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*J Immunol* 2004; 172:144-154; doi: 10.4049/jimmunol.172.1.144
http://www.jimmunol.org/content/172/1/144

**Supplementary Material**

http://www.jimmunol.org/content/suppl/2004/02/06/172.1.144.DC1

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Analysis of Gene Expression and Ig Transcription in PU.1/Spi-B-Deficient Progenitor B Cell Lines

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A number of presumptive target genes for the Ets-family transcription factor PU.1 have been identified in the B cell lineage. However, the precise function of PU.1 in B cells has not been studied because targeted null mutation of the PU.1 gene results in a block to lymphomyeloid development at an early developmental stage. In this study, we take advantage of recently developed mutant mouse lines, including PU.1/Spi-B double-deficient (d.d.d) IL-7 and stromal cell-dependent progenitor B (pro-B) cell lines to analyze the function of PU.1 and Spi-B in B cell development. We show that contrary to previously published expectations, PU.1 and/or Spi-B are not required for Ig H chain (IgH) gene transcription in pro-B cells. In fact, PU.1/Spi-B d.d.d pro-B cells have increased levels of IgH transcription compared with wild-type pro-B cells. In addition, high levels of Igk transcription are induced after IL-7 withdrawal of wild-type or PU.1/Spi-B d.d.d pro-B cells. In contrast, we found that Igλ transcription is reduced in PU.1/Spi-B d.d.d pro-B cells relative to wild-type pro-B cells after IL-7 withdrawal. These results suggest that Igλ, but not IgH or Igk, transcription, is dependent on PU.1 and/or Spi-B. The PU.1/Spi-B d.d.d pro-B cells have other phenotypic changes relative to wild-type pro-B cells including increased proliferation, increased CD25 expression, decreased c-Kit expression, and decreased RAG-1 expression. Taken together, our observations suggest that reduction of PU.1 and/or Spi-B activity in pro-B cells promotes their differentiation to a stage intermediate between late pro-B cells and large pre-B cells.

The development of B lymphocytes from hemopoietic stem cells is a multistep process, involving the ordered expression of intracellular and surface-located marker proteins, and stepwise rearrangement of gene segments in the Ig H and L chain gene loci (reviewed in Refs. 1–4). Lymphoid progenitors, including the earliest detectable B cell progenitors, are found in a fraction of bone marrow cells which express the α subunit of the IL-7R (IL-7Ra), low levels of c-Kit, and variable levels of terminal deoxynucleotidyl transferase (4, 5). The expression of the recombination activating genes RAG-1 and RAG-2, as well as D μ JH rearrangement of IgH genes, may also occur at this stage. It is generally agreed that acquisition of surface expression of both B220 and CD19 signifies commitment to the B cell lineage. These cells are generally referred to as progenitor B (pro-B)3 cells (4). Pro-B cells differentiate into pre-B cells when they complete V μ to D μ JH recombination and begin to express cytoplasmic IgH protein. Small amounts of functionally rearranged IgH protein can pair with the surrogate L chain components Vα and Aα and be deposited on the cell surface as a pre-B cell receptor (pre-BCR) (1). Pre-BCR-expressing cells are termed large pre-B cells, because these cells undergo a burst of proliferation stimulated by combined signaling from the pre-BCR and IL-7R (reviewed in Ref. 6). Dilution of surrogate L chain proteins over a series of cell divisions, as well as down-regulation of IL-7R expression, results in a halt to VH D H J H recombination and a return to proliferative quiescence (1, 7). These quiescent cells shrink in size and are referred to as small pre-B cells (1, 3). Finally, reactivation of RAG gene transcription and rearrangement of the IgL genes are induced by this proliferative quiescence (1). Functional rearrangement of Igk or Igλ and successful pairing with IgH proteins results in surface deposition of a functional BCR (1). BCR-expressing cells are considered to be immature B cells. There are at least three distinct classification schemes which define these stages (reviewed in Refs. 1–4). In this paper, we will use the classification terminology recently reviewed by Lu and Osmond (3).

Several transcription factors play important roles in B cell development, including PU.1, Ikaros, E2A, EBF, and Pax-5 (reviewed in Refs. 8 and 9). Mice homozygous for a null mutation in the PU.1 gene die during fetal development by 18.5 days postcoitum (d.p.c.) and lack B, T, and myeloid progenitors (10, 11). PU.1−/− fetal liver contains reduced numbers of multipotential lymphoid-myeloid progenitors (AA4.1+, Lin−). Furthermore, these mutant progenitors fail to proliferate and differentiate into pro-B cells in response to stromal cell contact and IL-7 (11). This is in part because PU.1−/− fetal liver progenitors fail to express the IL-7Ra gene (12). An alternative knockout model of the PU.1 gene, generated by insertional mutation of the PU.1 DNA-binding domain, results in a different phenotype such that homozygous null mice survive until birth and generate small numbers of thymic T cells and neutrophil-like cells (13, 14). However, B cells and B cell progenitors are undetectable in both PU.1 mutant mouse lines, demonstrating that PU.1 is absolutely required for B cell development (10, 11, 13).

PU.1 is a member of a subfamily of Ets transcription factors that includes two other closely related factors, Spi-B and Spi-C. Spi-B exhibits 43% overall amino acid sequence identity to PU.1, but the Ets domain is 67% identical to PU.1 (15). Spi-C (also called Prf) shares 40% overall sequence identity with PU.1, and shares 59%
identity in the DNA binding domain (16, 17). PU.1 and Spi-B function as strong transcriptional activators, while Spi-C has weak transcription activation ability (17). PU.1 is expressed in multiple hemopoietic lineages, including all stages of B cell development, while the expression of Spi-B is restricted to B cells and T cells (18–20). Targeted null mutation of Spi-B does not affect B cell development, but results in defective BCR-mediated responses (21, 22). Both PU.1 and Spi-B are expressed in the B cell lineage beginning at the pro-B cell stage and appear to interact with identical DNA binding sites (15). Therefore in developing B cells, the available evidence indicates that PU.1 and Spi-B are functionally interchangeable (22, 23).

