA	CTTGGGCTGACCTAGGATTG	337
VpreB	CGTCTGTCCTGCTCATGCT	ACGGCACAGTAATACACAGCC	342
Rag-1	TGCAGACATTCTAGCACTCTGG	ACATCTGCCTTCACGTCGAT	556
Rag-2	CACATCCACAAGCAGGAAGTACAC	TCCCTCGACTATACACCACGTCAA	515
Mb-1	GCCAGGGGGTCTAGAAGC	TCACTTGGCACCCAGTACAA	310
Bcl-2	TCGCTACCGTCGTGACTTC	AAACAGAGGTCGCATGCTG	315
PLRLC	CAATGTCTCCATCAGGCCTT	CCTCCTCACTGAACCGGTC	413

All oligonucleotides were designed to amplify sequences that contain introns in order to discriminate signals resulting from contaminating DNA. The size of the amplified fragment is also given.

Rearrangement Assay. 2×10^5 cells sorted according to phenotype directly into a microcentrifuge tube were pelleted, washed in Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 8.0) at 4°C, and then digested with 0.5 mg/ml proteinase K for 2 h at 50°C in buffer A (0.5% sodium lauroyl sarkosinate, 10 mM EDTA, 50 mM Tris, pH 8.0) containing 1% low-gelling temperature agarose. After digestion samples were allowed to gel on ice for 5 min, dialyzed against TE buffer (three changes in 36 h), and then stored at 4°C. Before use, DNA samples were melted at 65°C, treated with RNase (10 ng, 37°C for 2–4 h), then diluted 1:5 with 65°C ddH_2O (final volume 150 μl) and stored at 4°C. One fifth of the sample was used for each PCR reaction. Most of the PCR oligonucleotides (Table 2) have been described previously. Each set detecting a particular rearrangement was coamplified with a normalizing set that amplifies a fragment from the α-actin gene. This serves to control for variation in efficiency of DNA preparation, PCR amplification, pipetting, and transfer. Conditions for PCR were denaturation at 95°C for 1 min, annealing at 63°C for 30 s, and polymerization at 72°C for 1.5 min. Aliquots were withdrawn at 22 and 26 cycles for separate analysis to ensure that amplification was within the linear range and care was taken to use relatively comparable levels (within a threefold range) of starting DNA. PCR samples were then separated electrophoretically and blotted as described above. Filters were hybridized overnight (at 55°C) with random primed probes (α-actin and pJ11 or α-actin and pECk), then washed and analyzed as for the RT-PCR assay. Radioactivity

Table 2. *Oligonucleotides Used to Amplify Ig Rearrangements from DNA*

Fragment	Oligo	Sequence	Reference
α-actin	5′	GGCATCGTGTTGGATTCTG	This paper
	3′	CACGAAGGAATAGCCACGC	This paper
D-J_H4	5′	ACAAGCTTCAAAGCACAATGCCTGGCT	33
	3′	CTCTCAGCCGGCTCCCTCAGGG	33
7183-J_H4	5′	GCAGCTGGTGGAGTCTGG	30
	3′	Same as D-J_H4	
Q52-J_H4	5′	TCCAGACTGAGCATCAGCAA	This paper
	3′	Same as D-J_H4	
J558-J_H4	5′	CAGGTCCAACTGCAGCAG	29
	3′	Same as D-J_H4	
$V_κ$-$J_κ$	5′	G A T A GGCTGCAGCTTCAGTGGCAGTGGGTCAGGGAC	31
	3′	TTCCAACTTTGTCCCCGAGCCG	45

Many oligonucleotides have been used in previously published work and appropriate references are indicated.

in individual bands representing each PCR product was measured, expressed as a ratio to the signal derived from the coamplified α-actin normalizing band and then the ratio was expressed as a percentage of the level seen in mature B cells. For V_H-D-J_H rearrangement we have multiplied this value times the expected frequency of cells bearing rearrangements for the particular family to derive an estimate of the frequency of cells possessing a rearrangement. Each sample was separately amplified two-to-four times and several independently sorted samples of each cell phenotype were analyzed.

