The Ets Factors PU.1 and Spi-B Regulate the Transcription in Vivo of P2Y10, a Lymphoid Restricted Heptahelical Receptor*

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To investigate the in vivo functions of PU.1 and Spi-B, two highly related Ets transcription factors, we previously generated PU.1+/−/Spi-B−/− and PU.1+/−/Spi-B+/− mice and demonstrated a significant decrease in B-cell receptor (BCR) signaling in mutants. Major components of BCR signaling appear to be expressed at normal levels in these mice, implying that PU.1 and Spi-B cooperate in the transcription of additional target genes important for antigen receptor signaling. We used subtractive hybridization to identify novel in vivo PU.1/Spi-B target genes and determined that the expression of a heptahelical receptor, P2Y10, is dramatically reduced in PU.1+/−/Spi-B−/− B-cells. Further analysis shows that P2Y10 expression is restricted to lymphoid cells and parallels that of Spi-B in B-lymphocytes. Lastly, the P2Y10 promoter contains a PU.1/Spi-B binding site functionally required for efficient transcription in B-cells. Thus, P2Y10 is likely to be a direct in vivo transcriptional target for PU.1 and Spi-B and provide a unique model to explore transcriptional regulation by this Ets factor subfamily. Furthermore, P2Y10 suggests an intriguing connection between heterotrimeric G-proteins and BCR signaling.

B-cell development represents a complex series of steps during which a single, pluripotent stem cell gives rise to a mature B-lymphocyte with rearranged heavy and light chain loci. Transcription factors play a crucial role in this process by regulating gene expression which affects both lineage specification and function (1, 2). Ets DNA-binding proteins represent a key family of transcription factors implicated in lymphoid development and activity. Ets proteins consist of monomeric transcription factors which bind the DNA site AGAA/T through their Ets domain, a unique winged helix-turn-helix motif (3–6). PU.1, Spi-B, and the recently described Spi-C (7) are a divergent group of Ets proteins due to unique amino acids in their Ets domain relative to other family members. Because of these differences, PU.1 and Spi-B proteins can also bind the non-canonical AGAA (8, 9).

PU.1/Spi-1 was cloned as a B-cell- and macrophage-restricted DNA binding activity (10) and as an oncogene which causes erythroleukemias (11). PU.1 was eventually shown to transactivate a large number of B-cell genes such as those encoding CD72 (12), LSP1 (13), CD20 (14), Btk (15), J-chain (8), mb-1 (16), μ heavy chain (17), κ (18, 19), and λ (20) light chains. Spi-B was cloned based upon its homology to PU.1 (90% similar in the Ets domain), and has been shown to bind many of the same sites as PU.1 (although with different affinities) and transactivate some of the same genes as PU.1 (9, 21, 22). While PU.1 is expressed in B-cells, early T-cells, megakaryocytes, granulocytes, mast cells, immature erythrocytes, and myeloid cells (10, 21, 23), Spi-B is restricted to B-cells and immature T-cells (9, 24). Thus, B-lymphocytes express at least two Ets proteins which can bind to similar DNA sites and transactivate similar genes.

To better understand the roles of PU.1 and Spi-B in B-lymphocyte development, function, and transcription, we created targeted disruptions of both genes in mice. PU.1+/− animals die at approximately day 16.5 of gestation and lack lymphoid and myeloid cells (25) due to a cell intrinsic defect (26) of a recently described lymphoid-myeloid common progenitor (27). Mice with a different targeted allele of PU.1 display a less severe phenotype, but also lack B-cells (28, 29). In contrast to the dramatic phenotype presented by the PU.1+/−/Spi-B−/− animals, Spi-B−/− mice are viable and have no defect in B- or T-cell numbers (30). However, Spi-B−/− B-cells exhibit an interesting defect in BCR2 signaling which appears to be due to misexpression of one or more novel signaling molecules (31).

PU.1+/− and Spi-B−/− mice present two of the problems commonly observed when analyzing the phenotype of knockout animals. The PU.1+/− mice completely lack B-cells, and thus it is impossible to use these animals as a tool for exploring the role of PU.1 in a mature B-cell. In contrast, Spi-B−/− mice may exhibit a mild phenotype because their B-cells express wild-type levels of PU.1 protein, which could compensate for the lack of Spi-B (31). To assess functional redundancies of PU.1 and Spi-B, we generated PU.1+/−/Spi-B−/− mice (31). These mice are viable, but unlike the PU.1+/−/Spi-B−/− animals, they exhibit perturbed B-cell numbers as well as a more severe decrease in BCR-mediated signaling. Thus, PU.1+/−/Spi-B−/− mice act as a genetic model which allows us to explore the roles of this subgroup of Ets proteins in B-cell transcription and function.