A number of presumptive target genes for PU.1 and/or Spi-B have been identified in vitro in the B cell lineage. First, binding sites are found in a number of Ig promoters and enhancers, including Igκ V region promoters (24), the Igκ 3’ enhancer (25), the Igλ 2–4 enhancer (26), the IgH intronic enhancer (27), and the Ig J-chain promoter (28). Other lymphoid and B cell-specific genes regulated by PU.1 and/or Spi-B include c-rel (29), CD20 (30), mb-1 (31), P2Y10 (23), Bruton’s tyrosine kinase (Btk) (32), germin center nuclear-associated protein (33), and CD72 (34). These target genes have been identified by analysis of promoter and/or enhancer elements involved in driving their tissue-specific transcription. However, because mutation of the PU.1 gene results in complete loss of B cell development, it has been unclear whether PU.1 is required for transcription of any of these genes in B cells. We have recently shown that retrotransduction of PU.1/fl or PU.1/fl–Spi-B/fl– progenitors with IL-7Rα cDNA restores IL-7 and stromal cell-dependent proliferation. In addition, IL-7Rα transduction promotes low-frequency differentiation into CD19+ pro-B cells (12). We have therefore been able to establish IL-7-dependent PU.1/fl– and PU.1/fl–Spi-B/fl– pro-B cell lines and clones. The comparison of gene expression in wild-type and PU.1/fl–Spi-B/fl– pro-B cell lines represents a novel opportunity to investigate the target genes of PU.1 and/or Spi-B in the B lineage.

In this study, we compare wild-type and PU.1/fl–Spi-B/fl– pro-B cell lines to analyze the function of PU.1 and/or Spi-B in B cell development. We examined PU.1/fl–Spi-B/fl– pro-B cell lines due to possible functional redundancy of PU.1 and Spi-B in B cells. We show that contrary to previously published predictions, transcription of IgH is increased in PU.1/fl–Spi-B/fl– pro-B cells relative to wild-type pro-B cells. After IL-7 withdrawal, transcription of Igκ, but not Igλ, can be induced in PU.1/fl–Spi-B/fl– pro-B cells. Phenotypic characterization of PU.1/fl–Spi-B/fl– pro-B cell lines suggests that these cells are further advanced in their development than wild-type pro-B cell lines. We demonstrate that we can reproduce these results by interfering with PU.1 and/or Spi-B activity in wild-type cell lines using ectopic expression of an inactive mutant form of PU.1. Taken together, these results suggest that reduction of PU.1 and/or Spi-B activity in pro-B cells promotes their differentiation to a stage intermediate between late pro-B cells and large pre-B cells.

Materials and Methods

Cell lines

The retroviral packaging cell lines Phoenix (35) and GP+E86 (36) were maintained in complete DMEM medium. S17 stromal cells (37) and J558-IL-7 cells (38) were maintained in complete RPMI 1640 medium. All complete medium contained 10% FBS (S17 stromal cells, 5%), 100 U/ml penicillin/streptomycin, 2 mM L-glutamine, 5 × 10–5 M 2-ME, and 0.5 mM HEPEs (Invitrogen, San Diego, CA).

Construction of retroviral vectors and packaging cell lines

MIG-PU.1 retrovirus containing PU.1 and an internal ribosomal entry site green fluorescent protein (IRES-GFP) has been described previously (39, 40). PU.1AN100 was generated as described previously (40). PU.1AN100 was ligated into the XhoI and EcoRI sites of the MIGR1 vector after digestion from the MSCV-EGFP vector using digestion with XhoI and EcoRI. To establish retroviral packaging cell lines, retrovirus produced by transient transfection of Phoenix cells was used to infect GP+E86 cells after overnight treatment with tunicamycin. After 2 days, the brightest GFP-expressing cells were sorted using the BD Biosciences FACSVerse system (San Diego, CA) (Cincinnati Shriners Hospital, Cincinnati, OH) and expanded under gpt selection. Sorted cell lines were analyzed periodically to ensure continued GFP expression. Expression of PU.1 and PU.1AN100 were confirmed by Western blotting using an anti-C-terminal PU.1 Ab (Santa Cruz Biotechnology, Santa Cruz, CA).

Isolation of lineage-depleted fetal hemopoietic progenitors

PU.1/fl+ or PU.1/fl–Spi-B/fl– mice were mated to produce PU.1/fl– and PU.1/fl–Spi-B/fl– fetuses as well as control littersmates. The presence of a vaginal plug on the morning after matings was taken as 0.5 d.p.c. Genotyping of PU.1 alleles was performed by PCR as previously described (12). Lineage-depleted (Lin–) hemopoietic progenitors were isolated from 14.5 d.p.c. fetal liver suspensions as also described previously (40).

Infection and culture of lineage-depleted fetal hemopoietic progenitors

GP+E86 lines were irradiated using 2000 rad from a Cesium-137 source and plated at 1.5 × 105 cells/well in a 24-well plate (Corning–Costar, Cambridge, MA) and incubated overnight. Lin– fetal liver progenitors (105) were added to each well and infected by coculture for 2 days in complete IMDM medium and 5% conditioned medium from J558-IL-7 cells. Infected cells were removed by gentle pipetting and transferred onto a monolayer of irradiated (2000 rad) S17 stromal cells. Cultures were fed with fresh IL-7-containing complete IMDM medium every 4 days and analyzed after 10–14 days.

Establishment of IL-7-dependent cell lines

Cells from 10–14 day cultures of PU.1/fl– and PU.1/fl–Spi-B/fl– hemopoietic progenitors resuspended with IL-7Rα cDNA were expanded and passaged by plating on monolayers of irradiated S17 stromal cells in IL-7 containing medium as described above. IL-7-dependent cell lines were passaged by plating 5 × 105 cells/ml every 3–4 days.

Infection and establishment of retrovirally infected pro-B cell lines

Irradiated (2000 rad) GP+E86 packaging cell lines were plated into six-well plates at a concentration of 5 × 105 cells/well in a 24-well plate (Corning–Costar, Cambridge, MA) and incubated overnight. Lin– fetal liver progenitors (105) were added to each well and infected by coculture for 2 days in complete IMDM medium and 5% conditioned medium from J558-IL-7 cells. Infected cells were removed by gentle pipetting and washed three times in flow cytometry buffer (Dulbecco’s PBS (Life Technologies) supplemented with 0.5 mM EDTA and 0.5% BSA fraction V (Fisher, Pittsburgh, PA)) and sorted for the brightest 40% GFP-expressing cells. These cells were sorted into gentamicin-containing (10 μg/ml) IL-7-containing complete IMDM medium and plated into 25-cm2 flasks containing 7 × 105 irradiated (2000 rad) S17 stromal cells. Flow cytometric analysis was performed at each passage to ensure maintenance of GFP expression.

