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Identification and Characterization of the Hematopoietic Cell-Specific Enhancer-Like Element of the Mouse *Hex* Gene

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***Hex* is one of the homeobox genes suggested to be important for hematopoietic cell differentiation. However, its biological function and mechanism of transcriptional regulation in hematopoietic cells remain elusive. We have identified the regulatory region necessary for transcription of the mouse *Hex* gene in K562 leukemia cells through transient reporter assays involving various deletion mutants. This region, comprising +775 to +1177 in the first intron, had enhancer-like properties and showed high activity in other hematopoietic cell lines such as U937, HEL, and RAW264.7, but little activity in other *Hex*-expressing cell lines such as MH_1C_1 and H4IIE hepatoma cells, suggesting that this region functions as a hematopoietic cell-specific enhancer-like element. Binding site mutation of hematopoietic transcription factors, such as GATAs and c-Myb present in the enhancer-like element, significantly decreased the luciferase reporter gene expression in K562 cells. Electrophoretic mobility shift assays showed that GATA-1, GATA-2, or c-Myb actually binds to three of these putative binding sites, and also suggested that several unidentified factors might interact with the enhancer-like element. Overexpression of GATA-1, GATA-2, or c-Myb stimulated the enhancer-like activity *via* these three binding sites. Thus, we conclude that *Hex* expression in hematopoietic cells is mainly regulated by GATA-1, GATA-2, and c-Myb *via* this intronic enhancer-like element.**

Key words: c-Myb, GATA, hematopoiesis, Hex, transcriptional regulation.

Abbreviations: E, embryonic age; EMSA, electrophoretic mobility shift assay; M-PK, M-type pyruvate kinase; PMA, phorbol 12-myristate 13-acetate; TK, thymidine kinase.

Homeobox genes belong to an important gene family that encodes transcription factors which can regulate tissue morphogenesis or cell differentiation during development (*1*). These proteins are distinguished by a highly conserved 60 amino acid homeodomain motif which can mediate sequence-specific binding to DNA. The hematopoietically expressed homeobox (*Hex*) gene is also known as the proline-rich homeobox (*Prh*), as its N-terminal is rich in proline residues. *Hex* is a divergent homeobox gene and its cDNA has been isolated from several animal species (*2–8*). The genomic organization of *Hex* has been previously determined and it has also been shown that Hex protein functions as a transcriptional repressor, its repressor domain being located in the N-terminal region (amino acids 45–136) from the homeodomain (amino acids 137–196) (*7*, *9*). On the other hand, the function of Hex as a transcriptional activator has also been reported (*10*). The molecular basis for these dual functions of Hex remains elusive.

Hex expression has been observed in the visceral endoderm in the distal region of the egg cylinder at embryonic age (E) 5.5, developing thyroid, liver, and lung during murine development (*11*, *12*), as well as in several adult tissues including liver, lung, and spleen (*2–4*). Our gene targeting study has shown that a *Hex*-deficiency in mice resulted in embryonic lethality around E10.5, due to a lack of substantial liver formation, as shown by the absence of *albumin* expression (*13*). As for hematopoiesis, monocyte differentiation was impaired in mutant embryos whereas erythroid- and mixed-colony formation were unaffected. These results indicate that *Hex* is essential for early embryonic liver development and also for differentiation of the monocyte lineage. Another group has reported that *Hex* is essential for normal development of the forebrain and thyroid gland in addition to the liver, based upon their targeting study (*14*).

Hex is also expressed in a range of multipotent hematopoietic progenitor cells and restricted cell lineages, namely in B-cell but not T-cell lineages. In addition, the *Hex* transcript is generally reduced during terminal hematopoietic cell differentiation (*15*). This expression pattern suggests that *Hex* may be involved in the early stages of hematopoietic cell differentiation by acting as a transcriptional regulator. In fact, when Hex was overexpressed in Myb-Ets transformed chicken blastoderm cells, which exhibit many characteristics of multipotent hematopoietic cells, its transformation and/or proliferation were inhibited (*16*). In addition, a gene targeting

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Table 1. **Oligonucleotide sequences used for site-directed mutation and EMSAs.** These are antisense oligonucleotides designed with the Künkel method. Mutated bases are underlined. Some of these oligonucleotides were also used for EMSAs after annealing to complementary sense oligonucleotides.

Element (position)	Oligonucleotide sequence (5′ to 3′)
mG-1 (+856 to +879)	GATTTACTCCTGCAGAGAACTCAA
mG-2 (+954 to +982)	GTCTGTATTGCCCCGGGAAATAAAATAAT
mG-3 (+995 to +1020)	GGATCCTGGCTGCAGGACGTCGAGTG
mG-4 (+1018 to +1043)	GAAATGAACACTGCAGGAAAAACGGA
mG-5 (+1111 to +1136)	CAGCCTCCAACTGCAGGGGTGGGGGG
mM-1 (+828 to +853)	TTTCCTGTATGTCGACAAAGGGTCAG
mM-2 (+1005 to +1034)	AATATCAGAACCCGGGATCCTGGCTATCAG

study revealed that Hex is necessary for B-cell development and function (*17*). However, its transcriptional role and gene regulating mechanism in hematopoiesis have never been established.

Recently, Denson *et al*. reported that hepatocyte nuclear factor 3β and GATA-4 transactivated the *Hex* expression in HepG2 hepatoma cells (*18*). We have also shown that the 5′-flanking region up to –199 contains four GC boxes necessary for high promoter activity in MH_1C_1 hepatoma and K562 leukemia cells, and ubiquitously expressed transcription factors, Sp1 and Sp3, bind to these boxes to stimulate transcription in both cell types (*19*). To determine the tissue specificity of this –199 promoter, we produced transgenic mice which express enhanced green fluorescent protein under the control of the *Hex* –199 promoter. However, it turned out that this promoter region was not responsible for hematopoietic- or hepatic-specific expression of *Hex* (our unpublished results). On the other hand, Rodriguez *et al*. showed, using a transgenic approach, that the dynamic patterns of *Hex* expression during early murine development were regulated by a number of distinct *cis*-acting elements, and suggested that a blood island enhancer was present between positions +56 and +3045 (referred to as +27 and +3016 downstream of the *Hex* ATG in their report) (*20*). However, further identification of the possible hematopoietic enhancer has never been conducted, since their study focused on an anterior visceral endoderm/anterior definitive endoderm enhancer. All these findings strongly suggest that hematopoietic cell-specific transcriptional regulatory elements are present in addition to the promoter region previously reported.