Although a large number of B-cell PU.1/Spi-B target genes have been identified, all major signaling molecules are expressed at normal levels in PU.1+/−/Spi-B−/− mice (31). We hoped to identify novel PU.1/Spi-B target genes important for BCR signaling using subtractive hybridization on PU.1+/−/Spi-B−/− B-cells. These mice provided an attractive population to

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The abbreviations used are: BCR, B-cell receptor; EMSA, electromobility shift assay; NE, nuclear extracts; IVT, in vitro transcription and translation; TA, transactivation domain; PCR, polymerase chain reaction; kb, kilobase pair(s).
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perform the subtraction because they are phenotypically identical to PU.1\(^{+/+}\)-Spi-B\(^{+/+}\) B-cells based on fluorescence-activated cell sorter analysis except for the loss of Spi-B and PU.1. One cDNA corresponding to an mRNA underexpressed in PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) B-cells encodes a lymphoid-restricted, heptahelical receptor (P2Y10) that couples through heterotrimeric G-proteins. The promoter of P2Y10 contains a functionally important PU.1/Spi-B-binding site critical for its transcriptional activity in B-cells. We, therefore, identify an in vivo PU.1/Spi-B target gene dependent on wild-type levels of PU.1 and Spi-B for its transcription. P2Y10 is unique in the way it is the first such target gene shown to be regulated by PU.1/Spi-B, to our knowledge. We, therefore, identify an additional target gene, P2Y10, the transcription of which is dependent on wild-type levels of PU.1 and Spi-B for its transcriptional activity in B-cells. We, therefore, identify an in vivo PU.1/Spi-B-binding site critical for its transcriptional activity in B-cells. We, therefore, identify an in vivo PU.1/Spi-B target gene dependent on wild-type levels of PU.1 and Spi-B for its transcription. P2Y10 is unique in the way it is the first such target gene shown to be regulated by PU.1/Spi-B, to our knowledge.

Experimental Procedures

Cell Purification and RNA Extraction—Spleenic B-lymphocytes were purified from mice of the appropriate genotypes by subjecting ammonium chloride-lysed splenocytes to anti-Thy1.2 plus rabbit complement mediated cytolysis. Both the wild-type and mutant spleens were treated with 0.25% (wt/vol) of B220 staining. Spleenic CD3\(^+\) T cells were purified via magnetic cell sorting (PerSeptive Biosystems). Total RNA was isolated from all cells and tissues using the Trizol reagent (Life Technologies, Inc.) according to manufacturer’s protocols.

Subtractive Hybridization—The subtractive hybridization procedure was performed using the PCR-cDNA Subtraction and PCR-Select Differential Screening kits (CLONTECH), as per the manufacturer’s protocols. Due to the limited amount of total RNA isolated from purified B-lymphocytes, the cDNA used in the subtraction was prepared by the SMART PCR cDNA Synthesis kit (CLONTECH).

Northern Analysis—All Northern blots utilized 10–20 \mu g of total RNA and were prepared as described previously (9). For P2Y10, the probe was a 900-base pair Real fragment contained in the 5\’-untranslated region of the cDNA which was isolated by the subtractive hybridization. The PU.1 probe was a 350-base pair ApaI digested fragment of the 3\’-untranslated region, and the Spi-B probe has been previously described (9). Quantification of signal intensity was done on a Molecular Dynamics PhosphorImager using Image Quant software.

cDNA Library Screening and Promoter Isolation—To isolate the full-length cDNA, the 900-base pair fragment generated from the subtractive hybridization was used as a probe to screen a C57/BL6 splenocyte library (Stratagene). Pure phage were isolated according to the manufacturer’s protocols and sequenced to confirm their identity.

