Transactivation of an Intronic Hematopoietic-specific Enhancer of the Human Wilms’ Tumor 1 Gene by GATA-1 and c-Myb*

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The Wilms’ tumor 1 gene (WT1) encodes a zinc-finger transcription factor which is expressed in a tissue-specific manner. Our studies indicate that in addition to the promoter, other regulatory elements are required for tissue-specific expression of this gene. A 258-base pair hematopoietic-specific enhancer in intron 3 of the WT1 gene increased the transcriptional activity of the WT1 promoter by 8–10-fold in K562 and HL60 cells. Sequence analysis revealed both a GATA and a c-Myb motif in the enhancer fragment. Mutation of the GATA motif decreased the enhancer activity by 60% in K562 cells. Electrophoretic mobility shift assays showed that the GATA-1 protein in K562 nuclear extracts binds to this motif. Cotransfection of the enhancer containing reporter construct with a GATA-1 expression vector showed that GATA-1 transactivated this enhancer, increasing the CAT reporter activity 10–15-fold. Similar analysis of the c-Myb motif by cotransfection with the enhancer CAT reporter construct and a c-Myb expression vector showed that c-Myb transactivated the enhancer by 5-fold. A DNase I-hypersensitive site has also been mapped in the 258-base pair enhancer region. These data suggest that GATA-1 and c-Myb are responsible for the activity of this enhancer in hematopoietic cells and may bind to the enhancer in vivo.

Wilms’ tumor is a pediatric nephroblastoma that is one of the most frequent solid tumors in children (1). Mutation or deletion of both copies of the Wilms’ tumor 1 (WT1) gene is associated with Wilms’ tumor, implying that WT1 is a tumor suppressor gene (2, 3). Mice with homozygous WT1 mutations fail to develop kidneys and gonads (4), suggesting that WT1 has a crucial role in early urogenital development. However, WT1 mutations have also been associated with other tumors (3), including mesotheliomas (5), juvenile granulosa cell tumor of the ovary (6), and secondary acute myelogenous leukemia (7). Recently, five WT1 mutations were found in four of 36 patients with sporadic acute leukemia (8), which is a mutation rate comparable to that found in Wilms’ tumors.

WT1 has been mapped to human chromosome 11p13, cloned, and shown to encode a zinc-finger transcription factor (9, 10). Recent studies have shown that WT1 is expressed in normal hematopoietic cells, at higher levels in immature hematopoietic cells (e.g. in CD34+/bone marrow and fetal liver cells) (8, 11, 12) than in differentiated mature blood cells (12). It has been reported that in normal human bone marrow, fluorescence-activated cell sorted CD34+/CD33+/Lin- cells have levels of WT1 gene expression a hundred times greater than those of fluorescence-activated cell sorted CD34+/CD33+/Lin- cells (11). WT1 is strongly expressed in the peripheral blood of many patients with acute leukemia and in the blast-crisis (BC) phase of chronic leukemias but is absent in the chronic phase of chronic leukemias (13). The average levels of WT1 expression were more than 20 times higher for CD19+/CD20-/B-cell acute lymphoblastic leukemia than for CD19+/CD20+/B-cell intermediate B-cell acute lymphoblastic leukemia, indicating that WT1 expression is associated with immature B phenotypes of acute lymphoblastic leukemia cells (11). In chronic myelogenous leukemia (CML), WT1 expression levels are clearly associated with the clinical phase, and the levels increase as the clinical phase progresses. This indicates that in CML, WT1 gene expression is also associated with immature leukemic cells. Thus, WT1 may be a new prognostic factor and a new marker for the detection of minimal residual disease in acute leukemia (11). It has also been reported that the WT1 gene is down-regulated during terminal differentiation of both K562 cells (a human erythroleukemia cell line derived from a patient with BC-CML) and HL60 cells (a human myelocytic leukemia cell line) (14, 15). It appears, therefore, that WT1 gene expression is associated with the immature cells from which leukemic cells originate.

To understand the regulation of WT1 in hematopoiesis and leukemia, we examined the elements involved in the transcriptional control of the WT1 gene (16). We have identified three transcription start sites and an essential promoter region of the WT1 gene. The WT1 promoter is a member of the GC-rich, TATA-less, CCAAT-less class of polymerase II promoters (16). Whereas the WT1 promoter is similar to other GC-rich tumor suppressor gene promoters, the WT1 expression pattern is tissue restricted, unlike RB and p53 patterns. However, the WT1 GC-rich promoter functions in all cell lines tested, independent of WT1 expression. This finding suggests that the tissue-specific expression of WT1 is modulated by additional regulatory elements. We previously identified a transcriptional enhancer at the 3’ end of the gene, more than 50 kb downstream of the promoter (16). This 3’-enhancer increases the basal transcription rate of the WT1 promoter in the human erythroleukemia cell line K562 but not in the non-hematopoietic cell lines tested. The hematopoietic transcription factor GATA-1 binds and transactivates the 3’-enhancer (17).

