PU.1 Activates Transcription of SHP-1 Gene in Hematopoietic Cells*

Pawel Wlodarski§, Qian Zhang†, Xiaobin Liu†, Monika Kasprzycka§, Michal Marzec‡, and Mariusz A. Wasik‡¶

From the §Department of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104 and the †Departments of Histology and Embryology, and ¶Clinical Immunology, Warsaw Medical University, 02004 Warsaw, Poland

Protein-tyrosine phosphatase SHP-1 is the key negative regulator of numerous signaling pathways. SHP-1 is expressed in the hematopoietic and epithelial cells as two structurally similar mRNA transcripts controlled by two different promoters designated P2 and P1, respectively. Whereas the transcriptional regulation of the SHP-1 gene P1 promoter has been partially elucidated, the structure and functional control of the P2 promoter remain unknown despite the critical role played by SHP-1 in the normal and malignant lymphoid and other hematopoietic cells. Using luciferase reporter assays with the set of constructs that contained a gradually truncated intron 1 of the SHP-1 gene, we identified the minimal (<120 bp) fragment that is able to fully activate expression of the reporter gene. Furthermore, we found that PU.1 (a member of the Ets transcription factor family that plays a crucial role in differentiation and function of the lymphoid and myeloid cells) binds to the identified P2 promoter both in vitro and in vivo. PU.1 also activates the promoter in the sequence specific manner and is critical for its expression as evidenced by the profound suppression of the SHP-1 gene transcription upon the siRNA-mediated depletion of PU.1. These findings provide an insight into the structure of the hematopoietic cell-specific P2 promoter of the SHP-1 gene and identify PU.1 as the transcriptional activator of the P2 promoter.

SHP-1 tyrosine phosphatase (also known as SHP1-PTP, SHPTP1, SHP-1C, PTP1C, Hep, HepH, HPTP1C, and HCP) is encoded by the PTEN6 gene and expressed primarily in the hematopoietic and epithelial cells (1, 2). SHP-1 plays a particularly important role in the maturation and functional differentiation of lymphoid and myeloid cells as evidenced by the aberrant immunoproliferation and impaired hematopoiesis in the “motheaten” mice that display defects in the SHP-1 gene expression (3, 4). SHP-1 acts in the immune and other hematopoietic cells by inhibiting signaling through receptors for cytokines, growth factors, and chemokines as well as receptors directly involved in the immune responses and programmed cell death (5). SHP-1 down-regulates cell activation by binding and de-phosphorylating the receptors, receptor associated tyrosine kinases, and the down-stream signaling molecules such as Vav1 (6) and src kinase substrates (7). SHP-1 acts as tumor suppressor and loss of its expression has been identified in the whole spectrum of lymphoid and myeloid cell malignancies (8–11). The SHP-1 gene, located on chromosome 12p13, is comprised of 17 exons and activated from two different promoters (5). Whereas the distal promoter, P1, located upstream of the very short exon 1 (sometimes designated also exon 1a) is active in epithelial cells, the proximal promoter P2 that initiates gene transcription from exon 2 (alternatively known as exon 1b), is utilized by the hematopoietic cells. Whereas function of the P1 promoter has been partially elucidated (12), the structure and regulatory mechanisms of the P2 promoter remain essentially unknown.

Using a series of reporter assays, we identified the minimal, <120-bp-long, P2 promoter located immediately upstream of the exon 2 that is capable of driving full expression of the reporter luciferase gene. By using the combination of “in silico” analysis, electromobility shift assay (EMSA),2 chromatin immunoprecipitation (ChIP), and siRNA-based depletion experiments, we identified PU.1, the member of the Ets factor family important for lymphoid and myeloid cell maturation, as the transcriptional activator of the P2 promoter. This new insight into the structure and regulation of the P2 promoter expression may contribute to a better understanding of the role played by PU.1 and SHP-1 in normal and malignant hematopoietic cells.