The promoter of P2Y10 was initially isolated using the Genome Walker kit (CLONTECH) and yielded fragments from 0.6–2.2 kb in size. These clones were sequenced and used to generate PCR primers to direct the synthesis of each fragment or a 0.10-kb of the promoter with the following primers: 5\’ primer (0.6 kb) CTGTCGAGATCCTATTTCACCCACCAATGC, 5\’ primer (0.1 kb) GGAGCTTACCTACGTCTCTGCAACAGGAAGTCC, 3\’ primer (wild-type PU.1 site) CTGGCTGATACGTGGAGAAGAATAATGGTGG, 3\’ primer (mutant PU.1 site) GAAATAATGTTGAAACACCGGGTAGAGCACATTAATGTC. Both 3\’ primers were chosen in the 5\’-untranslated region of the P2Y10 cDNA. PCR was performed using the high-fidelity polymerase Bio-X-Act (Bioline) with C57/BL6 mouse genomic DNA as a template for a limited number of cycles. These promoter fragments (referred to as 0.6 kb wt and 0.6 kb mut) were subcloned into the Sall-BamHI site of the promoter-less reporter construct pGL4H (Nichols Institute) which expresses the human growth hormone (human growth hormone) cDNA. Promoter constructs were sequenced and revealed no nucleotide differences from a consensus sequence developed from 5 individual PCR isolates.

Transient Transfections—A20 and EL-4 cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (Life Technologies, Inc.), 100 units/ml penicillin, 100 \mu g/ml streptomycin, and 0.1 \mu g/ml deoxyribonuclease I (Life Technologies), 100 units/ml penicillin, and 100 \mu g/ml streptomycin. For MEL, EL-4, and A20 transfections, 20 \mu g of reporter DNA and 2 \mu g of the luciferase expressing plasmid pGL2 Control (Promega) were added to 10\(^6\) cells in 0.4 ml of media without additives in a 0.4-cm gap electroporation cuvette (Bio-Rad) and electroporated at 250 V, 960 microfarads using a Bio-Rad Gene-Pulser. Cells were then incubated for 10 min on ice and replated in normal growth media. Cell supernants and cell extracts were prepared 24 h after transfection and assayed for human growth hormone (Nichols Institute) and luciferase activity (Promega) as a monitor of transfection efficiency following the manufacturer’s protocols.

For NIH 3T3 co-transfections, cells were transiently transfected using the Superfect reagent (Qiagen) according to the manufacturer’s protocols. For each co-transfection, 1 \mu g of reporter plasmid (0.6 kb wt or 0.6 kb mut), 1 \mu g of a \(\beta\)-galactosidase expressing plasmid (pMSV\(\beta\)gal), and 3 \mu g of an expression plasmid for PU.1, Spi-B, or Spi-B\Delta\(\alpha\), all with a hemagglutinin epitope tag which have been described previously (22). 48 h after transfection, supernatants were collected and assayed for human growth hormone production and transfection efficiency was monitored by \(\beta\)-galactosidase activity as described previously (32).

Electrophoretic Mobility Shift Assays (EMSA)—Nuclear extracts (NE) and in vitro Transcription and Translation (IVT) proteins were prepared as described previously (22).

Binding reactions were performed for 30 min at room temperature and contained equimolar amounts of IVTs, 10 \mu g of A20 NE or 2.5 \mu g of primary B-cell NE, 10 nm Tris, 1 nm EDTA, 1 nm dithiothreitol, 75 nm KCI, 4% Ficol, 5 \times 10\(^6\) cm^3/ml of \(32^P\)-labeled double-stranded oligonucleotide probe, and either 12.5 \mu g/ml (IVTs) or 60 \mu g/ml (NE) poly(dI:dC) (Amersham Pharmacia Biotech). At this time, cold competitors or anti-P2Y10 antisera was added. The anti-P2Y10 antiserum has been previously described (9); the anti-PU.1 antibody was purchased from Santa Cruz. Protein-DNA complexes were resolved on a 6% (19:1) polyacrylamide/bisacrylamide (Bio-Rad), 0.5 \times TBE gel at 200 V for 3.5 h, dried, and subjected to autoradiography.

The following double-stranded synthetic oligonucleotides were used (top strand) 5\’-CTAGCGGAAGATAAAAGAATGGAAACACGATG-3\’, GALA 5\’-GACCAGGACTTCTCTTCTCCAG-3\’, P2Y10 5\’-TCTGCCATTACTCTCTTCTCAACATT-3\’, P2Y10 mut 5\’-TGTGCTCTACCCGGTTGTCAAACATT-3\’.