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Here we report the identification of a hematopoietic-specific enhancer in the third intron of the WT1 gene. While both GATA-1 and c-Myb could transactivate this intronic enhancer, the previously identified 3’-enhancer lacks c-Myb binding motifs (17). Consequently, the two WT1 enhancers functioned differently in hematopoietic cells of various lineages. The intronic enhancer was very active in myelocytic leukemia HL60 cells but only weakly active in erythroleukemia HEL cells. Conversely, the 3’-enhancer is very active in HEL cells but only weakly active in HL60 cells.

**EXPERIMENTAL PROCEDURES**

Cell Culture—K562 cells, a human cell line with erythroleukemia characteristics, and derived from a patient with BC-CML (CCL243; American Type Culture Collection (ATCC), Rockville, MD); HEL cells, a human erythroleukemia cell line (TIB180, ATCC); HL60 cells, a human myelocytic leukemia cell line (CCL240, ATCC); and CEM cells, a human T-cell acute lymphoblastic leukemia cell line (CCL119, ATCC), were maintained in RPMI 1640 medium containing 10% fetal calf serum. HeLa, a human cervical carcinoma cell line (CCL2, ATCC) and 293 cells, derived from adenovirus 5-transformed human embryonic kidney cells (CRL1573, ATCC) were grown in Eagle’s minimal essential medium supplemented with 10% fetal calf serum (18); a rat melanoma cell line, were maintained in Dulbecco’s minimal essential medium/F-12 supplemented with insulin, transferrin, selenium, and hydrocortisone. Saos-2 cells, a human osteosarcoma cell line (HTB85, ATCC), were grown in McCoy’s 5a medium with 10% fetal calf serum.

**Construction of WT1 Enhancer Plasmids**—The cosmid cB5-2 containing WT1 5’-flanking sequence and the first three introns (16) was digested with BamHI and the 6.4-, 3.4-, 2.4-, 1.0-, and 1.5-kilobase (kb) fragments were separately inserted into the WT1 promoter-chloramphenicol acetyltransferase (CAT) construct, pCB.7PH (16) to generate plasmids pCB.7e6.4, pCB.7e3.4, pCB.7e2.4, pCB.7e1.0, and pCB.7e1.5, respectively (Fig. 1A). The cosmid cB5-2 was also cleaved with BglII, and the resulting 3.0-kb BglII enhancer fragment was cloned into pCB.7PH to generate plasmid pCB.7e3.0 (Fig. 1A). To verify the promoter independence of the 1.5-kb enhancer fragment, the 1.5-kb BamHI fragment from pCB.7e1.5 was also subcloned into pCAT®-Promoter to generate pCAT®-e258 (Promega Corp.), which contains the simian virus (SV40) promoter, to generate pCATP1.5 (Fig. 1B). To localize the enhancer region within the 1.5-kb fragment, pCATP1.5 was cut with SphI to release a 678-bp SphI fragment, and the remaining plasmid, containing the 833-base pair (bp) SphI/BamHI fragment was religated to generate pCAT®-p33. The 678-bp SphI fragment was gel purified and then subcloned into pCAT®-Promoter to generate pCATP678. Further deletion within the 833-bp SphI/BamHI fragment was performed by partial digestion with HindIII. A 575-bp HindIII fragment was released from pCAT®-p33 to produce a 258-bp HindIII/BamHI fragment that remained in the digested pCAT®-Promoter construct pCAT®-p258. The pCAT®-p258 construct which contains the 258-bp enhancer fragment, was sequenced (Fig. 2).

The 258-bp HindIII/BamHI enhancer fragment was amplified by polymerase chain reaction (PCR). The 258-bp intron enhancer fragment was PCR amplified using the pCAT®-p258 plasmid as template with the 5′-prime primers T1e258A (5′-GATGCCGAGCTCCTGCAGTGGGGAAC-3′) and T1e258B (5′-GCTCGAGGATCCCTACAATTGTTGG-3′), which contains an introduced BamHI site, and the 3′-vector primer CPB (5′-GATCTAGATTCTGTCGTCGGCCTG-3′); digested with BamHI; and subcloned into pCB.7PH in both orientations to create pCB.7mGATA-Ae258. The sequence of each PCR product was then PCR amplified with primers WT1e258A and pCB.7e258, respectively.

