Spi-C Has Opposing Effects to PU.1 on Gene Expression in Progenitor B Cells

Brock L. Schweitzer, Kelly J. Huang, Meghana B. Kamath, Alexander V. Emelyanov, Barbara K. Birshtein and Rodney P. DeKoter

*J Immunol* 2006; 177:2195-2207; doi: 10.4049/jimmunol.177.4.2195

http://www.jimmunol.org/content/177/4/2195

References

This article cites 64 articles, 37 of which you can access for free at: http://www.jimmunol.org/content/177/4/2195.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Spi-C Has Opposing Effects to PU.1 on Gene Expression in Progenitor B Cells

Brock L. Schweitzer,* Kelly J. Huang,* Meghana B. Kamath,* Alexander V. Emelyanov,† Barbara K. Birshstein,* and Rodney P. DeKoter2*†

The Ets transcription factor Spi-C, expressed in B cells and macrophages, is closely related to PU.1 and has the ability to recognize the same DNA consensus sequence. However, the function of Spi-C has yet to be determined. The purpose of this study is to further examine Spi-C activity in B cell development. First, using retroviral vectors to infect PU.1−/− fetal liver progenitors, Spi-C was found to be inefficient at inducing cytokine-dependent proliferation and differentiation of progenitor B (pro-B) cells or macrophages relative to PU.1 or Spi-B. Next, Spi-C was ectopically expressed in fetal liver-derived, IL-7-dependent pro-B cell lines. Wild-type (WT) pro-B cells ectopically expressing Spi-C (WT-Spi-C) have several phenotypic characteristics of pre-B cells such as increased CD25 and decreased c-Kit surface expression. In addition, WT-Spi-C pro-B cells express increased levels of IgH sterile transcripts and reduced levels of expression and transcription of the FcγRIIb gene. Gel-shift analysis suggests that Spi-C, ectopically expressed in pro-B cells, can bind PU.1 consensus sites in the IgH intronic enhancer and FcγRIIb promoter. Transient transfection analysis demonstrated that PU.1 functions to repress the IgH intronic enhancer and activate the FcγRIIb promoter, while Spi-C opposes these activities. WT-Spi-C pro-B cells have reduced levels of dimethylation on lysine 9 of histone H3 within the IgH 3′ regulatory region, indicating that Spi-C can contribute to removal of repressive features in the IgH locus. Overall, these studies suggest that Spi-C may promote B cell differentiation by modulating the activity of PU.1-dependent genes. The Journal of Immunology, 2006, 177: 2195–2207.

B cell commitment is characterized by DNA rearrangement at the IgH locus and expression of signaling molecules associated with the BCR. The earliest committed B cell is termed the progenitor B (pro-B) cell, which is identified by rearrangements in the D-J segments of the IgH locus. These cells maintain expression of lymphoid progenitor cell markers, such as c-Kit and IL-7R. The pro-B cell can also be maintained ex vivo in culture with IL-7 (1). V-DJ rearrangements in the IgH locus give rise to functional μ protein, which can be expressed on the surface of the cells in conjunction with the surrogate L chain proteins, Vpre-B and A5, along with the signaling adaptor proteins, Igδ and Igβ, comprising the pre-BCR (2). Cells that express the pre-BCR on their surface are termed pre-B cells. Pre-B cells initiate rearrangement of the Ig L chain loci, either κ or λ, which results in surface expression of a functional BCR complex and progression to the immature B cell stage. Alternative splicing of the IgH transcript results in the expression of the IgD isoform of IgH protein on the surface, which, in addition to IgM expression, defines the mature B cell stage.

Several transcription factors play important roles early in B cell development (reviewed in Ref. 3). In the early stages, these factors promote commitment to the B cell fate by activating B cell-specific genes and suppressing genes of other cell lineages. As the B cell develops, transcriptional regulation of the Ig loci becomes central because rearrangement and expression of the Ig loci are critical for development of B cells once they are generated (24, 26, 27). One of the consequences of PU.1 deletion in committed B cells is a

1 Abbreviations used in this paper: pro-B, progenitor B; HS, hypersensitive site; IRF, IFN regulatory factor; WT, wild type; HA, hemagglutinin; IRES, internal ribosomal entry site; FLP, fetal liver progenitor; MF, mean fluorescence.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact. The work was supported by National Institutes of Health Grants 1R01AI052175 (to R.P.D.) and AI13509 (to B.K.B.).

Received for publication February 23, 2006. Accepted for publication May 27, 2006.

The Journal of Immunology
switch from a B-2 to a B-1 phenotype (27). However, it is not yet clear what the underlying changes in gene expression are upon PU.1 deletion. PU.1−/− or PU.1−/−Spi-B−/− pro-B cells can be generated by retroviral transduction with the IL-7Rα cDNA and grown in IL-7 (28). We have previously shown that PU.1−/−Spi-B−/− pro-B cells have a phenotype similar to pre-B cells with increased CD25 and decreased c-Kit surface expression relative to wild-type (WT) pro-B cells. In addition, PU.1−/−Spi-B−/− pro-B cells express increased IgH sterile transcripts relative to WT pro-B cells (29). The PU.1−/−Spi-B−/− phenotype could be recapitulated by ectopic expression of a mutant form of PU.1 lacking an activation domain (29). Based on these observations, the inhibition of PU.1 appears to promote a pre-B cell-like phenotype, which is advanced over the pro-B cell stage.

