THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 274, No. 16, Issue of April 16, pp. 11115–11124, 1999
Printed in U.S.A.

SPI-B Activates Transcription via a Unique Proline, Serine, and Threonine Domain and Exhibits DNA Binding Affinity Differences from PU.1*

(Received for publication, May 4, 1998, and in revised form, December 29, 1998)

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SPI-B is a B lymphocyte-specific Ets transcription factor that shares a high degree of similarity with PU.1/SPI-1. In direct contrast to *PU.1*$^{-/-}$ mice that die *in utero* and lack monocytes, neutrophils, B cells, and T cells, *Spi-B*$^{-/-}$ mice are viable and exhibit a severe B cell proliferation defect. Since PU.1 is expressed at wild type levels in *Spi-B*$^{-/-}$ B cells, the mutant mice provide genetic evidence that SPI-B and PU.1 have at least some non-redundant roles in B lymphocytes. To begin to understand the molecular basis for these defects, we delineated functional domains of SPI-B for comparison to those of PU.1. By using a heterologous co-transfection system, we identified two independent transactivation domains in the N terminus of SPI-B. Interestingly, only one of these domains (amino acids 31–61), a proline/serine/threonine-rich region, unique among Ets proteins, is necessary for transactivation of the immunoglobulin λ light chain enhancer. This transactivation motif is in marked contrast to PU.1, which contains acidic and glutamine-rich domains. In addition, we describe a functional PU.1 site within the c-*FES* promoter which SPI-B fails to bind efficiently and transactivate. Finally, we show that SPI-B interacts with the PU.1 cofactors Pip, TBP, c-Jun and with lower affinity to nuclear factor interleukin-6β and retinoblastoma. Taken together, these data suggest that SPI-B binds DNA with a different affinity for certain sites than PU.1 and harbors different transactivation domains. We conclude that SPI-B may activate unique target genes in B lymphocytes and interact with unique, although currently unidentified, cofactors.

Hematopoiesis represents the coordinated development of all blood cell lineages (granulocytes, monocytes, lymphocytes, erythrocytes, and platelets), which arise from a self-renewing, pluripotent stem cell. This complex developmental process is guided by interactions between extracellular signals, cell-surface receptors, cell-cell interactions, and the regulation of gene expression by transcription factors (reviewed in Refs. 1 and 2). Transcription factors play a crucial role in hematopoiesis due to their ability to regulate gene expression controlling the eventual differentiation and development of distinct cell types.

One family of transcription factors thought to play a pivotal role in hematopoiesis is the Ets DNA-binding proteins. This family of transcription factors consists of approximately 30 different proteins that bear a high degree of similarity to the founding member, Ets-1. Ets proteins are monomeric transcription factors that bind to the purine-rich element of GGA(A/T) through their Ets domain (3–6). Based upon differences within the Ets and other domains, Ets proteins can be divided into a series of subfamilies consisting of the Ets-1, PU.1, Elf-1, Fli-1, and GABPα groups. The PU.1 subgroup consists of PU.1/SPI-1 and SPI-B and represents the most divergent members of the Ets family due to many differences in the Ets domain (40% similarity to Ets-1). In contrast to other Ets proteins, both PU.1 and SPI-B can bind the non-canonical DNA sequence GCAGAA (7).

In addition to having a distinct DNA binding domain compared with other Ets family members, PU.1 possesses several protein motifs unique among Ets proteins (Fig. 1). PU.1 has a C-terminal Ets domain that is involved in both DNA binding as well as protein-protein interactions involving AP-1 family members (8, 9), NF-IL6β[1] (C/EBPδ) (10), and other Ets proteins (8, 11, 12). Immediately adjacent to the Ets domain is a proline-, glutamic acid-, serine-, and threonine-rich (PEST) region, which is involved in protein-protein interactions with the lymphoid-specific co-activator Pip/IRF4/NF-EM5 and other IRF proteins (13, 14), but does not destabilize protein as other PEST sequences do (15). The PU.1-Pip interaction is crucial for the transcription of immunoglobulin light chain loci (13) and CD20 (16) and requires PU.1 binding to DNA with subsequent recruitment of Pip via a phosphorylated serine residue (Ser-148) in the PEST region (17, 18). At the N terminus of PU.1 resides a series of three independent transcriptional activation domains, including two acidic subdomains and one glutamine-rich domain (19). In addition to activating transcription, the N terminus of the protein has been shown to interact with Rb and TBP (20).

In contrast to PU.1, very little is known about the functional domains of SPI-B (Fig. 1). The two proteins are 60% similar overall, with the N terminus of SPI-B being highly diverged from PU.1 (20% similarity) but presumed to contain the tran-

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[1] The abbreviations used are: NF-IL6β, nuclear factor interleukin-6β; Rb, retinoblastoma protein; PEST, proline-, glutamic acid-, serine-, and threonine-rich, aa, amino acid(s); B4, tetramerized λB element; TK, herpes simplex virus thymidine kinase promoter; GH, human growth hormone protein; PCR, polymerase chain reaction; CMV, cytomegalovirus; GAL4, GAL4 DNA binding domain; EMSA, electrophoretic mobility shift assay; GST, glutathione *S*-transferase protein; IVT, *in vitro* transcribed and translated protein; HA, the hemagglutinin epitope YPYDVPPDYA; PAGE, polyacrylamide gel electrophoresis.

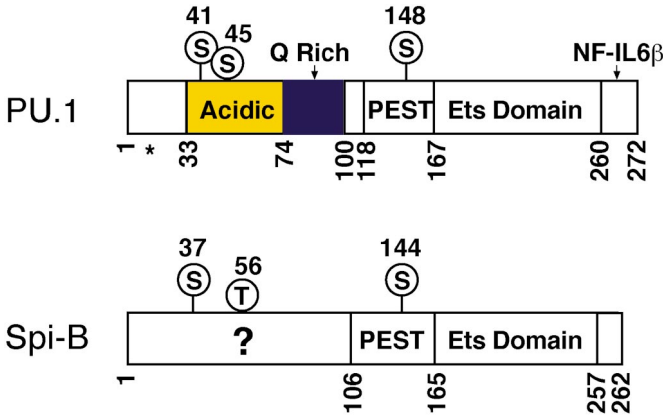

FIG. 1. **Important functional domains of PU.1 and SPI-B for DNA binding, protein-protein interactions, and transactivation.** Critical phosphorylation sites in the two proteins are also shown. * indicates this domain is weakly acidic.

scriptional activation domain. The SPI-B Ets domain is 90% similar to that of PU.1, whereas the PEST region exhibits 70% similarity (21). SPI-B binds to the same DNA elements as PU.1 *in vitro* and interacts with Pip to transactivate a target site in the λ enhancer (21–23). Furthermore, SPI-B has been shown to bind Rb via its N terminus which requires a single threonine residue (Thr-56) whose phosphorylation by ERK1 abolishes this interaction (24). It has not been determined if SPI-B can also bind to other PU.1 interacting proteins such as c-Jun, TBP, NF-IL6β, or other Ets proteins.