**Transfections and CAT Assays**—K562 cells were transfected by electroporation using a modification of the protocol of Chou et al. (19) adapted to other cell lines (5×10^4 cells) with 0.5 μg of plasmid DNA, or 50 μg of expression vector DNA. The 5′ end of the enhancer (Fig. 2), but no potential GATA-binding sites in the 104-bp I fragment was gel purified and then subcloned into pCAT®-Promoter to generate pCAT®-p1.5 (Fig. 1B). To verify the promoter independence of the 1.5-kb enhancer fragment, the 1.5-kb BamHI fragment from pCB.7e1.5 was also subcloned into pCAT®-Promoter (Promega Corp.), which contains the simian virus (SV40) promoter, to generate pCATP1.5 (Fig. 1B). To localize the enhancer region within the 1.5-kb fragment, pCATP1.5 was cut with SphI to release a 678-bp SphI fragment, and the remaining plasmid, containing the 833-base pair (bp) SphI/BamHI fragment was religated to generate pCAT®-p33. The 678-bp SphI fragment was gel purified and then subcloned into pCAT®-Promoter to generate pCATP678. Further deletion within the 833-bp SphI/BamHI fragment was performed by partial digestion with HindIII. A 575-bp HindIII fragment was released from pCAT®-p33 to produce a 258-bp HindIII/BamHI fragment that remained in the digested pCAT®-Promoter construct pCAT®-p258. The pCAT®-p258 construct which contains the 258-bp enhancer fragment, was sequenced (Fig. 2).

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**Nuclear Extract Preparations and Electrophoretic Mobility Shift Assays**—Crude nuclear mini-extracts were prepared from exponentially growing K562 cells according to the method of Schreiber et al. (25) as modified by Aggarwal et al., (26). EMSAs were performed in 10-μl reaction mixtures containing 15 μg Tritc (pH 7.5), 6.5% glycerol, 90 μM KCl, 0.7 μM EDTA (pH 8.0), 0.2 mM dithiothreitol, 1 mM MgCl2, 90 μM bovine serum albumin, 0.25 μg of poly(dI-dC), 4 μg of K562 nuclear extract, and 50–100 fmol (5×10^4 cpm) of the double-stranded probe, GATA-A (5′-GGCCCGGGCGCCCTGCAGTGGGTG-3′) and GATA-A (5′-GGCCCGGGCGCCCTGCAGTGGGTG-3′), respectively. For analysis of the 258-bp enhancer fragment (Fig. 3), the most 152-bp fragment was generated by PCR amplification using the 258-bp enhancer fragment as template with primers T1e258A and T1e258B (5′-GCGGCCGATCCCTGTCGTCGGCCTG-3′) and CPB (5′-GCGGCCGATCCCTGTCGTCGGCCTG-3′). The 258-bp enhancer fragment was PCR amplified using the pCAT®-p258 plasmid as template with primers T1e258A and T1e258B (5′-GATGCCGAGCTCCTGCAGTGGGGAAC-3′) and T1e258B (5′-GATGCAG ATTCTGTCGTCGGCCTG-3′). The most 152-bp fragment was generated by PCR amplification using the 258-bp enhancer fragment as template with primers T1e258A and T1e258B (5′-GCGGCCGATCCCTGTCGTCGGCCTG-3′) and CPB (5′-GCGGCCGATCCCTGTCGTCGGCCTG-3′). The 258-bp enhancer fragment was PCR amplified using the pCAT®-p258 plasmid as template with primers T1e258A and T1e258B (5′-GCGGCCGATCCCTGTCGTCGGCCTG-3′) and CPB (5′-GCGGCCGATCCCTGTCGTCGGCCTG-3′).
Santa Cruz, CA) were added to each reaction mixture first. After incubation with the K562 nuclear extracts for 15 min at room temperature, the end labeled probe was added and the mixture was incubated an additional 15 min at room temperature and analyzed as described above. For antibody ablation experiments, 0.5 and 1.0 μg of anti-c-Myb rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-GATA-1 rat monoclonal antibody (Santa Cruz Biotechnology), anti-GATA-2 goat polyclonal antibody (Santa Cruz Biotechnology), or anti-GATA-3 mouse monoclonal antibody (Santa Cruz Biotechnology) were added before the addition of labeled probe and incubated for 15 min at room temperature. After the addition of labeled probe, the reaction mixture was incubated for an additional 15 min at room temperature and analyzed as described above.