Spi-C/Prf (PU.1-related factor) was identified simultaneously in two independent laboratories through a yeast-one hybrid method using the PU.1-binding sites of the Igκ 3′ enhancer and SP6 κ promoter sequences as bait (30, 31). Although the DNA-binding domain of Spi-C shares 59% aa identity to the DNA-binding domain of PU.1, the transactivation domains are divergent (30, 31). Spi-C is predicted to be a 28-kDa protein, which is smaller than the 31-kDa PU.1 protein (32). Northern blot analysis shows high levels of Spi-C in the spleen (30, 31). Specifically, Spi-C is expressed in macrophage cell lines, pre-B cell lines, and mature B cell lines, but is not found in pro-B cell lines or Igκ− immature B cell lines (30). This pattern of expression partially overlaps the other members of the Spi family, PU.1 and Spi-B, which are expressed throughout B cell development (33–35). Gel mobility shift analysis shows that Spi-C can bind to PU.1-binding sites in the Igκ locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).

In this study, we investigate a biological function for Spi-C. We show that, in contrast to PU.1, Spi-C is inefficient at restoring B cell development in PU.1−/− fetal liver progenitors. Ectopic expression of Spi-C in WT fetal liver-derived pro-B cells (WT-Spi-C) induces an advanced pre-B cell-like phenotype characterized by increased expression of CD25 and reduced expression of c-Kit. In addition, Steele transcription from the locus (32). However, Spi-C cannot form a ternary complex with IFN regulatory factor (IRF)-4 on DNA (32). This is in contrast to PU.1, which cooperates with IRF-4 to efficiently bind Ets-IRF composite elements (36).
II/III receptor (2AG2), c-Kit (2B8), CD25 (7D4), IgM (II/41). Cells were pelleted by centrifugation and washed three times before staining with an appropriate dilution of secondary for 20 min on ice. Cells were pelleted by centrifugation, washed three times, and finally diluted to 748 mM propidium iodide solution. Cells were analyzed by a BD Biosciences FACSCalibur system. The final gating procedure involved either staining from PE or allophycocyanin based on the presence of endogenous GFP expression. Western blot was performed using standard methods with rabbit anti-PU.1 peptide Ab (Santa Cruz Biotechnology), goat anti-actin peptide Ab (Santa Cruz Biotechnology), mouse anti-HA peptide Ab (12CA5; Roche), and HRP-conjugated anti-rabbit or anti-goat secondary Abs (Pierce).

PCR analysis

Total RNA was extracted from cultured IL-7-dependent pro-B cell lines using RNA-Be (Tel-test). Total RNA was used as template for cDNA synthesis using a cDNA synthesis kit (BD Biosciences) according to manufacturer’s protocol. cDNA was analyzed using primers spanning intronic sequence for β-actin to verify lack of genomic DNA contamination and equal cDNA amplification from various template RNAs. Primers for target sequences were designed to span introns or splice junctions using Stratagene PCR primer designer. PCR was performed on a Robocycler (Stratagene) using the following reaction cycle. This includes a 5-min melting step at 95°C followed by 25–30 cycles of amplification at the optimum annealing temperature, followed by an extension step of 2 min at 72°C. PCR products were run on agarose gels and visualized by ethidium bromide staining. The following primers were used: 5′-ACCTGGGAGATATGGTTGGCCTTT-3′ and 5′-ATGACATGTGTTTGCCTCC-3′ (40), FcγRIIB: 5′-CAGACTCAGGATCTTCTACCC-3′ and 5′-AGGCTGCTGATCAACCTACCA-3′, and 5′-GGCGGCTCAAC-3′ and 5′-CATCCTCCGAGTCTCAACCA-3′, or mutant PU.1-binding site (mutant Pu.1-binding site, 5′-TCGAATTCTTTTCACCCAT-3′, or a mutated PU.1-binding site, 5′-TCGAATTCTTTTCACCCAT-3′, or 5′-AGAACGGCACCATCCTAAACT-3′). Primers were washed in Dulbecco’s PBS following centrifugation. Washed cells were suspended in buffer (10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 0.5 mM PMSF, 0.5 mM DTT) and lysed with a glass homogenizer on ice. Lysed cells were centrifuged and dialyzed for 5 h at 4°C using the Slide-A-Lyzer Mini Dialysis Unit (Pierce). Synthetic complementary oligonucleotides were annealed and labeled with [α-32P]dCTP using the Klenow fragment. DNA-binding reactions were performed for 30 min at 25°C and contained 2.5 × 106 cpm of probe, 2 μl of in vitro-translated PU.1 or 10 μg of nuclear lysate, and 100 ng of poly(dI-dC) in a final volume of 30 μl. Supershifting reactions were performed using 1 μl of anti-HA Ab (Covance), anti-PU.1 Ab (Santa Cruz Biotechnology), or control rabbit serum. Abs were added to nuclear lysates and incubated for 20 min on ice before addition of radiolabeled probe and binding reactions. Binding reactions were resolved on a 5% native polyacrylamide gel using 1× Tris-glycine-EDTA buffer, at 150 V for 2.5 h. Double-stranded oligonucleotide probes contained the PU.1-binding site in the: IgH intronic enhancer (μB): 5′-TGCACAGGCTATTTGGGGAG-3′, or a mutated PU.1-binding site (mutant μB), 5′-TGCACAGGCTATTTGGGGAG-3′; FcγRIIB promoter, 5′-TCGACATTCTTTGCTCCCAT-3′, or a mutated Pu.1-binding site (mutant FcγRIIB), 5′-TCGACATTCTTTGCTCCCAT-3′. A 2% agarose gel was placed on a Santa Cruz Biotechnology, mouse anti-HA peptide Ab (12CA5; Roche), and HRP-conjugated anti-rabbit or antigoat secondary Abs (Pierce).