In the present study, we have identified a hematopoietic cell-specific enhancer-like element located at +775/+1177 in *Hex* gene intron 1. This enhancer-like element spans about 400 bases and contains several binding sites for hematopoietic transcription factors such as GATA-1, GATA-2, and c-Myb, which are essential for *Hex* expression in hematopoietic cells.

MATERIALS AND METHODS

Cell Culture—K562 (human chronic myelogenous leukemia), U937 (human promonocytic leukemia), and HEL (human erythroleukemia) cell lines were maintained in RPMI1640 (Nissui Pharmaceuticals, Tokyo). RAW264.7 (mouse macrophage-like leukemia), MH_1C_1 (rat hepatoma), H4IIE (rat hepatoma), and HeLa (human cervical carcinoma) cell lines were maintained in Dulbecco's modified Eagle's medium (Nissui Pharmaceuticals). Both media were supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), 0.4% (v/v) amphotericin B (Sigma, St Louis, MO, USA), and 10% (v/v) fetal bovine serum (Sigma), and all cell lines were maintained in a 37°C/5% CO_2 humidified incubator. RAW264.7 cells were obtained from the RIKEN Cell Bank (Ibaraki), and other cell lines from the American Type Culture Collection (Manassas, VA, USA).

Plasmids—All restriction and modification enzymes, and polymerases were obtained from New England Biolabs (Beverly, MA, USA). We cloned various mouse *Hex* genomic fragments and determined their sequences previously (*9*). *Hex* promoter-luciferase plasmid pHluc67, which contains the –67/+46 fragment of the mouse *Hex* gene at the *Bgl*II site of pGL3-Basic vector (Promega, Madison, WI, USA), was previously described (*9*). Construction of deletion reporter constructs involved the ligation of various *Hex* genomic fragments into the *Sma*I site of pHluc67. The *Apa*I/*Hin*cII fragment of *Hex* intron 1 (+544/+2317) was inserted into the *Sma*I site of pHluc67 to generate pH(544/2317)luc67. Similarly, the construction of pH(544/1453)luc67, pH(1454/2317)luc67, pH(1016/1453)luc67, pH(775/1177)luc67, pH(808/1453)luc67, and pH(892/1071)luc67 involved the ligation of the *Apa*I/*Hin*cII (+544/+1453), *Hin*cII/*Hin*cII (+1454/+2317), *Bam*HI/*Hin*cII (+1016/+1453), *Ava*I/*Ava*I (+775/+1177), *Hin*dIII/*Hin*cII (+808/+1453), and *Alu*I/*Alu*I (+892/+1071) fragments into pHluc67, respectively. Fragments with overhangs were filled-in using *Klenow* fragment before ligation into the *Sma*I site of pHluc67.

The putative 403 bp *Ava*I/*Ava*I enhancer-like fragment, +775/+1177, was inserted into the *Sma*I (upstream from the *Hex* –67 promoter) or *Sal*I (downstream from the luciferase gene) site of pHluc67 in both the forward and reverse orientations to create pH(+E403)luc67, which is similar to pH(775/1177)luc67, pH(–E403)luc67, pHluc67(+E403), and pHluc67(–E403). The pTKluc reporter vector was generated by ligating the 164 bp herpes simplex virus thymidine kinase (TK) promoter fragment into the *Bgl*II site of pGL3-Basic. pTK(+E403)luc contains the 403 bp putative enhancer-like fragment ligated into the *Sma*I site of pTKluc.

For the generation of various mutation constructs, site-directed mutagenesis was performed according to the Künkel method with some modifications (*21*). The blunt-ended 403 bp putative enhancer-like fragment was subcloned into the *Sma*I site of the pBluescript®II-SK+ vector (Stratagene, La Jolla, CA, USA). Single-stranded

Table 2. **Oligonucleotide sequences used for EMSAs.** The sense strand sequences are shown. Lowercase letters indicate *Eco*RI enzyme site linker sequences.

Element (position)	Oligonucleotide sequence (5′ to 3′)
G-2 (+957 to +982)	gatcATTTTATCGACATCCTCAATACAGAC
G-3 (+995 to +1020)	gatcCACTCGACGTCTGATAGCCAGGATCC
M-1 (+828 to +853)	gatcCTGACCCTTTCCGTTCATACAGGAAA
GATA-consensus	CACTTGATAACAGAAAGTGATAACTCT
c-Myb-consensus	TACAGGCATAACGGTTCCGTAGTGA

template DNA prepared in *Escherichia coli* strain CJ236 was separately annealed to phosphorylated mutagenic oligonucleotides, each putative binding site being replaced with a *Pst*I, *Sma*I, or *Sal*I enzyme site (Table 1). The complementary DNA strands were synthesized with T4 DNA polymerase and T4 DNA ligase. The resulting heteroduplex DNAs were used to transform *Escherichia coli* strain DH5α. Each mutated putative enhancer-like fragment derived from these constructs was ligated into the *Sma*I site of pHluc67. GATA-1 and GATA-2 expression plasmids, pEF-mGATA-1 and pEF-mGATA-2, and GATA-positive control reporter plasmid pRBGP3-Mαp (*22*) were generously provided by Dr. Masayuki Yamamoto (Tsukuba University, Ibaraki). c-Myb expression plasmid pact-c-myb was generously provided by Dr. Shunsuke Ishii (The Institute of Physical and Chemical Research, RIKEN, Ibaraki). All plasmids were prepared with a Plasmid Midi-kit (Qiagen, Valencia, CA, USA) and confirmed by DNA sequencing.

