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PU.1 Regulates the Expression of the *Vav* Proto-Oncogene

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Abstract *Vav* is a guanine nucleotide exchange factor for the rho/rac GTPases that is upregulated in the embryo during the transition from primitive to definitive hematopoiesis. It is one of several genetic markers that correlates with the differentiation of the intraembryonic definitive hematopoietic stem cell. Subsequently, in the adult, *vav* is expressed predominantly in cells of the hematopoietic system. A heat-resistant protein complex that bound to a 23-bp segment, which is essential for *vav* promoter activity, was found to be present in myeloid cells but not T-cells. The complex was absent in non-hematopoietic cells which normally do not express *vav*. Using a saturation mutagenesis method, Mutex, a ''footprint'' of the protein binding site (AGAGGAAGT) was obtained that was consistent with the consensus binding site for PU.1. A specific antibody to PU.1 supershifted the complex and identified the presence of PU.1 within the complex. A GST fusion protein of the human PU.1 bound to the same consensus sequence as the heat-resistant complex from myeloid lineages. Specific mutation of the GGAA PU.1 core binding site silenced *vav* promoter activity and a dominant negative PU.1 inhibited the transactivation of PU.1 at the *vav* promoter as measured by the expression of the EGFP reporter gene. In addition, PCR analysis of immunoprecipitated chromatin using specific antibodies for PU.1 detected the co-immunoprecipitation of DNA containing the *vav* promoter. These results suggest that PU.1 is essential for transcriptional activity of the *vav* promoter in myeloid cells. J. Cell. Biochem. 84: 772–783, 2002.

Key words: hematopoiesis; stem cell; transcription; development; embryogenesis

The *vav* proto-oncogene encodes a guanine nucleotide exchange factor for the rho/rac GTPases [Bustelo, 2000]. During the switch from primitive to definitive hematopoiesis, the expression of *vav* is upregulated in the aorta-gonad-mesonephros (AGM) region of the embryo [Okada et al., 1998] and is subsequently expressed within the cells of the adult hematopoietic system [Katzav et al., 1989; Adams et al., 1992]. The AGM is an important intraembryonic source of definitive hematopoietic stem cells and the appearance of the stem cells correlates with the upregulation of *vav* expression. Definitive hematopoietic stem cells appear to differentiate from the ventral hemogenic endothelium of the dorsal aorta and enter the developing circulatory system to seed the fetal liver [Jaffredo et al., 1998; North et al., 1999; de Bruijn et al., 2000]. In the microenvironment of the fetal liver, definitive erythrocytic, myeloid, and lymphoid lineages develop. In newborn and adult mice, *vav* is expressed specifically in hematopoietic cells from the thymus, lymph node, bone marrow, and spleen.

Previous studies with somatic cell hybrids support the hypothesis that the hematopoietic cell specific expression of *vav* is regulated in part by the interaction of transcriptional activators that are also specifically expressed in hematopoietic cells [Denkinger and Kawahara, 1997]. In the mouse, five DNase hypersensitive sites in the *vav* promoter and first intron have been identified [Ogilvy et al., 1998]. Cells which did not express *vav* lacked these hypersensitive sites. A *vav* genomic construct containing four of the five hypersensitive sites was able to confer hematopoietic cell specific expression of human

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CD4 [Ogilvy et al., 1999] in transgenic mice. The expression of human CD4 was detected in definitive, but not primitive mouse hematopoietic cells. This pattern of expression mimicked the natural expression of *vav* and delineated the boundaries for the future identification of important control elements.

The expression of the human *vav* proto-oncogene has been studied using promoter deletion mutants [Denkinger et al., 2000]. A 23-bp segment of the *vav* promoter near the transcriptional start site was found to be essential for activity in U937 cells. The same *vav* promoter constructs were inactive in HeLa cells. This suggested that one or more hematopoietic cell specific transcription factors bound to sequences within the 23 bp DNA segment to activate *vav* transcription. Specific transcription factors that regulate the expression of *vav* have not been previously identified and the mechanism by which *vav* is induced during the genesis of the hematopoietic stem cell remains unknown. In this study, we report on the identity and characterization of a protein complex from myeloid cell lines that recognizes specific sequences within the critical 23-bp segment of the *vav* promoter.

MATERIALS AND METHODS

Materials

Cell lines were purchased from American Type Culture Collection (Rockville, MD): human histiocytic lymphoma, U937 (CRL-1593.2), human AML, THP-1 (TIB 202), human AML, KG-1a (CCL-246.1), human CML, K562 (CCL 243), human acute T-cell lymphoblastic leukemia, MOLT-3 (CRL-1552), and mouse myeloblast, M1 (TIB-192). The mouse lymphoma, S49 *cyc*−, was obtained from the UCSF cell culture facility. U937, THP-1, K562, MOLT-3, M1, and Jurkat cells were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum (FBS). Media for the THP-1 cells included 0.05 mM 2-mercaptoethanol. KG-1a cells were maintained in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% FBS. HeLa and S49 *cyc*− cells were maintained as previously described [Denkinger and Kawahara, 1997].

Reverse Transcriptase (RT) and Polymerase Chain Reaction (PCR) products were subcloned into vector pCMV5, the kind gift of David W. Russell, PhD (University of Texas, Southwestern Medical Center, Dallas, TX), pcDNA3.1 (Invitrogen Corp., Carlsbad, CA) or pGEX2T (Amersham Pharmacia Biotech, Piscataway, NJ).

Oligonucleotide Probes

Oligonucleotide probes for the *vav* promoter region were synthesized by PCR as previously described [Denkinger et al., 2000] using promoter deletion construct JD-4 as a template for the following primer pairs: 14-15 (JD-14, ACAGTTACAGGCAAAGAAGAGG and JD-15, GCACGCCTGCCCTGCTCGCCTG); 14-23 (JD-14 with the reverse primer JD-23, CAGCTAGTGCTACCACTTCCTC); 10-15 (JD-10, GCACTAGCTGTCGCTCCACAGG and JD-15); 30-29 (JD-30, AGGGCGACAGTTACAGGCAA with JD-29, AGTGCTACCACTTCCTCTTC); and 31-29 (JD-31, CAGGCAAAGAAGAGGAAGTGG and JD-29). Probes 14-22 and 31-32 contained JD-14 and JD-31 annealed to their complementary primers.