Results

Subtractive Hybridization Identifies Genes Poorly Expressed in PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) B-Lymphocytes—To identify B-cell target genes of PU.1 and Spi-B, we chose a PCR-based subtractive hybridization approach (Fig. 1) based upon the fact that resting B-cells from PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) mice are essentially phenotypically normal (31). PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) B-cells, because of reduced levels of PU.1 and Spi-B, should express lower levels of any target genes critically dependent on normal levels of PU.1 and Spi-B for their transcription. Therefore, spleenic B-cells from a PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) mouse and a wild-type littermate were purified and cDNA prepared as described under “Experimental Procedures.” These cDNAs were then used in a subtractive hybridization protocol (33). After a preliminary screen, 42 individual clones were isolated, sequenced, and subjected to analysis by either Northern blot or reverse transcriptase-PCR based approaches to identify which ones were differentially expressed. Of these 42, only 3 cDNA fragments appeared to be expressed at lower levels in PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) B-cells (data not shown). One cDNA (3H6) was further characterized because it was consistently underexpressed in PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) B-cells prepared from multiple mice (data not shown). As shown in Fig. 2, this mRNA species was reduced approximately 2-fold in PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) B-cells and 8-fold in PU.1\(^{-/-}\)-Spi-B\(^{-/-}\) B-cells as compared with PU.1\(^{+/+}\)-Spi-B\(^{+/+}\) B-cells. The isolated cDNA fragment was used to obtain cDNAs from a mouse splenocyte library and yielded three overlapping phage clones. One clone contained an open reading frame of 328 amino acids with strong homology to the human purinergic receptor P2Y1 (GenBank accession number AF000545), a heterotrimeric G-protein coupled, heptahelical receptor (Fig. 2A). Because the coding regions of the two genes are highly related (83% identical, 88% similar), it appears that 3H6 is the mouse homologue of human P2Y10. An alignment of mouse and human P2Y10 amino acids, the most closely related family member P2Y5, and the founding family member P2Y1 are shown in Fig. 3. 1. 2. 3. 4. A, B, homology between these four receptors is greatest.

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B-characterize the murine P2Y10 expression in B-cells.

levels in the seven transmembrane domains.

Therefore, by using the PU.1+/−/Spi-B+/− B-lymphocytes in a subtractive hybridization we identified the heptahelical receptor P2Y10 as a possible novel target gene of PU.1 and Spi-B in vivo. In addition, it appears that P2Y10 transcription is dependent on maximal levels of both PU.1 and Spi-B for its expression in B-cells.

P2Y10 Expression Is Restricted to Lymphocytes—To further characterize the murine P2Y10 gene, we examined its tissue and cell line distribution using Northern blot analysis. Using organs isolated from adult mice, P2Y10 mRNA is expressed predominantly in the spleen and thymus, with very low levels of expression detected in bone marrow (Fig. 4A and data not shown). When murine cell lines were tested, P2Y10 appeared to be exclusively expressed in immature and mature T-cells, immature and mature B-cells, and faint expression was detected in one of the two pre-B cell lines tested (Fig. 4B). However, it was not expressed in macrophage, erythroid, or fibroblast cell lines.

When these same cell lines were tested for Spi-B and PU.1 expression, PU.1 appears to be expressed at all stages of B-cell development, whereas Spi-B is expressed exclusively in immature and mature B-cells (Fig. 4B). Neither PU.1 nor Spi-B is expressed in the T-cell lines tested, but PU.1 is expressed in the macrophage cell line (Fig. 4). The erythroid cell line used (MEL) has been shown by others to express low levels of PU.1 (10, 34, 35). Thus, it appears that P2Y10 mRNA expression mirrors that of Spi-B in B-cells, but is also expressed in T-cells where neither PU.1 nor Spi-B is expressed. This data indicates that in B-cells P2Y10 may be a direct in vivo transcriptional target of PU.1 and Spi-B. To investigate if P2Y10 expression was altered in T-cells harvested from PU.1+/+Spi-B−/− (data not shown) or PU.1−/−Spi-B−/− mice, we probed the Northern blot represented in Fig. 4C. As shown in Fig. 4, the expression of P2Y10 in T cells is not reproducibly affected by the genotype at either the PU.1 and Spi-B loci. These results indicate that P2Y10 is regulated by other factors in T lymphocytes (see below).