**EXPERIMENTAL PROCEDURES**

Cells—Immature T-cell, acute leukemia/lymphoblastic lymphoma-derived Jurkat, mature T-cell, anaplastic lymphoma kinase (ALK)-expressing SUP-M2 and Karpas 299, mature B-cell, Burkitt’s lymphoma-derived BJAB and diffuse large B-cell lymphoma-derived LY18 and VAL, and the breast carcinoma-derived MDA-453 cell lines were cultured in the RPMI medium supplemented with 2 mM l-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin (all from Invitrogen) and 10% fetal bovine serum (Cellgro/Mediatech Inc., Herndon, VA). The cells were maintained in 5% CO2 atmosphere at 37 °C. At the time of transfection the cells were cultured in the serum-free Opti-MEM medium (Invitrogen).

**Reporter Constructs**—Fragments of intron 1 and exon 2 were amplified by PCR using specific primers and the Expand High

---

*This work was supported in part by the NCI/National Institutes of Health Grants R01-CA89194 and R01-CA96856. 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.

†To whom correspondence should be addressed: Dept. of Pathology and Laboratory Medicine, University of Pennsylvania Medical Center, 3400 Spruce St., 7106 Founders Pavilion, Philadelphia, PA 19104-4283. Tel.: 215-662-3467; Fax: 215-662-7594; E-mail: wasik@mail.med.upenn.edu.

2 The abbreviations used are: EMSA, electromobility shift assay; ChIP, chromatin immunoprecipitation; ALK, anaplastic lymphoma kinase; PMSF, phenylmethylsulfonyl fluoride; pcv, volume of pelleted cells; pnv, volume of pelleted nucleic; siRNA, small interfering RNA; RT, reverse transcription.
Site-directed Mutagenesis—To analyze the functional role of the identified transcription factor binding site in the SHP-1 gene P2 promoter, the selected PU.1b site was mutated by QuikChange® II site-directed mutagenesis kits (Stratagene, La Jolla, CA) according to the product manual. pGL3Enh -266 +97 construct was used as a template. Plasmids obtained by site directed mutagenesis were purified with a QiAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA) and sequenced to confirm the presence of the mutation. Primers used for the mutagenesis reactions were as follows: 5′-GTG CTC TAA AAC ACA CTA CAA GTG AGT TCC CCC (forward) and 5′-GGG GGA ACT CAC TGT TAG TGT GTT TTA GAG CAC (reverse; with the mutated sequence being underlined).

Reporter Assays—Jurkat cells were transiently co-transfected with fragments of SHP-1 gene P2 promoter cloned into pGL3Enh vector and pRL-TK vector (both vectors were from Promega, Madison, WI) using 10:1 molar ratios of these DNA, respectively. All transfections were performed in triplicates. After 24 h, the cells were lysed in Passive Lysis Buffer (Promega). Activity of the firefly and Renilla luciferases was evaluated in 20 μl of the cell lysate using the reagents included in the Dual-luciferase Reporter Assay System (Promega) and the Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The results are presented as the activity ratio of firefly luciferase activity induced by the pGL3-SHP-1-promoter to the activity of the Renilla luciferase induced by the constitutively active pRL-TK vector.

Nuclear Protein Extraction—Cells were collected by centrifugation and washed with phosphate-buffered saline. Volume of the pelleted cells (pcv) was measured using the graduation on the tube. The cells were next, briefly washed in a hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol) with the volume of the buffer being equal to 5 pcv and centrifuged. The pellets were suspended in a 3-fold pcv volume of hypotonic buffer, incubated for 10 min on ice, transferred into a precooled Dounce homogenizer, and disrupted with a type B pestle. Nuclei were pelleted by centrifugation (15 min, 3300 × g), and their volume (pcv) was determined. Nuclei were then suspended in a 0.5 pcv of the low salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 0.02 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 dithiothreitol). Next, 0.5 pcv of the high salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 dithiothreitol) was added for a 30-min incubation with continuous, gentle mixing. The extracts were cleared by centrifugation (30 min, 25,000 × g).