Affymetrix GeneChip analysis

Affymetrix GeneChip analysis (Santa Clara, CA) was performed by the Cincinnati Children’s Hospital Research Foundation Affymetrix core facility. Total RNA was extracted from IL-7-dependent pro-B cells using RNA-Bee (Tel-Test, Friendswood, TX). RNA was chloroform extracted a Cincinnati Children’s Hospital Research Foundation Affymetrix GeneChip analysis (Santa Clara, CA) and plated at 1.5 × 105 cells/well in a 24-well plate (Corning–Costar, Cambridge, MA) and incubated overnight. Lin– fetal liver progenitors (105) were added to each well and infected by coculture for 2 days in complete IMDM medium and 5% conditioned medium from J558-IL-7 cells. Infected cells were removed by gentle pipetting and washed three times in flow cytometry buffer (Dulbecco’s PBS (Life Technologies) supplemented with 0.5 mM EDTA and 0.5% BSA fraction V (Fisher, Pittsburgh, PA)) and sorted for the brightest 40% GFP-expressing cells. These cells were sorted into gentamicin-containing (10 μg/ml) IL-7-containing complete IMDM medium and plated into 25-cm2 flasks containing 7 × 105 irradiated (2000 rad) S17 stromal cells. Flow cytometric analysis was performed at each passage to ensure maintenance of GFP expression.

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PCR analysis

Total RNA was extracted from culture of IL-7-dependent pro-B cell lines using RNA-Ace (Tel-Test). Total RNA at 1 μg/μl was used as template for cDNA synthesis using a cDNA synthesis kit (Clontech Laboratories, Palo Alto, CA). This cDNA was analyzed using primers spanning intronic sequence for β-actin to verify lack of DNA contamination and equal cDNA amplification from various template RNAs. Primers for various genes were designed to span introns or splice junctions using Stratagene PCR primer designer. For real-time PCR, 25 μl PCR consisted of 100 pmol of primers, 1×Taq buffer (GeneTaq, Frederick, MD), 0.5× of 50,000× stock of SYBR Green I (Molecular Probes, Eugene, OR), 100 pmol of MgCl₂, 1× standard PCR buffer, and nuclease-free deionized water. All amplification protocols used a 1-min melting step of 90°C followed by 45 cycles of amplification. Each cycle for various primer sets used an initial melt of 95°C for 15 s, an annealing step of 7 s at optimized temperature, followed by amplification. Each cycle for various primer sets used an initial melt of 95°C for 15 s, an annealing step of 7 s at optimized temperature, followed by amplification. Each cycle for various primer sets used an initial melt of 95°C for 15 s, an annealing step of 7 s at optimized temperature, followed by amplification.

Flow cytometric analysis

Pro-B cells (0.5 × 10⁶) were washed three times in flow cytometry wash buffer. Following the third wash, cells were suspended in 100 μl of a 1/100 dilution of the appropriate Abs and stained for 30 min on ice. Cells were spun out, washed three times, and finally diluted into 300 μl of 748 mM propidium iodide solution. Cells were analyzed using a BD Biosciences FACScalibur system by gating on forward and side scatter characteristics for optimal size and shape of cells. Secondary gating was done based on propidium iodide staining for viable cells. The final gating procedure involved either staining from PerCP-PE or allophycocyanin based on the presence of endogenous GFP expression.

Western blots

Lysates were generated from pro-B cells and S17 stromal cells using Laemmli buffer according to standard methods. Lysates were run on SDS-PAGE gel using the Mighty Small Electrophoresis System (Hoeffer, San Francisco, CA). Proteins were transferred to nitrocellulose and stained with anti-IgM or anti-PU.1 using standard procedures. Anti-β-actin was used as control for lysate concentration. HRP secondary Abs were visualized using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and used to expose x-ray film (Kodak, Melville, NY).

Statistics

All statistics were performed on separate sets of cell lines when possible. The figures show results of representative experiments.

Results

Phenotypic characterization of PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ and Spi-B⁻⁻/⁻⁻/⁻⁻ pro-B cell lines

PU.1 and Spi-B are thought to directly regulate a number of target genes in the B cell lineage. However, homozgyous null mutation of the PU.1 gene results in an early block to B cell development. We have previously shown that retroviral transduction of PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ and Spi-B⁻⁻/⁻⁻/⁻⁻ fetal liver progenitors with IL-7Rα cDNA, followed by culture in IL-7 on S17 stromal cells, results in differentiation into CD19⁺ pro-B cells (12). These pro-B cells can be established as IL-7-dependent cell lines. We showed that by several criteria, such as expression of B cell-specific genes and active Dnmt1 DNA rearrangement, these cell lines appear to represent normal late pro-B cells (12). To investigate whether PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ and Spi-B⁻⁻/⁻⁻/⁻⁻ pro-B cells are phenotypically identical to wild-type pro-B cells, we characterized two sets of these cell lines further.

First, cell counting was used as a method of measuring the doubling time of wild-type and mutant cell lines. PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ pro-B cell lines had a significantly reduced doubling time than wild-type cell lines, indicating an increased proliferation rate (16.5 ± 0.6 h, n = 3; compared with 20.2 ± 1.1 h, n = 3) (Fig. 1A). Next, flow cytometric analysis was used to characterize wild-type and mutant cells. Expression of cell surface markers has been extensively used to classify distinct stages of B cell development (reviewed in Ref. 2). PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ pro-B cells had previously been shown to express CD19 (12). CD19 is a B cell-specific surface Ag that is expressed upon commitment to the B cell lineage and functions as an amplifier of BCR signaling (42). As expected, all PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ and wild-type pro-B cell lines expressed similar levels of CD19 (Fig. 1B, upper panels). In contrast, the low-affinity IgG FcR FcγRIIB, which is suspected to be a target gene of PU.1 (43), was expressed on wild-type pro-B cells but was reduced or undetectable on PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ pro-B cell lines (Fig. 1B, lower panels). Analysis of surface H chain expression showed that extracellular IgH levels were low on PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ pro-B cell lines (Fig. 1C, first row). CD43 (sialophorin) is an early B cell development marker that is lost in the transition from early large pre-B to late large pre-B cells, coinciding with surface expression of the pre-BCR (44). We found that CD43 was expressed at similar levels in wild-type and PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ cell lines (Fig. 1C, second row). The receptor for stem cell factor, c-KIT, is expressed throughout early B cell development but is lost during the transition from pro-B to the large pre-B stage (reviewed in Ref. 1). Although wild-type pro-B cells were positive for c-KIT (mean fluorescence (MF) 109 ± 7.0, n = 4), PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ pro-B cells had reduced levels of expression (MF 5 ± 1.4, n = 4) (Fig. 1C, third row). Despite retroviral expression with IL-7Rα cDNA, the PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ pro-B cell lines had greatly reduced expression (MF 7 ± 1.7, n = 3) compared with wild-type pro-B cells (MF 33 ± 1.7, n = 3) (Fig. 1C, bottom row). Finally, we examined expression of CD25 (IL-2Rα, Tac), which is a marker for differentiation to the pre-B cell stage (45). PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ pro-B cell lines expressed significantly higher levels of CD25 (MF 53 ± 3.9, n = 3) than did wild-type cell lines (MF = 14 ± 0.9, n = 3) (Fig. 1C, bottom row). In summary, PU.1⁻¹⁻⁻/⁻⁻/⁻⁻ pro-B cell lines proliferate at a
greater rate; yet express lower levels of c-Kit and IL-7Rα than wild-type pro-B cell lines. Other surface markers, such as CD19, CD43, and H chain, are comparable to wild-type pro-B cell lines. Expression of CD25 on PU.1+/− Spi-B−/− cells suggests that these cells may be further advanced in their differentiation than wild-type cell lines.