Results and Discussion

Gene Expression in B Lineage Fractions in BM

TdT, λ5, and VpreB. TdT mediates addition of nongermline encoded nucleotides at the V-D and D-J junctions of Ig H chain genes (6, 7). The presence of such N regions at junctions of H chains and absence (or rarity) from κ L chain V-J junctions has been presumed to reflect the expression of TdT early in B cell differentiation and its absence in later stages where most L chain rearrangement is thought to occur. In agreement with this model, as shown in Fig. 2 and summarized in Fig. 3, we detect high expression of TdT in Fr. B and C, then absence of message (a 50-fold decrease) from small Pre-B cells (Fr. D). Two other genes, λ5 and VpreB, also show a very similar expression pattern. The molecules encoded by these genes together form the "surrogate light chain" which is thought to play an important role in B cell differentiation before L chain gene rearrangement (11, 13). Indeed, the expression of both genes is largely (by at least 10-fold) restricted to fractions before L chain rearrangement, the Pro-B cell stages. It is interesting to note that all three genes share a common promoter structure lacking the TATA element and bearing a motif recognized by the transcriptional activating protein LyF-1 (23), suggesting that these genes might be coregulated during early B cell differentiation.

Rag-1 and Rag-2. In contrast with the pattern seen for TdT, λ5, and VpreB, the expression of two other genes, Rag-1 and Rag-2, critical for both H and L chain rearrangement (8, 9), occurs at high levels in Pre B (Fr. D) as well as in Pro-B (Fr. B and C). This is expected since the bulk of L chain gene rearrangements are taking place in this latter fraction. Throughout these early B lineage fractions, we detect similar regulation of both Rag-1 and Rag-2, without either being expressed alone (Figs. 2 and 3). This is in contrast to B cell development in the chicken where Rag-2 alone is expressed in a cell fraction thought to be undergoing gene conversion (24). We also continue to detect low levels of Rag-1 and Rag-2 (5–10% of maximum) in immature (IgM^+IgD^-) B cells, but not in IgD^+ cells. We have not yet determined whether this lower level represents uniformly decreased expression in all cells or continued high level in a small subset of the fraction. Nevertheless, this downregulation of recombinase activating genes in immature B cells mirrors a similar finding in newly generated intrathymic T cells (25). Furthermore, there is a report that crosslinking of surface Ig on certain B cell lines that express Rag-1 and Rag-2 results in downregulation of their expression (26). As with T cells, it will be interesting to test whether IgM crosslinking modulates the expression of Rag-1 and Rag-2 in normal cells in vitro.

Mb-1 and Bcl-2. Finally, two additional genes show very different patterns of expression. Mb-1 is the gene encoding Ig-α, one of the molecules associated with the Ig complex and critical for surface expression (10). We find that Mb-1 expression is present from a very early stage of B cell differentiation, consistent with an earlier report (11). Curiously, Mb-1 is expressed at high levels even before most cells have completed a productive H chain rearrangement. In contrast, Bcl-2, thought to encode an antiapoptotic activity (14, 16, 27), is present at high levels in the earliest fraction (Fr. A), but then at very low levels throughout the pathway, even through the immature B cell stage, and finally higher at the mature (IgD^+) B cell stage reminiscent of the change in expression of Bcl-2 during T cell development in the thymus (17). Although the reason for the low level of Bcl-2 expression in immature B lineage cells and its increase in mature B cells is not established, there are several possible interpretations based on the antiapoptotic activity of Bcl-2: (*a*) the large numbers of B lineage cells that fail to complete productive H and L chain rearrangements or that generate H/L chain combinations that do not pair well are fated to die apoptotically in the BM; (*b*) autoreactive B cells are eliminated at this immature stage (28) and only afterwards is Bcl-2 upregulated; (*c*) mature B cells detected in the BM may represent a further selected population that has recirculated back after maturation (and concomitant Bcl-2 upregulation) in the peripheral lymphoid organs [29]); or (*d*) feedback regulation of export from the BM results in the apoptotic death of most emerging B cells once the spleen is stably populated in the adult. Fur-