EMSA—The nuclear extracts were prepared as described above. Twenty fmol of biotin-labeled probe was incubated with 6.0 μg of the nuclear extract for 20 min at room temperature. To confirm the specificity of binding, the extract-probe mixtures were either further admixed with a 200-fold excess of the unlabeled, consensus nucleotide or 2 μg of the PU.1-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The formed protein-DNA complexes were resolved from the free probe in the 7% non-denaturing polyacrylamide gel by electrophoresis, transferred onto positively charged Nylon membrane (Amersham Biosciences) and UV-cross-linked (Stratalinker, Stratagene). The membrane was then subjected to a Lightshift chemiluminescent EMSA kit (Pierce) following the manufacturer’s instructions. The nucleotides were custom synthesized by IDT Inc. (Corvalle, IA). Probes used in the electrophoretic mobility shift assay were double-stranded biotin-labeled nucleotides. The sequences of the sense strands, 5′-biotinated, were

\[
luciferase induced by the constitutively active pRL-TK vector.\]

\[
\text{SHP-1 mRNA splice variants. mRNA harvested from an immature lymphoid T-cell line Jurkat and mature B-cell line BJAB as well as the control breast carcinoma cell line MDA-453 was examined by RT-PCR using the depicted PCR primers to identify the I and II SHP-1 mRNA transcripts.} \]

\[
\text{Fidelity PCR System (Roche Applied Bioscience). The forward primers recognized sites located -6266 bp, -5266 bp, -4266 bp, -3266 bp, -2266 bp, -1266 bp, -266 bp, and -66 bp upstream from the transcription initiation site, whereas the reverse primer was common and included the nucleotide 97 (+97) downstream form the site. Later, additional fragments starting at -1066 bp, -876 bp, -766 bp, -266 bp, and -166 bp were generated in the same manner. All forward primers contained KpnI restriction site in their 5′-end. The HindIII restriction site was included in the reverse primer. PCR products were first cloned into the pCR4.0 vector (Invitrogen), then subcloned with KpnI and HindIII into respective sites of pGL3Enh plasmid. The resulting constructs were named according to the nucleotide location of the 5′-end of the forward primer used in the PCR reaction.} \]

\[
\text{Site-directed Mutagenesis—To analyze the functional role of the identified transcription factor binding site in the SHP-1 gene P2 promoter, the selected PU.1b site was mutated by QuikChange® II site-directed mutagenesis kits (Stratagene, La Jolla, CA) according to the product manual. pGL3Enh -266 +97 construct was used as a template. Plasmids obtained by site directed mutagenesis were purified with a QiAprep Spin Miniprep kit (Qiagen Inc., Valencia, CA) and sequenced to confirm the presence of the mutation. Primers used for the mutagenesis reactions were as follows: 5′-GTG CTC TAA AAC ACA CTA CAA GTG AGT TCC CCC (forward) and 5′-GGG GGA ACT CAC TGT TAG TGT GTT TTA GAG CAC (reverse; with the mutated sequence being underlined).} \]

\[
\text{Reporter Assays—Jurkat cells were transiently co-transfected with fragments of SHP-1 gene P2 promoter cloned into pGL3Enh vector and pRL-TK vector (both vectors were from Promega, Madison, WI) using 10:1 molar ratios of these DNA, respectively. All transfections were performed in triplicates. After 24 h, the cells were lysed in Passive Lysis Buffer (Promega). Activity of the firefly and Renilla luciferases was evaluated in 20 μl of the cell lysate using the reagents included in the Dual-luciferase Reporter Assay System (Promega) and the Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). The results are presented as the activity ratio of firefly luciferase activity induced by the pGL3-SHP-1-promoter to the activity of the Renilla luciferase induced by the constitutively active pRL-TK vector.} \]