Comparison of gene expression profiles of wild-type and PU.1+/− Spi-B−/− pro-B cell lines

Affymetrix GeneChip analysis was used to comprehensively compare patterns of gene expression in PU.1+/− Spi-B−/− and wild-type pro-B cells. Total RNA was prepared from two sets of independently derived wild-type and PU.1+/− Spi-B−/− pro-B cell lines. One RNA set was probed once and the other twice using the U74Av2 GeneChip (Affymetrix). The data was analyzed using MicroArray Suite 5.0 software (Affymetrix). Data from the three independent analyses were compiled into lists of genes that were either increased or decreased at least 2-fold in the PU.1+/− Spi-B−/− pro-B cells relative to the wild-type (see full list in supplemental Tables I and II). Genes were broken down into classifications using GeneSpring software (Silicon Genetics, Redwood City, CA). Of the genes increased in expression in PU.1+/− Spi-B−/− pro-B cell lines, the greatest proportion was comprised of genes encoding enzymes (28%) and signal transduction molecules (30%). Genes encoding DNA binding proteins such as transcription factors made up 5% of those increased, whereas genes encoding cell surface Ags and receptors also made up 5%. Increased unassigned expressed sequence tags made up a large proportion (13%). Genes encoding cell cycle factors and other various proteins made up only a small fraction (5%). The most unexpected finding in the increased gene list was the percentage of immunity related proteins (14%), which included multiple IgH transcripts (11 probe sets) (supplemental Table I and Table I).

Genes decreased in expression may represent genes that require PU.1 or Spi-B function either directly or indirectly. Similar to those increased in PU.1+/− Spi-B−/− pro-B cells, the majority of the genes decreased in expression in PU.1+/− Spi-B−/− cell lines were those encoding enzymes (34%) and signal transduction molecules (27%). More genes encoding cell surface proteins were decreased (15%) than increased (5%). The proportions of genes encoding DNA binding proteins (5%) and cell cycle components (1%) decreased in expression were similar to those increased. The number of unassigned expressed sequence tags found to be decreased in expression (12%) was very similar to those increased. Despite the lower percentage of immunity related molecules (6%) decreased relative to those increased, an interesting result was that multiple Igκ L chain transcripts were significantly decreased in expression (nine probe sets) (supplemental Table II). Table I lists several genes that have B cell developmental relevance, which were selected for further analysis.

PU.1+/− Spi-B−/− cell lines have increased levels of IgH transcription and protein expression

Functional rearrangement and expression of the IgH locus is required to generate a functional BCR. Steady state levels of IgH transcripts were unexpectedly increased in PU.1+/− Spi-B−/− cell lines relative to wild-type cell lines. The increase in IgH transcripts in the PU.1+/− Spi-B−/− cell lines was observed with probe sets representing transcription from multiple regions of the IgH locus including those initiating in V regions and the Dκ-Jκ regions (Table I). The largest fold increase was seen in probe set 100361_f_at (53.1 ± 5.7-fold) representing germline IgH transcripts initiating in the Dκ-Jκ region. To confirm increased levels of H chain transcript expression, we used the ΔΔCt real-time RT-PCR method (41) to quantitate Igκ transcripts, which initiate within the IgH intronic enhancer (46). We found that Igκ transcripts were increased 23.5 ± 2.5-fold (n = 3) in PU.1+/− Spi-B−/− cell lines relative to wild-type cell lines (Fig. 2A). We also performed Western blotting to investigate whether increased concentrations of IgH protein were detectable in PU.1+/− Spi-B−/− cell lines. Cytoplasmic IgH
protein is normally detectable before assembly with surrogate L-chain components to form a functional pre-BCR (1). The results shown in Fig. 2B show that these cell lines express high concentrations of full-length IgH protein, whereas wild-type pro-B cells expressed low concentrations. Because most PU.1−/−/Spi-B−/− cells do not express detectable IgH on their surface (see Fig. 1C, first row) this likely represents protein accumulated in the cytoplasm of these cells. In summary, PU.1−/−/Spi-B−/− cell lines have increased levels of IgH transcription and cytoplasmic IgH protein compared with wild-type cell lines. These results indicate that PU.1 and/or Spi-B are not required for IgH transcription in pro-B cell lines.

**PU.1−/−/Spi-B−/− cell lines have decreased levels of Igκ transcription**

Results from nine independent probe sets on the U74A arrays indicated that steady state levels of Igκ transcripts were decreased in PU.1−/−/Spi-B−/− pro-B cells relative to wild-type cells (Table I). To confirm this result, real-time PCR was performed to quantitate levels of Igκ germline transcripts. ακ transcripts initiate at a site 5′ to the germline Jκ1 gene (47, 48). We found that ακ transcripts were decreased by 2.9-fold in PU.1−/−/Spi-B−/− pro-B cells relative to wild-type cells (Fig. 2A). Although there are two probe sets representing Igκ transcripts on the U74A arrays, these transcripts were scored as absent in all cell lines and also could not be detected by RT-PCR (data not shown). In summary, Igκ germline transcription is decreased in PU.1−/−/Spi-B−/− pro-B cells relative to wild-type cells, suggesting that PU.1 and/or Spi-B might be required for optimal levels of Igκ transcription in IL-7-dependent pro-B cells.