Transfection and Luciferase Assay—K562 cells were transfected with 2 μg of various firefly luciferase reporter plasmids and 40 ng of pRL-SV40 *Renilla* luciferase expression plasmid (Promega), as an internal control, using DMRIE-C Reagent (Invitrogen) according to the manufacturer's protocol. U937, HEL, and RAW264.7 cells were transfected with 1 μg of various firefly luciferase reporter plasmids and 0.1 μg of *Renilla* luciferase expression plasmid using SuperFect® Transfection Reagent (Qiagen) according to the manufacturer's protocol. Transfection of MH_1C_1, H4IIE, and HeLa cells was performed by the calcium phosphate method (*23*). Cells were seeded at 60–80% density on 6- or 12-well plates for 24 h before transfection. Various reporter plasmids and a 1/50 amount of pRL-SV40 were transfected into each cell line and the medium was changed at 5 h post-transfection. In the case of overexpression assays of HeLa cells, various amounts of effector plasmids were co-transfected and the total amount of DNA was adjusted by the addition of the corresponding empty vector. Cells were harvested after 48 h (for MH_1C_1, H4IIE, and HeLa) or 24 h (for other cell lines) to determine luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega). The firefly luciferase activity of the reporter plasmids was normalized as to the *Renilla* luciferase activity of pRL-SV40.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Assay (EMSA)—K562 nuclear extracts were prepared as previously described (*24*). EMSAs were performed as previously described (*25*) with some modifications. The oligonucleotides used in EMSAs are listed in Table 1 and 2. Double-stranded oligonucleotides were 5′ end-labeled with [γ-^{32}P]ATP (3,000 Ci/mmol, Amersham Biosciences, Little Chalfont, Bucks., UK) by T4 polynucleotide kinase (New England Biolabs). Ten microgram aliquots of K562 nuclear extracts were incubated for 15 min at room temperature with 0.5 ng of ^{32}P-labeled probe (>5 × 10^5 cpm) in a reaction mixture comprising 15 mM Tris-HCl (pH 7.5), 6.5% glycerol, 50 mM KCl, 0.7 mM EDTA (pH 8.0), 0.2 mM dithiothreitol, 1 mg/ml bovine serum albumin, 4% Ficoll, 1 μg of poly(dI-dC), and 0.2 μg of salmon sperm DNA. For competition assays, a 200-fold molar excess of unlabeled oligonucleotides was first added to the binding mixture. For supershift assays, 2 μg of anti-GATA-1 rat monoclonal antibodies (N6, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-GATA-2 mouse monoclonal antibodies (CG2-96), or anti-c-Myb mouse monoclonal antibodies (C-2) was added to the binding mixture before the addition of the ^{32}P-labeled probe, followed by pre-incubation for 15 min at room temperature. After the binding reaction, the mixture was subjected to electrophoresis on a 4–6% polyacrylamide gel (19:1, acrylamide: *N,N′*-methylenebis-acrylamide) in 0.5× Tris borate/EDTA buffer at 200 V for 1 h. The gel was dried before exposure to a phosphorimaging plate (Fuji Film, Tokyo).

Statistics—The relative luciferase activity values represent the means ± SEM for at least more than three independent experiments. Statistical significance was determined by means of Student's *t*-test or Welch's *t*-test.

RESULTS

Identification of the 403 bp Hematopoietic Regulatory Region in Hex Intron 1—Based on a recent report on a putative *Hex* blood enhancer which spans a 3 kb region (*20*), we focused on the *Hex* intron 1 region, as it is part of the reported region and also because introns often contain regulatory elements. *Hex* intron 1 spans about 1.9 kb and thus the 1.8 kb *Apa*I/*Hin*cII (+544/+2317) fragment containing most of the intron 1 sequence was used for further analysis (Fig. 1A). To determine whether or not this region contains regulatory elements, the 1.8 kb fragment was inserted upstream of pHluc67, and the resultant plasmid, pH(544/2317)luc67, was subsequently transfected into various cell lines. We chose K562 cells as a model of multipotent hematopoietic cells since they highly express *Hex* and can differentiate into progenitors of the erythrocytic, granulocytic, and monocytic series (*26*). In addition, we used three other cell lines as controls, MH_1C_1 and H4IIE hepatoma cells, both of which express *Hex*, and HeLa cells, which do not express *Hex*. The pHluc67 activity was very low in all cell lines tested. However, the 1.8 kb region increased the basal transcription level of pHluc67 by 3.8-fold in K562 cells, but not in MH_1C_1 or H4IIE hepatoma cells (Fig. 1B), or HeLa cells (data not shown). This result suggests that the 1.8 kb region may contain regulatory elements with hematopoi-

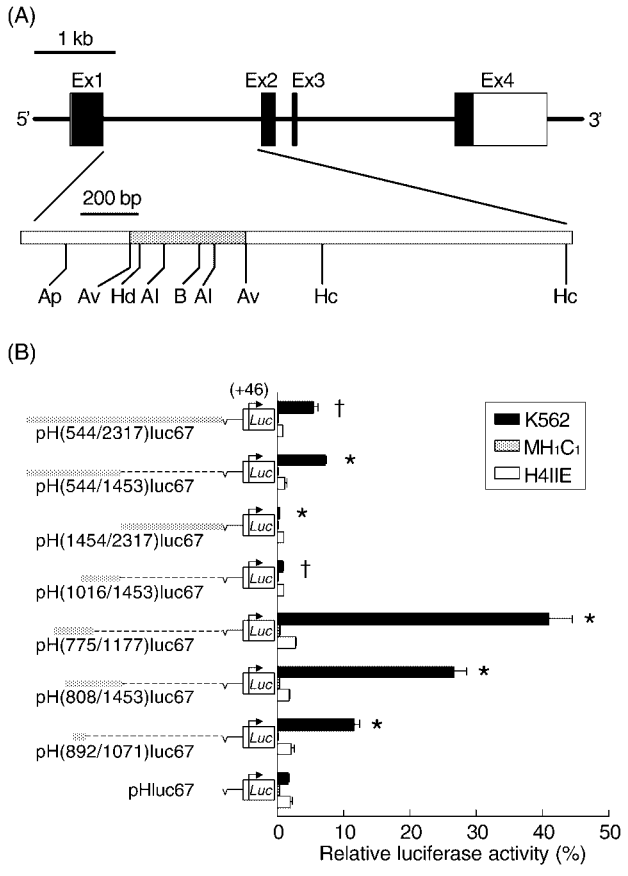

Fig. 1. **Identification of a hematopoietic cell-specific regulatory region of the *Hex* gene.** (A) Genomic structure of the mouse *Hex* gene. Exons are represented by boxes. Solid and open boxes represent coding and non-coding regions, respectively (upper panel). A restriction map of the *Hex* intron 1 is also shown and the putative regulatory region is indicated by a shaded box (lower panel). The restriction enzyme sites are indicated: Al, *Alu*I; Ap, *Apa*I; Av, *Ava*I; B, *Bam*HI; Hc, *Hin*cII; Hd, *Hin*dIII. (B) Expression of *Hex*-luciferase fusion genes in various cell lines. Various deleted *Hex* intron 1 gene fragments were ligated to pHluc67, which showed almost no promoter activity. The deletion constructs, shown on the left, were transfected into K562 (solid bars), MH_1C_1 (shaded bars), and H4IIE (open bars) cells. All firefly luciferase activities were normalized as to *Renilla* luciferase activity of pRL-SV40. The relative luciferase activities of the deletion constructs are expressed as percentages of that of pGL3-Control. The results are the means ± SEM for more than three independent experiments. *$p < 0.001$ and †$p < 0.01$ indicate significant changes in reporter gene expression of each deletion construct compared with pHluc67 in K562 cells. The straight thin and shaded thick lines indicate the *Hex* –67 minimum promoter sequence and *Hex* intron 1 sequence, respectively. The notches represent the sequence of the polylinker site of pGL3-Basic. *Luc*, firefly luciferase gene.

etic cell-specific activity. Up to 6.5 kb upstream of the *Hex* 5′-flanking region was also examined in K562, MH_1C_1, and H4IIE cells using luciferase reporter assays, but no regulatory elements for hematopoietic-specific activity were found (data not shown).