\[
\text{Nuclear Protein Extraction—Cells were collected by centrifugation and washed with phosphate-buffered saline. Volume of the pelleted cells (pcv) was measured using the graduation on the tube. The cells were next, briefly washed in a hypotonic buffer (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.2 mM PMSF, 0.5 mM dithiothreitol) with the volume of the buffer being equal to 5 pcv and centrifuged. The pellets were suspended in a 3-fold pcv volume of hypotonic buffer, incubated for 10 min on ice, transferred into a precooled Dounce homogenizer, and disrupted with a type B pestle. Nuclei were pelleted by centrifugation (15 min, 3300 × g), and their volume (pcv) was determined. Nuclei were then suspended in a 0.5 pcv of the low salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 0.02 mM KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 dithiothreitol). Next, 0.5 pcv of the high salt buffer (20 mM HEPES, 25% glycerol, 1.5 mM MgCl₂, 1.2 M KCl, 0.2 mM EDTA, 0.2 mM PMSF, 0.5 dithiothreitol) was added for a 30-min incubation with continuous, gentle mixing. The extracts were cleared by centrifugation (30 min, 25,000 × g).} \]

\[
\text{EMSA—The nuclear extracts were prepared as described above. Twenty fmol of biotin-labeled probe was incubated with 6.0 μg of the nuclear extract for 20 min at room temperature. To confirm the specificity of binding, the extract-probe mixtures were either further admixed with a 200-fold excess of the unlabeled, consensus nucleotide or 2 μg of the PU.1-specific antibody (Santa Cruz Biotechnology, Santa Cruz, CA). The formed protein-DNA complexes were resolved from the free probe in the 7% non-denaturing polyacrylamide gel by electrophoresis, transferred onto positively charged Nylon membrane (Amersham Biosciences) and UV-cross-linked (Stratalinker, Stratagene). The membrane was then subjected to a Lightshift chemiluminescent EMSA kit (Pierce) following the manufacturer’s instructions. The nucleotides were custom synthesized by IDT Inc. (Corvalle, IA). Probes used in the electrophoretic mobility shift assay were double-stranded biotin-labeled nucleotides. The sequences of the sense strands, 5′-biotinated, were} \]
PU.1 Activates P2 Promoter of the SHP-1 Gene

as follows: 5′-GCT GTG CTT CTC TCC CCC (PU.1a/Ets2) and 5′-TAA AAC GAG AAG TAC AAG (PU.1b). The reverse comple-
mentary and unlabeled nucleotides were coupled to form double-stranded probes. Competitor unlabeled, double-
stranded oligonucleotides (PU.1a and PU.1b cold probes) were

ChIP Assay—To cross-link DNA and DNA-binding proteins, 1 × 10⁶ of the Jurkat and BJAB cells were fixed with 1% formal-
dehyde at room temperature for 15 min and then at 4 °C for 45 min. The fixed cells were lysed with the buffer containing pro-
aldehyde at room temperature for 15 min and then at 4 °C for 45

FIGURE 2. Structural delineation of the SHP-1 gene P2 promoter. Progressi-

sively truncated fragments of intron 1 of the SHP-1 gene were inserted into

the luciferase gene-containing pGL3Enh vector and examined for their ability to activate transcription of the reporter gene in the lymphoid Jurkat cells. As

depicted in the figure, the fragments varied in length with the longest cover-

ning essentially the entire intron 1 (−6266 +97) and the shortest (−66 +97) containing the RNA polymerase binding complex region located immedi-

dately upstream of the transcription initiation site. The construct designated as

“promoterless” represents the pGL3Enh vector containing only the luciferase
gene but no promoter. The results reflect the calculated ratios of the luciferase
activity as compared with activity of the co-transfected renilla gene-conta-
novector. The depicted data (mean value ± S.E.) are representative of
three totally separate experiments performed in triplicates. A, rough charac-
terization of the P2 promoter position using the large, gradually truncated DNA fragments of the intron 1. B, refined analysis of the P2 location and size
using the smaller, progressively truncated fragments with the largest one

FIGURE 3. Western Blot analyses. A, Western Blot analysis of the SHP-1 pro-
moter activity (see Western Blot for details). B, Western Blot for structural delineation of the SHP-1 gene P2 promoter (see Western Blot for details).
TBST (20 mM Tris, pH 7.5, 0.15 M NaCl, 0.1% Tween 20), the blots were probed with the primary and secondary (horseradish peroxidase-conjugated) antibodies. The primary antibodies used were against PU.1, SHP-1, β-actin, and phosphorylated Y41 (all from Santa Cruz Biotechnology). Membranes were washed in TBST buffer, and proteins of interest were detected by ECL Plus chemiluminescence substrate (Amersham Biosciences). In some experiments, intensity of the signal was measured using scanner HP ScanJet3500c and ImageJ 1.32j software developed at the National Institute of Health, Bethesda, MD.