Nuclear Extract Preparation

Nuclear extracts were prepared from U937, HeLa, K562, KG-1a, MOLT-3, THP-1, Jurkat, M1 and S49 *cyc*− cell lines. Cells in suspension were harvested by centrifugation and washed twice with PBS. Cells adherent to the plate were washed with PBS and removed using a cell scraper in fresh PBS. Cells were centrifuged and pellets were resuspended in five packed volumes of Buffer A (10 mM Hepes pH 7.9, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.1 mM PMSF, and 1 μg/ml Pepstatin) and set on ice for 10 min. The cells were lysed with a minimum of 15 strokes through a 22 gauge needle and centrifuged for 5 min at 1,500*g* at 4°C. The pellet containing the nuclei was resuspended in one packed volume of buffer C (20 mM Hepes pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT, 0.1 mM PMSF, and 1 μg/ml Pepstatin) and incubated on ice for 30 min with occasional stirring. Insoluble nuclear debris was removed by centrifugation at 12,000*g* for 5 min at 4°C. The nuclear extract was then dialyzed for 2–4 h against Buffer D (20 mM Hepes pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 0.1 mM PMSF). Nuclear extracts were diluted to a protein concentration of 200 μg/ml and stored at −70°C.

Electro-Mobility Shift Assay (EMSA)

Double stranded oligonucleotides or gel purified PCR products were labeled with γ-[^{32}P]

ATP using T4 Polynucleotide Kinase (Life Technologies, Inc., Grand Island, NY) for 1 h at 37°C. The labeled probe was separated by centrifugation through a Bio-Spin 6 Chromatography Column (Bio-Rad Laboratories, Hercules, CA). Radioactive probe (25,000–50,000 cpm) was incubated with 4 μl of 5 × binding buffer (50 mM Tris pH 8, 100 mM KCl, 5 mM $MgCl_2$, 1 mM $Zn(C_2H_3O_2)_2$, 5 mM DTT, 50% glycerol), 5 μg of poly (dI–dC) and 1 μg of protein from nuclear extracts in a final volume of 20 μl for 20 min at room temperature. For experiments evaluating the heat stability of the protein complex, nuclear extracts were heated for 10 min at 100°C in a Perkin Elmer Cetus DNA Thermal Cycler 480 prior to setting up the reaction mixture. Reactions were separated on a 6% polyacrylamide gel using 0.25 × TBE as the running buffer. Radioactive gels were dried and bands were detected using a Molecular Dynamics Storm 860 PhosphorImager. Quantitative results were obtained using the ImageQuant software program.

Mutex EMSA Competitions

Wild-type or mutant double stranded oligonucleotides for EMSA competitions against the labeled 31-32 probe were prepared by annealing forward and complementary primers. Each mutant probe consisted of a double stranded oligonucleotide with a single base pair transversion or transition of the 21 bp wild-type 31-32 probe. For example, for one set of transversions, if the wild-type base was a C, it was changed to an A (C > A). Similarly, the other bases were modified from A > C, G > T, and T > G. Once the location of the DNA binding region was determined (bases 8 through 19), additional 21 bp primers were synthesized with single point mutations introduced in positions from bases 8 through 19. This resulted in the generation of two additional sets of 12 primer pairs. The first set contained primers for transversions: A > T, G > C, and T > A. The second set consisted of primers containing transitions: A > G, G > A, and T > C. There were no cytidines present in bases 8 through 19. EMSA data was quantitated using the ImageQuant software from Molecular Dynamics. For wild-type or each mutant competition, the percent of maximum binding (M_i) was calculated by dividing the level of EMSA binding in the presence of a double stranded oligonucleotide competitor by the total binding of the labeled probe in the absence of unlabeled competitor. M_i values were averaged and the frequency weight (F_i) for each of the four bases at each position was calculated by subtracting M_i from 1 and dividing by the sum of the $1-M_i$ values for all four bases. For any given base i, the frequency weight for that base is calculated by:

$$F_i = \frac{1 - M_i}{\sum 1 - M_i}$$

Cloning and Preparation of the Dominant Negative Human PU.1 Gene

Total RNA from THP-1 cells was isolated [Chomczynski and Sacchi, 1987] and reverse transcribed at 42°C for 45 min with antisense oligonucleotide primer PU.1-R2 (CCGGATCCTCAGTGGGGCGGGTGGCGCCGCTCGGCCAG). PCR amplification of the reaction was performed with the addition of the sense primer PU.1-F2 (GGGAATTCTGCAAAATGGAAGGGTTTCCCCTCGTCCCC) for 40 cycles at 94°C for 1 min and 60°C for 1 min. The final cycle was followed by a 7 min extension at 60°C. The gel purified cDNA was restriction digested with EcoRI and BamHI, ligated into the pCMV5 vector, and transformed into JM109 cells (Stratagene, La Jolla, CA). DNA for transfection was prepared using a Qiagen column (Qiagen Inc., Valencia, CA) and the insert was verified to be human PU.1 by sequencing.

A dominant negative PU.1 was prepared by subcloning the DNA binding domain in the absence of the transcriptional activation domain. The insert was prepared by PCR using sense primer HPU.1-F (GGGGATCCGCCACCATGAAGAAGAAGATCCGCCTGTAC) with antisense primer PU.1GST-R (CCGAATTCTCAGTGGGGCGGGTGGCGCCGC). The reaction was denatured at 95°C for 4 min and amplified for 31 cycles at 95, 55, and 72°C for 1 min at each temperature. This was followed by an extension for 6 min at 72°C. The final product was restriction digested with BamHI and EcoRI, and cloned into pcDNA3.1. The plasmid was Qiagen purified for transfection into U937 cells.

Preparation of the PU.1 Recombinant GST Fusion Protein

Utilizing the human PU.1 insert as the template, sense primer PU.1GST-F (GGGGATCCTGCAAAATGGAAGGGTTTCCCC) and antisense primer PU.1GST-R were used to prepare a PCR insert for subcloning using the

same conditions as used to create the dominant negative insert. The cDNA was restriction digested with BamHI and EcoRI, and ligated into pGEX2T. The resultant plasmid was transformed into SG1611 for expression of the GST fusion protein.