To define the critical region for this activity, various reporter deletion mutants of pH(544/2317)luc67 were generated and transfected into K562, MH_1C_1, and H4IIE cells (Fig. 1B). Interestingly, pH(775/1177)luc67 containing the 403 bp *Ava*I/*Ava*I (+775/+1177) fragment (shaded box in Fig. 1A) exhibited the strongest activity in K562 cells, corresponding to 41% that of the pGL3-Control vector (Fig. 1B). Further deletion of the 403 bp region, pH(892/1071)luc67, or other constructs containing various parts of the 403 bp region resulted in reduction of the reporter activity. In contrast, deletion mutant reporters such as pH(1454/2317)luc67 and pH(1016/1453)luc67 caused reduced activity, which may indicate the presence of repressor elements in these regions. No activity was found for all constructs in HeLa (data not shown), MH_1C_1, and H4IIE (Fig. 1B) cells when compared to pHluc67.

Table 3. **The activity of the 403 bp region in several hematopoietic cell lines.** The activity of the 403 bp region observed in K562 cells was also examined in several other hematopoietic cell lines. The results are the means ± SEM for more than three independent experiments. *$p < 0.001$ and ‡$p < 0.05$ indicate significant changes in reporter gene expression of pH(775/1177)luc67 compared to pHluc67.

Cell line	Relative luciferase activity	
	pHluc67	pH(775/1177)luc67
K562	1.00	27.1 ± 2.35*
U937	1.00	6.58 ± 0.31*
HEL	1.00	7.95 ± 1.19‡
RAW264.7	1.00	9.76 ± 0.43*

To further confirm that the 403 bp region is a hematopoietic cell-specific regulatory element, its activity was also analyzed in several hematopoietic cell lines other than K562, such as U937, HEL, and RAW264.7, which are derived from different species or lineages. The expression of *Hex* in U937 and HEL cells has been reported (*15*), and we also detected its expression in RAW264.7 cells using the reverse transcriptase-polymerase chain reaction (data not shown). When pH(775/1177)luc67 was transfected into these cells, the 403 bp region significantly activated the reporter gene expression in all hematopoietic cell lines tested when compared to pHluc67 (Table 3). However, its activation in K562 cells was higher than that in these three cell lines. Thus, we used K562 cells for further analyses. All the results suggest that the 403 bp region (+775/+1177) within *Hex* intron 1 is a hematopoietic cell-specific regulatory element.

Functional Analyses of the 403 bp Hex Regulatory Element—To determine the function of the 403 bp region as an enhancer, it was inserted either upstream or downstream of pHluc67, and in both the forward and reverse orientations. All constructs containing the 403 bp region in various orientations and positions significantly activated the reporter gene expression when compared to pHluc67, although the activation levels differed (Fig. 2A). Highest activation occurred when the 403 bp region was positioned upstream of the promoter and in the forward orientation, whereas this region showed weak but significant activation when placed downstream in the reverse orientation. Similar effects were observed when the 403 bp fragment was linked to pHluc199 containing the –199/+46 *Hex* promoter region and previously shown to exhibit high promoter activity (*19*) (data not shown). To determine whether or not the 403 bp region can function in the context of a heterologous promoter, this region was

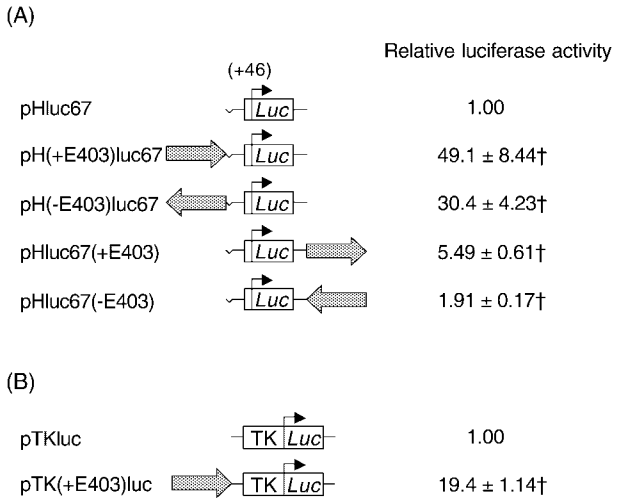

Fig. 2. **Positional and orientational effects and promoter independence of the *Hex* regulatory element.** (A) The putative 403 bp regulatory fragment was ligated upstream of the *Hex* –67 promoter or downstream of the luciferase gene in both the forward and reverse orientations. (B) The putative 403 bp regulatory fragment was ligated upstream of the heterologous TK promoter. The constructs, shown on the left, were transfected into K562 cells. All firefly luciferase activities were normalized as to *Renilla* luciferase activity of pRL-SV40. The relative luciferase activities of the various constructs are expressed relative to that of pHluc67 (A) or pTKluc (B). The results are the means ± SEM for more than three independent experiments. †$p < 0.01$ indicates significant changes in reporter gene expression of each construct compared with pHluc67 (A) or pTKluc (B). The putative regulatory element and its orientation are indicated by shaded arrows.

inserted upstream of the heterologous TK promoter. This resulted in an increase in reporter activity by about 20-fold (Fig. 2B). These results indicate that the 403 bp region of *Hex* intron 1 has enhancer-like properties, although it does not function like a typical enhancer. Thus, we refer to this region as an enhancer-like element.

GATA-1, GATA-2, and c-Myb Are Involved in the Enhancer-Like Activity—A search for transcription factor binding sites within the 403 bp *Hex* intron 1 region by computer analysis (TFSEARCH ver.1.3) (*27*) revealed many potential binding sites for GATAs and c-Myb, both of which are important for hematopoiesis. The 403 bp enhancer-like element of *Hex* contains five GATA and two c-Myb putative binding sites, designated as G-1, G-2, G-3, G-4, G-5, M-1, and M-2, respectively (Fig. 3). To determine whether or not these binding sites are responsible for the hematopoietic cell-specific enhancer-like activity, binding site mutation analyses were carried out on K562 cells. All mutations of pH(+E403)luc67 resulted in significant reduction of the reporter gene expression except for the G-4 and G-5 mutations (mG-4 and mG-5), which caused no significant changes (Fig. 4). The G-2 mutation (mG-2) resulted in a 91% reduction in transcriptional activity when compared to the wild-type pH(+E403)luc67, whereas the G-1, G-3, M-1, and M-2 mutations (mG-1, mG-3, mM-1, and mM-2) resulted in 49, 41, 44, and 65% reduction, respectively. These results suggest that several binding sites at which mutation influenced reporter gene expression could be responsible for the enhancer-like activity.