Results

PU.1−/− mice fail to produce B cells and macrophages in vivo, and cultured PU.1-deficient progenitors also fail to do so. However, if PU.1 is reintroduced by retroviral transduction, B cell and macrophage development can be rescued (42). This system provides an assay for transcription factor redundancy in vivo. To determine whether the other two Ets subfamily members, Spi-B and Spi-C, could rescue development as efficiently as PU.1, we retrovirally transduced PU.1−/− fetal liver progenitors with GFP-expressing retroviruses that encoded PU.1, Spi-B, or Spi-C (Fig. 1A). Equivalent numbers of infected fetal liver cells were plated in colony-forming assays of murine embryonic fibroblasts containing M-CSF to support the generation of macrophage colonies, or methylcellulose containing IL-7 and stromal cells to support the generation of pro-B cell colonies (Fig. 1B). Colonies were scored based on morphology using microscopy as performed previously (28). As shown in Table I, infection of progenitors with a PU.1 retrovirus generated a high number of macrophage colonies in M-CSF, as well as significant numbers of pro-B cell colonies in IL-7 and stromal cells. Spi-B was less efficient than PU.1 at inducing macrophage and pro-B cell colonies with 38% of macrophage rescue and 56% of B cell rescue, respectively. Infection with Spi-C cDNA was extremely inefficient at rescuing either macrophage or pro-B cell colony formation, producing only 1% of the macrophage colonies and 3% of the pro-B cell colonies relative to PU.1-rescued colony numbers (Table I). In parallel experiments, infected cells were also placed in liquid cultures containing S17 stromal cells and IL-7. Under these culture conditions, CD19+ pro-B cells can proliferate continuously whereas CD11b+ macrophages terminally differentiate and stop proliferation after the first 5–7 days. After 10–11 days, these cultures were analyzed by flow cytometry for CD19 and CD11b (Fig. 1C). Both PU.1 and Spi-B were able to promote macrophage growth in culture, with ~22 and 27% of the cells positive for CD11b, respectively (Fig. 1C, bottom panels). However, Spi-C inefficiently rescued macrophage growth with only 4% positive for CD11b (Fig. 1C, bottom panels). In contrast,
FIGURE 1. Spi-C is inefficient at rescuing $PU.1^{+/+}$ progenitor development. A, Schematic of retroviral constructs used to infect $PU.1^{-/-}$ fetal liver progenitors. Each viral construct contained an IRES upstream of DNA sequence encoding the enhanced GFP (EGFP). B, Diagram outlining procedure for assaying progenitor rescue. Progenitors from heterozygous or null fetal livers were retrovirally infected and plated on either methylcellulose + M-CSF or methylcellulose + IL-7 and stromal cells for colony formation assay. Retrovirally infected progenitors were also placed in liquid culture with M-CSF or IL-7 and stromal cells for analysis by flow cytometry. C, Spi-C was inefficient at promoting growth of macrophages, but efficient at promoting B cell growth. Retrovirally infected $PU.1^{-/-}$ fetal liver progenitors were grown in culture for 7 days and analyzed by flow cytometry for B cells (CD19) and macrophages (CD11b).
the relative frequency of pro-B cell growth in Spi-C-rescued progenitors was nearly twice (66%) that of PU.1 (37%) and Spi-B (30%) (Fig. 1C, top panels). In summary, we found from the colony rescue experiments that Spi-C is inefficient at rescuing progenitor development into B cells and macrophages relative to PU.1 and Spi-B. However, once they are generated, Spi-C-rescued pro-B cells proliferate well in response to IL-7 in liquid culture.