The highly related PU.1 and SPI-B proteins share overlapping patterns of expression. PU.1 is expressed in granulocytes, monocytes, immature erythroid cells, mast cells, megakaryocytes, B cells, and early in T cells (21, 25, 26). Although previously thought to have a similar tissue distribution as PU.1 (21), it has been shown that SPI-B is expressed only in B cells and immature T cells but not monocytes or neutrophils (23, 27). PU.1-binding sites are important for the transcriptional activity of a large number of myeloid genes such as *CD11b* (28, 29), *MCSF-R* (30), interleukin 1β (31), *GM-CSFR* (32), scavenger receptor (33), and macrophage mannose receptor (34) as well as B cell targets such as the immunoglobulin light chain loci (14, 35), *mb-1* (36), μ heavy chain (37), and J chain (7). In contrast to PU.1, the only confirmed mammalian target gene of SPI-B is the λ_{2-4} enhancer (23).

To address the *in vivo* functional differences between PU.1 and SPI-B, we have generated mice with targeted mutations in both loci. *PU.1*$^{-/-}$ mice die at approximately day 16.5 of gestation (38) and lack monocytes, neutrophils, B, and T cells but do possess erythroid cells, megakaryocytes, and immature mast cells. Mice with a different *PU.1*$^{-/-}$ allele display a similar but less severe phenotype (39–41). The loss of both lymphoid and myeloid cells suggests that PU.1 is required for the survival and/or differentiation of a multipotential lymphoid/myeloid precursor (42). In contrast to *PU.1*$^{-/-}$ mice, *Spi-B*$^{-/-}$ animals are viable and display a normal number of B and T cells (43). However, upon stimulation of B cells either *in vitro* or *in vivo*, they exhibit a proliferation defect (43) due to decreased signaling through the B cell receptor and inappropriate apoptosis.[2] One interesting issue raised by the *Spi-B*$^{-/-}$ mice is that PU.1 is obviously unable to complement this defect since it is present at wild type levels (43).

The genetic evidence that SPI-B and PU.1 are not completely redundant implies that they (i) regulate different target genes and/or (ii) bind different cofactors. To distinguish between these two possibilities, we attempt to elucidate differences between SPI-B and PU.1 which may alter their transcriptional activity. These studies reveal that SPI-B contains two N-terminal activation domains which are highly divergent from PU.1. In addition, the affinity of SPI-B for certain DNA sites appears to be different from PU.1, affecting the ability of SPI-B to transactivate the c-*FES* promoter, a known PU.1 target gene. Finally, SPI-B is shown to interact with Pip as well as other proteins in a manner similar to PU.1, suggesting that these interactions are critical for the proper function of the PU.1/SPI-B Ets subfamily but that interactions with other cofactors may contribute to differences in their ability to activate target genes.

EXPERIMENTAL PROCEDURES

Plasmids and Site-directed Mutagenesis—A tetrameric λ_{2-4} enhancer element (referred to as the λB site) (13) was subcloned immediately upstream of a TK (2) promoter in the pTKGH plasmid (Nichols Institute) to form the reporter construct B4TKGH. The 450-base pair promoter element from the human c-*FES* gene (44) was subcloned into the promoterless pϕGH vector (Nichols Institute) to form the reporter construct c-*FES* GH. The pGAL4GH (45) reporter and the Pip-CMV vector (23) have been previously described. The human SPI-B (21) and murine PU.1 (25) cDNAs have also been previously reported. Of note, the human SPI-B is 95% similar to the murine form, with only conservative differences in the Ets domain.

All plasmids for cDNA expression in mammalian cells used the CMV promoter-based pCDNA3 vector (Invitrogen). Constructs were generated by PCR and confirmed by sequencing. Deletion mutants are named based upon the amino acids that are missing from the protein. Hemagglutinin (HA) epitope-tagged cDNAs were generated by cloning PCR-generated fragments into the previously described vector pcDNA3-HA (18). SPI-B/PU.1 Ets and PU.1/SPI-B Ets contain *Xho*I and *Hin*dIII linkers between domains which insert two in-frame codons (LG and KL, respectively). ΔPEST (aa 107–165), Δ31–62, Δ31–106, Δ64–106, ΔEts (aa 166–257), and Δ257–262 contain an internal *Xho*I site within the deleted region.

GAL4 fusion proteins were constructed by inserting PCR-generated fragments of SPI-B into the pGAL4 vector (45) that contains the DNA binding domain of the GAL4 protein (aa 1–147) followed by a multiple cloning site and in-frame stop codons. Point mutations were introduced by overlapping PCR mutagenesis (46). The mutations are named by the normal amino acid, position, and the new amino acid.

Cells, Transfections, and Reporter Assays—Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units of penicillin, and 100 μg of streptomycin. NIH 3T3 fibroblasts were transfected with Lipofectin (Life Technologies, Inc.) according to manufacturer's protocol. For co-transfection experiments, 2 μg of reporter plasmid and 2 μg of a β-galactosidase expressing plasmid (pMSVβgal) were used. Transfections using the B4TKGH and c-*FES* GH reporters utilized 20 μg of expression plasmid; 8 μg of expression plasmid were used for the transfections with the GAL4GH reporter. 48 h after transfections, supernatants were collected for human growth hormone assay using a commercially available radioimmunoassay (Nichols Institute). Transfection efficiencies were measured by β-galactosidase activity in cell extracts as described previously (47).

COS-7 cells were transfected with Lipofectin and 20 μg of expression plasmid. Nuclear extracts were prepared 48 h after transfection using the method of Andrews and Faller (48).

In Vitro Transcription and Translation—[^{35}S[Methionine incorporated IVT proteins were generated using a commercially available TNT-coupled rabbit reticulocyte lysate kit (Promega). Proteins were resolved by SDS-PAGE and quantitated by PhosphorImager analysis (Molecular Dynamics).

Electrophoretic Mobility Shift Assays—Binding reactions were performed at room temperature for 30 min and contained equimolar amounts of IVT protein or 10 μg of nuclear extracts, 10 mM Tris, 1 mM EDTA, 1 mM dithiothreitol, 75 mM KCl, 4% Ficoll, 12.5 mg/ml poly(dI·dC) (Amersham Pharmacia Biotech), and 5×10^5 cpm/ml of ^{32}P-labeled double-stranded oligonucleotide probe. Protein-DNA complexes were resolved on a 6% (19:1) acrylamide:bisacrylamide (Bio-Rad), 0.5× TBE gel at 200 V for 4.5 h, dried, and subjected to autoradiography.

The following double-stranded synthetic oligonucleotides were used

[2] L. Garrett-Sinha, G. Su, S. Rao, Z. Hao, M. Clark, and M. C. Simon, submitted for publication.