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+775  CCGGGCGGTTCCTGGGCTGGGCTTGGTTCAACAAG
+810  CTTGTGCAGTGAAGGAGCCTGACCCTTTCCGTTCA
                                 c-Myb (M-1)
+845  TACAGGAAATCTTGAGTTCTCGATAGGAGTAAATC
                          GATA (G-1)
+880  TGGTTTCTGAAGCTGTTAAGTGTTTTCCTGGCTGC
+915  ATGAAGCAGACCCTTCCCCCGGAGTAGTGCACGCT
+950  GCTGATTATTTTATCGACATCCTCAATACAGACAA
                     GATA (G-2)
+985  ATTCAGGAAACACTCGACGTCTGATAGCCAGGATC
                               GATA (G-3)
+1020 CGTTTTTCTGATATTGTTCATTTCCTCTGTGTGGG
     c-Myb (M-2)  GATA (G-4)
+1055 ATGTGACTTTATGGCAGCTAAAATAACCAGTTGC
+1090 TTCCTCTCTGCCTCTCCCGCCACCCCCCACCCCCAT
                                    GATA (G-5)
+1125 CTTTGGAGGCTGTTTTGCACCAAGTCTGAAGCCTG
+1160 ACGCAAGCAAAATCTCGG
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Fig. 3. **Nucleotide sequence of the 403 bp enhancer-like element of the mouse *Hex* gene.** The sequence of the 403 bp enhancer-like element isolated by *Ava*I digestion that was used in this study is shown. Position +1 refers to the transcription start site. The GATA and c-Myb putative binding sites are underlined, and designated as G-1, G-2, G-3, G-4, G-5, M-1, and M-2, respectively.

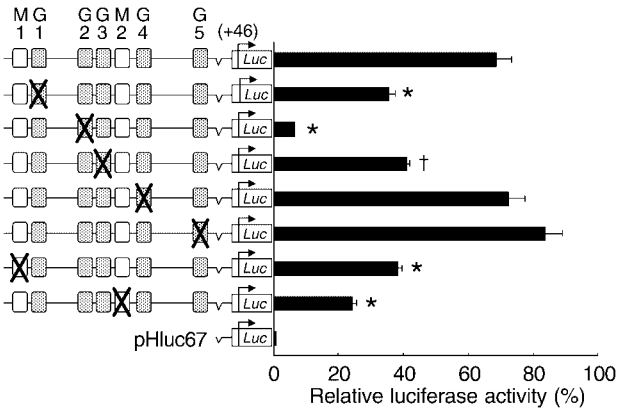

Fig. 4. **Effects of mutation at the GATA and c-Myb putative binding sites within the *Hex* enhancer-like element in K562 cells.** Various reporter plasmids with site-directed mutations of pH(+E403)luc67 to individually disrupt the five GATA and two c-Myb putative binding sites were constructed as described in the Materials and Methods section, and then transfected into K562 cells. The relative luciferase activities of these constructs are expressed as percentages of that of pGL3-Control after normalization as to *Renilla* luciferase activity. The results are the means ± SEM for more than three independent experiments. *$p < 0.001$ and †$p < 0.01$ indicate significant changes in reporter gene expression of each mutated construct compared with pH(+E403)luc67. The straight lines indicate the sequence of the *Hex* gene. Shaded and open boxes represent the GATA and c-Myb putative binding sites, respectively.

GATA-1 and GATA-2 Interact with the 403 bp Enhancer-Like Element in K562 Cells—EMSAs involving K562 nuclear extracts were performed to determine whether or not GATAs and c-Myb can bind to the sites tested above. When the end-labeled G-1 probe was incu-

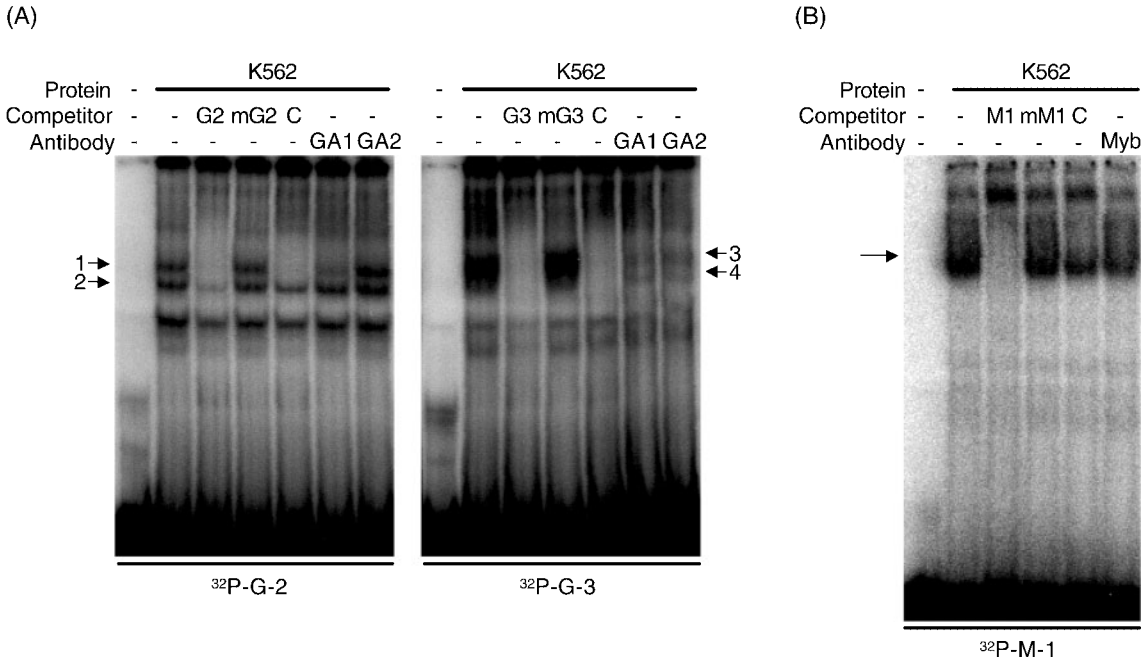

Fig. 5. **Electrophoretic mobility shift assay of the *Hex* enhancer-like element.** Double-stranded oligonucleotides, G-2, G-3 (A), and M-1 (B), were 5′-end-labeled with [γ-^{32}P]ATP and then incubated with 10 μg of K562 nuclear extract for 15 min. A 200-fold molar excess of the unlabeled competitor DNA or 2 μg of specific antibodies indicated was preincubated with nuclear extracts for 15 min prior to the addition of the probe DNAs. Protein-DNA complexes were separated on a 6% (A) or 4% (B) polyacrylamide gel. The arrows indicate the positions of the major specific protein-DNA complexes. Probe DNAs are indicated at the bottom, and the probe and competitor DNA sequences are shown in Tables 1 and 2. G2, G-2; mG2, mG-2; G3, G-3; mG3, mG-3; C, consensus; M1, M-1; mM1, mM-1; GA1, anti-GATA-1 antibody; GA2, anti-GATA-2 antibody; Myb, anti-c-Myb antibody.