**Ectopic expression of Spi-C in pro-B cells advances the phenotype toward pre-B cells**

RT-PCR analysis of IL-7-dependent pro-B cells demonstrates minimal to no expression of Spi-C. However, Spi-C is detected in pro-B cell and mature B cell lines (28, 30, 31). When IL-7-dependent pro-B cells are cultured in the absence of IL-7 for 3 days, they differentiate and rearrange the Ig L chain genes (1, 43). When we examined Spi-C transcripts by real-time RT-PCR, we found that IL-7 withdrawal induced Spi-C expression 2-fold compared with pro-B cells maintained in IL-7 (data not shown). These observations suggest that Spi-C might be involved in B cell differentiation.

To determine whether ectopic expression of Spi-C can induce marks of differentiation in pro-B cells, we infected WT fetal-liver derived pro-B cells with a GFP-containing retrovirus expressing the Spi-C cDNA (Fig. 2A). After sorting, flow cytometric analysis confirmed that these cells maintained GFP expression indefinitely, indicating that the control virus and Spi-C are not detrimental to cell growth (Fig. 2A). These cell lines are hereafter referred to as WT-Spi-C. Real-time RT-PCR analysis confirmed increased expression of Spi-C in the WT-Spi-C pro-B cells relative to WT-R1 pro-B cells (Fig. 2B). When Western blot analysis was performed against the HA tag on the retroviral Spi-C, we detected a protein at the predicted molecular mass for Spi-C only in nuclear lysates obtained from WT-Spi-C pro-B cells (Fig. 2C). Interestingly, analysis by real-time RT-PCR indicated increased transcript expression of PU.1 in WT-Spi-C pro-B cells relative to WT-R1 pro-B cells (Fig. 2D). However, Western blot analysis indicated equal PU.1 protein expression in WT-R1 and WT-Spi-C pro-B cells (Fig. 2E). Also of note, the polyclonal PU.1 Ab also detected a faster migrating band in the WT-Spi-C nuclear lysate that corresponds to the protein detected by the HA Ab (Fig. 2E). Based on the high homology between the PU.1 and Spi-C DNA-binding domains, this result suggests that the polyclonal Ab is cross-reactive with Spi-C.

We further characterized the WT-Spi-C pro-B cells by flow cytometry to observe any phenotypic differences induced by the ectopic expression of Spi-C. Levels of CD19 on the surface of WT-R1 pro-B cells (99.8 ± 0.4%) and WT-Spi-C pro-B cells (99.9 ± 0.3%) were equivalent (Fig. 3A). Both CD43 (99.9 ± 0.2%) and IgH (0.4 ± 0.6%), on the surface of WT-Spi-C pro-B cells relative to WT-R1 pro-B cells, were unchanged (99.7 ± 0.6% and 0.7 ± 1.2%, respectively, Fig. 3A). However, CD25 expression was increased to a mean fluorescence (MF) of 44.0 ± 6 from 10.2 ± 2 for WT-R1 pro-B cells, while c-Kit was reduced (MF = 47.2 ± 4) relative to WT-R1 pro-B cells (MF = 84.7 ± 2, Fig. 3B). These markers were also altered at the transcript level where CD25 was increased in WT-Spi-C pro-B cells relative to WT-R1 pro-B cells and c-Kit was reduced in Spi-C pro-B cells relative to WT-R1 pro-B cells as determined by RT-PCR analysis (Fig. 3C). Increased CD25 and reduced c-Kit are markers of differentiation into pre-B cells (44). In addition, B220 and FcγRIIb expression was severely reduced by Spi-C ectopic expression (MF = 81.6 ± 9 and 167.3 ± 7, respectively) compared with WT-R1 pro-B cells (MF = 164.3 ± 10 and 281.5 ± 8, respectively, Fig. 3D). Overall, WT-Spi-C pro-B cells exhibit an intermediate developmental phenotype between pro-B and pre-B based on the increase in CD25 and decrease in c-Kit on the surface and at the transcript level. In addition, the B cell markers B220 and FcγR are severely reduced in WT-Spi-C pro-B cells.

**Increased IgH sterile transcripts and reduced FcγRIIb transcripts in pro-B cells caused by ectopic expression of Spi-C**

To further characterize the phenotype of WT-Spi-C pro-B cells, we examined gene expression by RT-PCR. Similar to the phenotype of PU.1−/− Spi-B−/− pro-B cells (29), we found that steady-state levels of sterile transcripts initiating from the IgH intronic enhancer (Iμ) were increased relative to WT-R1 pro-B cells (Fig. 4A). Increased sterile transcripts are indicative of an active IgH locus, which is observed in total spleen containing mature B cells (Fig. 4A). Quantitation of this induction by real-time PCR showed that Iμ transcripts were 4.5-fold higher in WT-Spi-C pro-B cells relative to WT-R1 pro-B cells (Fig. 4B). We also examined FcγRIIb transcripts, because we had previously observed a reduction in these transcripts in PU.1−/− Spi-B−/− pro-B cells (29) and a corresponding reduction in FcγRIIb/III surface expression (Fig. 3C). The FcγRIIb gene encodes an inhibitory FcR, which is expressed on the surface of all stages of B cell development and mature B cells. The FcγRIIb receptor can block proliferation in B cells through the inactivation of MAPKs. In addition, mice deficient for FcγRIIb are susceptible to induced autoimmune disease (reviewed in Ref. 45). WT-Spi-C pro-B cells have a severe reduction in the levels of FcγRIIb transcripts relative to WT-R1 pro-B cells (Fig. 4C). Quantitatively, this reduction was 6-fold relative to WT-R1 pro-B cells as determined by real-time PCR (Fig. 4D).