The P2Y10 Promoter Is Lineage Restricted and Contains a Functionally Important PU.1/Spi-B-binding Site—To ensure that P2Y10 is a direct transcriptional target of PU.1 and Spi-B, the promoter of P2Y10 was isolated as described under “Experimenal Procedures,” and a 0.6-kb fragment was sequenced to identify possible transcription factor-binding sites (Fig. 5A). The transcriptional start site was confirmed by performing 5′-rapid amplification of cDNA ends, no additional sequences upstream of the cDNA isolated from the splenocyte library were detected (data not shown). Sequence analysis of the region immediately upstream of the transcriptional start site revealed no consensus PU.1/Spi-B-binding site located at −11 base pairs (36).

To test whether the putative PU.1/Spi-B-binding site actually binds these factors in vitro, EMSAs were performed using NE prepared from A20 cells, a mature (IgG2a) B-cell line. As demonstrated in Fig. 5B, a 32P-labeled probe containing the PU.1/Spi-B site from the P2Y10 promoter produced a single DNA-protein complex when incubated with A20 NE (lane 2). This complex was specific to the PU.1/Spi-B-binding site since it was not affected by a nonspecific cold competitor to the GAL4 transcription factor-binding site (lane 3) or a cold competitor P2Y10 probe in which the PU.1/Spi-B-binding site was mutated (GAGGAA → CACCGG, lane 5). In contrast, the A20 complex was efficiently competed by either the unlabeled P2Y10 probe (lane 4) or an unlabeled probe to the Aβ site from the Aβ enhancer which has been shown previously (22) to efficiently bind both PU.1 and Spi-B (lane 6). To confirm that PU.1 and/or Spi-B were present in the DNA-protein complex, supershift experiments were performed using antisera to both PU.1 and Spi-B. Antiserum to Spi-B caused a small increase in the mobility of the DNA-protein complex (lanes 8 and 9) that was not observed in the preimmune sera, and an antibody to PU.1 reduced the mobility and intensity of the complex (lane 10). Importantly, addition of antiserum to both PU.1 and Spi-B completely abolished the shift (lane 11), implying that the binding activity in A20 nuclear extracts to the P2Y10 promoter contains almost exclusively PU.1 and Spi-B. The most likely ex-
planation for the increase in mobility seen with the anti-Spi-B antiserum (lane 9) or the decrease in mobility seen with the anti-PU.1 antiserum (lane 10) is that blocking the binding of either PU.1 or Spi-B allows for better binding of the other transcription factor to the site. Since Spi-B DNA complexes migrate more slowly than PU.1 DNA complexes (Fig. 6B), the antisera are affecting mobility simply by allowing only one of the two transcription factors to form a protein-DNA complex.

To explore the binding activity of PU.1 and Spi-B from primary cells, NE were prepared from purified splenic B-cells and used in an EMSA. As demonstrated in Fig. 5C, a probe to the P2Y10 site generates a specific protein-DNA complex from these NE (lanes 2–6). This complex contains exclusively PU.1, as evidenced by the complete ablation of shift using an anti-PU.1 antibody (lane 10) and is essentially unaffected by the anti-Spi-B antiserum (lane 9). Thus, in both A20 and primary B-cell NE, PU.1 is the predominant component of the DNA binding activity for the P2Y10 site.

To confirm that PU.1 and Spi-B could bind to the Ets site in the P2Y10 promoter, equimolar amounts of IVT PU.1 and Spi-B were used in EMSAs. As shown in Fig. 6A, both PU.1 and Spi-B efficiently bound the P2Y10 site (lanes 2 and 3). Binding to the P2Y10 site was not competed by either a nonspecific cold competitor probe (GAL4, lanes 4 and 5) or an unlabeled P2Y10 probe containing a mutated PU.1/Spi-B site (lanes 8 and 9) but was efficiently competed by unlabeled P2Y10 probe (lanes 6 and 7) or the Ets site (lanes 10 and 11).