bated with K562 nuclear extracts, no specific binding was observed under the conditions used (data not shown), although it was shown that the G-1 site might also be involved in the transcriptional activation by the reporter gene assays with K562 cells (Fig. 4). This might be the result of the mG-1 sequence that affected the transcriptional activation by unknown endogenous factors other than GATAs in K562 cells.

In contrast, when the end-labeled G-2 and G-3 probes were used, two major specific complexes were formed (Fig. 5A). Complex 1 formed using the G-2 probe, and complexes 3 and 4 formed using the G-3 probe decreased when a 200-fold molar excess of the GATA-consensus oligonucleotide, as well as each unlabeled probe oligonucleotide, was added to the binding mixture before the incubation with the end-labeled probe oligonucleotide. The formation of complex 2 with the G-2 probe was slightly inhibited by the addition of the GATA-consensus oligonucleotide, but was greatly decreased with the G-2 unlabeled probe oligonucleotide. This suggests that complex 2 was formed with a non-GATA-binding site within the G-2 probe oligonucleotide. Both oligonucleotides containing mutations at the putative GATA binding site, mG-2 and mG-3, which have the same mutated sequence as the reporter gene constructs used in Fig. 4, failed to compete in the formation of protein complexes with each end-labeled probe. When GATA-1-specific antibodies were added to the reaction mixture prior to the reaction with the end-labeled probe, formation of complex 1 with the G-2 probe and complexes 3 and 4 with the G-3 probe was prevented. Formation of complexes 3 and 4 with the G-3 probe was prevented on the addition of GATA-2-specific antibodies, but not that of complex 1 with the G-2 probe. The addition of M-type pyruvate kinase (M-PK) antibodies as non-specific antibodies did not affect the complexes (data not shown). Neither GATA-1- or GATA-2-specific antibodies prevented the formation of complex 2 with the G-2 probe. This further indicates that complex 2 did not contain GATA-1 or GATA-2. All these results suggest that GATA-1 can bind to the G-2 site, and that both GATA-1 and GATA-2 can bind to the G-3 site under the binding conditions used.

c-Myb Can Also Interact with the 403 bp Enhancer-Like Element in K562 Cells—Similar EMSA analyses were performed for the putative c-Myb binding sites found within the 403 bp region. When the end-labeled M-1 probe was used, one major specific complex was formed (Fig. 5B). There was competition with this complex by the M-1 unlabeled probe oligonucleotide, but not by the mM-1 oligonucleotide containing a mutation at the putative c-Myb binding site. The c-Myb-consensus oligonucleotide and also c-Myb-specific antibodies partly prevented formation of the complex. However, the addition of M-PK antibodies did not affect the complex formation (data not shown). These results suggest that c-Myb interacts with the putative binding site within the 403 bp enhancer-like region but that other unknown proteins may also be involved in the formation of the complex.

No specific binding to the end-labeled M-2 probe was observed (data not shown), although the initial reporter gene assay results with K562 cells suggest involvement of this site. However, like the G-1 site, the M-2 mutation

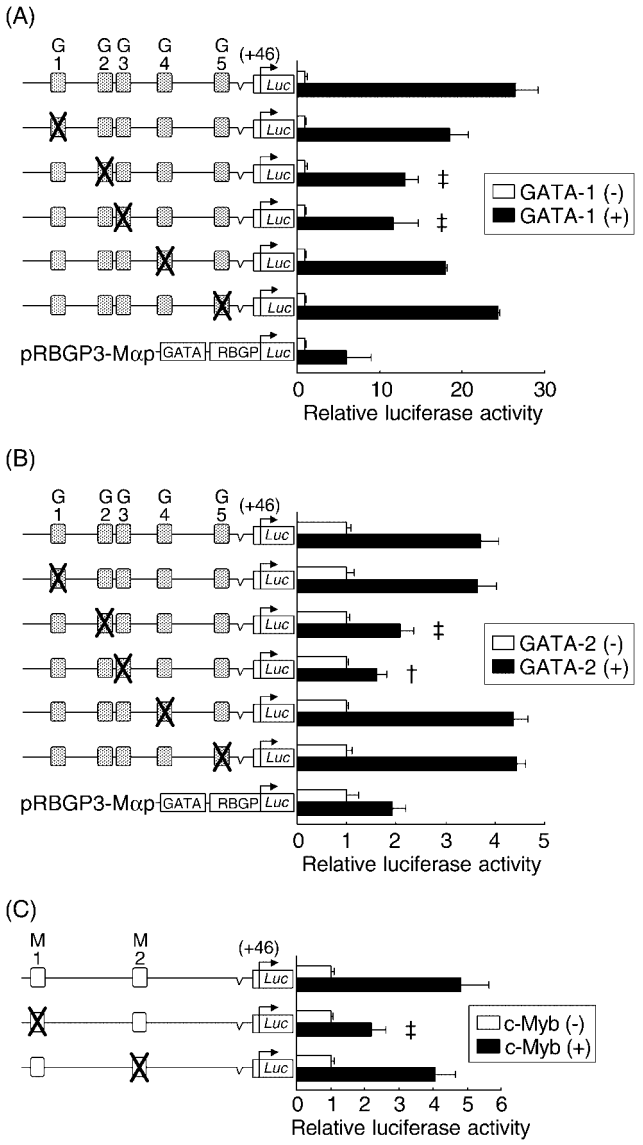

Fig. 6. **GATA-1, GATA-2, and c-Myb can transactivate the *Hex* enhancer-like element.** Effects of mutation in the GATA or c-Myb putative binding sites within the 403 bp *Hex* enhancer-like element in HeLa cells. The GATA-1 (A), GATA-2 (B), or c-Myb (C) expression plasmid (0.4 μg, solid bars), or the empty vector (0.4 μg, open bars) was co-transfected with 0.4 μg of the various reporter plasmids shown on the left into HeLa cells. The relative luciferase activities are expressed as the fold number relative to that of each reporter plasmid co-transfected with the empty vector after normalization as to *Renilla* luciferase activity. The activity of the pRBGP3-Mαp reporter plasmid containing the GATA binding sequence is shown as a positive control (A and B). The results are the means ± SEM for more than three independent experiments. †$p < 0.01$ and ‡$p < 0.05$ indicate significant changes in reporter gene expression of each mutated construct compared with pH(+E403)luc67. The GATA and c-Myb putative binding sites, and reporter constructs are depicted as in Fig. 4.

may have affected the transcriptional activation by other unknown endogenous factors in K562 cells.