RESULTS

Identification of the P2 Promoter of the SHP-1 Gene—As shown in Fig. 1A, exons 1 and 2 of the SHP-1 gene are separated by an intron that contains almost 6300 bp, which is believed to contain the hematopoietic cell-specific promoter P2. We have confirmed the presence of the P2 promoter in the intron 1 by demonstrating that, in contrast to the epithelial MDA-453 cells, the immature Jurkat T lymphocytes and mature BJAB B lymphocytes express SHP-1 mRNA encoded in part by exon 2 but not exon 1 (Fig. 1B). To determine the exact localization and size of P2, we developed a gene expression reporter system comprised of the promoterless vector pGL3Enh containing fragments of the intron 1 cloned in upstream of the luciferase gene. The constructed twelve fragments varied in length from over 6300 bp that cover essentially the entire intron 1 to ~160 bp (Fig. 2A). They all contained the transcription initiation site (+1) and the adjacent, 97-bp-long fragment of the coding DNA. The pGL3Enh vector that contained only the luciferase gene but no intronic DNA served as a negative control. As shown in Fig. 2A, most of the progressively truncated intron 1 fragments analyzed displayed a very similar, strong transcriptional activity. The only exceptions that generated much weaker signals in a highly reproducible manner were the second longest (5266 bp to 97 bp) and the shortest (66 bp to 97 bp) fragments. Whereas the result with the longer fragment suggests the existence of a long-range transcriptional repressor, the result with the shortest fragment, in particular when compared with the intact, full transcriptional activity of the second shortest fragment (~5266 + 97 bp) and the shortest (~66 + 97 bp) fragments. Whereas the result with the longer fragment suggests the existence of a long-range transcriptional repressor, the result with the shortest fragment, in particular when compared with the intact, full transcriptional activity of the second shortest fragment (~166 + 97 bp), indicated that the P2 promoter is rather small and located nearby the transcription initiation site. To determine the more exact size of the promoter, we prepared the second set of the intron 1 fragments starting at 141, ~116, and ~91. The additional fragments starting at ~1066 and ~166 served as positive controls, and the fragment starting at ~66 and the intronless vector served again as negative controls. No fragment that was shorter than the one starting at ~66 was prepared based on the premise that the region immediately upstream of the transcription initiation site contains the core promoter that binds the RNA polymerase complex (please see also below). As shown in

FIGURE 3. Identification of PU.1 binding site in the SHP-1 gene P2 promoter. A, analysis of the sequence of the SHP-1 P2 promoter for the transcription binding sites. Transcription element search software program revealed the depicted, putative binding sites for PU.1 (two sites designated PU.1a and PU.1b) and an Ets-2 site adjacent to the PU.1a site. Binding sites for the members of the RNA polymerase-containing transcription initiation complex TFII B and TFID were also identified. B, expression of PU.1 protein in various human lymphoid cell lines: ALK + TCL, T-cell lymphoma expressing ALK; DLBCL, diffuse large B-cell lymphoma; T-ALL, T-cell acute leukemia/lymphoblastic lymphoma; BL, Burkitt (B-cell) lymphoma. C, EMSA using Jurkat cell nuclear extract and biotin-labeled probes containing the PU.1a (left panel) and PU.1b (right panel) binding sites. The same but unlabeled (cold) probes were used as the additional binding specificity controls (the middle lanes in both panels). In the supershift EMSA (right panel) the anti-PU.1 antibody was added to the complexes containing the nuclear extract, and the labeled appropriate binding site-specific probe.
PU.1 Activates P2 Promoter of the SHP-1 Gene

Fig. 2B, transcriptional activity of the −141 +97 and −116 +97 regions was not significantly diminished when compared with the positive controls. In contrast, the activity of −91 +97 fragment was decreased but still much stronger than that of the −66 +97 core promoter indicating that important regulatory element(s) is (are) contained within the short −116 to −66 region.