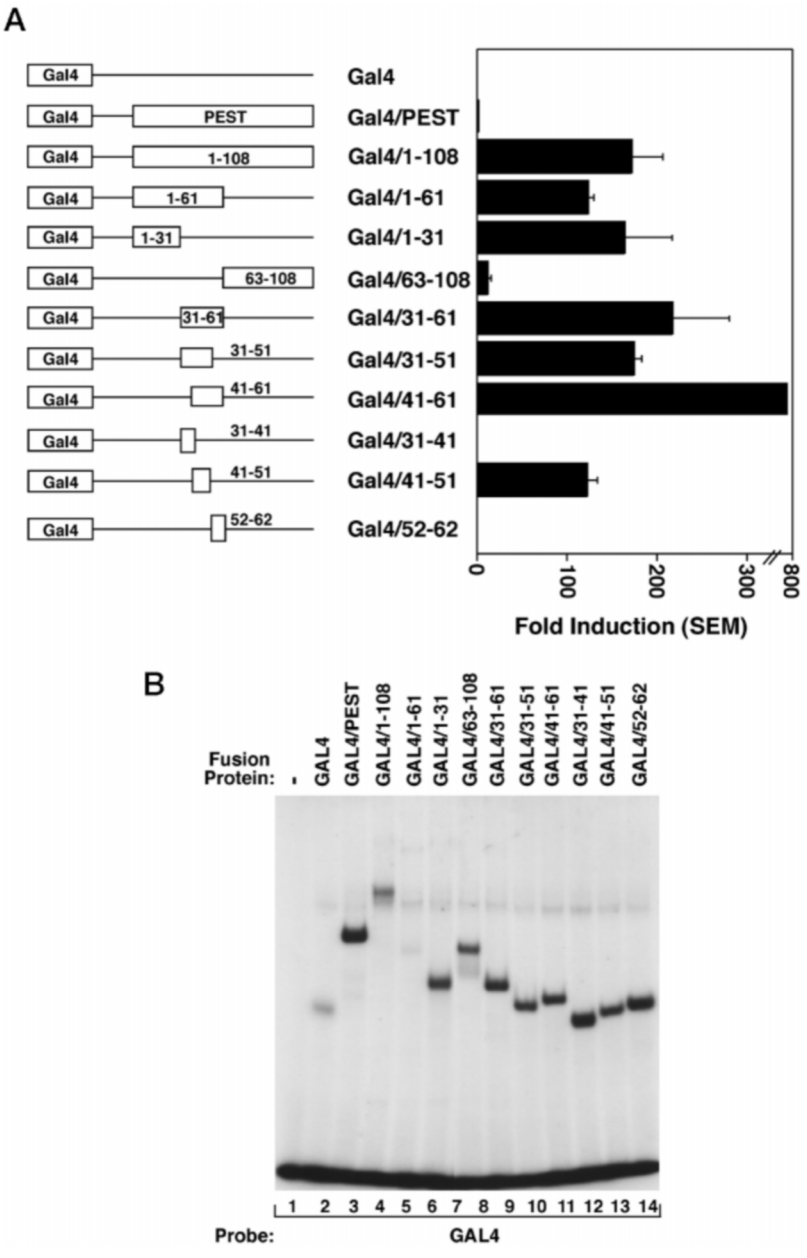

FIG. 2. *A*, 8 μg of expression plasmid (pCDNA3 based) encoding GAL4/SPI-B fusion proteins were transiently co-transfected with 2 μg of the pGAL4GH reporter and 2 μg of the pMSVβgal reference plasmid into NIH 3T3 cells. Media and cells were harvested 48 h after transfection and assayed for growth hormone activity. Data are expressed as fold induction (mean ± S.E. of four independent experiments) normalized to the pCDNA3 expression plasmid. *B*, EMSAs were performed to ensure that all GAL4 fusion proteins were capable of binding DNA using 10 μg of nuclear extracts from COS cells transiently transfected with 20 μg of the expression plasmids shown in *A* and a radiolabeled double-stranded oligonucleotide containing the consensus GAL4-binding site.

(top strand): λB, 5′ CTAGCGAGAAATAAAAGGAAGTGAAACCAAGT 3′; GAL4, 5′ GAGCGGAGTACTGTCCTCCGAG 3′; c-*FES*, 5′ CGGAATCAGGAACTGGCCGGGG 3′.

GST Affinity Chromatography—The GST fusion proteins were created by inserting the entire coding sequence of SPI-B or PU.1 into the pGEX vector multiple cloning site (Amersham Pharmacia Biotech). DH5α cultures expressing the fusion proteins were grown to saturation, diluted 1:10 in Luria broth, grown for 1 h at 30 °C, and induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4 h at 30 °C. Cells were then pelleted, washed once with NETN buffer (20 mM Tris, 100 mM NaCl, 1 mM EDTA, 0.2% Nonidet P-40), and sonicated on ice with 3–15-s bursts. Cell debris was then pelleted, and the fusion proteins were bound to pre-swelled glutathione-agarose beads (Sigma) for 30 min at 4 °C. Beads were then washed 3 times with NETN.

[^{35}S[Methionine-incorporated IVTs were pre-cleared for 1 h with glutathione-agarose beads at 4 °C. Equivalent amounts of fusion protein as judged by Coomassie staining were incubated with equal counts of IVT proteins for 1 h at 4 °C in NETN buffer. Beads were then washed 5 times with NETN, boiled in loading dye, fractionated by 10% (37.5:1) acrylamide:bisacrylamide (National Diagnostics) SDS-PAGE, and subjected to autoradiography.

Western Blot—3T3 cells were transfected with 24 μg of expression plasmids coding for HA epitope-tagged versions of each construct (18), and protein extracts were made 24 h post-transfection. Extracts were subjected to Western blot analysis using standard techniques and probed with an anti-HA antibody (Babco).

RESULTS

Identification of Independent Transactivation Domains—To identify regions of the SPI-B protein that could function as independent transactivation domains, portions of the SPI-B cDNA were fused in frame to the C terminus of the GAL4 DNA binding domain and tested for their ability to transactivate five linked copies of the GAL4 DNA-binding site upstream of a minimal TK promoter and the human growth hormone cDNA (pGAL4GH) when co-transfected into NIH 3T3 cells (Fig. 2*A*). As demonstrated in Fig. 2*A*, the PEST region of SPI-B exhibited no transactivation potential (GAL4/PEST), but the N-terminal 108 amino acids (aa, GAL4-(1–108)) potently activated the expression of the pGAL4GH reporter. C-terminal deletions within this region revealed that even the very N-terminal 30 aa (GAL4-(1–31)) could function as an independent transcriptional activator. N-terminal truncations of this region showed that aa 31–108 could also function as a transcriptional activator (data not shown), but aa 63–108 (GAL4-(63–108)) were

unable to activate transcription. The small stretch of aa contained in the GAL4-(31–61) was able to transactivate the reporter, thereby localizing two independent transactivation domains of SPI-B to aa 1–31 and aa 31–61.

To delineate further the domain contained between aa 31–61 (which conferred transcriptional activity at the λB site, see below), a series of smaller fusion proteins were generated. As demonstrated in Fig. 2*A*, GAL4-(31–51) and GAL4-(41–61) still functioned as transcriptional activators, and in fact only 10 aa (GAL4-(41–51)) retained some transactivation potential even though the flanking aa (GAL4-(31–41) and GAL4-(52–62)) did not. However, due to the strong potential exhibited by GAL4-(41–61) over GAL4-(41–51), it appears that the full transcriptional activation domain resides between aa 41 and 61. To ensure that all constructs were stably expressed and capable of binding to the GAL4 DNA element, nuclear extracts were prepared from COS cells transfected with all constructs shown in Fig. 2*A* and assayed for their ability to bind DNA in an electrophoretic mobility shift assay (EMSA *lanes 2–14,* Fig. 2*B*).

Based upon the GAL4 fusion protein analyses, it appears that SPI-B contains two independent transcriptional activation domains. The N-terminal domain (aa 1–31) has a calculated pI of 3.8, making it an acidic domain, similar to a motif found in PU.1 (19) and other Ets proteins (49). However, aa 41–61 most resemble a proline-, serine-, and Threonine (PST)-rich domain since they comprise almost 40% of the amino acids in this region, although it also has some acidic characteristics. PST activation domains have also been identified in GATA factors (45) as well as the homeodomain protein Pax6 (50). Interestingly, computer based alignment of the PST activation domain of SPI-B yielded no similarity with PU.1 or any other Ets family member (data not shown).