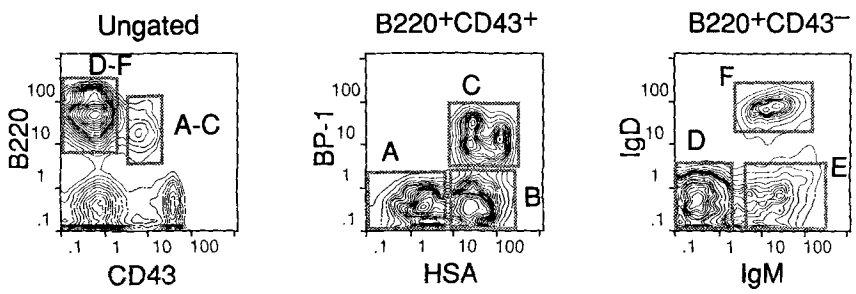

Figure 1. Flow cytometry of BM showing separation of subsets. Four color combinations of reagents specific for B220, IgM, IgD, CD43, HSA, and BP-1 are used to discriminate six fractions, labeled Fr. A (the most immature) through Fr. F (the most mature).

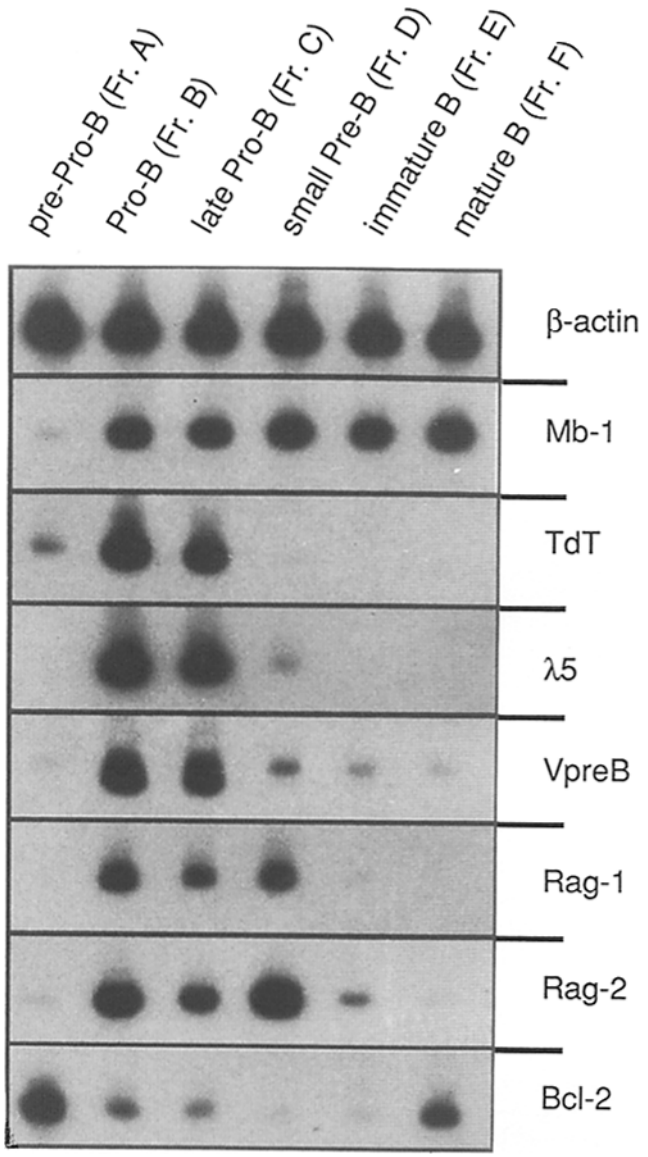

Figure 2. Representative autoradiographs of RT-PCR analysis of gene expression in the six BM B lineage fractions. 2-h exposure of 22 cycle amplifications. (*Fr. A* and *B*) Prepared by incubating BM cells with a combination of fluorescein (FL)-S7, PE-BP-1, PE-anti-IgM, biotin (BI)-30F1, and allophycocyanin (APC)-6B2, then sorted as $S7^+B220^+30F1^-BP\text{-}1^-IgM^-$ and $S7^+B220^+30F1^+BP\text{-}1^-IgM^-$. (*Fr. C*) Prepared by staining BM cells first with FL-S7, PE-anti-IgM, BI-30F1, and APC-6B2, sorting $S7^+B220^+30F1^+IgM^-$ cells, then restaining with PE-BP-1 and sorting $S7^+B220^+30F1^+BP\text{-}1^+$ cells. (*Fr. D–F*) Prepared by staining with FL-S7, PE-anti-IgM, APC-anti-IgD, and BI-6B2 followed by TR-avidin, then sorting $B220^+S7^-IgM^-IgD^-$, $B220^+S7^-IgM^+IgD^-$, and $B220^+S7^-IgM^+IgD^+$ cells.

ther investigation of the expression of Bcl-2 should help to decide between these alternatives.

Ig Gene Rearrangement in B Lineage Fractions in BM