Mapping of the Putative Transcription Activator Binding Sites within the SHP-1 Gene P2 Promoter—We next addressed the question of which transcription factor(s) may be involved in activation of the P2 promoter. To accomplish this aim, we performed an “in silico” structural analysis of the identified P2 promoter for potential transcription factor binding sites. Using the transcription element search software, we identified within the key −116 to −66 region three sequences specific for two members of the Ets transcription factor family with two sites potentially binding PU.1 (13) and one Ets-2 (14). It is noteworthy that in contrast to the well established critical role of PU.1 in normal and malignant hematopoiesis (15, 16), the potential involvement of Ets-2 in the process is less well documented (17, 18) and seemingly controversial (19). As shown in Fig. 3A, the potential PU.1 binding sites, designated PU.1a and PU.1b, are located at positions −103 −98 and −75 −70, respectively. The hypothetical Ets-2 site is located at −97 −93, directly adjacent to the PU.1a site. The most proximal region of the P2 promoter contained the BRE-TFIIB and INR-TFIID binding sites consistent with its role as the core promoter responsible for docking the RNA polymerase complex involved in the initiation of gene transcription.

Expression and Binding of PU.1 to the P2 Promoter—To confirm the expression of PU.1 by the Jurkat cells, we performed Western blot analysis. As shown in Fig. 3B, these immature T-cell line expressed substantial amount of the PU.1 protein comparable with the one seen in the mature B-cell line BJAB and, to a lesser degree, two other mature B-cell lines examined. In contrast, two mature T-cell lines did not express the protein. To determine whether PU.1 binds to the identified PU.1a and PU.1b sites within the P2 promoter, nuclear protein extracts from the lymphoid Jurkat cells were evaluated in EMSA for PU.1b sites within the P2 promoter, nuclear protein extracts obtained from Jurkat and another lymphoid cell line BJAB were examined in the ChIP assay using antibodies reactive with PU.1 and, as a negative control, ALK. The sonicated DNA fragments (depicted in the left panels) present in the immunoprecipitates were amplified in PCR using primer pairs corresponding to either the P2 promoter (−166 −59; Fig. 3A) or, as an additional control, exon 16 (Ex. 16) of the SHP-1 gene. Results of the PCR amplification of the control whole cell lysates (input) and the immunoprecipitates are shown in the right panels. B, PU.1-dependent activation of the P2 promoter. The effect of the base-substitution mutagenesis of the identified PU.1 binding sites PU.1a (CTTCTC to CACACA) and PU.1b (GAGAAG to ACACTA) within the −266 +97 P2 promoter region was evaluated in the luciferase reporter assay. The depicted results (mean ± S.E.) represent the luciferase/Renilla activity ratio and are representative of three independent experiments performed in triplicates.

FIGURE 4, PU.1 binds to and activates the P2 promoter in vivo. A, binding of PU.1 to the P2 promoter. Cell lysates prepared from Jurkat cells and another lymphoid cell line BJAB were examined in the ChIP assay using antibodies reactive with PU.1 and, as a negative control, ALK. The sonicated DNA fragments (depicted in the left panels) present in the immunoprecipitates were amplified in PCR using primer pairs corresponding to either the P2 promoter (−166 −59; Fig. 3A) or, as an additional control, exon 16 (Ex. 16) of the SHP-1 gene. Results of the PCR amplification of the control whole cell lysates (input) and the immunoprecipitates are shown in the right panels. B, PU.1-dependent activation of the P2 promoter. The effect of the base-substitution mutagenesis of the identified PU.1 binding sites PU.1a (CTTCTC to CACACA) and PU.1b (GAGAAG to ACACTA) within the −266 +97 P2 promoter region was evaluated in the luciferase reporter assay. The depicted results (mean ± S.E.) represent the luciferase/Renilla activity ratio and are representative of three independent experiments performed in triplicates.