**PU.1−/−/Spi-B−/− cell lines have decreased levels of RAG-1, Btk, and B cell linker protein (BLNK) transcription**

Besides Igκ, several lymphocyte-restricted genes were decreased in expression in PU.1−/−/Spi-B−/− pro-B cells. As suggested by the flow cytometric analysis and the large fold change using Affymetrix GeneChip analysis, FcyRIIb was expressed in wild-type pro-B cell lines but was undetectable in PU.1−/−/Spi-B−/− cell lines (Fig. 2B). Rearrangement of the Ig loci requires the action of the recombining enzymes RAG-1 and RAG-2 (reviewed in Ref. 2). As measured on the U74A arrays, steady state levels of both RAG-1 and RAG-2 transcripts were reduced in the PU.1−/−/Spi-B−/− cell lines. Using real-time RT-PCR, we found that RAG-1 transcripts were decreased by 4.1-fold in PU.1−/−/Spi-B−/− relative to wild-type cell lines (Fig. 2A). Btk and BLNK transcripts were reduced by 4.5 ± 1.9-fold (one probe set, n = 3) and 4.8 ± 1.4 (two probe sets, n = 3) respectively, in PU.1−/−/Spi-B−/− cells. Using real-time RT-PCR, we found that levels of BLNK transcription were decreased by 4.0-fold in PU.1−/−/Spi-B−/− cells relative to wild-type cells (Fig. 2A). These results suggest that PU.1 and/or Spi-B might be required for optimal transcription of the FcyRIIb, RAG-1, btk, and BLNK genes in IL-7-dependent pro-B cells.

**Igκ but not Igλ transcription and rearrangement is increased in PU.1−/−/Spi-B−/− cells after IL-7 withdrawal**

Culture in IL-7 arrests developing B cells at the late pro-B cell stage (49). When IL-7-dependent cell lines are cultured on stromal cells in the absence of IL-7, they initiate apoptotic cell death but also initiate differentiation, by rearrangement of L chain genes,
into surface Ig-expressing B cells (49, 50). Therefore, we withdrew IL-7 from culture media of wild-type and PU.1\(^{−/−}\)Spi-B\(^{−/−}\) pro-B cells for 72 h to test the ability of these cells to successfully rearrange and express Igκ L chain on their surface relative to wild-type pro-B cells. Igκ expression was measured using flow cytometry or using real-time RT-PCR for κ\(^{\text{V}}\) transcripts. In the presence of IL-7, few wild-type (0.3 ± 0.1%, n = 3) or PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cells (0.9 ± 0.2%, n = 3) expressed Igκ on their surface (Fig. 3A, upper panels). After IL-7 withdrawal, an increased fraction (2.5 ± 0.8%, n = 3) of wild-type pro-B cells expressed Igκ on their surface, similar to what has previously been reported (49). In contrast, a larger proportion of PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cells rearranged and expressed Igκ on their surface (10.9 ± 1.5%, n = 3) compared with wild-type cell lines (Fig. 3A, lower panels). These results demonstrate that PU.1\(^{−/−}\)Spi-B\(^{−/−}\) pro-B cells have an increased ability to rearrange and express IgH and Igκ genes after IL-7 withdrawal.

Germline (“sterile”) transcription of the Igκ and Igλ genes has been shown to be increased in pro-B cells after IL-7 withdrawal (51). Therefore, we performed standard RT-PCR and relative quantitation real-time RT-PCR to visualize the changes in Igκ and Igλ transcription after IL-7 withdrawal. As suggested by the cell surface expression of Igκ, PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cells increased their levels of κ\(^{\text{V}}\) transcription more than wild-type pro-B cells after IL-7 withdrawal (Fig. 3B, upper panels). Real-time RT-PCR analysis of κ\(^{\text{V}}\) transcripts confirmed that IL-7 withdrawal stimulated transcription at the Igκ locus to a greater degree in PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cells (14.6 ± 2.0-fold, n = 2) than in wild-type cells (5.5 ± 2.1-fold, n = 2) (Fig. 3C). The Igλ locus contains three functional genes, termed λ1, λ2, and λ3 (51). We analyzed germline transcription from the λ2 gene (51) as well as transcription of the VJ-rearranged λ1 gene (52). After IL-7 withdrawal, wild-type pro-B cells increased germline A2 as well as VJ-rearranged λ1 transcription (Fig. 3B, second and third rows). In contrast, in PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cells, VJ-rearranged λ1 transcription was undetectable, and germline A2 transcription was greatly impaired. In summary, PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cell lines actually increase their level of Igκ transcription and V-J rearrangement to greater than wild-type levels after IL-7 withdrawal. In contrast, Igλ transcription is absent or greatly impaired, demonstrating that PU.1 and/or Spi-B are important for Igλ transcription.

FIGURE 2. Analysis of gene expression and H chain protein levels in PU.1\(^{−/−}\)Spi-B\(^{−/−}\) pro-B cells. A. Real-time RT-PCR analysis of gene transcripts in pro-B cells. Total RNA from PU.1\(^{−/−}\)Spi-B\(^{−/−}\)and wild-type pro-B cells was used to generate cDNA that was analyzed by real-time RT-PCR. cDNA concentrations were normalized to G6PDH expression. Pro-B cells was used to generate cDNA that was analyzed by RT-PCR. Upper panel, FcγRIIb transcripts; lower panel, β-actin transcripts. C. Western blot analysis of Ig H chain protein levels in pro-B cells. Western blot analysis was performed on lysates from wild-type pro-B cells, PU.1\(^{−/−}\)Spi-B\(^{−/−}\) pro-B cells, and 317 stromal cells as a control. Blots were probed with anti-IgM and anti-β-actin as control.

The PU.1\(^{−/−}\)Spi-B\(^{−/−}\) phenotype can be recapitulated by ectopic expression of an inactive form of PU.1

To confirm that the phenotype displayed by PU.1\(^{−/−}\)Spi-B\(^{−/−}\) pro-B cells is primarily the result of loss of expression of PU.1 and/or Spi-B, we considered that ectopic expression of a mutant form of PU.1 lacking the N-terminal 100 amino acids might interfere with endogenous PU.1 and/or Spi-B activity in wild-type pro-B cell lines. Deletion of the first 100 amino acids of PU.1 (PU.1\(^{ΔN100}\)) removes both the acidic and the glutamine-rich activation domains of the protein, as measured on multimerized PU.1 binding sites (53). PU.1\(^{ΔN100}\) is also incapable of rescuing B cell or macrophage development from PU.1\(^{−/−}\) progenitors (40).