Our EMSA results with B-cell NE (Fig. 5, B and C) imply that Spi-B did not bind to the P2Y10 site at significant levels either because it has weak affinity for the site or because it is expressed at much lower levels than PU.1. To directly assess the DNA binding affinities of PU.1 and Spi-B for the P2Y10 site, we performed EMSA under conditions where the radiolabeled P2Y10 probe would saturate the binding of either PU.1 or

![Fig. 3. Clone 3H6 is the heptahelical purinergic receptor, P2Y10. A, deduced amino acid sequence of P2Y10. The putative transmembrane domains are underlined and numbered. B, alignment of the murine P2Y10 protein with its human homologue, its most closely related family member P2Y5, and the founding member of the P2Y family P2Y1. Red indicates identity, blue indicates similarity.](image-url)
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Fig. 4. P2Y10 is lymphoid restricted. A. examination of the tissue expression pattern of P2Y10 by Northern blot analysis. RNA was prepared from the indicated tissues of adult mice and total RNA was extracted. 18 S ribosomal RNA is shown to control for loading. B, examination of P2Y10. PU.1, and Spi-B expression in various murine cell lines by Northern blot analysis on total RNA extracted from Pro-B (NFS 70 c/10), pre-B (70 z/3 and 38 B9), immature B (WEHI 231), mature B (A20), mature T (RMA), immature T (EL-4), macrophage (J774.1), erythroid (MEL), and fibroblasts (3T3). 18 S ribosomal RNA is shown to control for loading. 28 S ribosomal RNA was equivalently loaded (data not shown). C, P2Y10 expression in PU.1+/+ Spi-B+/+ B cells, PU.1+/+ Spi-B−/− T-cells, and PU.1−/− Spi-B+/+ T-cells. 18 S RNA and actin serve as loading controls.

Spi-B and added increasing amounts of unlabeled probe (Fig. 6B). The reduction in radiolabeled DNA-protein complex was then quantified to generate a binding affinity curve for the two proteins (Fig. 6C). Based upon these results, PU.1 and Spi-B have a comparable binding affinity for the P2Y10 site (Kd ~ 3 nM). Therefore, the lack of Spi-B binding activity seen in B-cell extracts is most likely because it is expressed at much lower levels than PU.1. Unfortunately, no antibody for Western blotting of murine Spi-B is currently available, making it impossible to directly assess the protein levels of PU.1 and Spi-B in B-cells.

To directly address whether this promoter fragment depends on an intact PU.1/Spi-B site for proper transcription, the 0.6-kb fragment was subcloned into a reporter construct expressing the human growth hormone reporter gene with either a wild-type or a mutant PU.1/Spi-B-binding site (GAGGAA → CAC-CGG). Transient transfections with the wild-type (0.6 kb wt) and mutant promoter (0.6 kb mut) constructs were performed using three hematopoietic cell lines: EL-4 (immature T) and A20 (mature B) which both normally express P2Y10, and MEL (erythroid) which does not express P2Y10. As indicated in Fig. 7A, the 0.6-kb promoter fragment was not active in MEL cells, and only weakly active in EL-4 cells, although this activity was not affected by a mutation in the PU.1/Spi-B site. Because both PU.1 and Spi-B are not expressed in T-cells it is likely that other transcription factors are responsible for P2Y10 transcription in this lineage (see Fig. 4C). The Et cell factor, Elf-1, is present in T-cells and binds to similar DNA elements (2), making Elf-1 a candidate for regulating T-cell-specific P2Y10 expression. However, this seems less likely given that the Pu.1/Spi-B-binding site mutation failed to decrease promoter activity in EL-4 cells. A more plausible explanation is the existence of a second promoter that is active in T-cells and independent of PU.1 and Spi-B. In direct contrast, the 0.6-kb promoter was extremely active in A20 B-cells and mutation of the PU.1/Spi-B-binding site decreased its activity by 50–75%. Thus, the 0.6-kb P2Y10 promoter is lineage restricted to B-cells and the activity of this promoter depends on a functional PU.1/Spi-B-binding site.

To further examine the role of PU.1 and Spi-B in transcription of the P2Y10 promoter, co-transfection experiments were performed by transiently transfecting NIH 3T3 cells with a smaller reporter construct (0.1 kb) containing the wild-type PU.1/Spi-B-binding site and expression plasmids containing epitope tagged versions of PU.1, Spi-B, and two transcriptionally inactive deletions of Spi-B which either remove the DNA-binding domain (Spi-B ΔEts) or the transactivation domain (Spi-B ΔTA). Similar results were obtained with the 0.6 kb wt reporter construct (data not shown). As shown in Fig. 7B, either PU.1 or Spi-B caused strong transactivation of the reporter in this heterologous cell type. This effect is specific since Spi-B ΔEts and Spi-B ΔTA caused no transactivation of the reporter. Interestingly, the same promoter with a mutant PU.1/Spi-B was weakly transactivated by PU.1 or Spi-B (data not shown), implying that there may be other low affinity binding sites for these transcription factors in this DNA fragment. Transactivation of the P2Y10 0.6-kb promoter was also examined by reconstituting NIH 3T3 cells with both PU.1 and Spi-B. We found that transactivation by PU.1, Spi-B, or a combination of the two factors resulted in statistically similar levels of P2Y10 promoter activity (data not shown). These results are consistent with a model where P2Y10 is regulated by both PU.1 and Spi-B.