Isolation of Nuclei and DNase I Digestion—Nuclei were isolated essentially as described by Levy-Wilson et al. (27). Suspension cells and confluent monolayers from the K562 and HeLa cells were harvested by centrifugation at 2,000 rpm. The cell pellet was washed in 20 ml of reticulocyte standard buffer (RSB)/sucrose (10 mM Tris, pH 7.5, 10 mM NaCl, 1 mM MgCl2, 0.25 mM sucrose) containing 0.1 mM phenylmethylsulfonyl fluoride by gentle homogenization with a Dounce homogenizer, followed by a low-speed spin as described above. Cells were suspended in 3 volumes of RSB/sucrose/phenylmethylsulfonyl fluoride and lysed by the addition of Nonidet P-40 at a final concentration of 0.5% for 5 min on ice. The nuclear pellet was recovered by centrifugation at 4,000 rpm. Nuclei were washed once with RSB/sucrose/phenylmethylsulfonyl fluoride, and their integrity and purity were checked by light microscopy after they had been stained with trypan blue (0.4%).

Intact nuclei were suspended at a DNA concentration of 1 mg/ml in RSB/sucrose/phenylmethylsulfonyl fluoride. DNase I (10 units/μl, Boehringer Mannheim) were added to various final concentrations, and the mixtures were incubated for 10 min at 37°C. The reactions were terminated by the addition of an equal volume of 2× lysis buffer (0.6 M NaCl, 20 mM EDTA, 20 mM Tris hydrochloride, pH 7.5, 1% sodium dodecyl sulfate). RNase A (U. S. Biochemical Corp.) was added at 40 μg/ml for 30 min at 37°C, and then proteinase K was added at 100 μg/ml for 4–6 h at 37°C. DNA was purified by extraction with an equal volume of phenol, phenol/chloroform (1.1, v/v), and chloroform, followed by precipitation with isopropanol alcohol at room temperature. The DNA was recovered by centrifugation, washed once with 70% ethanol, and suspended in 100 μl of H2O.

Gel Electrophoresis and Southern Blotting—Aliquots containing 30 μg of DNA digested as above were digested with both EcoRI and XhoI. DNA was purified by extraction with an equal volume of phenol/chloroform and chloroform followed by precipitation with ethanol at −20°C for 2 h. The DNA was recovered by centrifugation, washed once with 70% ethanol, and suspended in 30 μl of TE. Purified DNA were electrophoresed in 1.2% agarose gels, and transferred by vacuum blotter (Bio-Rad) onto Zeta-Probe® GT blotting membranes (Bio-Rad) according to the manufacturer’s manual. All hybridizations were done according to the manufacturer’s instruction manual. The probe (EcoRI/SphI fragment, Fig. 2B) was 32P-labeled using the Megaprime DNA labeling system kit (Amersham). The filters were exposed to x-ray film for 1–3 days. The sizes of the hybridizing bands appearing in the autoradiogram were determined by using λ HindIII/EcoRI-digested marker DNA fragments as standards in each gel.

RESULTS

Identification of an Intronic Hematopoietic-specific Enhancer—Analysis of distant regulatory elements and intragenic enhancers for WT1 have been hampered by the size of the WT1 gene. We therefore focused on the region often reported to contain regulatory elements: the 3′- and 5′-flanking regions and the first few introns (Fig. 1A). We determined the BamHI and BglII sites in the WT1 genomic sequence in the 5′-flanking region cosmid CB5–2 (Fig. 1A) and subcloned small BamHI fragments from the cosmid into pCB.7PH, in which the CAT gene is under control of the WT1 promoter. Initially, we identified an enhancer element in the region between the 3′ end of exon 2 and 3′ of 5′ end of intron 3 (Fig. 1A). This 1.5-kb enhancer increased the basal transcription from the WT1 promoter by 5.84-fold in the human BC-CML cell line K562 but not in 293 (a human embryonic kidney cell line) or II-14 (a rat mesothelioma cell line), which also expressed WT1 (Fig. 1A). This suggested that the activity of the 1.5-kb fragment was hematopoietic specific. We also screened about 10 kb of the 3′-flanking region of WT1 in 293 and II-14 cells but found no enhancer elements (data not shown).

Deletion Analysis of the Intronic Enhancer—To delineate the enhancer region, the 1.5-kb fragment was subcloned into pCAT®.Promoter to generate pCATp1.5, and the deletion constructs derived from it were transfected into K562 cells (Fig. 1B). We found that the 1.5-kb fragment could enhance the activity of the WT1 promoter, but not the SV40 promoter. This lack of activity can be partially explained by the presence of an SV40 repressor in the 5′-enhancer region in pCATp678 (Fig. 1B). Therefore, we examined the 3′-region (in, pCATpS33), which had strong enhancer activity (4.54 times to that of the SV40 promoter). Further deletion of the 833-bp fragment showed that the most 3′ 258-bp fragment was sufficient for enhancer activity in K562 cells. When the fragment was transfected into K562 cells, it increased the basal transcription level of the SV40 promoter by 3.29 times and had enhancer activity nearly equivalent to that of the SV40 enhancer (data not shown). The 258-bp enhancer was located in WT1 intron 3, approximately 11 kb downstream of the promoter (Fig. 1A). Sequence analysis showed that this 258-bp fragment contains many potential binding sites for transcription factors, including Ets-1, GATA, c-Myb, and AP-2 (Fig. 2). It is possible that one or more of these transcription factors facilitate the expression of WT1 in hematopoietic cells.