In summary, Iμ sterile transcript was significantly increased in WT-Spi-C pro-B cells, but expression of the FcγRIIb gene was impaired by the ectopic expression of Spi-C. These changes in Iμ and FcγRIIb expression are similar to those detected in PU.1−/−Spi-B−/− pro-B cells (29).

### Table I. Colony forming assays results for PU.1−/− fetal liver progenitors infected with retroviral vectors

<table>
<thead>
<tr>
<th>Retrovirus</th>
<th>Colonies per 10⁵ infected cells</th>
<th>No. of experiments</th>
<th>Colony forming assays results for PU.1−/− fetal liver progenitors infected with retroviral vectors</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIGR1</td>
<td>0</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>MIG-PU.1</td>
<td>558 ± 16</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>MIG-Spi-B</td>
<td>214 ± 12</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>MIG-Spi-C</td>
<td>9 ± 3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MIGR1</td>
<td>0</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>MIG-PU.1</td>
<td>1635 ± 235</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>MIG-Spi-B</td>
<td>920 ± 71</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>MIG-Spi-C</td>
<td>51 ± 9</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

* Progenitors from PU.1−/− fetal liver progenitors were infected with the indicated retroviral vectors and plated on either methylcellulose + M-CSF or methylcellulose + IL-7 and stromal cells for colony formation assay. Results are expressed as the mean number of colonies ± SD for n experiments.
FIGURE 2. Generation of pro-B cells ectopically expressing Spi-C. A, WT fetal liver-derived pro-B cells were retrovirally infected with either a control GFP retrovirus or a Spi-C-expressing GFP retrovirus and grown in IL-7-containing medium on irradiated stromal cells. Flow cytometry was used to sort cultures for infected pro-B cells with GFP fluorescence. B, Relative quantitation real-time PCR was used to confirm the ectopic expression of Spi-C transcripts following infection and sorting. C, Western blot analysis was performed against the HA tag on the retroviral Spi-C to confirm ectopic protein expression. D, Relative quantitation real-time PCR was used to measure PU.1 expression in pro-B cells ectopically expressing Spi-C. E, Western blot analysis was used to confirm that levels of PU.1 protein were unchanged by Spi-C ectopic expression. *, A nonspecific band detected by the PU.1 polyclonal Ab.
Spi-C can bind to PU.1 sites within the IgH intronic enhancer and FcγRIIb promoter

Previous studies showed that Spi-C bound to PU.1 consensus sites, such as those in the IgH locus in vitro (32). Because we observed altered patterns of expression from the IgH intronic enhancer and the FcγRIIb promoter in WT-Spi-C pro-B cells, we set out to examine whether Spi-C was capable of binding PU.1 consensus sites within these two regulatory elements. Using an oligonucleotide containing a PU.1 consensus site previously shown to bind PU.1 in the IgH intronic enhancer core (11), we examined the binding potential of nuclear lysates from WT-R1 pro-B cells and WT-Spi-C pro-B cells. An oligonucleotide containing a consensus E2A-binding site, used as a loading control, demonstrated that the nuclear lysates contained equal amounts of E2A protein (Fig. 5 A, lanes 16–18, B, lanes 12–14). In vitro-translated PU.1 produced a unique complex that was not present in reactions with a μB probe mutated at the Ets site (Fig. 5 A, lanes 4 and 5). In vitro translation reactions charged with only the pcDNA3 vector generated nonspecific complexes present on both the WT and mutated μB probes (Fig. 5 A, lanes 2 and 3). When compared with in vitro-translated PU.1 protein, nuclear lysates from the WT-R1 pro-B cells produced a similar complex that could be disrupted by Ab specific to PU.1 (Fig. 5 A, lanes 4, 6, and 10). When nuclear lysates from the WT-Spi-C pro-B cells were incubated with the μB probe, additional faster migrating complexes appeared (Fig. 5 A, lane 8). The unique complexes were, along with the complex corresponding to PU.1, absent in reactions with a μB probe mutated at the Ets site (Fig. 5 A, lanes 5, 7, and 9). Ab specific to PU.1 disrupted the complex containing PU.1 from nuclear lysates of WT-Spi-C pro-B cells, but not the unique complexes (Fig. 5 A, lane 11). By incubating with an Ab specific to the HA tag on the retrovirally expressed Spi-C, the unique complexes could be disrupted, and a slower migrating complex appeared (Fig. 5 A, lane 13). In addition, the band corresponding to PU.1 increased in intensity (Fig. 5 A, compare lane 8 to lane 13). Control antiserum did not disrupt the Spi-C or the PU.1 complexes (Fig.