Previously we described a "deletional" approach to determine the status of Ig gene rearrangement in these B lineage fractions in BM (2). This technique used PCR to amplify

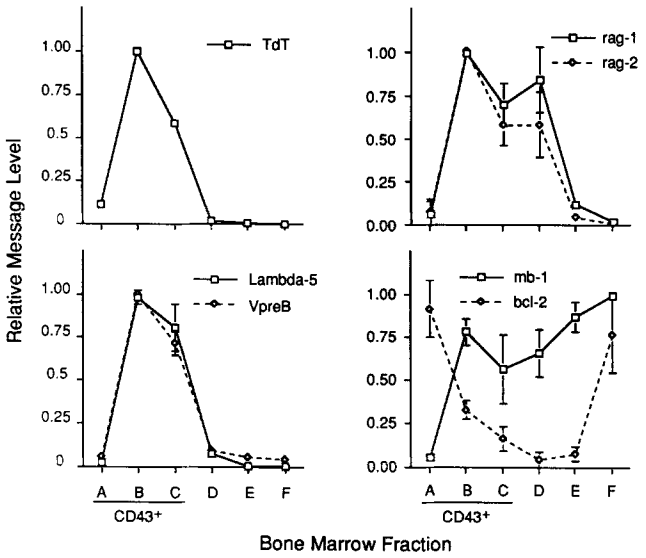

Figure 3. Plot of RT-PCR results for gene expression in BM fractions. Error bars show standard deviation for four analyses from two different sets of sorted samples. Amplitude of message is reported relative to the maximum value obtained in the analyses. See Materials and Methods for details.

segments of DNA that are always deleted upon rearrangement regardless of which V_H, D, or J_H is involved. A complementary technique used previously by others for investigating Ig gene rearrangement takes a "generational" approach: amplify newly generated D-J_H, V_H-DJ_H, or V_κ-J_κ segments that can be detected because rearrangement brings them close enough together to permit amplification (30, 31). The deletional PCR analysis has the advantage that it should detect any rearrangement comparably with no bias due to the particular V_H, D or J_H involved in the rearrangement. However, any retention of excised DNA within the cell would serve to mask the onset of rearrangement, and inversional rearrangement, a feature of approximately half of κ rearrangements (32), could be missed. The generational PCR does not suffer from these disadvantages, but is subject to potential biases in amplifying genes most homologous to the primers employed in the assay.