Transcriptional Activity at the λ Enhancer—It has previously been demonstrated that PU.1 or SPI-B in conjunction with the lymphoid-specific co-activator Pip (13) effectively transactivates a DNA element from the λ_{2-4} enhancer (5′ AAAAGGAAGTGAAACC 3′), termed the λB site, which is required for maximal activity of the enhancer (23). Full-length PU.1, SPI-B, and N-terminal deletions of SPI-B were tested for their ability to transactivate a tetramer of the λB site upstream of a minimal TK promoter driving growth hormone expression (pB4TKGH). As shown in Fig. 3*A*, both PU.1 and SPI-B, in combination with Pip, efficiently transactivated (15–20-fold over the empty mammalian expression vector pCDNA3) pB4TKGH. Deletion of the entire N terminus of the protein, leaving only a PEST and Ets domain (Δ2–106), produced a construct that did not transactivate pB4TKGH. Deletion of the first 30 aa of SPI-B (Δ2–30) yielded a construct with almost wild type levels of transactivation, but subsequent deletion of the first 61 aa (Δ2–62) resulted in a construct with no transactivation potential (Fig. 3*A*). This implies that only aa 31–61 are required for transactivation at the λ enhancer.

To define further the λB transactivation domain, small deletions were generated in the N terminus of SPI-B. Deletion of aa 31–62 (Δ31–62) or 31–107 (Δ31–107) sharply reduced transactivation, whereas deletions adjacent to aa 31–62 (Δ64–107) only modestly affected transactivation potential. These data demonstrate that aa 31–62 of SPI-B function as the primary transcriptional activation domain at the λ enhancer. The residual activity observed in Δ31–62 and Δ31–107 is most likely due to the N-terminal acidic domain defined by the GAL4 analysis, although it cannot compensate for the loss of the PST domain contained in aa 31–62.

To analyze the importance of other SPI-B domains for transactivation of the λB site, further deletions were made within the PEST (ΔPEST) and Ets (ΔEts) regions. Deletion of the PEST region reduced the transactivation potential to levels detected if full-length SPI-B were transfected without Pip (data not shown). This loss of transactivation potential could be due to the inability of this construct to recruit Pip to the λ enhancer that requires an intact PEST sequence (14). However, we determined that ΔPEST was poorly expressed in 3T3 cells, making it difficult to interpret these results (Fig. 3*D*). As expected, deletion of the Ets domain, which blocks the ability to bind DNA, completely abolished transactivation. Finally, deletion of the very C terminus of SPI-B (Δ257–262), which is not conserved with PU.1, did not significantly alter transactivation from the full-length SPI-B.

To confirm the ability of these SPI-B plasmids to be expressed in mammalian cells, nuclear extracts were prepared from COS cells transfected with each of the above constructs and tested for their ability to bind to a single copy of the λB DNA site by EMSA. As demonstrated in Fig. 3*B* (*lanes 2–13*), all constructs, except for the Ets deletion mutant (Δ166–256, *lane 12*), produced a protein-DNA complex not observed in COS cells transfected with empty expression vector (pCDNA3, *lane 2*), and these constructs migrated more rapidly than the full-length SPI-B. All mutant proteins (except for ΔPEST and ΔEts) were found to interact equivalently with Pip using *in vitro* transcribed and translated (IVT) plasmids and EMSA (Fig. 3*C*). Finally, all constructs that failed to transactivate the λB reporter element were assayed for stable expression in 3T3 cells by HA epitope tagging (Fig. 3*D, lanes 2–10*). All proteins (with the exception of ΔPEST) were found to be expressed at least as well as the positive control PU.1 protein (Fig. 3*D*). Of note, HA Δ31–62, which defines the minimal domain required for transactivation of the λB reporter element, was expressed at levels identical to HA SPI-B (Fig. 3*D, lanes 3* and *6*). Thus, SPI-B seems to require not only its PEST and Ets domains for transactivation of the λ enhancer, but also a small number of amino acids (31–61) in its N terminus which corresponds to the PST activation domain.

Importance of Specific Amino Acids for Transactivation Potential of SPI-B—To identify important residues of SPI-B required for interactions with Pip, DNA, and other factors, a series of non-conservative mutations in SPI-B were created by site-directed mutagenesis. One important residue in the PEST domain of PU.1 is Ser-148, whose phosphorylation by casein kinase II is critical for proper recruitment of Pip and transactivation of the λ enhancer (13, 14, 17, 18). The analogous residue in SPI-B (Ser-144) was mutated to alanine to test whether phosphorylation of this residue is also critical for the SPI-B-Pip interaction. Much like the PU.1 S148A construct, the SPI-B S144A protein reduced the transactivation from the wild type protein by approximately 2-fold (Fig. 4*A*). However, this transactivation is still higher than when the entire PEST region of SPI-B is deleted (ΔPEST, Fig. 3*A*). This implies that there may be contacts other than Ser-144/Ser-148 made between SPI-B or PU.1 with Pip that are important for transactivation in this system.

Next, the importance of a specific amino acid in SPI-B for contact with DNA and proper transactivation of the λB element was tested by mutating Lys-242 to glycine. Based on the co-crystal data of the Ets domain of PU.1 with DNA (51), this mutation in the Ets domain of SPI-B should abolish DNA binding by preventing DNA bending toward the recognition helix. As demonstrated (Fig. 4*B*), the K242G mutation completely abolishes transactivation at the λ enhancer, implying that DNA binding is prevented (see below). Importantly, K242G is expressed at wild type levels (see Fig. 3*D*).

Further analysis of important phosphorylation events was performed by the creation of two separate point mutations