Stimulation of the SG1611 transformants with 0.1 mM IPTG for approximately 4 h at 37°C induced production of the PU.1 recombinant protein. Bacterial cells were centrifuged and the pellet resuspended in B-PER Reagent (Pierce, Rockford, IL) according to the manufacturer's protocol. The control GST protein (pGEX2T vector) was harvested from the soluble fraction, while the PU.1 GST fusion protein was found in the insoluble fraction. Purification of the PU.1 GST fusion protein from the inclusion bodies was accomplished by the addition of lysozyme (200 μg/ml) followed by three rounds of sonication for 30 s. The fusion proteins were isolated by adding glutathione sepharose 4B (Amersham Pharmacia Biotech, Piscataway, NJ) and incubated at 4°C with gentle rocking for a minimum of 2 h. After several washes of the beads with PBS, the purified protein was eluted off the gel matrix with 10 mM reduced Glutathione.

Transfection of Cells

U937 cells were transfected with Lipofectamine Reagent (Life Technologies) as described previously [Denkinger et al., 2000]. The pcDNA3.1-PU.1 dominant negative construct was transfected at a ratio of 5 pmoles to 1 pmole of linearized JD-4 construct or circular pEGFP-C1. Control transfections with JD-4 or pEGFP-C1 alone were conducted with 16 μl of Lipofectamine reagent, whereas, transfections with JD-4 or pEGFP-C1 and the dominant negative PU.1 were conducted with 36 μl to maintain the lipid to DNA ratio.

EGFP Reporter Gene Analysis

Two days post-transfection, plates were viewed on a Zeiss confocal microscope with a 20 × objective using the standard FITC filter set. Comparisons were normalized by using a contrast setting of 375 and a brightness setting of 9797 for each digital image. Images were quantitated using the ImageQuant software from Molecular Dynamics. The Quattro-Pro spreadsheet was used to tabulate and store data. The dynamic range of fluorescence ranged from 0 to 256 units.

Chromatin Immunoprecipitation Assay

U937 and HeLa cells were harvested and centrifuged at 140*g* for 5 min to remove the media. The chromatin was crosslinked by resuspending the cells in PBS with 1% formaldehyde for 10 min at room temperature. Crosslinking was stopped by adding glycine dissolved in PBS to a final concentration of 0.125 M. The cells were centrifuged at 7,000*g* for 5 min and the supernatant discarded. The cells were washed twice with cold PBS containing 0.125 M glycine and resuspended in solution A (10 mM Tris pH 8, 0.25% Triton X-100, 10 mM EDTA, 0.5 mM EGTA) for 10 min. The cells were homogenized using a microtube pestle and centrifuged at 2,000*g* for 10 min. The supernatant was discarded and the nuclei were washed with solution A. The nuclei were resuspend in solution B (10 mM Tris pH 8, 100 mM NaCl, 0.25% Triton X-100, 0.025% Tween-20, 1 mM EDTA, 0.5 mM EGTA) and sonicated twice for 30 s. The sonicated chromatin solution was precleared by adding sepharose blocked with 10 mg/ml sheared herring sperm DNA and 0.5% BSA. After rotating the tubes for 2 h at 4°C, the beads were removed by centrifugation at 7,000*g* for 10 min. For immunoprecipitations, 1–2 μg of antibody or non-immune serum was added with 100 μl of a 50% slurry of blocked protein A/G Sepharose in solution B to the sonicated crosslinked chromatin. The tubes were rotated for 5 h or overnight at 4°C. The beads were washed five times with 1 ml ice cold solution B and separated from the supernatant by centrifugation at 7,000*g* for 5 min. To reverse the crosslinks, the beads were resuspended in solution C (10 mM Tris pH 8, 25 mM EDTA, 100 mM NaCl, 0.5% SDS, 500 μg/ ml proteinase K) and incubated at 50°C overnight. An aliquot of crosslinked chromatin was processed in parallel to isolate the total DNA used in the immunoprecipitations. The temperature was increased to 65°C for 5 h to complete the reversal of the crosslinks. The supernatant was then phenol extracted and ethanol precipitated with glycogen. The immunoprecipitated DNA was dissolved in 20 μl of water for PCR analysis. *Vav* promoter DNA was detected by PCR using primers JD-14 and JD-9 (GGCTACCGCCTG-CCCAGGGCCG). PCR amplifications were initiated by denaturation for 5 min at 95°C followed by primer annealing at 55°C for 30 s, primer extension at 72°C for 30 s and 95°C for

30 s. After 40 cycles, the final product was incubated at 55°C for 1 min and 72°C for 7 min. The products were then separated on a 4% Amresco (Solon, OH) 3:1 agarose gel.

RESULTS

Protein Complex from Myeloid Cell Lines Binds to the Critical 23 bp *Vav* Promoter Segment

A series of genomic fragments of the human *vav* promoter (Fig. 1A) were used as probes to screen for differential protein binding between nuclear extracts from U937 cells, which express *vav*, and HeLa cells, which do not. Fragment 14-15 detected a protein complex from U937 cells that was absent in nuclear extracts from HeLa cells (Fig. 1B). Comparison of the EMSA gel shift pattern of nuclear extracts from U937 and HeLa cells also identified a low mobility band present in both extracts, a slightly faster migrating band specific to HeLa cells and several bands that migrated faster than the band specifically detected in U937 cell extracts. DNA fragment 14-15 was thought to be important because it contained a 23-bp segment that had been previously shown to be necessary for *vav* expression [Denkinger et al., 2000] and included the tandem *vav* transcriptional start sites. Since a clear footprint of the interacting complex could not be obtained, a series of shorter DNA fragments (Fig. 1A) were used in EMSA competitions to define the protein binding site. Unlabeled DNA fragments from the *vav* promoter were added at increasing concentrations to compete with radioactive probe 14-15. Binding to the specific complex found in U937 nuclear extracts (Fig. 1B, arrow) was inhibited at lower concentrations of unlabeled 14-15 as compared to the other DNA complexes. Fragment 14-22, which included