D-J_H. We utilized an oligonucleotide primer that can amplify 10/12 of the D segments to assess D-J_H rearrangement (33). This primer together with a primer complementary to a segment of DNA 3′ of J_H4 can amplify a ladder of rearranged bands. We reveal this amplified DNA ladder by hybridization with the pJ11 probe which preferentially detects rearrangements to J_H1, J_H2, and J_H3 (there is little homologous sequence for D-J_H4 rearrangements). Furthermore, because of their size, it is likely that D-J_H1 rearrangements are underrepresented. However, these caveats apply for all samples and so comparisons between fractions should yield relative rearrangement levels among the subsets. We also coamplified a fragment from the α-actin gene to permit controlling for variation in input DNA or amplification efficiency. A representative set of autoradiographs is shown in Fig. 4 and the data are summarized in Fig. 5.

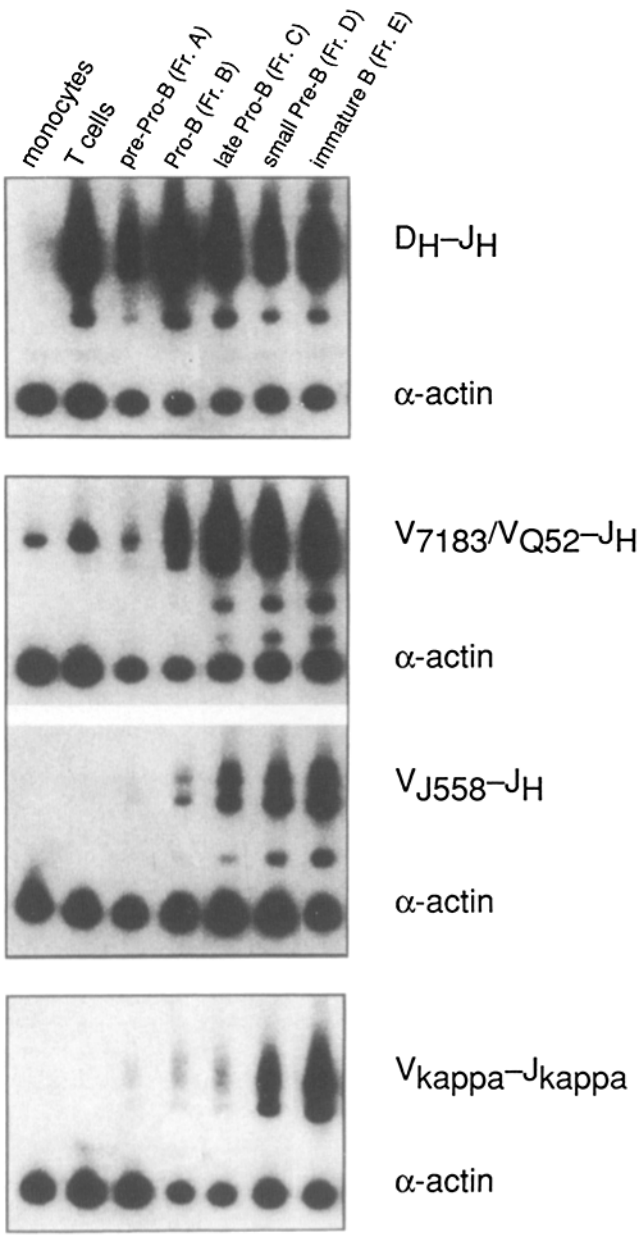

Figure 4. Representative autoradiographs of PCR generational rearrangement assays for five BM fractions and for nonlymphoid and splenic T cells as controls. B lineage cells were sorted as described in Fig. 2. Nonlymphoid cells were sorted as a $B220^-CD43^+$ fraction. T cells were sorted as $CD5^+B220^-IgM^-$.