Lineage Specificity of the Intronic and 3′-WT1 Enhancers—To determine the tissue specificity of the intronic enhancer, the 258-bp enhancer was cloned into pCB.7PH 3′ of the WT1 promoter to generate the construct pCB.7e258. The 258-bp enhancer increased basal transcription of the WT1 promoter in K562, HL60, and HEL cells but not in 293, HeLa, and CEM cells (Table I). This indicates that the 258-bp enhancer was hematopoietic specific. Although the 258-bp fragment functioned in three hematopoietic cell lines (K562, HL60, and HEL), the enhancer had different degrees of activity in each cell line: it increased the basal transcription levels of the WT1 promoter by 8–9-fold in K562 cells, 5–6-fold in HL60 cells, and only 2–3-fold in HEL cells. This suggested the intronic enhancer had the strongest activity in myeloid-lineage uncommitted progenitor cells.

To compare the tissue specificity of the intronic enhancer and the 3′-enhancer previously characterized by Wu et al. (17), the 3′-enhancer was transfected into HL60 and HEL cells. The 3′-enhancer increased the basal transcription levels of the WT1 promoter 6-fold in HEL cells but only 2-fold in HL60 cells (Table I). This indicates that unlike the intronic enhancer, the 3′-enhancer functioned better in cells with erythroid characteristics (K562 and HEL) than in cells with only myelocytic characteristics (HL60).

Identification of the Minimal WT1 Intronic Enhancer—To determine whether the intronic enhancer can function in an orientation-independent manner, the 258-bp fragment was cloned into pCB.7PH in both the normal and reverse orientations to generate pCB.7e258+ and pCB.7e258−. Both constructs had strong activity (greater than 8-fold activation) in K562 cells (Fig. 3). We also examined the effect of the intronic enhancer on the minimal promoter, independent of the multiple regulatory elements located within the full-length promoter. The 258-bp fragment was subcloned into pCB.1 (17) to generate pCB.1e258, which contains a 104-bp minimal promoter. The intronic enhancer increased the activity of the minimal promoter by 12.1 times (Fig. 3). To define the minimal enhancer region of the 258-bp fragment, we dissected it into four overlapping pieces: the 127-bp 5′-portion, 129-bp middle portion, 154-bp 3′-portion, and 62-bp most 3′-portion. These fragments were subcloned into pCB.7PH to generate
pCB,7e127, pCB,7e129, pCB,7e154, and pCB,7e62, respectively. None of these fragments had strong enhancer activity in K562 and HL60 cells (Fig. 3), indicating either that the 258-bp fragment cannot be further subdivided without total loss of activity or that all the cleavage sites are at positions essential for activity in K562 and HL60 cells.

GATA-1 Bound to the WT1 Intronic Enhancer—
GATA-1 is a zinc-finger transcription factor believed to play an important role in gene regulation during the development of erythroid cells (28). A computer search for transcription factor-binding sites in the intronic enhancer revealed potential GATA-1 and c-Myb binding sites located near the transcription start site of the WT1 gene.

**Fig. 1. Identification of a tissue-specific enhancer of the WT1 gene.** A, restriction map of the 5'-flanking region and the first three exons (indicated by solid boxes labeled E1, E2, and E3) and introns. Bg, BgII; N, NotI; and B, BamHI. One of the major transcription start sites is indicated by an arrow. The location and sizes of each of the fragments tested are marked. Enhancer activity was tested in hematopoietic (K562), kidney (293), and mesothelial (II-14) cells. CAT activity of each of the fragments is expressed relative to that of pCB.7PH, which contains the WT1 promoter but no enhancers. ND, not determined.

**Fig. 2. Sequence of the 258-bp intronic enhancer of WT1.** Potential transcription factor-binding sites are underlined. GATA-binding sites are located at bp 108 (A), 207 (B), and 254 (C), and a c-Myb binding site is located 10 bp 3' of the GATA-A site.