Therefore, we constructed a recombinant retroviral vector containing a cDNA encoding the ΔN100 mutant form of PU.1 (MIG-PU.1\(^{ΔN100}\)). A stably infected MIG-PU.1\(^{ΔN100}\) packaging cell line was then used to infect wild-type pro-B cell lines by coculture. Infected pro-B cell lines that expressed high levels of GFP protein were selected using flow cytometry and propagated in cultures containing IL-7 and stromal cells. As controls, wild-type pro-B cell lines were infected with control (MIGR1) retrovirus or retrovirus encoding wild-type PU.1 (MIG-PU.1). Infection of wild-type pro-B cell lines with control retrovirus had no effect on proliferation of the cells. In contrast, infection with MIG-PU.1 retrovirus blocked proliferation of wild-type pro-B cells, resulting in the rapid loss of sorted cell lines (data not shown). Strikingly, infection with PU.1\(^{ΔN100}\) resulted in cell lines with significantly increased proliferation over control-infected cell lines (doubling time of 19.3 ± 0.9 h, n = 8, compared with 22.0 ± 1.2 h, n = 8). High levels of PU.1\(^{ΔN100}\) expression relative to expression of the wild-type protein was confirmed by Western blot analysis using an Ab recognizing the C terminus of PU.1 (Fig. 4A).

To further characterize the wild-type-PU.1\(^{ΔN100}\) cell lines, these cells were analyzed for cell surface marker expression by flow cytometry and for gene expression using real-time RT-PCR analysis. We found that FcγRIIb expression was reduced on WT-PU.1\(^{ΔN100}\) cells (MF = 101 ± 4.9, n = 2) relative to wild-type cells (MF = 205 ± 12.1, n = 2), indicating that PU.1 and/or Spi-B activity was reduced in these cells. We also observed that WT-PU.1\(^{ΔN100}\) cells expressed lower levels of c-Kit and IL-7Rα than did the control infected wild-type cells. c-Kit levels on the WT-PU.1\(^{ΔN100}\) cells had a MF of 125 ± 4.3 (n = 3) while the wild-type levels were 130 ± 2.8 (n = 3). IL-7Rα levels on the WT-PU.1\(^{ΔN100}\) cells had a MF of 32.6 ± 5.5 (n = 3) while the wild-type levels were 46.0 ± 5.7 (n = 3). CD19 and CD43 expression levels were equivalent on wild-type and WT-PU.1\(^{ΔN100}\) cells. Taken together, the phenotype exhibited by the WT-PU.1\(^{ΔN100}\) cells follows the trend of the PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cells, although the differences in MF were smaller.

PU.1\(^{ΔN100}\) cell lines showed some similar patterns of gene expression to the PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cells (Fig. 4B). Although smaller than in PU.1\(^{−/−}\)Spi-B\(^{−/−}\) cells, IgH germline transcripts were increased (1.4 ± 0.3-fold, n = 2) in WT-PU.1\(^{ΔN100}\) cell
lines relative to wild-type cell lines. Transcription of Igκ was similarly reduced in WT-PU.1ΔN100 cell lines relative to control cells (4.9 ± 1.6-fold, n = 3). Finally, transcription of RAG-1 and BLNK were also reduced in WT-PU.1ΔN100 cell lines relative to control cells (1.58 ± 0.5-fold, n = 2; 1.6 ± 0.5-fold, n = 4). These changes followed the trend seen in wild-type vs PU.1−/− Spi-B−/− cells, as shown in Fig. 2, and therefore suggest that PU.1 and/or Spi-B are important for transcription of Igκ, RAG-1, and BLNK genes in pro-B cells. The milder phenotype suggests that ectopic expression of PU.1ΔN100 does not result in a complete block of PU.1 and/or Spi-B activity.

IL-7 withdrawal assays were used to induce Igκ expression in wild-type and WT-PU.1ΔN100 cells. Resembling the phenomenon seen in PU.1−/− Spi-B−/− cells, neither control-infected nor WT-PU.1ΔN100-infected cell lines contained significant numbers of Igκ-expressing cells when cultured in IL-7 (0.3 ± 0.2%, n = 2 and 0.5 ± 0.4%, n = 4, respectively) (Fig. 5A, upper row). However, a higher frequency of WT-PU.1ΔN100 cells expressed Igκ on their surface after 72-h withdrawal of IL-7 than control-infected wild-type cells (9.3 ± 2.0%, n = 4; compared with 1.4 ± 0.9%, n = 2) (Fig. 5A, middle row). IL-7 withdrawal from control-infected wild-type pro-B cells resulted in an increase in Igκ germline transcription (4.9 ± 1.2-fold, n = 2), based on real-time analysis. IL-7 withdrawal of WT-PU.1ΔN100-infected cells induced greater expression of Igκ germline transcripts than did IL-7 withdrawal of control-infected wild-type pro-B cells (8.9 ± 1.3-fold, n = 2) (Fig. 5B). In contrast, germline transcription of the λ2 gene was increased after IL-7 withdrawal of WT-PU.1ΔN100-infected pro-B cells (38.0 ± 7.0-fold, n = 2), but not to the same degree as in control cells (50.3 ± 3.8-fold, n = 2) (Fig. 5C). We note that in experiments performed with one particular wild-type cell line we were able to observe similar changes in Igκ and Igλ germline transcription in WT-PU.1ΔN100-infected cells after IL-7 withdrawal, but were unable to detect increased cell surface expression of Igκ using flow cytometry. Others have reported similar findings and suggest that this might be due to the particular cell line having a tendency to undergo unproductive IgV_{μ} - J_{μ} rearrangements (54).

In summary, the observation that the PU.1−/− Spi-B−/− phenotype can be reproduced in wild-type pro-B cells through expression of an activation-deficient form of PU.1 confirms that the phenotype seen in PU.1−/− Spi-B−/− pro-B cells is directly due to loss of function of PU.1 and/or Spi-B.

### Discussion

In this study, we have analyzed IL-7-dependent, PU.1−/− Spi-B−/− cell lines for their phenotypes and patterns of gene expression relative to wild-type cell lines. PU.1 has been implicated in transcriptional regulation of the IgH, Igκ, and Igλ loci. Functional PU.1 binding sites have been extensively characterized in the IgH intronic enhancer (27); in Igκ V region promoters (24); in the Igκ 3’ enhancer (24, 25); and in the Igλ 2–4 enhancer (26). Our most striking finding is that instead of being impaired, germline transcription of the IgH locus is significantly increased in the absence of PU.1 and Spi-B. The IgH transcripts that are increased were detected by multiple probe sets on the Affymetrix GeneChip, representing transcripts from the V region, the J region, and the intronic enhancer (Iμ). We confirmed, using relative quantitation real-time PCR, that levels of Iμ transcripts are up-regulated at least 10-fold in...
PU.1/−/− Spi-B−/− cell lines. Therefore, these results suggest that PU.1 and/or Spi-B are not required for IgH transcription.