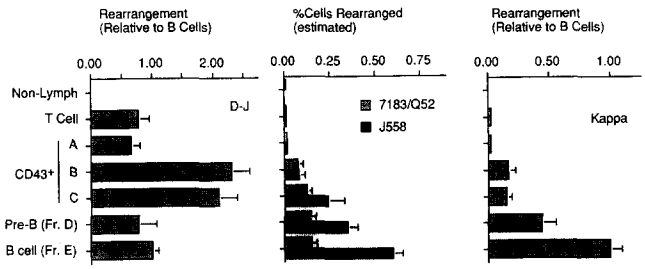

Figure 5. Plot of results of rearrangement assays for BM fractions. Error bars show standard deviation for three to five PCR analyses from two to three sorted samples of each subset. Values are reported relative to the level obtained with IgM^+IgD^- B cells in BM for D-J_H and V_κ-J_κ rearrangement. For V_H-DJ_H rearrangement, we report the estimated frequency of cells in the fraction bearing 7183/Q52 or J558 rearrangements by multiplying the relative intensity times the expected frequency of cells bearing such rearrangements in B cells. See Materials and Methods for details.

Using this approach we find that the maximal level of D-J_H rearrangement is seen in Fr. B and C, the subsets that we reported previously as consisting largely of D-J_H rearranged cells. It is reasonable that, as shown above, these fractions possess high levels of TdT, Rag-1, and Rag-2, important for rearrangement. Furthermore, we were able to detect some D-J_H rearrangement in Fr. A, the earliest fraction analyzed. The failure to previously detect this D-J_H rearrangement by deletional analysis may be due to the persistence of deleted DNA fragments after initial rearrangement, masking some early rearrangement (34). Alternatively, all of this early D-J_H rearrangement might be inversional, although H chain rearrangement is thought to occur exclusively by a deletional mechanism (35–37). Nevertheless, since the level of rearrangement we observed in Fr. A is also seen in splenic T cells (consistent with previous reports of some D-J_H rearrangement in T cell lines; 38), whereas nonlymphoid cells are truly negative, we favor the idea that this level of rearrangement occurs before the branch point between B and T lineage cells in a lymphoid progenitor. The levels of TdT, Rag-1, and Rag-2 are all very low in Fr. A as compared with Fr. B. Thus, it will be interesting to compare the nature of D-J_H rearrangements in Fr. A with those from Fr. B to determine whether such differences in gene expression affect the nature (such as D or J_H usage, reading frame, levels of N addition) of the rearrangements. Curiously, the level of D-J_H rearrangement as assessed by the generational assay appears to diminish as cells progress to the pre-B and latter stages. This decreased signal could be explained by lessened homology with the D primer as V_H-DJ_H rearrangement becomes predominant in these latter fractions.

V_H-DJ_H. V_H-DJ_H rearrangement was assessed by a generational approach employing primers that amplify members of the 7183/Q52 (J-proximal) and J558 (J-distal) V gene families. As described above, amplification together with a primer 3′ of J_H4 will generate a ladder similar to that seen with the D-J amplification (Fig. 4). Interestingly, our results revealed differences in the accumulation of cells with J-proximal versus J-distal rearrangements. That is, the signal seen with a mixture of 7183 and Q52 primers appears in Fr. B and rapidly reaches (at Fr. C) the level seen in B cells. In contrast, the signal from the J558 primer appears in Fr. B at a low level and accumulates more slowly. These relative intensities can be converted into estimated frequencies by assuming that the levels of 7183/Q52 and J558 rearrangements are 20 and 60% in mature B cells, estimates based on analyses of Ig repertoire in cDNA libraries (39). Then, as shown in Fig. 5, 7183/Q52 rearrangements account for 10% of Fr. B (50% level × 20% frequency) whereas J558 rearrangements account for 12% of

Fr. B (20% level × 60% frequency). Thus, in Fr. B, the ratio of J-distal (J558) to J-proximal (7183/Q52) rearrangements is about 1:1, whereas in mature B cells, it is about 3:1 (see Fig. 5). These data support earlier work (40–43) that suggested a preference for members of the J-proximal V gene families early in B cell development. Our results also imply that the "normalization" (i.e., increased representation of J558 genes) occurs before the mature B cell stage and so does not simply reflect antigenic selection between the immature and mature B cell repertoires as was previously hypothesized (44).