To provide evidence that the identified PU.1 binding sites are transcriptionally active, we performed a luciferase reporter assay with the P2 promoter constructs that contained mutation of the PU.1a or PU.1b binding site. To preserve the overall structure and spatial relations of the site, we performed substitutions rather than deletions of the target binding site nucleotides leaving intact the remaining sequence of the examined −266 +97 fragment. As shown in Fig. 4B, the targeted six-base
PU.1 Activates P2 Promoter of the SHP-1 Gene

FIGURE 5. PU.1-dependent activation of the SHP-1 gene. A, Jurkat and BJAB cells were transfected with the PU.1-specific and control siRNA and examined for the expression of PU.1 protein and SHP-1 mRNA by Western blot and RT-PCR, respectively, with β-actin expression serving as positive control in both assays. B, PU.1-specific and control siRNA-treated BJAB cells were analyzed for changes in the protein-tyrosine phosphorylation using the phospho-specific 4G10 antibody with expression of PU.1 and SHP-1 proteins serving as controls. C, the changes in the protein expression and phosphorylation in the PU.1 siRNA-treated cells as compared with the control siRNA-treated cells was quantified by densitometry. Cont., control.

substitution of the PU.1a binding site markedly diminished transcriptional activity of the entire fragment. The effect of substitution of the PU.1b site was even more profound and decreased the transcriptional activity almost to the baseline level of the RNA polymerase-binding core promoter. The combination of both PU.1a and PU.1b binding site substitutions had no additional inhibitory effect. These findings indicate that PU.1 is the transcriptional activator of the P2 promoter.

PU.1-dependent Transcription of the SHP-1 Gene—To demonstrate that PU.1 binding to the P2 promoter and activation of the promoter as documented in the luciferase gene reporter assay also impacts on the expression of the endogenous, native SHP-1 gene, we inhibited expression of PU.1 using the siRNA technology. Treatment of both Jurkat and BJAB cells with the PU.1-specific but not the control, non-targeting siRNA markedly diminished expression of PU.1 in these cells. The inhibition of PU.1 expression resulted in the profound suppression of transcriptional activity of the SHP-1 gene as determined by marked decrease in the amount of the SHP-1 mRNA (Fig. 5A) and protein (Fig. 5B). These observations provided the final evidence for the key role of PU.1 in activation of the SHP-1 gene. Of note, the PU.1 depletion and the related down-regulation of SHP-1 expression were associated with the substantial increase in tyrosine phosphorylation of several intracellular proteins (Fig. 5B) in agreement with the well defined role of SHP-1 as the tyrosine phosphatase of a number of key proteins in the in the hematopoietic cells.

DISCUSSION

The important role of SHP-1 phosphatase in cell physiology and malignant transformation is well recognized. Studies with two different strains of the motheaten mice that display different degrees of SHP-1 impairment and as a result suffer from dysfunction of the immune system and aberrant hematopoiesis clearly demonstrated that SHP-1 activity is particularly critical for the maturation and function of lymphoid and myeloid cells. SHP-1 acts mainly by negatively regulating cell signaling by a large spectrum of receptors for cytokines, growth factors, chemokines, and antigenic and programmed cell death stimuli (1–7). Notably, SHP-1 expression is dysregulated in such diverse processes as neoplasia and parasitic infection. Whereas in malignant cells SHP-1 expression is often inhibited to facilitate the persistent and, hence, oncogenic signal transduction (5, 8–11), leishmania, and other organisms secrete proteins that up-regulate SHP-1 expression in the host cells and, consequently, attenuate immune response (20). Despite this wealth of data pointing to the critical role of SHP-1 in the cells of hematopoietic origin, very little is known about the mechanisms that govern SHP-1 expression in these cells.