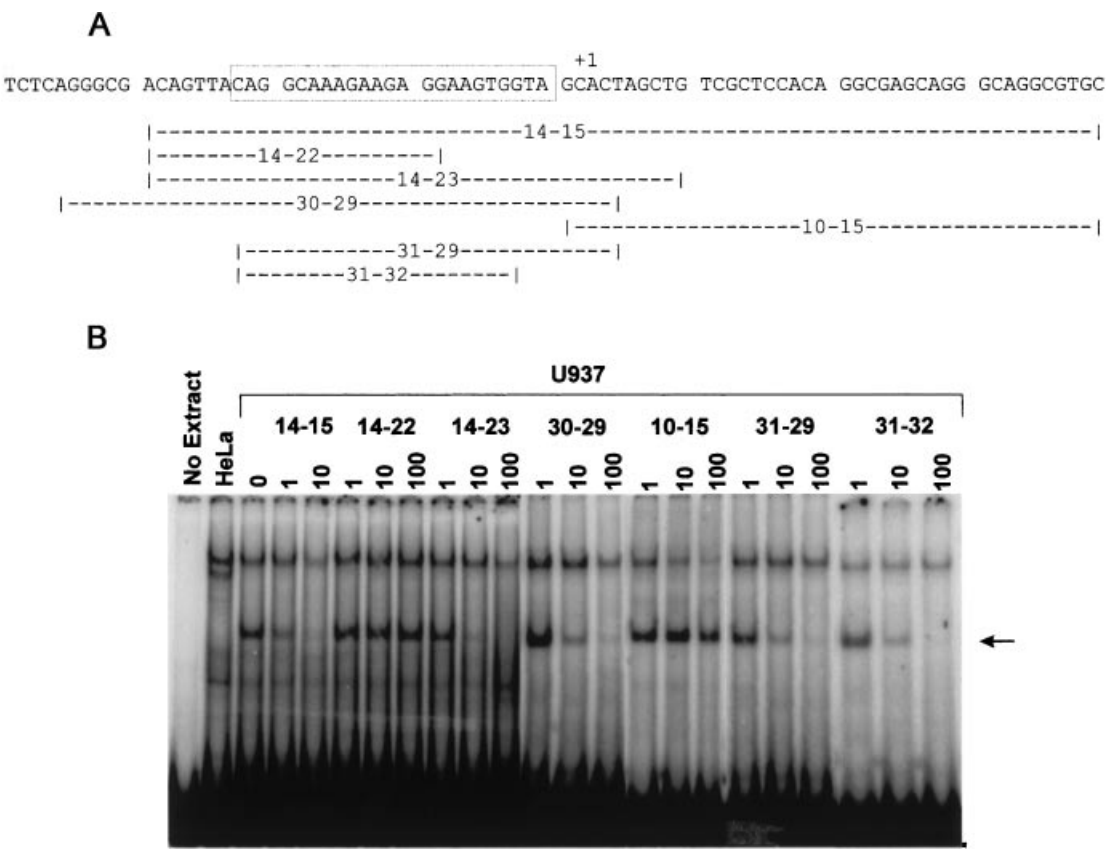


Fig. 1. **A**: Location and composition of *vav* promoter DNA segments used in EMSA competitions. DNA segments were synthesized by PCR or annealing two complementary oligonucleotides. The critical 23 bp *vav* promoter segment is shown in an enclosed rectangle. **B**: EMSA competition of labeled 14-15 with unlabeled 14-15, 14-22, 14-23, 30-29, 10-15, 31-29, and 31-32. Labeled 14-15 probe was incubated in the absence (no extract) or presence of nuclear extracts from HeLa or U937 cells. U937 nuclear extract was incubated in the absence (0) or presence of competitor DNA at the indicated molar excess (1, 10, or 100 ×) over the labeled 14-15 probe. The arrow shows a protein complex found in U937 but not HeLa cells.

15 bp of the 23-bp segment, did not effectively compete for 14-15 binding. However, fragments 14-23, 30-29, and 31-29, which included the entire 23-bp segment, effectively competed for radioactive 14-15 binding. In each case, binding to the U937 specific complex was inhibited at lower concentrations of the competitor as compared with the slower migrating complex common to both U937 and HeLa cell lines. Fragment 10-15, which contained the 5′ untranslated region of *vav*, did not compete for the U937 specific complex, but did inhibit the formation of the slower mobility complex present in both U937 and HeLa extracts. Fragment 31-32, which did not include the entire potential Runx1/AML-1 binding site [Denkinger et al., 2000], was an effective competitor and suggested that the U937 specific protein complex probably did not include the Runx1/AML-1 transcription factor. The same complex found in U937 cells was also detected in K562, KG-1a, THP-1, and M1 cells (Fig. 2) using 31-32 as the radioactive probe. A relatively large quantity of the complex was detected in THP-1 cells, whereas KG-1a and M1 cell lines contained the least complex formation. The complex was not detected in MOLT-3, Jurkat, HeLa, or S49 cell extracts. This suggested that the protein complex was present in myeloid but not T-lymphocyte derived cell lines. MOLT-3, Jurkat, and S49 are T-cell derived leukemia or lymphoma cell lines, whereas U937, K562, KG-1a, THP-1, and M1 are derived from cells of the myeloid/erythroid lineages. The complex was also unusual because it was found to be partially stable when heated to 100°C for 10 min (Fig. 2B). The percentage of DNA binding to the radioactive 31-32 probe after heating ranged from 71% in THP-1 extracts, 34–35% in U937, K562, KG-1a extracts and 23% in M1 extracts.

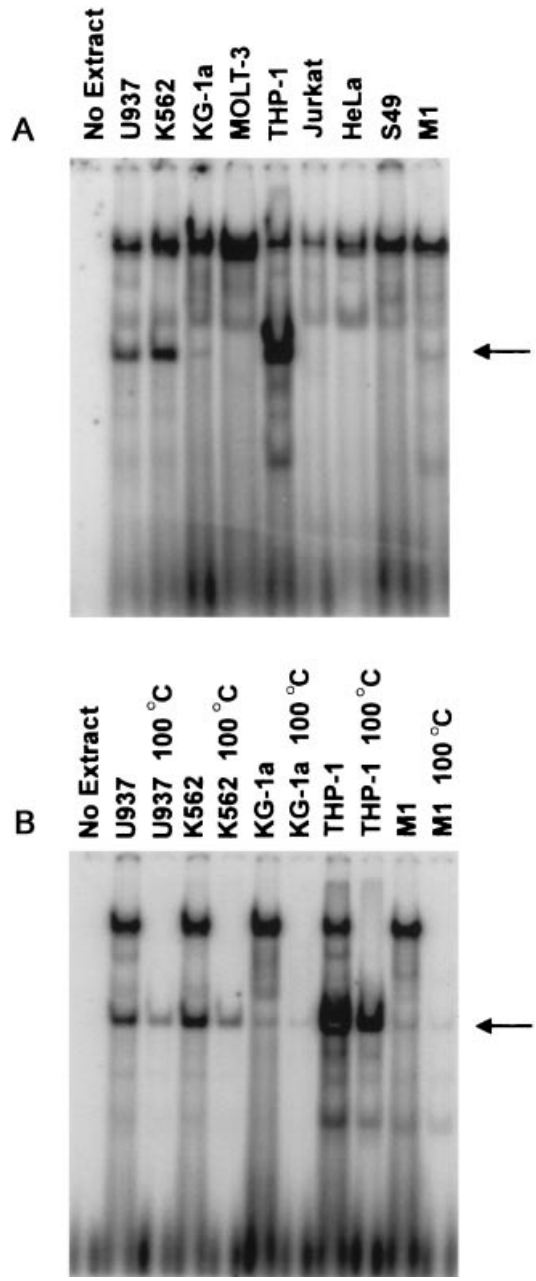


Fig. 2. **A**: EMSA with labeled probe 31-32 in the absence (no extract) or presence of nuclear extracts from the indicated cell lines. **B**: EMSA with labeled probe 31-32 and nuclear extracts that were either unheated or heated at 100°C for 10 min. The arrow indicates the heat resistant band found in myeloid cells, but not in T-cells or HeLa cells.