V_κ-J_κ. The onset of κ L chain rearrangement was measured by using a V_κ primer reported previously to amplify 80% of κ variable genes (31) together with a primer to $J_\kappa 3$, the nonfunctional joining segment. This allows detection of both $J_\kappa 1$ and $J_\kappa 2$ rearrangements with no penalty for amplifying sequences that are too long (45). This analysis (Figs. 4 and 5) showed that the bulk of κ rearrangements occur in the $CD43^-$ fractions, in agreement with our earlier data. Unexpectedly, we could detect some κ rearrangements very early (Fr. B). Levels seen in $CD43^+$ fractions are low (10% of mature cells), but there is a clear onset of L chain rearrangement simultaneous with V_H-DJ_H H chain rearrangement in some cells. Since we have demonstrated high level TdT expression in Fr. B and C, it will be interesting to determine whether these early κ rearrangements (in Fr. B and C) have greater N addition as compared with the more typical κ rearrangements (in Fr. D).

Similarities and Differences in Gene Expression between Fetal and Adult B Lymphopoiesis

We have extended our analysis of gene expression to ask whether there are discernible differences in B lymphopoiesis at distinct ontogenic timings, comparing phenotypically similar Pro-B cells isolated from fetal liver with those in adult BM (Fig. 6). As predicted from previous reports of diminished N addition at the H chain junctions in fetally generated B cells (18, 46), there is a complete absence of TdT expression in Fr. B/C in day 16 gestation fetal liver. In contrast, the other genes examined, λ5, VpreB, Rag-1, and Rag-2, are all expressed comparably, confirming our previous assignment of functional status with surface phenotype.

Recently, another gene whose expression differs between fetal and adult development has been described, PLRLC (20), whose function may relate to the structural organization of the cell surface. This gene was shown to be expressed in early B and T lineage cells, but absent from more mature populations. Further, it was undetectable in Abelson lines derived from fetal liver or IL-7 cultured fetal liver. Our analysis, shown in Fig. 7, confirms and extends the initial characterization by showing that PLRLC is largely restricted to the Pro-B cell stage (Fr. B in Fig. 7) of early B cell differentiation in BM and is completely absent from the corresponding fraction in fetal liver. These results demonstrate clearly that two otherwise very similar stages of B cell differentiation can differ strikingly in the expression certain genes, depending on ontogenic timing.

Concluding Remarks

Quantitative RT-PCR can be used to determine the levels of B lineage associated gene products during B cell differentiation. We feel that this approach is important because determining expression using transformed cell lines or lymphomas selected as representative of stages in normal cell differentiation might easily misrepresent the regulated expression of genes. Indeed, this appears to be true for λ5/VpreB which are downregulated more rapidly in normal differenti-

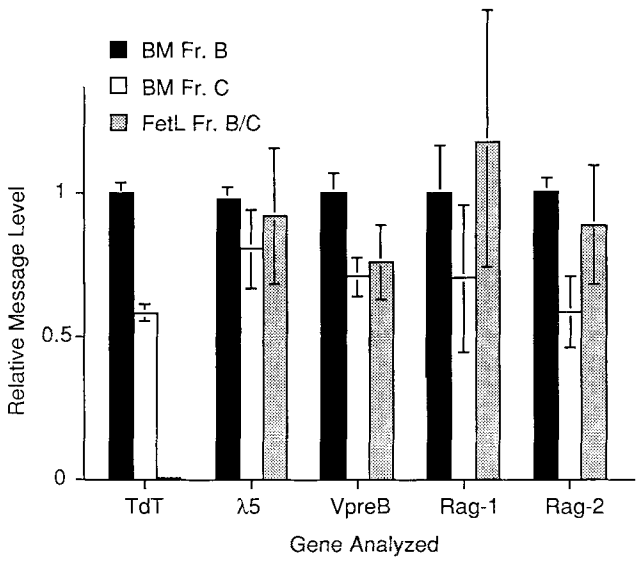

Figure 6. TdT is highly expressed in Pro-B fractions from BM, but absent from Pro-B cells of fetal liver (*FetL*). In contrast, λ5, VpreB, Rag-1, and Rag-2 are expressed at comparable levels in both fractions. For fetal liver, Fr. B and C were not resolved. Error bars show standard deviation for two to three PCR analyses from two to three sorted samples of each subset.