In contrast to the finding with IgH transcription, Igk transcription was reproducibly decreased 2- to 3-fold in PU.1/−/−Spi-B−/− pro-B cells relative to wild-type pro-B cells. When PU.1/−/−Spi-B−/− cells were subjected to IL-7 withdrawal they exhibited increases in Igk germline transcription and in VκJκ recombination relative to wild-type cell lines, as demonstrated by increased numbers of cells expressing surface Igk protein. These results imply that PU.1 and/or Spi-B are dispensable for Igk transcription and rearrangement in the developing B cell. Sakanos and colleagues (55) have previously suggested that PU.1 might have negative regulatory function in the Igk 3′ enhancer. They found that mutation of the PU.1 binding site in transgenic Igk 3′ enhancer constructs resulted in increased Igk transcription and VκJκ DNA rearrangement in pro-B cells, instead of restriction to the pre-B cell stage when it is normally initiated (55). These results, and our observations, suggest that PU.1 is not required to activate IgH and Igk transcription and VDJ recombination, and that PU.1 may actually inhibit these functions in pro-B cells.

We found that Igλ transcription was severely reduced in PU.1/−/−Spi-B−/− cell lines relative to wild-type cell lines after IL-7 withdrawal. The PU.1 binding sites in either the Igλ 2–4 or the Igλ 3–1 enhancers have been shown to be essential for the function of these enhancers when tested in transient transfection assays (26). Also, ectopic expression of a PU.1-IFN regulatory factor (IRF)-4 forced dimer lacking activation domains has been shown to silence Igλ transcription in a B cell line (56). Therefore our results provide further evidence that PU.1 and/or Spi-B are important for Igλ transcription and VκJκ rearrangement. We speculate that the reason for the differential requirement of PU.1 and/or Spi-B for the Igλ vs the Igk and IgH genes might be the nature of the enhancers present in these loci. The Igλ locus contains two enhancers that are nearly identical, and are believed to have arisen by gene duplication (57). Both of these enhancers contain PU.1-IRF-4 composite binding sites and therefore the cumulative effect of loss of PU.1 and/or Spi-B activity may be significant impairment of enhancer function (26). In the Igk locus PU.1 interacts with the 3′ enhancer but not the intronic enhancer (25), and therefore the intronic enhancer may compensate for the loss of activity in the 3′ enhancer. In the IgH locus PU.1 interacts with the intronic enhancer but not the 3′ enhancer region (27), and therefore the 3′ enhancer may likewise be able to compensate for loss of activity of the intronic enhancer. Additional experiments must be performed to test this hypothesis.

In further support of the validity of our results, we interfered with PU.1 and/or Spi-B function in wild-type pro-B cell lines by ectopic expression of an inactive mutant form of PU.1 (PU.1ΔN100). We expect that PU.1ΔN100 protein functions by displacement of PU.1 and/or Spi-B at DNA binding sites. Alternatively, ectopic expression of PU.1ΔN100 protein may result in formation of aberrant protein complexes due to interactions with other proteins. Therefore, a caveat of this approach is that PU.1ΔN100 protein may modulate the function of factors other than PU.1 and Spi-B. We found that ectopic expression of PU.1ΔN100 protein promoted proliferation of wild-type pro-B cell lines, whereas wild-type PU.1 protein inhibited their proliferation. As shown in Figs. 4 and 5, wild-type pro-B cells that ectopically express PU.1ΔN100 acquire a phenotype that is similar to that possessed by PU.1/−/−Spi-B−/− cell lines. In addition to increased proliferation, these cell lines have decreased germline Igk and RAG-1 transcription. Withdrawal of IL-7 from wild-type PU.1ΔN100 cell lines results in an increase in Igk transcription relative to wild-type cell lines, and increased VκJκ rearrangement as demonstrated by surface expression of Igk.

The phenotype of these cells is milder than that of PU.1/−/−Spi-B−/− cells, probably due to an incomplete block of PU.1 and/or Spi-B activity. In summary, interference with PU.1 and/or Spi-B activity does not inhibit but rather promotes Igk transcription and VDJ rearrangement.

PU.1/−/− Spi-B−/− and WT-ΔN100 (low/mutant) pro-B cell lines have altered phenotypic characteristics relative to wild-type pro-B cell lines. A hypothesis that integrates most of our observations is that the PU.1/Spi-B low/mutant cell lines are advanced in their state of differentiation relative to wild-type pro-B cell lines, for the following reasons (see Fig. 6). In vivo, large pre-B cells proliferate more rapidly than pro-B cells, as a result of signaling through the combination of the IL-7R and pre-BCR (6). This is consistent with the observation that PU.1/Spi-B low/mutant cell lines proliferate more rapidly than wild-type cells. Second, IL-7Rα and c-Kit expression are normally down-regulated during the pre-B to pre-B cell transition, consistent with our observations that they are down-regulated in PU.1/Spi-B low/mutant cell lines (Fig. 1). Third, CD25 expression is increased on PU.1/−/−Spi-B−/− cell lines, consistent with the observation that CD25 expression is initiated at the pre-B cell stage (45). Fourth, VκDμJκ recombination is normally completed during the pre-B to pre-B cell transition, followed by deposition of pre-BCR on the cell surface, consistent with our observation that the PU.1/Spi-B low/mutant cell lines have acquired large amounts of cytoplasmic H chain protein. Because the PU.1/Spi-B low/mutant cell lines have not yet acquired...
significant levels of surface IgH expression, this is consistent with the idea that they are not yet differentiated to pre-B cells. Fifth, expression of the RAG-1 and RAG-2 genes are normally down-regulated during the pro-B to pre-B cell transition, as VH-DH-JH recombination is completed and these cells enter a proliferative burst phase. As shown in Table I and in Figs. 2 and 4, PU.1/Spi-B low/mutant cells also have lower levels of RAG-1 transcription than wild-type cell lines. Finally, the expression of CD43 on PU.1 low/mutant cells (Fig. 1 and data not shown) indicates that these cells have not differentiated to the small pre-B cell stage. Therefore, although PU.1/Spi-B low/mutant cell lines have acquired many of the characteristics of large pre-B cells, they remain arrested in a stage of development just before the pre-B cell stage (Fig. 6). We suggest that lowering the level of PU.1 and/or Spi-B activity in late pro-B cells promotes differentiation to this later stage.