Complex Contains the Transcription Factor PU.1

To identify the DNA binding transcription factor that was responsible for the U937 specific heat-resistant complex, a saturation mutagenesis (Mutex) of the 31-32 DNA segment was conducted. Using nuclear extracts from THP-1 and K562 cells, EMSA competitions with radioactive 31-32 probe and a series of mutant probes containing single base pair transitions or transversions at each base pair position were conducted. The concentration of unlabeled 31-32 (wild-type) probe sufficient to reduce DNA binding to approximately 90% was chosen to permit the detection of potential mutations that resulted in the generation of competitors with higher DNA binding affinity as compared to wild-type. If mutations are introduced at critical positions, then a decrease in the ability of the unlabeled mutant probe to compete for factor

binding will be detected by the increase in band intensity as compared to the competition with wild-type 31-32. Mutations at nonessential bases are expected to demonstrate binding competition similar to wild-type. As shown in Figure 3, a specific "footprint" of the U937 heat-resistant complex could be detected and appeared bimodal. The complex appeared to be affected by changes in the sequence GAAG and the GGAAGT sequence that immediately follows it. The overall pattern of binding sensitivity to single base pair mutations suggests that the complex from THP-1 and K562 cells is likely to be identical. The entire Mutex data set was quantitated and is summarized graphically in Figure 4. The complex appears to be most sensitive to mutations that alter the core GGAA sequence as shown by the larger letters in Figure 4A. The purine rich motif suggested that the factor, which recognized this sequence, belonged to the *ets* family of transcriptional activators.

TEL and PU.1 are *ets* family members that are expressed specifically in the hematopoietic system and have been previously identified in the regulation of other genes expressed in hematopoietic cells. Comparison of the consensus binding sequence derived from Mutex analysis with the consensus derived from the study of other promoters, Multiplex and SELEX methods (Fig. 4B), showed a strong correlation with the binding site for PU.1 [Szymczyna and Arrowsmith, 2000]. Using specific antibodies to TEL and PU.1 in supershift assays, we tested the hypothesis that either TEL or PU.1 might be responsible for the formation of the heat-resistant complex. In Figure 5A, nuclear extracts from K562 and THP-1 cells were bound to the 31-32 probe in the absence or presence of purified non-immune IgG or antibodies raised against TEL or PU.1. The addition of anti-PU.1 resulted in the appearance of a supershifted complex and the corresponding disappearance of the heat-resistant complex. These results indicated that the heat-resistant complex contained PU.1, but not TEL. To further establish that PU.1 could bind to *vav* promoter sequences recognized by the heat stable complex, the full length human PU.1 cDNA was cloned by RT-PCR and expressed as a GST fusion protein. When the GST-PU.1 fusion protein was analyzed in Mutex EMSA competition assays using 31-32 as the radioactive probe and unlabeled 31-32 (wild-type) or single base pair mutants as competitors, a bimodal "footprint" similar to the heat stable complex from K562 and THP-1 cells was obtained (Fig. 5B).

Mutation of the Core PU.1 Binding Site Inhibits *Vav* Promoter Activity

Introduction of a TTC mutation in the GGAA PU.1 core binding site (Fig. 6A, JD-4mutPU) in the *vav* promoter construct JD-4 abolished all transcriptional activity of the *vav* promoter (Fig. 6B). The mutation introduced in JD-4mutPU did not alter the overlapping Runx1/AML-1 binding site and suggests that in myeloid cells, PU.1 is the major regulating factor that regulates the *vav* promoter in this region. This data agrees with the previously reported mutations for the *vav* promoter [Denkinger et al., 2000]. Deletion of the myb binding site in JD-4 (Fig. 6) did not affect *vav* promoter activity. However, the additional deletion of the Runx1/AML-1 binding site (myb-cbf) silenced the activity of the *vav* promoter [Denkinger et al., 2000]. This result can now be explained by the existence of a partial overlap of the Runx1/AML-1 binding site with the PU.1 GGAA core binding site (Fig. 6). Deletion of the final adenosine in the PU.1 GGAA core converts the PU.1 core to GGAG which significantly reduces PU.1 binding (Figs. 3 and 4). The del-1 mutant, which deletes the myb binding site and the sequence between the myb and the Runx1/AML-1 binding sites (Fig. 6A, dashed underlined), also resulted in the loss of *vav* promoter activity. The del-1 mutant of JD-4 deletes a significant portion of the PU.1 binding site and is consistent with the data presented in this article.

Dominant Negative PU.1 Inhibits Reporter Expression by the *Vav* Promoter

To demonstrate that PU.1 could regulate the *vav* promoter, a dominant negative PU.1 was constructed. This construct contained the *ets* binding domain, but lacked the transcriptional activation domain. Previous results have shown that the ets binding domain was insufficient to promote macrophage differentiation in the absence of the transactivation domain [Fisher et al., 1998]. Cotransfection of pEGFP-C1 driven by the CMV promoter with the PU.1 dominant negative construct did not affect EGFP reporter expression (Fig. 6B). However, the expression of the EGFP reporter driven by the JD-4 *vav* promoter construct was clearly