GATA-1, GATA-2, and c-Myb Activate the 403 bp Hex Enhancer-Like Element through Their Binding in HeLa Cells—To determine whether or not GATA-1, GATA-2, and c-Myb transactivate the *Hex* 403 bp enhancer-like region through their binding sites, co-transfection assays were performed on HeLa cells using mutated reporter constructs, similar to in Fig. 4, together with expression vectors of GATA-1, GATA-2, or c-Myb. As shown in Fig. 6A, expression of GATA-1 resulted in about 26-fold stimulation of the activity of the reporter containing the 403 bp enhancer-like region in HeLa cells. However, when the reporter construct carrying mG-2 or mG-3 was co-transfected with the GATA-1 expression vector into HeLa cells, reporter gene expression was significantly reduced to 49% or 44% compared to in the case of wild-type pH(+E403)luc67, respectively. No significant reduction in the reporter gene expression was observed with the other mutation constructs. These results confirmed that the G-2 and G-3 putative GATA binding sites were critical for the activation of the *Hex* gene by GATA-1 *via* its 403 bp hematopoietic enhancer-like element. The activity of the pRBGP3-Mαp reporter vector containing a known GATA binding site from the mouse α-1 globin gene was used as a positive control (*22*). GATA-1 activated this reporter gene expression by 6.0-fold, which corresponds to 23% of pH(+E403)luc67 induction. These results indicate that the *Hex* 403 bp enhancer-like element is strongly activated by GATA-1.

Similar analyses were also performed with the GATA-2 expression vector (Fig. 6B). GATA-2 expression caused about 3.7-fold stimulation of the activity of pH(+E403)luc67 in HeLa cells. However, when the reporter construct carrying mG-2 or mG-3 was co-transfected with the GATA-2 expression vector into HeLa cells, reporter gene expression was significantly reduced to 56% or 44% compared to that of the wild-type reporter plasmid, respectively. This suggests that GATA-2 also binds to the G-2 site, although this is inconsistent with the EMSA results suggesting that GATA-2 could not bind to the G-2 probe. GATA-2 enhanced the reporter gene expression of pRBGP3-Mαp by 1.9-fold, which corresponds to 52% of pH(+E403)luc67 induction. All these results strongly suggest that both GATA-1 and GATA-2 could activate the *Hex* expression through their binding to the 403 bp hematopoietic enhancer-like element, although the transcriptional activities differ.

The c-Myb expression caused an about 4.8-fold increase in the activity of the wild-type reporter plasmid (Fig. 6C). When co-transfection assays with the mutated reporter constructs were performed, only the reporter construct carrying mM-1 exhibited significantly reduced reporter gene expression, *i.e.* 46% compared to that of wild-type pH(+E403)luc67. This is consistent with the EMSA result that c-Myb could bind only to the M-1 probe. Thus, we have shown that c-Myb, as well as GATA-1 and GATA-2, can activate *Hex* expression in hematopoietic cells through its binding to the 403 bp enhancer-like element.

DISCUSSION

The hematopoietic cell-specific expression pattern of *Hex* suggested that unique regulatory mechanisms in hematopoiesis in addition to its ubiquitous promoter exist (*19*). Here we described the identification of the hematopoietic cell-specific enhancer-like element within *Hex* intron 1. This 403 bp region was shown to possess enhancer-like

properties; it could increase the transcription from the *Hex* promoter regardless of its orientation and position, and also activated the heterologous TK promoter, although the extent of the *Hex* enhancer-like activity was affected by its orientation and position. Thus, the 403 bp region should be active in the normal context of the *Hex* gene, although this has to be further examined. The activity of the 403 bp enhancer-like element was strong in hematopoietic cell lines, especially in K562 cells, but not detected in hepatoma cell lines in spite of *Hex* expression in these cells, and the entire 403 bp region was indispensable for maximum activation. On the other hand, a repressor element(s) seems to exist in the flanking region of the 403 bp element. However, the identity of the putative repressor element(s) remains to be determined.

The Myb-related proteins are a large family of transcription factors. c-Myb recognizes the AACNG sequence, and regulates the growth and differentiation of immature hematopoietic cells (*28*). When *Hex* expression was down-regulated during myelomonocytic differentiation of K562 cells induced by phorbol 12-myristate 13-acetate (PMA), c-*myb* expression was also down-regulated (*15*). We found that the down-regulation of c-*myb* occurred rapidly and dramatically, and was followed by a moderate reduction in *Hex* expression in less than 6 h after PMA treatment (our unpublished results). This expression pattern suggests that c-Myb may regulate the *Hex* gene, and the present study demonstrated that c-Myb binds to the 403 bp hematopoietic enhancer-like element within *Hex* intron 1 to activate its expression (Figs. 5B and 6C). During hematopoietic cell differentiation, some repression mechanisms for the *Hex* enhancer-like element must exist, and there is a possibility that the decrease in c-Myb is involved in the repression of the *Hex* enhancer-like element and subsequent decreased expression of *Hex*. We also tried to determine whether or not the activity of the 403 bp enhancer-like element is down-regulated during PMA-induced differentiation of K562 cells using a similar reporter gene assay approach. However, we failed to detect its down-regulation in this assay system because of the strong influence of PMA on the luciferase assay system used. Further analyses of the relationship between *Hex* down-regulation and this enhancer-like element are now under way.

The GATA transcription factor family, another family that is important for hematopoiesis, consists of GATA-1 to –6, and recognizes consensus sequence WGATAR (W = A or T and R = A or G), but it is known that many nonconsensus sequences also bind GATA proteins with high affinity (*29*, *30*). GATA-1, GATA-2, and GATA-3 are called "hematopoietic GATAs" as they are expressed predominantly in hematopoietic tissues and play important roles in hematopoiesis (*31*, *32*). Among these "hematopoietic GATAs," GATA-1 is essential for the differentiation of erythroid and some myeloid lineages, GATA-2 is crucial for the maintenance and proliferation of immature hematopoietic progenitor cells, and GATA-3 is essential for T-cell development and the nervous system (*33*–*37*). No *Hex* expression is found in the T-cell lineage (*15*), and down-regulation of *Hex* is essential for normal T-cell development (*38*), thus we regarded GATA-3 as not being important for the regulation of *Hex* gene expression, and only examined GATA-1 and GATA-2 in this study.