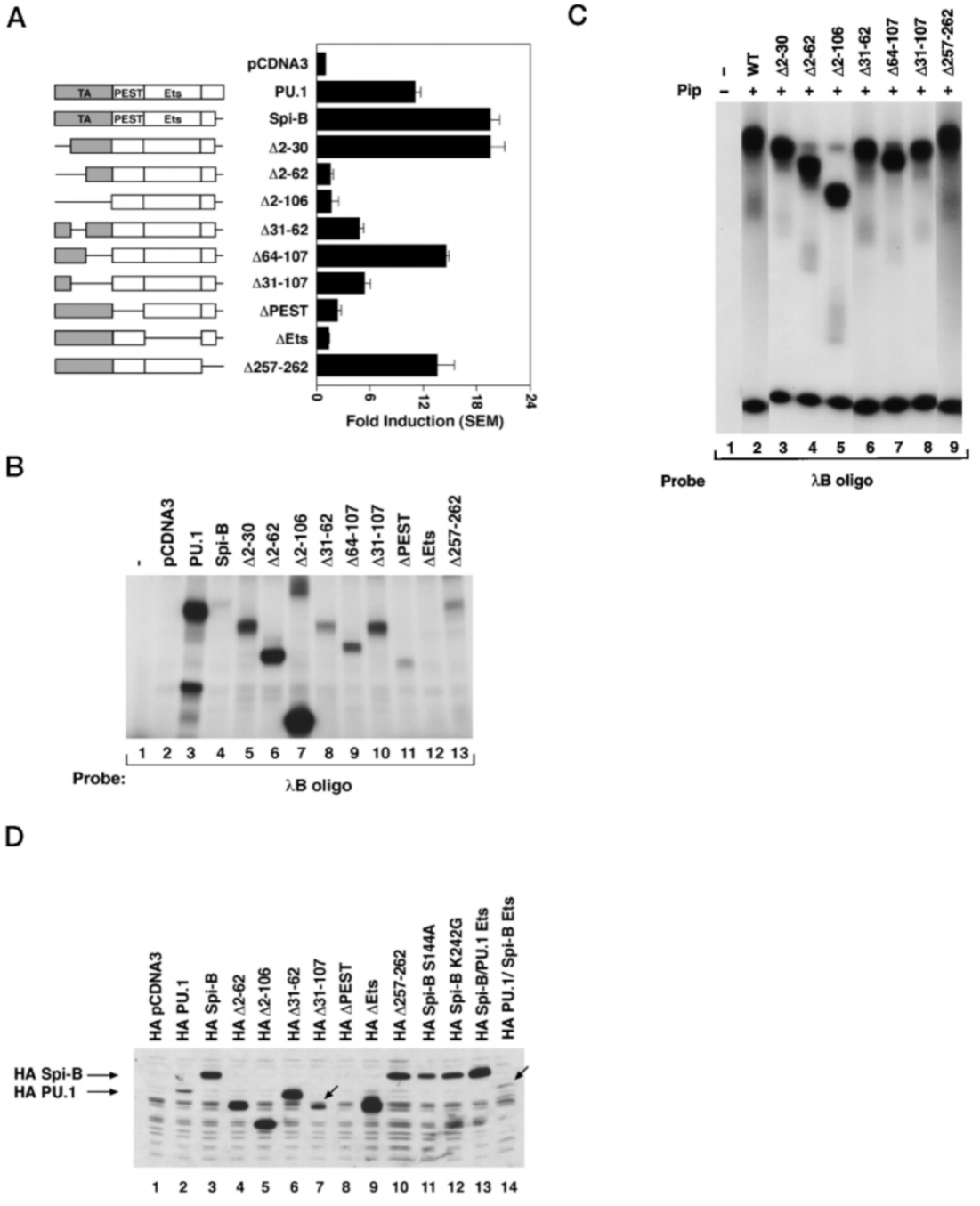

FIG. 3. *A*, 10 μg of expression plasmids (pCDNA3 based) encoding deletion mutant of SPI-B plus 10 μg of the Pip CMV expression plasmid were transiently co-transfected into NIH 3T3 cells with 2 μg of the B4TKGH reporter and pMSVβgal reference plasmid. 48 h after transfection cell media and cell extracts were prepared and assayed for growth hormone activity. The data are reported as fold induction (mean ± S.E. of four independent experiments) over the pCDNA3 alone. *B*, to confirm that all deletion mutants were still capable of binding DNA, 10 μg of nuclear extracts from COS cells transiently transfected with 20 μg of each expression plasmid was assayed for binding activity by EMSA using a double-stranded oligonucleotide probe containing the PU.1/Pip site from the λB site. *C*, to confirm that all deletion mutants were still capable of binding Pip, equimolar amounts of IVT proteins were used in an EMSA with an excess of Pip and a probe containing the PU.1/Pip site from the λB site as in *B*. *WT*, wild type. *D*, to assay directly steady-state protein levels, HA epitope-tagged versions of the indicated constructs were tested for expression in transiently transfected 3T3 cells by Western blot analysis, illustrating that all constructs (except ΔPEST) were expressed at least as well as PU.1.

within the PST domain that have been shown to affect SPI-B or PU.1 function. To test the importance of the interaction of SPI-B with Rb, Thr-56 was mutated to alanine to block its phosphorylation by ERK1, thereby allowing Rb to interact with SPI-B constitutively (24). This mutation had no effect on transactivation (Fig. 4*A*), implying that this amino acid does not play a role in the transcriptional activation of SPI-B at the λB site. Finally, mutation of two phosphorylation sites in the N terminus of PU.1 (Ser-41 and Ser-45) to alanine have been shown to inhibit macrophage proliferation *in vitro* (52). A similar phosphorylation site in SPI-B (S37), when mutated to alanine, caused no change in transactivation. All proteins were expressed in 3T3 cells, based on Western blot analysis (Fig. 3*D, lanes 2, 3, 11*, and *12*) and bound DNA (except K242G, *lane 7*) in EMSAs (Fig. 4*B, lanes 2–9*). Single mutations of all other possible phosphorylation sites to alanine between aa 31 and 61 (Ser-32, Ser-33, Tyr-34, Ser-43, Thr-48, and Tyr-58) of SPI-B showed only minor changes in transcriptional activity at the λB