**Table I**

| Cell line | Expression of a | Enhancer activity b |
|-----------|-----------------|---------------------|
|           | WT1             | GATA-1              | Intronic | 3' |
| K562      | Yes             | Yes                 | +++++    | +  |
| HEL       | Yes             | Yes                 | +        | +  |
| HL60      | Yes             | No                  | +++      | +/−|
| CEM       | Yes             | No                  | −        | −  |
| 293       | Yes             | No                  | −        | −  |
| HeLa      | No              | No                  | −        | −  |

a Reverse transcriptase PCR analysis of RNA from cell lines; yes, PCR products visible by ethidium bromide staining of agarose gels; no, PCR products not visible by ethidium bromide staining.

b CAT activity of enhancer relative to that of the 652-bp WT1 promoter. +++++, 8–9-fold; ++++, 5–6-fold; +++, 2–3-fold; ++++, 1.5–2.0-fold; +/-, <1.5-fold.
Adjacent GATA-1 and c-Myb Motifs in the Intrinsic WT1 Enhancer

Fig. 3. Deletion analysis of the 258-bp intronic enhancer. A series of deletion constructs was made to determine the essential region of the 258-bp intronic enhancer. The full-length WT1 promoter is shown by two open boxes flanking a shaded box (the 104-bp WT1 minimal promoter). GATA-binding sites are shown by filled circles. The 258-, 127-, 129-, 154-, and 62-bp fragments were generated by PCR amplification as described in the text and subcloned into pCB.7PH. The orientation of the fragments relative to the WT1 promoter is shown by arrows. The 258-bp fragment was also subcloned into pCB.1 containing the 104-bp minimal WT1 promoter alone (16), to generate pCB.1e258. The enhancer activity was tested in erythroid (K562) and myeloid (HL60) cells. CAT activity of each construct is expressed relative to that of the empty vector (pCB.7PH or pCB.1). ND, not determined.

| WT1 promoter | CAT reporter | Intronic enhancer | Size (bp) | K562 | HL60 |
|--------------|-------------|------------------|----------|------|------|
| WT1 pro      |             |                  |          | 1.00 | 1.00 |
| mini         | CAT         |                  | 258      | 8.70 | 6.05 |
|              | CAT         |                  | 258      | 8.60 | ND   |
|              | CAT         |                  | 127      | 1.29 | 0.76 |
|              | CAT         |                  | 129      | 1.58 | 1.21 |
|              | CAT         |                  | 154      | 1.10 | 2.73 |
|              | CAT         |                  | 62       | 1.75 | 1.06 |
|              | CAT         |                  | 258      | 12.10| ND   |

The non-consensus GATA-1-binding sites were previously identified as GATA-binding sites by oligonucleotide selection methods (29, 30). To determine whether GATA-1 can bind the WT1 site in the intronic enhancer, we performed EMSAs. K562 nuclear extracts were incubated with the end-labeled probe, GATA-A, which contains the most 5′-GATA motif in the intronic enhancer (Fig. 4A). Three major complexes were seen, complexes 1, 2, and 3 (Fig. 4B, lane 2). They were all diminished when 50 and 150 times molar excesses of unlabeled probes were added before the incubation of the radiolabeled probe (Fig. 4B, lanes 3 and 4). This suggested that these complexes were specific for the GATA-A probe; however, they might not be GATA specific. To determine which complex represents binding to the GATA-A motif, additional competition experiments were performed. Excess of the competitor mGATA-A (Fig. 4A), which contains the mutant 108-bp GATA motif, was added before incubation with the probe. We found that none of the complexes could be competed by mGATA-A (Fig. 4B, lanes 5 and 6), indicating that all of the complexes contained proteins which bound preferentially to the GATA-A probe. To determine whether the GATA-A motif binds GATA protein with the same affinity as the GATA consensus (GATA con) sequence (Fig. 4A), 50- and 150-fold molar excesses of double-stranded GATA consensus competitors were added before the incubation with the radioactive probe. We found that both complexes 2 and 3 were eliminated by the competition with GATA consensus oligonucleotide (Fig. 4B, lanes 7 and 8), suggesting that both of these complexes contained GATA-binding proteins but that complex 1 did not. To verify that complex 1 did not contain GATA-binding proteins, mGATA-A was radiolabeled and incubated with K562 nuclear extracts. The GATA-specific complexes 2 and 3 were not formed, only complex 1 was seen (Fig. 4C, lane 4). Overall, this indicates that complex 1 forms on a region of the oligonucleotides distinct from the GATA sites, thus contains proteins which are not GATA-binding proteins. The inability of the mGATA to compete off complex 1 when it is bound to the GATA-A oligonucleotide indicates it has a higher affinity to GATA-A than to the mGATA-A, possibly signifying cooperative interaction between the GATA complexes and complex 1.