A, lanes 14 and 15). This indicates that the μB-binding complex generated from nuclear lysates of WT-Spi-C pro-B cells contained functional Spi-C protein. In addition, the amount of PU.1 complex was reduced relative to the WT-R1 nuclear lysates (Fig. 5 A, compare lane 6 to lane 8).

We next wanted to know whether Spi-C was capable of binding to an Ets DNA-binding site in the FcγRIIb promoter. The FcγRIIb promoter had not been previously characterized, so sequence alignments were performed using TRAFAC software (46). A region upstream of the translation start site, highly conserved between mouse and human, contains a consensus Ets-binding site (M. B. Kamath and R. P. DeKoter, unpublished results). Nuclear lysates were prepared from WT-R1 pro-B cells or WT-Spi-C pro-B cells. Using gel mobility shift analysis, nuclear lysates were incubated with radiolabeled oligonucleotide probes containing a WT or a point mutant in the Ets site were identified within the FcγRIIb promoter (Fig. 5 B). In vitro-translated PU.1 produced a unique complex that was not present in reactions with a FcγRIIb probe mutated at the Ets site (Fig. 5 B, lanes 3 and 4). In vitro translation reactions charged with only the pcDNA3 vector generated nonspecific complexes present on both the WT and mutated μB probes (Fig. 5 B, lanes 2 and 8). When compared with in vitro-translated PU.1 protein, nuclear lysates from the WT-R1 pro-B cells produced a similar complex that could be disrupted by Ab specific to PU.1 (Fig. 5 B, lanes 3 and 4, and data not shown). When nuclear lysates from WT-Spi-C pro-B cells were incubated with the FcγRIIb promoter probe, additional faster-migrating complexes appeared (Fig. 5 B, lane 5). The unique complexes, as well as the complex corresponding to PU.1, were absent in reactions with an FcγRIIb promoter probe mutated at the Ets site (Fig. 5 B, lanes 9–11). By incubating with an Ab to the HA tag on the retrovirally expressed Spi-C, the unique complexes could be disrupted, and a slower migrating complex appeared (Fig. 5 B, lane 5). This indicated that the complexes generated from nuclear lysates of WT-Spi-C pro-B cells contained functional Spi-C. Control antisera did not disrupt the Spi-C or the PU.1 complexes (Fig. 5 B, lane 7). In addition, the amount of PU.1 complex was reduced relative to the WT-R1 pro-B nuclear lysates (Fig. 5 B, compare lane 5 to lane 4). However, Western blotting revealed that PU.1 protein levels were equal in WT-R1 and WT-Spi-C pro-B cells (Fig. 2 C).

**PU.1 and Spi-C exert opposite activities on the Eμ enhancer and FcγRIIb promoter**

Based on the similar effects of PU.1 deficiency and ectopic Spi-C expression in pro-B cells on IgH transcription, we investigated the activity of PU.1 and Spi-C on the IgH intrinsic enhancer and FcγRIIb promoter.
enhancer and the FcγRIIB promoter using transient transfection assays with luciferase reporter vectors driven by these regulatory elements. Using the 38B9 pro-B cell line, which expresses endogenous PU.1 but not Spi-C, we transiently transfected these reporters together with plasmids encoding PU.1, Spi-C, or both. When PU.1 was cotransfected with the IgH intronic enhancer reporter, we saw a 2.3-fold reduction in activity relative to the enhancer activity assayed in the presence of endogenous levels of PU.1 (Fig. 6A). This difference in activity was no longer detected when we analyzed a mutant form of the PU.1 (R232, 235A; Fig. 6B). This result suggests that endogenous PU.1 expression in 38B9 pro-B cells is not required for activation of this enhancer. Results were different with Spi-C. When Spi-C was cotransfected with the intronic enhancer, activity increased (Fig. 6A), an outcome opposite to that observed with PU.1. When both PU.1 and Spi-C were cotransfected with the IgH intronic enhancer, the reporter activity was reduced relative to the reporter transfected alone, but not reduced to the same degree as with PU.1 alone (Fig. 6A).

In accord with the observation that PU.1-deficient pro-B cells have reduced FcγRIIB expression, the FcγRIIB promoter was activated 1.7-fold by PU.1 when cotransfected into 38B9 pro-B cells (Fig. 6C). However, Spi-C did not significantly affect the activity of the FcγRIIB promoter (Fig. 6C). In summary, PU.1 repressed IgH intronic enhancer activity and activated FcγRIIB promoter activity, whereas Spi-C enhanced IgH intronic enhancer activity, but did not alter FcγRIIB promoter activity in transient transfection assays. Coexpression of PU.1 and Spi-C with the IgH intronic enhancer reporter produced an intermediate activity between PU.1 and Spi-C expression individually.