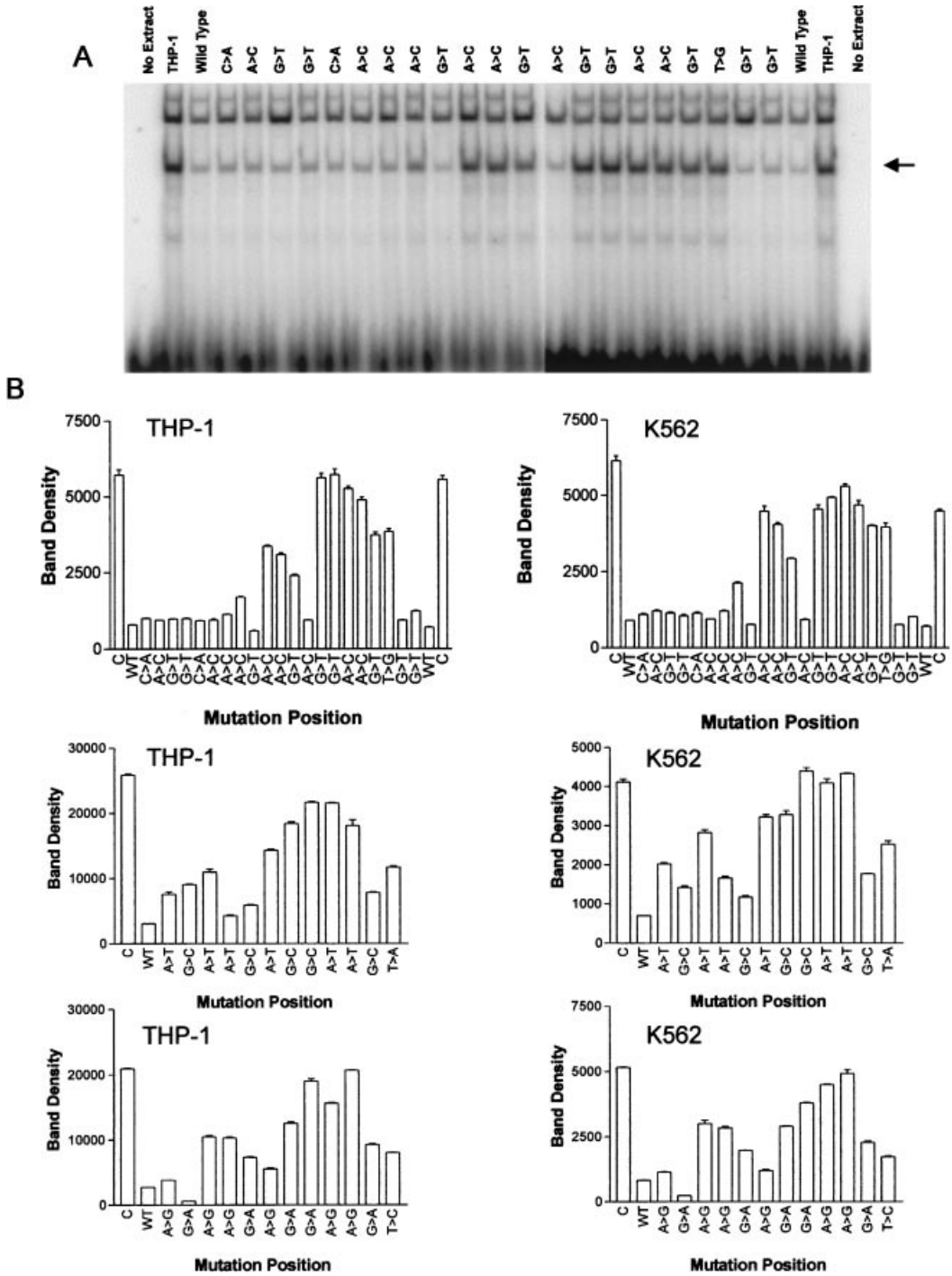
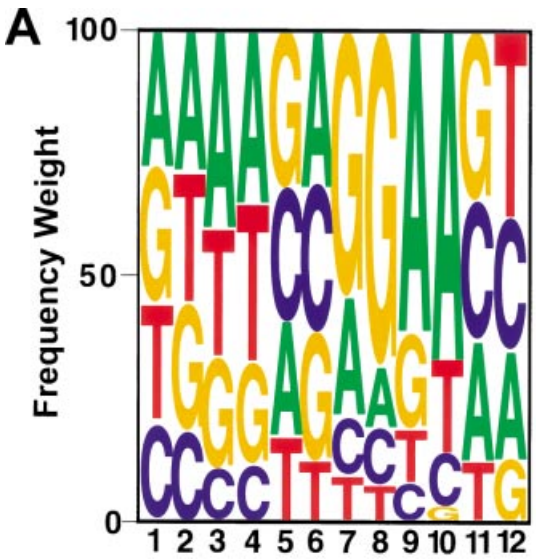


Fig. 3. **A**: Labeled 31-32 probe in the absence (no extract) or presence of nuclear extracts from THP-1 cells was used in EMSA competitions with unlabeled 31-32 double stranded oligonucleotides containing single point mutations. THP-1 nuclear extract was incubated with labeled 31-32 probe alone (THP-1) or with unlabeled 31-32 (wild type) probe or with a series of 31-32 probes with a single point mutation at each of the positions as indicated. In this panel, cytosine was replaced with adenosine (C > A), adenosine for cytosine (A > C), guanosine for thymidine (G > T), or thymidine for guanosine (T > G). Mutation of critical bases reduces the ability of the unlabeled probe to act as a competitor. The arrow indicates the heat-resistant complex. **B**: Quantitation of EMSA competitions with single base residues mutated to each of the other three bases. EMSA were conducted with nuclear extracts from THP-1 and K562 cells as described above. Experiments with nuclear extracts from THP-1 cells are shown in the left column and similar experiments with K562 nuclear extracts are shown on the right. EMSA phosphorimages were quantitated using the ImageQuant software from Molecular Dynamics.



B

Mutex	AT	Gca	ACg	G	G	A	A	G	T
Promoter	At	G	A	G	G	A	A	G	T
Multiplex	Tag	AG	Cag	G	G	A	A	AGc	Ct
SELEX	AT	Gc	Cga	G	G	A	A	Gc	T

Fig. 4. **A**: Graphical representation of the Mutex matrix for THP-1 cells. The data shown in Figure 3 was used to determine the frequency weight of the Mutex matrix. The frequency weight calculated for each base at a given position is proportionally represented by the height of each base in numerically descending order. The consensus purine rich binding site can be determined by reading the bases at the top of each column. Larger letters at the top indicate the preferred base at each position. **B**: Comparison of the consensus binding sequence for PU.1 as determined by Mutex, promoter consensus, Multiplex, and SELEX methods. Uppercase letters represent the preferred base over less preferred bases in lowercase letters. The promoter, Multiplex, and SELEX consensus sites were from Szymczyna and Arrowsmith [2000].

inhibited by the cotransfection of the dominant negative PU.1.