GATA-1 Transactivation of the WT1 Intronic Enhancer—To determine whether GATA-1 can also transactivate the intronic enhancer, we cotransfected the WT1 reporter construct containing the 258-bp WT1 intronic enhancer (with three GATA sites) and the minimal WT1 promoter (with no GATA-binding sites) with the mouse GATA-1 expression vector in HeLa cells (which do not express endogenous GATA-1 protein). In cotransfection assays using 5.0 μg of the WT1 reporter construct, pCB.1e258 and increasing amounts of a mouse GATA-1 cDNA expression construct (23), a dose-dependent increase in enhancer activity was observed (Fig. 5). Four micrograms of the mouse GATA-1 expression construct produced a 15-fold increase in WT1 promoter activity in HeLa cells and also transactivated pCB.1e258 in Saos-2 and 293 cells by 10-fold (data not shown).

GATA-A Is Responsible for the Enhancer Activity—There are three non-consensus GATA motifs in the 258-bp enhancer located at positions 108, 207, and 254. Of them, the GATA-A motif is most similar to the GATA consensus motif. Therefore,
we mutated this site by altering 2 bp (GA to CT) within the binding site by PCR amplification. The CAT reporter construct containing the WT1 promoter and intronic enhancer with the mGATA-A motif, pCB.7mGATA-Ae258, was transfected into K562, HL60, and HEL cells. The mutagenized enhancer (pCB.7mGATA-Ae258) was 60% less active in K562 and HL60 cells and 50% less active in HEL cells than the wild type enhancer (pCB.7e258) (Fig. 6).

Additive Effects of the Two Enhancers—Because both enhancers function in K562 cells, we examined whether activation by both the intronic and the 3′-enhancers has an additive or synergistic effect on the WT1 promoter in K562 cells. The 3′-enhancer was linked to the 258-bp intronic enhancer to generate the dual enhancer construct pCB.7e3⁹eint, which was then tested in K562 cells (Fig. 7). The construct containing both enhancers was much more active than the constructs that contained either enhancer alone and was slightly more active than the additive value of the two enhancers (Fig. 7). No synergistic or additive effects were observed in HL60 cells (data not shown), which is not surprising as the 3′-enhancer does not function in this cell line (Table I).

c-Myb Transactivation of the Intronic Enhancer—Because

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**Fig. 4. Electrophoretic mobility shift assay of the intronic enhancer.** A, the sequences of the GATA-A, mutant GATA-A (mGATA-A), and GATA consensus (GATA con) oligonucleotides are shown. GATA motifs are underlined. Asterisks indicate 2-bp mismatches. B, nuclear extracts of K562 cells were bound to radiolabeled oligonucleotides GATA-A and unlabeled competitors GATA-A, mGATA-A, and GATA con. Complexes 1, 2, and 3 are indicated by arrows. C, nuclear extracts of K562 cells were bound to radiolabeled GATA-A or mGATA-A. Complexes are labeled as for B. D, K562 nuclear extracts were incubated with c-Myb, GATA-1, -2, and -3 antibodies before binding to radiolabeled GATA-A oligonucleotides. Complexes are labeled as for B.
the intronic enhancer was very active in HL60 cells which lack GATA-1, we asked whether transcription factors other than GATA-1 are responsible for enhancer activity in HL60 cells. By computer analysis, we found a c-Myb-binding site in the intronic enhancer fragment (Fig. 2). High-level expression of c-myb in normal cells occurs primarily in immature hematopoietic progenitor cells of various lineages (31). The c-myb proto-oncogene is also expressed in both acute and chronic leukemias, and its expression overlaps the expression of WT1. Therefore, we tested c-Myb for its ability to transactivate the 258-bp intronic enhancer containing one c-Myb site. We transfected Saos-2 cells with 5.0 μg of CAT reporter pCB.1e258 (which contains the WT1 minimal promoter and the intronic enhancer) and 8.0 μg of the empty vector, wild type c-Myb expression vector, or a mutant c-Myb (lacking the DNA-binding domain) expression vector. We found that the wild-type c-Myb construct transactivated the reporter construct pCB.1 e258 nearly 5-fold more than the empty vector, while the mutant c-Myb did not significantly transactivate pCB.1 e258 (Fig. 8).