In this study we have structurally characterized the hematopoietic cell-specific P2 promoter of SHP-1 gene as well as identified the PU.1 transcription factor as the activator of the P2 promoter. In the series of luciferase reporter assays that examined 12 gradually truncated fragments of intron 1 of the SHP-1 gene (Fig. 1A) with the largest fragment (−6266 + 97) covering essentially the entire intron 1 and the shortest (−66 + 97) limited to the core promoter binding the RNA polymerase gene transcription complex (Fig. 3A) and, therefore, almost certainly devoid of any regulatory elements, we determined that even the second shortest fragment examined (−166 + 97) was able to fully induce activation of gene transcription (Fig. 2A). Further truncation of the −166 + 97 fragment demonstrated that the critical regulatory elements of the P2 promoter are located within the even smaller, 50-bp-long −116 −66 domain. The region contained two attach-
PU.1 Activates P2 Promoter of the SHP-1 Gene

ment sites for the PU.1 transcription factor binding of which to the sites was documented both in vitro and in vivo. The biological importance of PU.1 in activation of the P2 promoter was documented first by the directed mutational analysis of the PU.1b site and next by the siRNA-mediated PU.1 depletion. These findings define the exact location of the “minimal” fully active P2 promoter of the SHP-1 gene and identify PU.1 as the critical transcription activator of the P2 promoter. Binding of additional transcription factors to the P2 promoter has been suggested by other investigators (5) based on identification of the putative binding sites for GATA, AP2, and SP-1 within the broadly defined P2 promoter region. Of note, all the postulated binding sites fall outside of the −116/−66 domain identified by us as critical for the transcriptional activity of P2 (Fig. 2B). Further studies are needed to elucidate the putative role of these additional transcription factors in regulating the P2 activity.

Similar to SHP-1, PU.1 plays a critical role in hematopoiesis. Although somewhat discrepant, the studies with PU.1 gene knock-out mice have demonstrated the requirement for PU.1 expression in development of both myeloid and lymphoid cells (21, 22). Interestingly, the concentration of PU.1 in the progenitor cells seems to be important in determining the cell lineage. Whereas sustained, high PU.1 levels promote myeloid and, in particular, monocytic differentiation, lower levels favor lymphoid differentiation of the progenitor cells (23–26). PU.1 concentration is particularly high in the fully mature myeloid and lymphoid B cells. PU.1 expression is present in immature T lymphocytes but inhibited in the mature T cells with the apparent functional replacement of PU.1 by two other members of the Ets transcription factor family, GA-binding proteins a and b (27). Notably, recent studies identified PU.1 as a tumor suppressor. Mice with diminished (28) or absent (29) PU.1 expression in the hematopoietic progenitor cells developed an aggressive disease that resembled acute myelogenous leukemia. Furthermore, several mutations of the PU.1 gene have been identified in a subset of patients with the acute myeloid leukemia (30). PU.1 acts in part by inhibiting, rather than activating expression of the target genes (26). Recent findings (31) indicate that such inhibition may be due in some instances to the PU.1-induced epigenetic gene silencing.

Given the importance of SHP-1, it is likely that some of the PU.1 functions may be mediated through its effect on the SHP-1 expression in both normal and malignant cells. The above-mentioned causative relationship between the PU.1 loss and the development of acute myelogenous leukemia (28–30), on one hand, and the identified role of SHP-1 loss in the progression of myelodysplastic syndrome to an overt acute myelogenous leukemia (11), on the other hand, may represent one such possible link. Recent finding (32) that Friend spleen focus-forming virus-infected erythroleukemic cells express high concentrations of PU.1 that directly correlate with high concentration of SHP-1 that, in turn, seems to interfere with STAT1 phosphorylation may be another example for the important role of the PU.1-SHP-1 axis. Future, in-depth studies in these two and other disorders and experimental models are, however, needed to determine the exact contribution of the SHP-1 gene regulation to the role played by PU.1 in the hematopoietic cells, both normal and malignant. The structural characterization of the SHP-1 gene P2 promoter and, more importantly, identification of PU.1 as the transcription activator of the P2 promoter reported here, pave the road for such investigations.

REFERENCES