Ectopic Spi-C expression removes marks of inactive chromatin in the IgH 3’ regulatory region

Because we observed that IgH sterile transcription was increased by ectopic expression of Spi-C, we further characterized the IgH locus by chromatin immunoprecipitation assays on histone modifications. The IgH intronic enhancer is typically in an active state in pro-B cells, and the 3’ regulatory region appears to be active at various stages of B cell development. For example, the HS4 enhancer is active throughout development (48, 49) and a recently identified segment downstream of HS4 that contains additional DNase I hypersensitive sites is associated with marks of active chromatin beginning in pro-B cells (41). The 3’ regulatory region of the IgH locus plays an important role in the accessibility of IgH gene expression in differentiated B cells and has been implicated in sterile IgH μ0 transcription in pro-B cells (50–54). Sterile transcription within the IgH locus is an indicator of active chromatin. Posttranslational modification of histone N-terminal tails by acetylation provides access to chromatin remodeling factors that open regions to transcriptional machinery (55). Therefore, we analyzed this region by performing chromatin immunoprecipitation for the acetylated histones H3 and H4. The active IL-7Rx gene locus was used as a positive control for acetylation in our analysis. The 3’ regulatory region was associated with both acetylated histone H3 and H4 in WT-R1 pro-B cells, and no significant changes in either H3 or H4 histone acetylation profiles were detected in WT-Spi-C pro-B cells (Fig. 7A). Instead, Spi-C ectopic expression resulted in a significant reduction in dimethylation of lysine 9 (di-Me K9 H3) of histone H3 (Fig. 7B, right panel), a modification associated with repression because it prevents histone acetylation and recruits chromatin silencing factors (reviewed in Ref. 56). Although the entire 3’ regulatory region was associated with di-Me K9 H3 in WT-R1 pro-B cells, this mark was essentially absent in WT-Spi-C pro-B cells (Fig. 7B). In summary, these experiments show that ectopic Spi-C expression changed the status of the IgH 3’ regulatory region from a repressive to a permissive chromatin environment.

Discussion

Studies of the Ets transcription factor Spi-C have yet to determine a biological function for this protein. In an attempt to better understand its activity, we ectopically expressed Spi-C in WT IL-7-dependent
pro-B cells. Initially, using a progenitor rescue assay, we observed that Spi-C could not efficiently compensate for PU.1 loss in generating B cells and macrophages from progenitors. This suggests that Spi-C does not have the same transcriptional activation ability on genes necessary for development as the other members of this Ets subfamily, PU.1 and Spi-B. The increased frequencies of B cells in cultures of Spi-C-rescued cells are most likely due to the severely reduced numbers of macrophages because B cell populations grow continuously under IL-7 and stromal cell conditions. Once a B cell progenitor is generated, it can proliferate and express IL-7Rα without a requirement for either PU.1 or Spi-B (25). Spi-B is also less efficient than PU.1 at rescuing both B cells and macrophages. This is in contrast to previous observations that suggest Spi-B can substitute for PU.1 for myeloid development but not for lymphoid development (57). Our data provides the first quantitative comparison of transactivation potential between PU.1, Spi-B, and Spi-C in a biological setting. These results suggest that PU.1 possesses the strongest transactivation potential among this subfamily of Ets transcription factors.

When PU.1 is ectopically expressed in pro-B cells, their proliferation is rapidly arrested (Ref. 42 and our unpublished observations). Successful high-level ectopic expression of Spi-C in pro-B cells indicates that its biological activity differs from both PU.1 and Spi-B, and Spi-C in a biological setting. These results suggest that PU.1 possesses the strongest transactivation potential among this subfamily of Ets transcription factors.

FIGURE 6. Opposing effects of PU.1 and Spi-C on the IgH intronic enhancer and the FcγRIIb promoter. 38B9 pro-B cells were transiently transfected with luciferase reporters using electroporation. Renilla luciferase was used as a transfection control. A, A luciferase reporter driven by the IgH intronic enhancer was cotransfected alone or with PU.1, DNA-binding mutant of PU.1, Spi-C, or both PU.1 and Spi-C. B, A luciferase reporter driven by the IgH intronic enhancer or a version mutated at the PU.1 consensus site was transiently transfected into 38B9 pro-B cells alone. C, A luciferase reporter driven by the FcγRIIb promoter was cotransfected with PU.1 or Spi-C. *, $p < 0.05$, and ***, $p < 0.001$, as determined by Student’s t test assuming equal variances.
PU.1, but not Spi-C, activates transcription from the \( \text{Fc/H9253RIIb} \) promoter using a transient transfection assay (Fig. 6C). Although transient transfection of the minimal \( \text{Fc/H9253RIIb} \) promoter with Spi-C did not repress activity like that seen with reduced transcripts in WT-Spi-C pro-B cells, we speculate that the repressive activity of Spi-C on the \( \text{Fc/H9253RIIb} \) promoter may be mediated through another DNA-binding element not included in the minimal promoter used in transient transfection assays. However, our results indicate that PU.1 is a positive regulator of the \( \text{Fc/H9253RIIb} \) gene, while Spi-C functions as a negative regulator of this gene either as an active repressor or an inert inhibitor of PU.1 activity. The regulation of receptor genes plays an important regulatory role during B cell development. In addition, the FcγRIIb inhibitory receptor has been linked to various autoimmune diseases (reviewed in Ref. 61). Proliferation in response to functional pre-BCR signaling is necessary for progression to the immature stages, so down-regulation of the FcγRIIb inhibitory receptor would promote this proliferation and may play an important role in B cell development. The expression of Spi-C at particular stages requiring proliferation, such as pre-B and mature B cell stages, suggest that it functions to eliminate the antiproliferative effects of PU.1 and the FcγRIIb receptor.