We conclude that the putative PU.1/Spi-B site in the P2Y10 promoter efficiently binds both proteins, and this binding site is critical for P2Y10 transcription in B-cells. In addition, the P2Y10 promoter can be transactivated by PU.1 or Spi-B in heterologous cells. Given that PU.1+/+ Spi-B−/− B-cells express 50% as much P2Y10 as wild type cells and that PU.1+/− Spi-B+/− B-cells express 10–13% as much, we suggest that PU.1 is the dominant factor. Importantly, PU.1+/+ Spi-B−/− B-cells express wild type levels of P2Y10 (data not shown). Therefore, PU.1/Spi-B is necessary for 100% levels of P2Y10 transcription. Taken together, PU.1 and Spi-B regulate P2Y10 and it is likely to be a direct transcriptional target for these factors.

DISCUSSION

PU.1−/− mice fail to develop any mature B-cells (25, 28). Therefore, there is no way to assess the role of PU.1 in B-cell function without a conditional mutation in B-lymphocytes (37, 38). To explore the role of PU.1 in mature B-cells, we created a semi-epistatic condition in which B-cells expressed 50% of wild
type PU.1 protein levels and lacked Spi-B (PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup>). B-cells from PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup> mice contained sufficient PU.1 protein to support B-lymphocyte development but insufficient PU.1/Spi-B binding activity for efficient transcription of all target genes.

PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup> B-cells represent a tractable model system for analysis of these factors in vivo because they are phenotypically normal when unstimulated (31) but display signaling defects upon IgM cross-linking. As such, PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup> and PU.1<sup>−/−</sup>Spi-B<sup>−/−</sup> purified B-cells provide attractive substrates for subtractive hybridization, with the only difference in the two populations being disruption of the PU.1 and Spi-B loci. Our data indicate that PU.1 and Spi-B are necessary for efficient transcription of membrane proximal component(s) of the BCR signaling cascade. Importantly, the expression of several presumptive target genes of PU.1 and Spi-B such as mb-1, Btk, Blk, κ, and λ light chains and all known components of BCR signaling are unaffected by reduced levels of PU.1 and Spi-B (31). Therefore, PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup> B-cells could be used to clone novel PU.1/Spi-B targets important for BCR-mediated responses.

We show in this report that PU.1 and Spi-B are both necessary for efficient transcription of P2Y10 in B-cells. It is possible that PU.1 and Spi-B also regulate another transcription factor required for the proper expression of P2Y10. However, we have cloned the P2Y10 promoter and demonstrated that it contains a consensus PU.1/Spi-B-binding site important for promoter activity in B-cells. While these data cannot exclude the possibility that PU.1 and Spi-B also regulate additional gene(s) required for P2Y10 expression, our data illustrate that PU.1 and Spi-B directly control P2Y10 promoter activity. Other possible low affinity PU.1/Spi-B sites in the proximal P2Y10 promoter (Fig. 7B) may explain why the single mutation created in our reporter constructs (Fig. 7, A and B) does not completely abolish transcription in transient assays.