Antibodies Against PU.1 Co-Immunoprecipitate the *Vav* Promoter

Formaldehyde crosslinked chromatin from U937 and HeLa cells was sheared and immu-

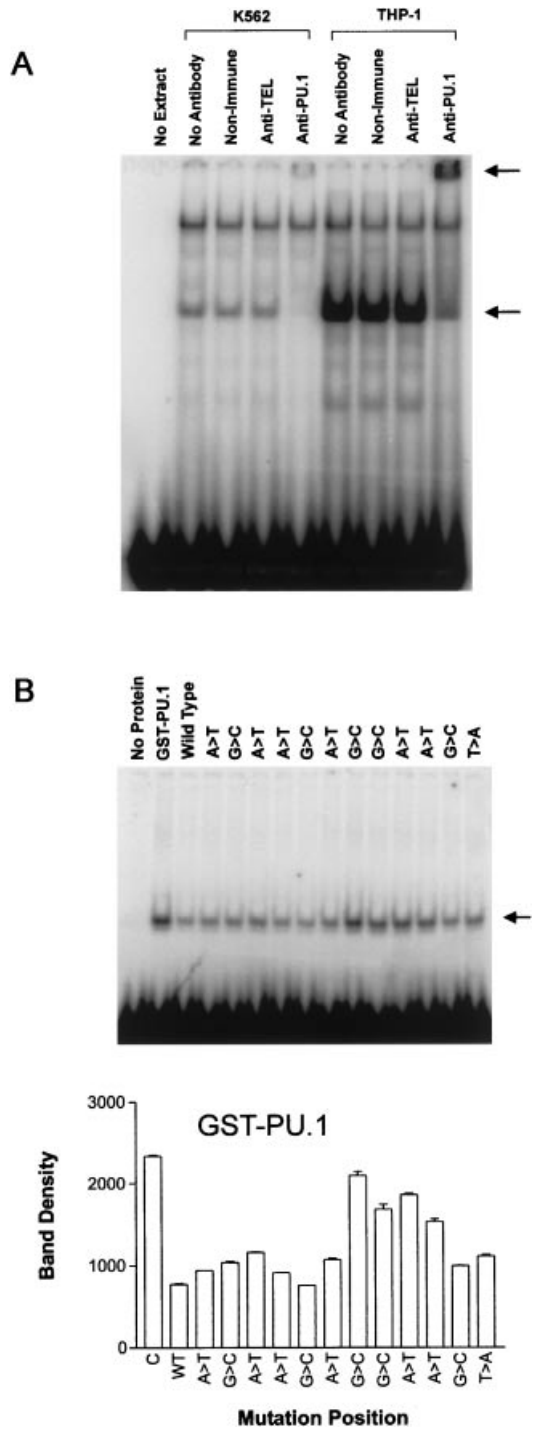


Fig. 5. **A**: Supershift of the complex indicates the presence of PU.1. Labeled 31-32 probe was incubated in the absence (no extract) or presence of nuclear extracts from K562 or THP-1 cells. Non-immune rabbit serum, anti-TEL, or anti-PU.1 antibodies were added as indicated. The top arrow displays the supershifted complex. The bottom arrow shows the normal position of the heat-resistant complex. **B**: Labeled 31-32 probe was incubated in the absence (no protein) or presence of GST-PU.1 fusion protein in EMSA competitions with unlabeled 31-32 probe containing single point mutations. Purified GST-PU.1 fusion protein was incubated with labeled 31-32 probe without competitor (GST-PU.1) or with unlabeled 31-32 (wild type) probe or 31-32 with a single point mutation at the indicated position. In this panel, adenosine was replaced with thymidine (A > T), guanosine for cytosine (G > C), or thymidine for adenosine (T > A). Quantitation of the EMSA competition data using the GST-PU.1 fusion protein is shown in the lower panel.

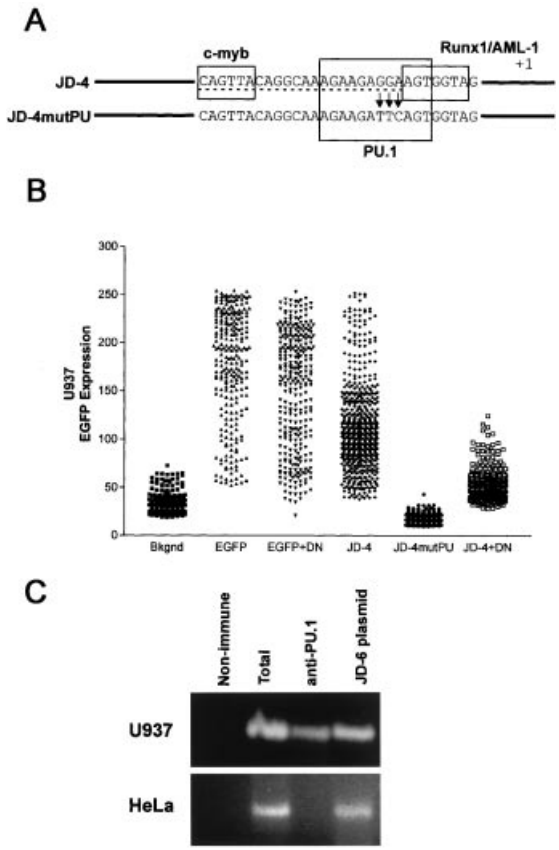


Fig. 6. **A**: The control elements of the *vav* promoter. Control elements c-myb, PU.1, and Runx1/AML-1 sites are shown in enclosed boxes. Previously published mutations [Denkinger et al., 2000] myb (deletion of the sequence within the c-myb box), myb-cbf (deletion of the sequences within the c-myb and Runx1/AML-1 boxes) and del-1 (deletion of the dotted underlined sequence) are shown relative to the mutation of the PU.1 binding site (shown by arrows). The start site of the RNA is denoted as +1. **B**: The mutation of the PU.1 binding site and dominant negative PU.1 inhibits *vav* promoter activity. U937 cells were transfected with: pEGFP-C1, EGFP driven by the CMV promoter (EGFP); pEGFP-C1 and the dominant negative PU.1 (EGFP + DN); *vav* promoter construct JD-4 alone (JD-4); JD-4 with the PU.1 binding site mutated (JD-4mutPU); or JD-4 with the dominant negative PU.1 (JD-4 + DN). Background (Bkgnd) represents the fluorescence of untransfected U937 cells. Fluorescent cells were quantitated with the ImageQuant software. **C**: Chromatin immunoprecipitation with anti-PU.1 antibodies. Chromatin crosslinked with formaldehyde from U937 or HeLa cells was sheared and immunoprecipitated with either rabbit non-immune serum or anti-PU.1 antibodies and analyzed by PCR. DNA from an aliquot of chromatin used in the immunoprecipitation was processed in parallel as a control. DNA from plasmid JD-6 [Denkinger et al., 2000] was also used as a PCR control. DNA from the *vav* promoter co-immunoprecipitated with the antibody raised against PU.1 was detected by PCR with primers JD-14 and JD-9. Lanes are as indicated: Non-immune, chromatin immunoprecipitated with non-immune serum; total, an aliquot of DNA from the total chromatin solution; anti-PU.1, DNA precipitated with the anti-PU.1 antibody; JD-6 plasmid, amplification of the vav promoter using plasmid JD-6 as a control template.