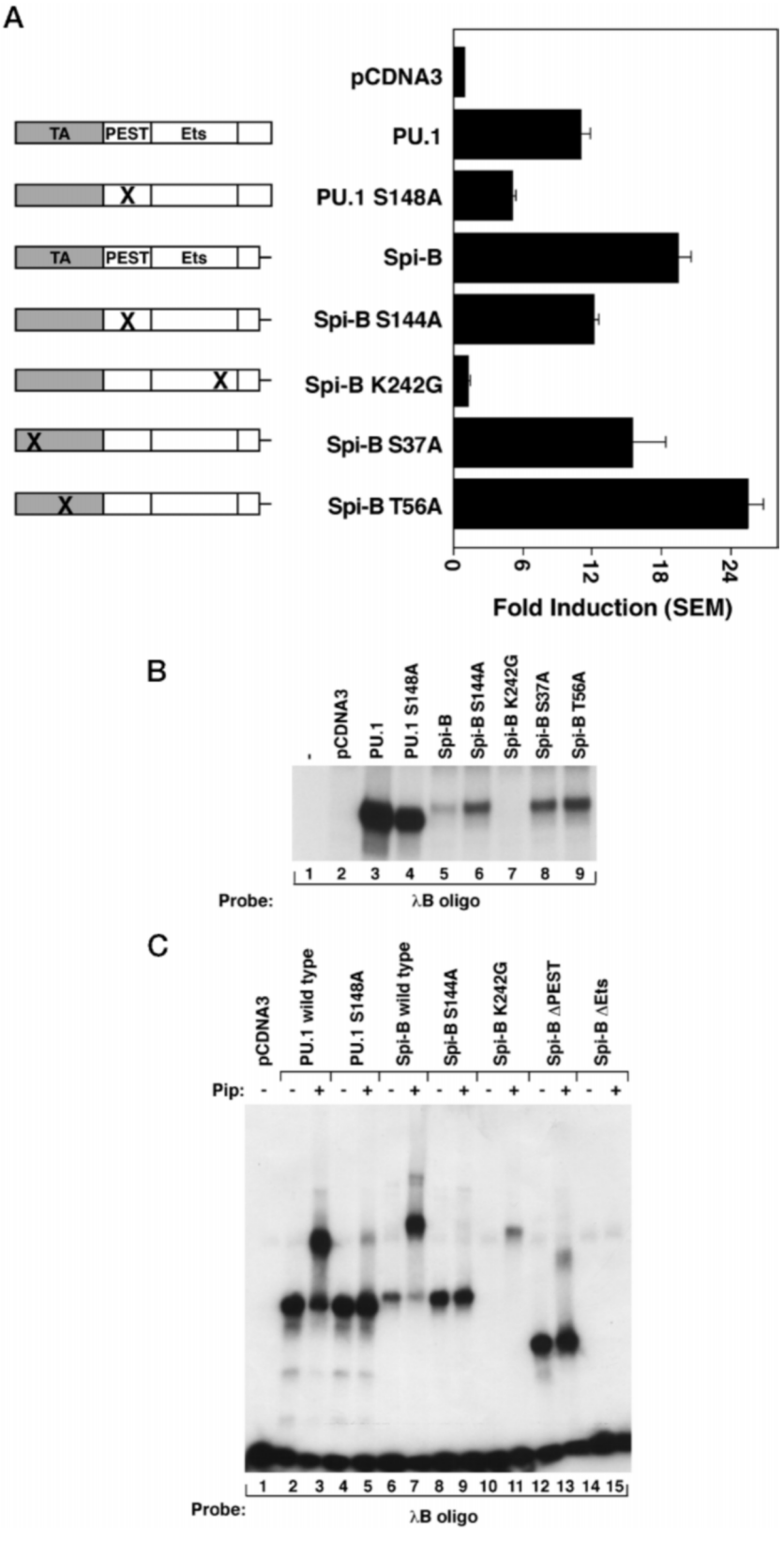

FIG. 4. *A*, point mutations in both PU.1 and SPI-B were generated by site-directed mutagenesis and tested for their ability to transactivate the λ enhancer element in co-transfection experiments performed as in Fig. 3*A*. Data represent fold induction (mean ± S.E. of four independent experiments) over the empty expression vector pCDNA3. *B*, EMSA was performed with nuclear extracts from transiently transfected COS cells with each construct to ensure DNA binding to the λB enhancer site by each construct. *C*, EMSAs were performed using equimolar amounts of IVT proteins of selected constructs from Figs. 3*A* and 4*A* with the λB site to examine SPI-B interactions with Pip.

element (data not shown).

To investigate further the interactions between SPI-B and Pip at the λB DNA element, IVT proteins were generated, and equimolar amounts were used in an EMSA assay at the λB site to test their ability to bind DNA and interact with Pip. Pip has previously been shown to bind very poorly to this site and requires recruitment via PU.1 (13, 14, 18). As shown in Fig. 4*C*, PU.1 efficiently binds to this DNA site (*lane 2*) and recruits Pip (*lane 3*) to form a ternary PU.1-Pip-DNA complex that migrates more slowly. A S148A mutation in PU.1 greatly reduces the recruitment of Pip to this site (*lanes 4* and *5*). Like PU.1, SPI-B is able to form a ternary complex with Pip (*lanes 6* and *7*) at this site, and the mutation S144A blocks the recruitment of Pip (*lanes 8* and *9*). Deletion of the entire PEST region of SPI-B

(ΔPEST) does not prevent DNA binding (*lane 12*) but does inhibit the recruitment of Pip to the site (*lane 13*).

Finally, two mutations that affect SPI-B DNA binding ability were tested for their ability to recruit Pip. First, the K242G mutation in SPI-B was found not to bind DNA by itself (*lane 10*), but a small amount of the ternary complex was formed when Pip was added (*lane 11*). This implies that a low affinity DNA-Pip interaction may be enough to allow the lower affinity K242G mutation to bind DNA. This type of mechanism has been observed with PU.1 and Pip at the CD20 promoter (16). However, a complete deletion of the Ets domain of SPI-B (ΔEts) prevents both DNA binding (*lane 14*) and formation of the ternary complex with Pip (*lane 15*).

Thus, while it appears that DNA binding and recruitment of Pip through a phosphorylated Ser-144 of SPI-B are important events for transactivation of the λB site, the mutation of any single Ser, Thr, or Tyr residue between aa 31 and 61 to alanine did not dramatically affect transactivation by SPI-B.

SPI-B Activity at the c-FES Promoter—To investigate the ability of SPI-B to transactivate a native promoter element that does not require Pip, a 450-base pair element derived from the c-*FES* promoter was tested. This promoter has been shown to require PU.1 (44) for maximal activity, as well as to be activated by SPI-B (21, 22). However, in our hands, SPI-B was unable to transactivate this promoter to the same levels as PU.1 (Fig. 5*A*). To test which domain(s) of SPI-B was responsible for this difference, a pair of chimeric molecules was generated where the PU.1 and SPI-B Ets domains were swapped. Thus, PU.1/SPI-B Ets is PU.1 with the SPI-B Ets domain, and SPI-B/PU.1 Ets is SPI-B with the PU.1 Ets domain. As demonstrated in Fig. 5*A*, the ability to transactivate this promoter segregated with the PU.1 Ets domain. This implies that either a difference in DNA binding or protein-protein interaction through the Ets domain of PU.1 are required for maximal activity of the c-*FES* promoter. Importantly, both the SPI-B/PU.1 Ets and PU.1/SPI-B Ets proteins efficiently transactivated the λB reporter plasmid (data not shown). These results are surprising because we assumed that the transcriptional activation domains of SPI-B, not its Ets domain, would prevent it from transactivating the c-*FES* promoter. To ensure that all constructs were capable of binding DNA, nuclear extracts from COS cells transfected with PU.1, SPI-B, and the two chimeric molecules were shown to contain proteins that bound to the λB site (Fig. 5*B*, *lanes 2–5*). Furthermore, each protein could also be detected by Western blot assay at levels comparable to the positive control PU.1 (Fig. 3*D, lanes 2, 3, 13*, and *14*).

To examine this further, equimolar amounts of IVT proteins were used in an EMSA (Fig. 5*C*) with oligonucleotides from both the λB site as well as the *PU.1* site from the c-*FES* promoter (−11 to +11, 5′ TCAGGAACTG 3′). All four proteins, PU.1, SPI-B, SPI-B/PU.1 Ets, and PU.1/SPI-B Ets, bound to the λB site efficiently (*lanes 2–5*) and interacted with Pip to form a ternary complex with DNA (*lanes 6–10*). However, only the PU.1 and SPI-B/PU.1 Ets constructs were able to efficiently bind to the c-*FES* site (*lanes 12–15*). By calculating binding constants for PU.1 and SPI-B at the λB and c-*FES* sites, PU.1 binds to the λB site 2-fold better than SPI-B but greater than 10-fold better to the c-*FES* site (data not shown). Together, these data indicate that although the SPI-B Ets domain seems to bind to the λB site, it binds at a much lower efficiency than PU.1 to the c-*FES* site. Therefore it appears that SPI-B has an overlapping but not identical DNA binding affinity to PU.1.

Protein-Protein Interactions of SPI-B and PU.1—PU.1 has been shown to interact with multiple proteins such as c-Jun (9), IRF family members (18), NF-IL6β (10), TBP (20), and other Ets proteins (8, 11, 12) to more potently activate transcription.