**FIGURE 7.** Chromatin immunoprecipitation of modified histones. A, Chromatin immunoprecipitation analyses of acetylated histones H3 and H4 were assayed by real-time PCR for various segments from the 3′ regulatory region of the IgH locus (enhancers HS3a, HS1,2, HS3b, and HS4; downstream DNase I hypersensitive sites HS5, HS6, and HS7, i.e., 34; and segments upstream of HS3a, i.e., 0.2; 3′ of HS1,2, i.e., 14; and downstream of HS7 at position 34, i.e., 38 and 48), and the intronic enhancer, as previously described (41). IL-7 receptor \( \alpha \) was used as positive control. B, Chromatin immunoprecipitation analysis of dimethylated lysine 9 on histone H3 was assayed by real-time PCR for various regulatory regions in the 3′ end of the IgH locus and the intronic enhancer.
In contrast to down-regulation of the FcγRIIib gene as a result of Spi-C expression, IgH sterile transcription (Igm) was increased. This is similar to our findings that IgH sterile transcripts were increased in PU.1+/−/Spi−/− pro-B cells relative to WT pro-B cells (29). Using transient transfection assays, we were able to demonstrate that PU.1 repressed activity of the Eμ enhancer, while Spi-C partially relieved this repression (Fig. 6A). Repression of Eμ by PU.1 was dependent on DNA-binding interactions (Fig. 6A). Finally, we found that mutation of the PU.1 consensus site in Eμ resulted in a slight, although statistically insignificant, activation of reporter gene activity (Fig. 6B). Taken together, these results suggest that PU.1 functions as a repressor for the Eμ enhancer. Although much of the literature describes PU.1 as a transcriptional activator, we note that PU.1 was recently found to participate in developmentally regulated repression of several regulatory elements, including the tal-1 silencer, the p1Gloko promoter, and the IgH 3′ regulatory region enhancer HS1.2 (14, 62, 63).

When examining the 3′ regulatory region of the IgH locus, we observed that acetylation of histones H3 and H4 was generally equivalent between WT-R1 pro-B cells and WT-Spi-C pro-B cells (Fig. 7A). In contrast, di-Me K9 H3 was significantly reduced in WT-Spi-C pro-B cells (Fig. 7B). This histone mark causes a condensation of chromatin and inhibits expression. The observation that this mark is removed by Spi-C ectopic expression suggests that this may help facilitate IgH expression, and is associated, directly or indirectly, with the increased IgH sterile transcription observed in WT-Spi-C pro-B cells. Perhaps, compared with PU.1, Spi-C has unique interactions with either histone methyltransferases or factors that recruit these enzymes, providing a mechanism of derepression at the IgH locus.

Our gel-shift results demonstrate that PU.1 and Spi-C can interact with a defined binding site in the Eμ enhancer and FcγRIIib promoter (Fig. 5). Although levels of PU.1 protein were found to be equivalent, gel-shift complexes corresponding to PU.1 are reduced in WT-Spi-C pro-B cell nuclear lysates relative to WT-R1 pro-B cell nuclear lysates. Combined with the results discussed above that PU.1 and Spi-C have opposing activities on the FcγRIIib and IgH genes, our data suggest that Spi-C can inhibit PU.1 DNA binding either directly or indirectly. This type of inhibition is also observed with Ets-2 repressor factor, which acts as a repressor to Ets-2 activity (64). Therefore, we propose that when Spi-C expression is induced during normal B cell development, it inhibits PU.1 binding to target genes. We expect that the activation domain of Spi-C functions differently from that of PU.1, such that Spi-C relieves PU.1-mediated repression of the IgH intronic enhancer, while interfering with PU.1-mediated activation of the FcγRIIib promoter. Because Spi-C expression is confined to particular stages of B cell development, beginning at the pre-B cell stage and ending in mature B cells, it may function in a developmental stage-specific manner. Taken together, our data lead us to propose that the biological function of Spi-C is to antagonize PU.1 to promote B cell differentiation.

Acknowledgments
We acknowledge Dr. Jerry Adams (Walter and Eliza Hall Institute, Melbourne, Australia) for his gift of the pEμ-SRA vector; William Miller (University of Cincinnati College of Medicine, Cincinnati, OH) for his gift of the monoclonal anti-HA Ab; Olivia Schneider for initial cloning of the FcγRIIib promoter; and Ryan Lorber and Sabrina Volpi for technical assistance. We acknowledge the flow cytometry core of the Cincinnati Children’s Hospital Research Foundation for assistance in cell sorting and the real-time PCR core of Albert Einstein College of Medicine for assistance in ChIP analysis. We thank Isaac Houston for critically reading the manuscript.

Disclosures
The authors have no financial conflict of interest.

References