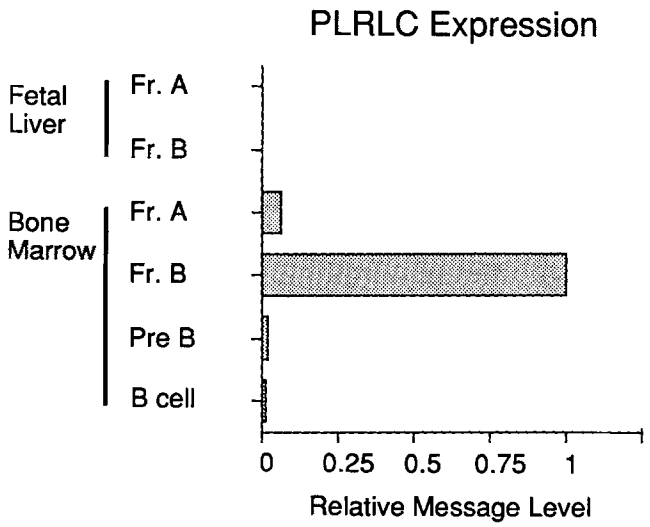

Figure 7. PLRLC expression is maximal in Pro-B cells from BM, but absent from the corresponding fractions from fetal liver. Representative data from several analyses.

ation than has been reported to be the case from work with cell lines (47). Our data with normal cells supports our previously proposed model of ordered B cell differentiation stages and reveals precise regulation of gene expression throughout this pathway. Furthermore, analysis of TdT expression permits correlation with the work of Park and Osmond (48) who have extensively characterized the population dynamics of BM B cell differentiation. Finally, our results with Bcl-2 expression are particularly intriguing, providing evidence for its role in the maturation of B cells similar to that proposed in the T lineage. Results with Bcl-2 transgenic mice showing expanded populations of B lineage cells in these animals also support this idea (15, 49, 50).

Our analysis of the status of Ig gene rearrangement in these fractions confirms the broad outlines of our previous assignments, but with a few notable differences that provide interesting points for further investigation. For example, although D-J_H rearrangement may appear one stage earlier than previously detected, the cells in this fraction may represent a largely uncommitted lymphoid progenitor population (pre-Pro-B cells), since comparable levels of D-J_H rearrangement are also detected in T cells and since the levels of TdT and Rag-1 and Rag-2 are all very low compared with their expression in Pro-B cells. In addition, some V_H-DJ_H rearrangement appears earlier than previously detected, but this is enriched for particular (J-proximal) gene families. Finally, the detection of some V_κ-J_κ rearrangements very early in the pathway, in a fraction expressing high levels of TdT, provides impetus for determination of Ig H and L chain sequences at the single cell level.

The detection of differences in expression of TdT and PLRLC in corresponding fractions depending on ontogenic timing is important in light of our previous finding that B cells with distinct phenotypes are generated from fetal and adult Pro-B cells in identical environments, suggesting a developmental switch in B lymphopoiesis (19). Our data suggest that the majority of these fetally generated B cells will have reduced N addition (at least those generated at day 16 of gestation) owing to the lack of TdT expression. Furthermore, it is interesting to identify another difference between fetal and adult B cell precursors related to the structural organization of the cell, but unrelated to Ig gene rearrangement, suggesting that numerous unrecognized differences between fetal and adult B cell differentiation await further investigation. Based on analysis of enrichment for certain types of autoreactive Abs in the CD5 B cell subset, we proposed previously that fetal-derived B cells are positively selected based on germline encoded self-reactivity (51, 52), unlike adult-derived B cells. Thus, additional candidates for differential expression may include molecules involved in signal transduction. We believe our approach provides a useful method to continue this investigation.

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