The importance of PU.1 and Spi-B for P2Y10 transcription is supported by their convergent expression patterns. In B-cells, P2Y10 expression essentially parallels that of Spi-B in that both are preferentially transcribed in immature and mature B-cells, whereas PU.1 is expressed throughout B-cell development. Importantly, one of the two pre-B cell lines producing P2Y10 (38 B9) also expresses low levels of Spi-B (9), and the other pre-B cell line lacks both P2Y10 (70 Z/3) and Spi-B. These data imply that Spi-B is important for P2Y10 expression (Fig. 4B). However, EMSA shows that PU.1 is the predominant DNA binding activity contained in A20 or primary B-cell nuclear extracts.
 extracts (Fig. 5, B and C). Based on these results and mRNA levels of P2Y10 from PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup> and PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup>-purified B-cells (Fig. 2), P2Y10 transcription in B-cells is dependent on the combined dosage of PU.1 and Spi-B, although PU.1 is more critical because it is expressed at higher levels than Spi-B. Transient transfection experiments in NIH 3T3 fibroblasts also support a combined role for PU.1 and Spi-B (Fig. 7). Our data are similar to results reported for bHLH proteins of the E2A family where B-cell development is affected (39). In this situation, combinations of mutations in E2A, E2-2, and HEB revealed that a critical dosage of all three genes is required for efficient B-cell formation and development. However, in our situation the dosage of PU.1 and Spi-B seems to effect the transcription of a specific target gene. One possible reason that P2Y10 transcription depends on adequate levels of PU.1 and Spi-B is that its proximal promoter contains a paucity of binding sites for other known transcription factors (data not shown). PU.1 and Spi-B are likely to be scaffolding proteins which bind DNA elements and create activation complexes that recruit the basal transcriptional machinery to promoters through multiple protein-protein interactions (40–44). When other factors are present, such protein-protein interactions stabilize PU.1 and/or Spi-B binding and low levels of PU.1/Spi-B are sufficient to create effective transcriptional activation complexes. The lack of binding sites for other transcription factors in the P2Y10 promoter could reduce the stability of activation complexes, requiring maximal levels of PU.1 and Spi-B for proper activation.

Another explanation for the observed gene dosage effects could be the presence of additional Ets sites outside of the 0.6-kb P2Y10 promoter that preferentially bind either PU.1 or Spi-B which are critical for P2Y10 transcription. Functional Ets sites in B-cell promoters or enhancers which selectively bind PU.1 or Spi-B have not been identified thus far. However, an Ets site in the myeloid cell-specific c-fes promoter appears to bind PU.1 at a significantly higher affinity than Spi-B (22). The loss of PU.1 or Spi-B would selectively reduce P2Y10 transcription through the activity of such DNA elements.

The ultimate goal of our studies was to understand how PU.1/Spi-B target genes contribute to the phenotype of mutant mice. The PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup> B-cell defect is striking: they exhibit poor proliferation in response to antigenic stimulation both in vivo and in vitro, decreased levels of substrate tyrosine phosphorylation, and a blunted calcium response upon IgM cross-linking. The most likely cause of reduced BCR signaling is decreased Syk phosphorylation of its downstream targets such as phospholipase C-γ2 and BLNK (31). Could P2Y10 be the in vivo target gene responsible for the PU.1<sup>+/−</sup>Spi-B<sup>−/−</sup>B-cell signaling defect? Classically, membrane proximal BCR
signaling events are thought to involve tyrosine phosphorylation, while activation of heterotrimeric G-proteins and calcium fluxes act downstream. Like other heptahelial receptors, P2Y family members couple to heterotrimeric G-proteins (usually Goq) and eventually lead to PLC activation (45). However, recent evidence indicates that Btk, a critical tyrosine kinase in BCR signaling and B-cell development (46, 47), is activated by both Goq and Gβγ subunits (48–54). Therefore P2Y10 could activate Btk via G-proteins, thereby influencing the tyrosine phosphorylation of membrane proximal components of BCR signaling.

If P2Y10 does influence BCR signaling, it must become activated by binding its cognate ligand. Unfortunately, the P2Y10 ligand remains unknown, and it may not be the classical ligand for most purinergic receptors: ATP/ADP or UTP/UDP (55–60). The identity of the ligand for P2Y5, the most closely related member of the P2Y family (32% identity), is still unclear (61, 62), and another family member, P2Y7, has been shown to bind leukotriene B4 (63, 64). Once the natural ligand or a chemical agonist of P2Y10 is discovered the role of this receptor in BCR signaling can be directly assessed.

This study represents a unique example of a targeted mouse as a model to identify in vivo target genes of two transcription factors which contribute to an observed lymphoid phenotype. P2Y10 appears to be a direct transcriptional target of both PU.1 and Spi-B in vivo and provides an interesting model of transcriptional activation by this subgroup of Ets proteins. In addition, P2Y10 could have important implications in coupling heterotrimeric G-proteins directly with membrane proximal BCR signaling events. It will be important to explore the transcriptional regulation of P2Y10 as well as its effects on antigen receptor signaling in B-cells.

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