noprecipitated with non-immune serum or with anti-PU.1 antibodies. The crosslinks were reversed and DNA that co-immunoprecipitated with PU.1 was isolated. The *vav* promoter was detected by PCR using primers JD-14 and JD-9. This primer pair resulted in a specific 129 bp product that was absent when herring sperm DNA was used as a template. As shown in Figure 6C, a 129 bp PCR product from the *vav* promoter was detected by gel electrophoresis from DNA co-immunoprecipitated with anti-PU.1 from U937 cells, but not with the non-immune serum. Chromatin immunoprecipitations from HeLa cells with the same anti-PU.1 antibody did not co-immunoprecipitate the *vav* promoter. A fraction of the DNA isolated from the sheared chromatin and the plasmid JD-6, which contains a copy of the *vav* promoter, were used as control templates for the PCR reactions. Both bands co-migrated with the PCR product detected from the DNA co-immunoprecipitated with the anti-PU.1 antibody. These results suggest that PU.1 is able to bind to the *vav* promoter in intact U937 cells.

DISCUSSION

A heat-resistant DNA binding protein complex that interacts within a 23-bp segment of the *vav* promoter has been identified. The 23-bp segment has been previously shown to be important for promoter activity [Denkinger et al., 2000]. The complex interacts strongly with a GGAAGT core sequence and is consistent with the DNA binding consensus sequence for the *ets* family transcription factor PU.1. The bimodal "footprint" of the heat-resistant complex was similar to the "footprint" derived from a human GST-PU.1 fusion protein. The heat-resistant complex was supershifted by antibodies to PU.1 and a dominant negative PU.1 construct inhibited *vav* promoter activity as determined by the loss of EGFP reporter gene expression. PU.1 was detected bound to the *vav* promoter by chromatin immunoprecipitation in intact U937 cells, but not HeLa cells. Previously reported [Denkinger et al., 2000] *vav* promoter mutations and specific mutation of the GGAA PU.1 core recognition sequence all support the importance of PU.1 for *vav* promoter activity in myeloid cells. Consistent with the known expression of PU.1, the heat-resistant complex was expressed in myeloid cells, but not in T-cells [Ray et al., 1992; Chen et al., 1995]. U937 cells,

neutrophils, and monocytes are known to express high levels of PU.1, but not Spi-B, a related member of the ets family [Chen et al., 1995]. Therefore, PU.1, not Spi-B, is important in the expression of *vav* in myeloid cell lineages.

The *vav* promoter has neither a TATA box nor an initiator (INR) sequence [Denkinger et al., 2000]. Since the essential 23-bp segment is immediately 5′ to the tandem transcriptional initiation sites, it is likely that PU.1 acts to recruit the basic transcriptional machinery to the *vav* promoter. TFIID is known to bind to the N-terminus of PU.1 [Hagemeier et al., 1993]. Many myeloid specific promoters such as the promoters for the M-CSF, GM-CSF, and G-CSF receptors also lack a TATA box or an INR sequence and contain functional PU.1 binding sites in close proximity to the transcriptional start site [Zhang et al., 1994; Hohaus et al., 1995; Smith et al., 1996]. The initiation of other TATA-less promoters is affected by SP1 cooperation with PU.1 [Chen et al., 1993; Honda et al., 1997]. Consensus SP1 binding sites have been identified both 5′ and 3′ from the PU.1 binding sites. Whether these sites are important and cooperate to enhance transcriptional initiation from the *vav* promoter remains to be determined. A highly conserved segment in the *vav* promoter that is identical in the mouse, rat, and human species, located downstream from the tandem transcriptional start site, plays a major role in the promoter activity of *vav* [Denkinger et. al., 2001]. Mutation of the CACCC core sequence resulted in significantly diminished *vav* promoter activity. One transcription factor that bound to this region was found to be a member of the krüppel-like factor family. Lung krüppel-like factor (LKLF) [Anderson et al., 1995] was identified by DNA crosslinking and antibody supershift experiments. A dominant negative LKLF was able to reduce the expression of EGFP driven by the *vav* promoter. The binding of PU.1 and LKLF are likely to cooperate to enhance the activity of the *vav* promoter. Both PU.1 and LKLF are expressed in hematopoietic cells.

The tissue specific expression of *vav* is determined in part by the hematopoietic specific expression of PU.1. PU.1 is expressed specifically in hematopoietic stem cells and is upregulated in differentiating myeloid and B-lymphoid progenitors. However, PU.1 is not expressed in T-cells and is downregulated in differentiating erythroid progenitors. Since *vav* is expressed in T-cells and is important in T-cell receptor signaling [Fischer et al., 1995; Tarakhovsky et al., 1995; Zhang et al., 1995; Turner et al., 1997], *vav* appears to be regulated by a PU.1 independent mechanism in T-cells. This appears contrary to the expectation that the expression of *vav* throughout the hematopoietic system is regulated by a single mechanism. Previous evidence has suggested that transcription in different hematopoietic lineages is initiated from a single promoter and contains the same pattern of tandem transcriptional start sites in different lineages [Katzav et al., 1991]. Recent evidence from our laboratory supports the hypothesis that *vav* is expressed by a different transcriptional control mechanism in T-cells and that another hematopoietic specific transcription factor substitutes in the absence of PU.1. Although *vav* is selectively expressed within cells of the hematopoietic system, the transcriptional control mechanisms which induce expression appear to differ between the hematopoietic lineages. The major finding of this work indicates that PU.1 plays a significant role in the transcriptional regulation of the *vav* promoter in myeloid cells.

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