**DISCUSSION**

Here we described the identification of a second hematopoietic-specific enhancer of the WT1 gene. This enhancer was both position- and orientation-independent and was capable of increasing basal transcription levels from both the SV40 and WT1 promoters in K562 cells. Although both this enhancer and the previously isolated 3′-enhancer were hematopoietic-specific, activating transcription in K562 cells, they differed in lineage specificity. The 3′-enhancer was very active in HEL cells with erythroid characteristics, whereas the intronic enhancer was very active in HL60 cells with myeloid characteristics. K562 cells are multipotent leukemic cells that have both myeloid and erythroid characteristics. K562 cells are multipotent leukemic cells that have both myeloid and erythroid characteristics and can differentiate into erythroid and megakaryocytic cells in vitro (14). HEL cells are erythroleukemic cells that can also be induced to differentiate along erythroid and megakaryocytic pathways (32). In contrast, HL60 cells are promyelocytic leukemia cells and can be induced to differentiate into monocytes and neutrophils (15). Interestingly, WT1 expression decreases after treatment of K562 and HL60 cells with differentiation inducers (14, 15). While both T- and B-cell leukemias express WT1 (11, 13), neither the 3′ nor the intronic enhancer was active in CEM cells, a T-cell leukemia cell line. We have not yet determined whether these enhancers function in cell lines of B-cell origin, but our results suggest that these two enhancers lack T-cell-specific WT1 regulatory enhancer elements.

The fact that GATA-1 is present in almost all erythroid-specific gene promoters suggests that in K562 and HEL cells, GATA-1 plays an important role in the activation of the WT1 promoter, possibly by directly contacting the basal transcriptional machinery and influencing the frequency of initiation. Two GATA-binding sites have also been found in the WT1 promoter, and GATA-1 transactivation increased the promoter activity 10-fold. 2 We hypothesize that interaction between GATA-binding proteins may help establish contact between the WT1 promoter and the two enhancers.

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2 G. C. Fraizer and G. F. Saunders, unpublished results.
Because c-Myb could also transactivate the intron enhancer and the c-Myb and the GATA-A motifs are only 10 bp apart (Fig. 2), it is possible that GATA-1 and c-Myb can form a functional complex and facilitate transcription. It has been reported that both GATA-1 and c-Myb can form heterodimers with other transcription factors. For example, GATA-1 activity can be enhanced by forming dimers or heterodimers with either Sp1 or erythroid Krüppel-like factor (33, 34). It has also been found that c-Myb and the core-binding factor act synergistically but bind independently to adjacent sites in the T-cell receptor γ-enhancer (35).

Although we showed that both GATA-1 and c-Myb could transactivate the intron enhancer, we cannot rule out the possibility that other transcription factors may also modulate the enhancer activity. In the EMSAs (Fig. 4C), we found that both complexes 2 and 3 could be eliminated by GATA-2 antibody, indicating that GATA-2 is probably involved. Consistent with these data, the activity of the intron enhancer with the mutant GATA-A motif was decreased by 60% in HL60 cells, which have no endogenous GATA-1 (Table I) but have other GATA-binding proteins, for example, GATA-2. Recently, we found that GATA-2 also could transactivate this enhancer by 8-fold in 293 cells.

FIG. 7. Additive effect of the two enhancers in K562 cells. The 258-bp intronic enhancer sequence was cloned into pCB.7e3', which contains the WT1 promoter and the 258-bp 3'-enhancer (17), to generate pCB.7e3'eint. The WT1 promoter and the GATA sites are depicted as in Fig. 3. The activities of the 3'-enhancer, the intronic enhancer, and both enhancers together were compared with that of pCB.7PH which contains just the WT1 promoter. One representative CAT assay is shown with the average percentage conversion of acetylated [14C]chloramphenicol from three different experiments listed on the right.

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It is important to establish the physiological role of this enhancer. Previously, we showed that (a) mature leukocytes in normal blood expressed low levels of WT1 and GATA-1, whereas the normal CD34+ bone marrow hematopoietic progenitors expressed a significant amount of WT1 and GATA-1 (36); (b) leukocytes from acute myelogenous leukemia patients are expressed a significant amount of both WT1 and GATA-1 (36). This suggests that the intronic enhancer may be responsible for the high expression of WT1 in normal bone marrow

3 X. Zhang and G. F. Saunders, unpublished results.
and acute myelogenous leukemia patients.

It is essential to determine whether GATA-1, c-Myb, or other transcription factors bind to the 258-bp minimal enhancer region in its natural context. We examined the DNase I-hypersensitive site in the 1.7-kb EcoRI/XbaI region which contains the 258-bp intronic enhancer. One hypersensitive site was mapped to the 258-bp enhancer region in K562 cells, but not in HeLa cells. This result not only confirmed our previous transfection data but also indicated that the transcription factors bind to the 258-bp minimal enhancer region in K562 cells, and not in HeLa cells. One hypersensitive site was sensitive in the 1.7-kb region in its natural context. We examined the DNase I-hypersensitive site in the 258-bp enhancer region in K562 cells, but not in HeLa cells.

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