There are several genes whose level of expression in wild-type vs PU.1/Spi-B low/mutant cell lines is not predicted according to the hypothesis described above. BLNK (SLP-65) is an adaptor protein required for signaling through the BCR and for normal B cell development (58, 59). There is no suggestion in the literature that BLNK transcription should be down-regulated during the pro-B to pre-B cell transition, but we found that BLNK transcription was reduced in PU.1/Spi-B low/mutant cells by an average of 3-fold. Therefore BLNK may represent a novel target of PU.1 and/or Spi-B during B cell development. Secondly, Btk is a B cell-restricted protein tyrosine kinase required for normal BCR signaling and for normal B cell development (60, 61). The btk gene is known to contain PU.1 binding sites in its promoter region (32, 62). Because btk is also not expected to be down-regulated during the pro-B to pre-B transition, our finding that btk transcription is decreased in PU.1/Spi-B low/mutant cells suggests that it is indeed

FIGURE 5. Induction of Igκ in PU.1ΔN100-expressing wild-type pro-B cells after IL-7 withdrawal. A, Flow cytometric analysis of surface Igκ expression in retrovirally infected pro-B cells. Retrovirally infected pro-B cells were analyzed for surface κ expression before and after culture in the presence or absence of IL-7 for 72 h; GFP expression is shown in the bottom panel as a control. B, Real-time RT-PCR analysis of κ transcripts in retrovirally infected pro-B cells. Retrovirally infected pro-B cells were cultured in the presence or absence of IL-7 for 72 h and expression of κ germ line transcripts are shown as fold change in retrovirally infected pro-B cells after IL-7 withdrawal relative to transcript levels in cells cultured in the presence of IL-7 using the ΔΔCt method (49). C, Real-time RT-PCR analysis of λ2 germine transcripts in retrovirally infected pro-B cells. Retrovirally infected pro-B cells were cultured in the presence or absence of IL-7 for 72 h and expression of λ2 germine transcripts are shown as fold change in retrovirally infected pro-B cells after IL-7 withdrawal relative to transcript levels in cells cultured in the presence of IL-7 using the ΔΔCt method (49).

FIGURE 6. Model of PU.1 function in early B cell development.
a target gene. Further biochemical experiments will have to be performed to confirm that BLNK and btk, as well as other genes shown in Table I, are targets of PU.1 and/or Spi-B in the B cell lineage.

What are the possible mechanisms by which interference with PU.1 and/or Spi-B activity promotes Ig transcription? First, PU.1 and/or Spi-B occupancy of Ig promoters and enhancers may directly inhibit transcription and VDJ rearrangement. This seems unlikely because most studies have demonstrated that these proteins function as transcriptional activators. Therefore it seems more likely that in the absence of PU.1 and/or Spi-B, other transcription factors might be increased in expression or activity such that they can compensate for the reduced PU.1 and/or Spi-B activity. We note that in the Affymetrix GeneChip analyses, the expression of several transcription factors, including IRF-4 and Pax-5, are significantly up-regulated in PU.1+/- Spi-B+/- cell lines (supplementary Table II). IRF-4 requires an interaction partner, such as PU.1, to efficiently bind DNA and activate transcription (63). It has recently been shown that E47 can bind to DNA cooperatively with IRF-4 to synergistically activate transcription of Ig germline transcription (64). Therefore it is possible that these types of transcription factor interactions could compensate for the loss of PU.1 and/or Spi-B activity. It is also possible that other uncharacterized Ets-family transcription factors might occupy PU.1 and/or Spi-B binding sites in their absence and bypass the normal requirement for these factors. Additional experiments will have to be performed to clarify which mechanisms explain the increased rates of Ig transcription.

Based on our suggestion that interference with PU.1 and/or Spi-B activity in pro-B cells promotes differentiation to a stage intermediate between late pro-B and large pre-B cells, we speculate that modulation of the activity of these factors may normally occur during the late pro-B to large pre-B cell transition in B cell development (Fig. 6). Intriguingly, the transcription factor Spi-C (Prf), which is closely related to PU.1 and Spi-B, was initially cloned in a screen for factors which could occupy PU.1 DNA binding sites (17). Spi-C has extremely weak transcription-promoting activity, and unlike PU.1 and Spi-B, cannot interact with IRF-4 to synergistically activate gene expression on Ets/IRF composite elements (17, 65). Spi-C has an extremely restricted pattern of expression: its transcription is initiated at the pre-B cell stage of B cell development, it is expressed in mature B cells, and its transcription is extinguished in plasma cells (16, 17). Taken together, these results suggest that the normal function of Spi-C might be as a negative regulator of PU.1/Spi-B activity (17, 65). We speculate that Spi-C may normally interfere with PU.1/Spi-B activity starting at the large pre-B cell stage of B cell development, and that this negative regulation might be required for normal B cell development.

In summary, we have demonstrated that contrary to what has been predicted, PU.1 and/or Spi-B do not seem to be required for transcription and VDJ rearrangement of the IgH and Igκ loci in pro-B cell lines. However, our evidence demonstrates that PU.1 and Spi-B are required for transcription of the Igλ locus. Taken together with other phenotypic changes in PU.1+/- Spi-B+/- and wild-type PU.1+/+λN100 cell lines relative to control cell lines, our observations suggest that reduction of PU.1 activity in pro-B cell lines promotes the differentiation of these cells to a stage intermediate between late pro-B cells and large pre-B cells (Fig. 6). We speculate that a reduction in PU.1 and/or Spi-B activity starting before the large pre-B cell stage might be a normal feature of B cell development. Additional experiments will have to be performed to test this hypothesis and distinguish between various mechanisms by which PU.1 activity might be regulated.

Acknowledgments

We thank Lee-Anh Garret-Sinha (State University of New York, Buffalo, NY) and Celeste Simon (University of Pennsylvania, Philadelphia, PA) for giving us PU.1+/- Spi-B+/- (PUB) mice and Antonius Rolink (University of Basel, Basel, Switzerland) for generously providing the J588-IL-7 cell line. We thank Harinder Singh and Jagan Pongubala (University of Chicago, Chicago, IL) for helpful comments and discussion, and Isaac Houston (University of Cincinnati, Cincinnati, OH) and Pierre-Yves Berclaz (Cincinnati Children’s Hospital, Cincinnati, OH) for critically reading the manuscript.

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