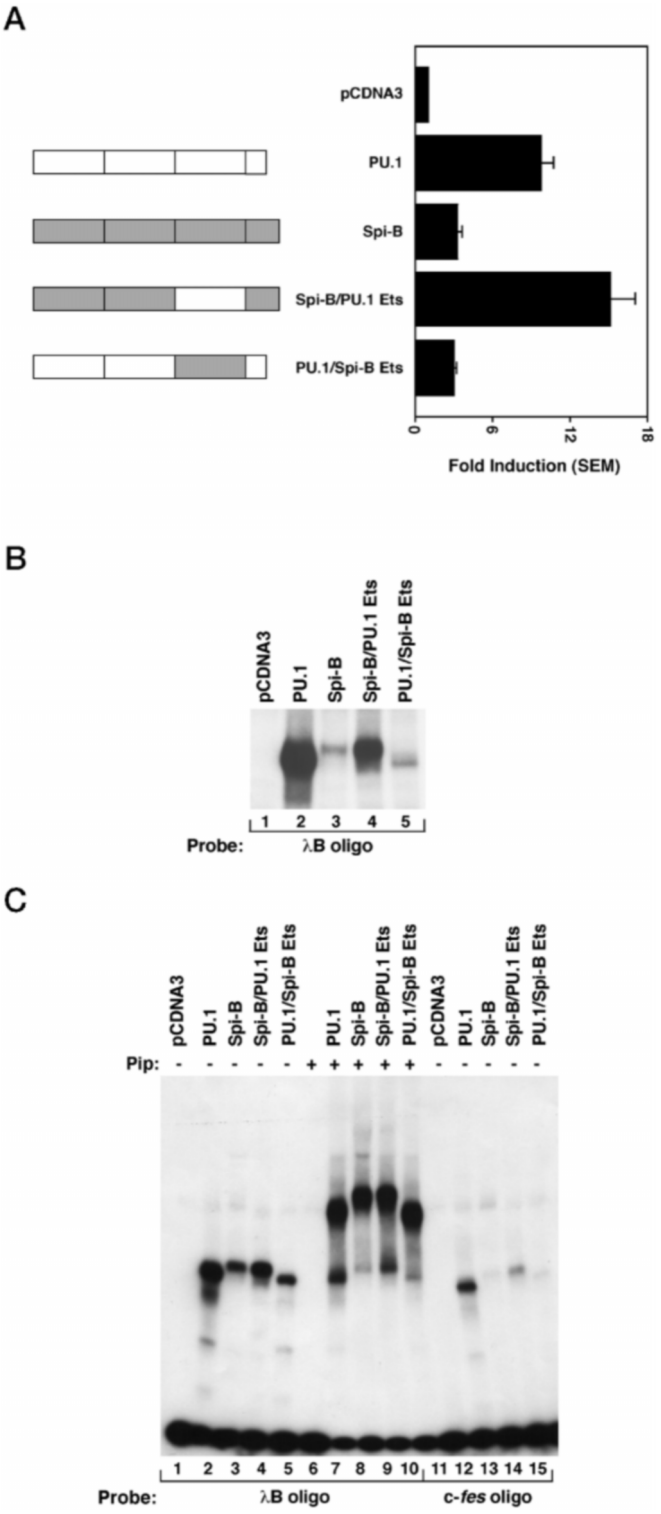

FIG. 5. *A*, 20 μg of expression plasmids for PU.1, SPI-B, and chimeric proteins where the Ets domains have been swapped were transiently co-transfected with 2 μg of the c-*FES* GH reporter and 2 μg of the pMSVβgal reference plasmid into NIH 3T3 cells. 48 h after transfection cell media were assayed for GH activity; data are represented as fold induction (mean ± S.E. of four independent experiments) over the empty mammalian expression vector pCDNA3. *B*, to ensure that all constructs could bind to DNA, EMSA was performed with 10 μg of nuclear extracts from transiently transfected COS cells and a radiolabeled double-stranded oligonucleotide from the λB site. *C*, EMSA was performed using equimolar amount of IVT proteins to show DNA binding activity to radiolabeled double-stranded oligonucleotides representing the λB site as well as the c-*FES* (−11 to +11) PU.1 sites for all constructs are shown in *A*.

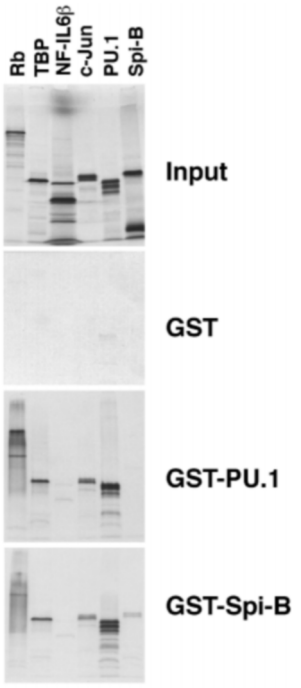

FIG. 6. **GST affinity chromatography was used to investigate the ability of [^{35}S]methionine IVT forms of Rb, TBP, NF-IL6β, c-Jun, PU.1, and SPI-B could interact with GST, GST-PU.1, or GST-SPI-B.** Approximately equal counts of each IVT was used in each binding reaction; 1/10 the IVT used in each binding reaction was resolved by SDS-PAGE for comparison. IVT proteins were incubated with equal amounts (as judged by Coomassie stain) of bacterially expressed GST, GST-PU.1, or GST-SPI-B immobilized on glutathione-agarose beads, washed vigorously, eluted from the beads by boiling in loading dye, fractionated by SDS-PAGE, and visualized by autoradiography.

GST affinity chromatography was utilized to explore which of the PU.1 interaction partners SPI-B could also bind. Full-length PU.1 and SPI-B cDNAs were fused to the C terminus of the GST protein, bound to glutathione-agarose beads, and tested for their ability to interact with [^{35}S[methionine-incorporated IVT forms of Rb, TBP, NF-IL6β, c-Jun, PU.1, and SPI-B. Although PU.1 and SPI-B appeared to react equivalently with TBP and c-Jun, SPI-B reproducibly did not bind NF-IL6β or Rb as well as PU.1 (Fig. 6 and data not shown). The most likely explanation for NF-IL6β is that while the Ets domain is required for interaction with C/EBP family members, sequences on the very C terminus of PU.1, which are not present in SPI-B, increase the affinity of this interaction. For Rb, it has been shown that the two strongly acidic domains of PU.1 are required for this interaction (20), whereas SPI-B has a single, weakly acidic domain. None of the IVT proteins bound significantly to GST alone; 1/10 the IVT used in each binding reaction is shown as input for comparison. A similar binding pattern was observed in the presence of 50 μg/ml of the DNA intercalating agent ethidium bromide (data not shown), proving that all observed interactions are DNA-independent.

Finally, SPI-B and PU.1 both seem to interact with themselves in a DNA-independent manner. However, an interaction between SPI-B and PU.1 was only detected with GST-SPI-B and IVT PU.1 but not GST-PU.1 and IVT SPI-B. One explanation for this observation is that GST-PU.1 may strongly dimerize with itself on the agarose beads and block the binding of IVT SPI-B. We were unable to reproducibly detect an interaction between either PU.1 or SPI-B with Ets-1 (data not shown). Based upon these results, SPI-B is able to interact with similar proteins as PU.1, although not with the same affinity in the case of NF-IL6β and Rb. However, we cannot rule out differences in interactions with other unknown cofactors that may contribute to the *in vivo* function of these two proteins.

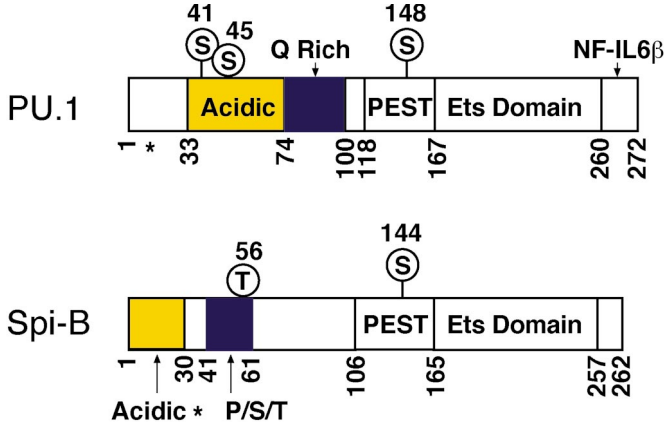

FIG. 7. **A revised domain structure of PU.1 and SPI-B showing the Ets, PEST, and transactivation domains of each.** *, this domain is only weakly acidic.

DISCUSSION

We reasoned, based on the phenotypes of the *PU.1*$^{-/-}$ and *Spi-B*$^{-/-}$ mice, that differences in DNA-binding sites, transactivation domains, or interactions with cofactors would exist between PU.1 and SPI-B. To define better these structural and functional differences, we have delineated functional domains of SPI-B that allow it to activate transcription, as well as to investigate its interactions with known cofactors of PU.1. Initial studies based upon GAL4 fusion proteins and activation studies at a GAL4 DNA site have allowed us to map two independent activation domains in the N terminus of SPI-B. The first domain, located between amino acids 1 and 31, displays potent activation potential in the GAL4 system and represents a weakly acidic motif similar to that of PU.1 and most other Ets proteins (49) (see Fig. 7). Interestingly, this region is dispensable for the ability of SPI-B to transactivate the λB site of the lambda enhancer. In direct contrast, the second activation domain of SPI-B located between amino acids 41 and 61 as defined by the GAL4 system is essential for transactivation of the λB site. A similar example of transactivation domain selectivity has been observed for PU.1 at the J chain promoter in which the glutamine-rich region of PU.1 is required for maximal activity, but the acidic domains are dispensable (7). The important difference is that the transactivation domain required for SPI-B activity is a PST domain, a motif not observed in PU.1 or any other Ets family member (49). Although it is not surprising that SPI-B and PU.1 have different activation domains given the lack of similarity (only 20%) in the N terminus, it is unexpected that the two proteins contain activation domains containing widely divergent amino acids. Thus, differences in which genes are activated by PU.1 and SPI-B may rest upon which cofactors are recruited by a glutamine-rich *versus* PST domain.

To investigate further the ability of SPI-B to transactivate the λB DNA element, we explored the interactions of SPI-B with Pip at this element. First, like PU.1, SPI-B must bind to the DNA element and subsequently recruit Pip to the element. This recruitment, based upon our EMSA data, requires a phosphorylated Ser-144. Interestingly, our transfection data imply that whereas the PU.1 or SPI-B interaction via Ser-148/Ser-144 is important for maximal activity, it is not absolutely required as has been previously reported by some (13, 17, 18). The most likely explanation for these data is that the conditions of our transient transfections promote high level expression of the cDNA constructs and reporter, allowing lower affin-

ity interactions between SPI-B or PU.1 and Pip to compensate for the loss of phosphorylated Ser-144/Ser-148 at the interaction site, as reported by Brass *et al.* (18). These interactions are difficult to detect by EMSA because very small amounts of each IVT protein (≈10 fmol) are used in each binding.

The inability of SPI-B to transactivate the myeloid c-*FES* promoter is perhaps most puzzling based upon the similarities in the Ets domain between PU.1 and SPI-B. Although it has been reported that PU.1 and SPI-B can transactivate this element (22), our data do not support this conclusion for SPI-B. One explanation is that Ray-Gallet *et al.* (22) observed lower transactivation (2-fold transactivation *versus* 12-fold from our data for PU.1) due to lower transfection efficiencies that may mask differences between constructs due to experimental error. Another explanation we cannot exclude is that the HeLa cells used by Ray-Gallet *et al.* (22) contained a cofactor required by the Ets domain of SPI-B for DNA binding and/or transcriptional activity at the c-*FES* promoter which is absent in our NIH 3T3 cells. Nonetheless, the evidence that SPI-B does not bind to the −11 to +11 PU.1 site in the c-*FES* promoter is surprising because multiple lines of evidence suggested that SPI-B had an identical DNA binding specificity to PU.1 *in vitro* (21, 23). However, based upon inspection of the 3′ flank for PU.1 and SPI-B consensus DNA-binding sites derived from site-selection experiments (22), the CTG contained in the 3′ flank of our c-*FES*-derived site (5′ TCAGGAACTG 3′) does not appear in any of the sequences recovered for SPI-B but is recovered for PU.1. This implies that differences in the 3′ flank of the GGAA core of the PU.1/SPI-B DNA-binding site may determine the relative affinities of the two transcription factors for DNA. Based upon these differences in the 3′-flanking sequences, our data suggest that subtle differences in the α1 helix of the Ets domain may be responsible for differences in DNA binding affinity. This helix positions a critical arginine residue (Arg-173 in PU.1 and Arg-170 in SPI-B) involved in neutralization of the phosphate backbone 3′ to the GGAA core which allows DNA bending toward the recognition helix (51, 53, 54). Further analysis is required to ensure that the observed differences in DNA binding affinity between PU.1 and SPI-B is truly due to a difference in specificity. Nonetheless, analysis of divergent amino acids within this region of the PU.1 and SPI-B Ets domains may provide biochemical explanations for differences of DNA binding specificities of not just the PU.1 subfamily but other Ets proteins as well.

One proposed function of PU.1 is to act not only as a transcriptional activator but as a "scaffolding protein" which binds DNA and then allows other transcription factors to bind through protein-protein interactions, thereby creating an activation complex that recruits the basal transcription machinery to a promoter (33, 34, 55–57) for potent transcription. In fact, recent reports have shown that some or all of the activation domains of PU.1 are dispensable for transactivation, as long as other factors such as c-Jun, Pip, bZIP proteins, or other Ets proteins are also present (7, 12, 31, 55). Since these combinatorial protein-protein interactions are important for proper tissue and temporal gene expression, we chose to examine the ability of SPI-B to interact with other proteins known to interact with PU.1. By using GST affinity chromatography, we have shown that SPI-B can interact with similar proteins to PU.1, although perhaps with lower affinity in the case of NF-IL6β and Rb. In addition, the ability of SPI-B to interact with TBP most likely allows it to transactivate the TATA-less c-*FES* promoter as long as it can bind to the promoter by using a PU.1 Ets domain. Thus, as with PU.1, SPI-B may transactivate TATA-less promoters in B lymphocytes by recruiting TBP and interact with other transcription factors to form an activation complex similar to PU.1.

Important differences in the transcriptional activation domains and DNA binding activity of SPI-B and PU.1 suggest that they have different target genes *in vivo* and therefore provide a framework to understand the non-redundancy presented by the *PU.1*$^{-/-}$ and *Spi-B*$^{-/-}$ animals. However, we cannot formally exclude the possibility that the genetic evidence of non-redundancy is due in part to post-translational modifications such as phosphorylation or interactions with currently unknown cofactors that might the modulate the activity of PU.1 and SPI-B *in vivo*. Future experiments to understand the role of SPI-B as well as the different activation domains of PU.1 will provide important insights into not only the biology of these two proteins and how they are regulated but also how the interactions between different transactivation motifs can interact with the basal transcription machinery to cause gene expression.

Acknowledgments—We thank A. Brass and H. Singh for multiple vectors and thoughtful advice; M. Atchison for the NF-IL6β cDNA; and E. Morrisey and M. Parmacek for the pGAL4 and pGAL4GH vectors. We thank L. Gottschalk, G. Schneider, and D. Wiler for expert preparation of the illustrations; Cheryl Small for secretarial assistance; and Donna Fackenthal for automated sequencing analysis. We also thank E. Morrisey, H. Ip, and T. McKeithan for critical review of the manuscript.

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