Initial binding site mutation analyses showed that mutation of the G-2 site (mG-2) within the *Hex* enhancer-like element drastically reduced the transcriptional activity in K562 cells (Fig. 4). However, further analyses involving EMSAs or co-transfection assays did not reveal that the involvement of GATA proteins in the G-2 site is more critical than in the G-3 site (Figs. 5A, and 6, A and B). The EMSA results also indicated the involvement of other factors within the G-2 site (Fig. 5A, complex 2). Therefore, the severe reduction in the activity of the mG-2 reporter is not due to elimination of only GATAs but also some unknown factors.

EMSA analyses involving the end-labeled G-2 or G-3 probe and GATA-2-specific antibodies with K562 nuclear extracts indicated that GATA-2 could only bind to the G-3 probe, i.e. not the G-2 one. This result differs with the finding that the G-2 mutation inhibited activation of the reporter gene by GATA-2 in HeLa cells (Figs. 5A and 6B). The cause of this discrepancy is unclear, but it may just be due to the different cell lines; that is, the results of reporter gene assays or EMSAs involving K562 cells reflect the effects of endogenous factors, whereas those of co-transfection assays involving HeLa cells reflect those of overexpressed exogenous factors. The mutations of the G-1 site (mG-1) and M-2 site (mM-2) caused significant decreases in the activity of the 403 bp region in K562 cells. However, EMSAs failed to show the binding of any protein to these sites. We suspect that the conditions used for EMSAs were not suitable for detecting proteins bound to G-1 and M-2, or that these proteins were simply inactivated during extraction. Our EMSA results indicate that some other factors besides GATA or c-Myb protein interact with the G-2 or M-1 site, respectively. Identification of these proteins is required for further understanding of the mechanism of hematopoietic specific expression of *Hex*.

We also examined the involvement of PU.1, which is an important transcriptional activator for B-cell or myelomonocytic differentiation (*39*), in the *Hex* enhancer-like activity. Co-transfection assays showed that the reporter gene expression of pH(+E403)luc67 was increased by exogenous PU.1 in HeLa cells, but that mutation of the putative binding sites had no effect on its transcriptional activation in either K562 or HeLa cells (data not shown). This suggests that PU.1 may interact with an unknown binding site if it is involved in the *Hex* enhancer-like activity. However, PU.1 is unlikely to be important for the regulation of *Hex* because its expression increases during myelomonocytic differentiation while *Hex* expression dramatically decreases (*40*).

One of the most critical points to be clarified is the down-regulatory mechanism(s) of *Hex* during hematopoietic cell differentiation, as mentioned above. Furthermore, it has recently been reported that Hex is a critical regulator of hemangioblast differentiation into definitive hematopoietic progenitor cells (*41*). Since the hemangioblast is the earliest common mesodermal precursor for hematopoietic and endothelial cells, well-regulated *Hex* expression is thought to be indispensable from an early stage of hematopoiesis. From these perspectives, *Hex* intron 1 containing the hematopoietic enhancer-like element and possible repressor elements is a very important region for analyses in order to reveal the gene regulatory

mechanism of *Hex*. Further analyses of this region will lead to a better understanding of the role of *Hex* in hematopoiesis.

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REFERENCES

1. Levine, M. and Hoey, T. (1988) Homeobox proteins as sequence-specific transcription factors. *Cell* **55**, 537–540
2. Crompton, M.R., Bartlett, T.J., MacGregor, A.D., Manfioletti, G., Buratti, E., Giancotti, V., and Goodwin, G.H. (1992) Identification of a novel vertebrate homeobox gene expressed in haematopoietic cells. *Nucleic Acids Res.* **20**, 5661–5667
3. Bedford, F.K., Ashworth, A., Enver, T., and Wiedemann, L.M. (1993) *HEX*: a novel homeobox gene expressed during haematopoiesis and conserved between mouse and human. *Nucleic Acids Res.* **21**, 1245–1249
4. Hromas, R., Radich, J., and Collins, S. (1993) PCR cloning of an orphan homeobox gene (*PRH*) preferentially expressed in myeloid and liver cells. *Biochem. Biophys. Res. Commun.* **195**, 976–983
5. Newman, C.S., Chia, F., and Krieg, P.A. (1997) The *XHex* homeobox gene is expressed during development of the vascular endothelium: overexpression leads to an increase in vascular endothelial cell number. *Mech. Dev.* **66**, 83–93
6. Ho, C.Y., Houart, C., Wilson, S.W., and Stainier, D.Y.R. (1999) A role for the extraembryonic yolk syncytial layer in patterning the zebrafish embryo suggested by properties of the *hex* gene. *Curr. Biol.* **9**, 1131–1134
7. Tanaka, T., Inazu, T., Yamada, K., Myint, Z., Keng, V.W., Inoue, Y., Taniguchi, N., and Noguchi, T. (1999) cDNA cloning and expression of rat homeobox gene, *Hex*, and functional characterization of the protein. *Biochem. J.* **339**, 111–117
8. Yatskievych, T.A., Pascoe, S., and Antin, P.B. (1999) Expression of the homeobox gene *Hex* during early stages of chick embryo development. *Mech. Dev.* **80**, 107–109
9. Myint, Z., Inazu, T., Tanaka, T., Yamada, K., Keng, V.W., Inoue, Y., Kuriyama, M., and Noguchi, T. (1999) Genomic organization and promoter analysis of a mouse homeobox gene, *Hex*. *J. Biochem. (Tokyo)* **125**, 795–802
10. Denson, L.A., Karpen, S.J., Bogue, C.W., and Jacobs, H.C. (2000) Divergent homeobox gene *Hex* regulates promoter of the Na^+-dependent bile acid cotransporter. *Am. J. Physiol. Gastrointest. Liver Physiol.* **279**, G347–G355
11. Thomas, P.Q., Brown, A., and Beddington, R.S.P. (1998) *Hex*: a homeobox gene revealing peri-implantation asymmetry in the mouse embryo and an early transient marker of endothelial cell precursors. *Development* **125**, 85–94
12. Keng, V.W., Fujimori, K.E., Myint, Z., Tamamaki, N., Nojyo, Y., and Noguchi, T. (1998) Expression of Hex mRNA in early murine postimplantation embryo development. *FEBS Lett.* **426**, 183–186
13. Keng, V.W., Yagi, H., Ikawa, M., Nagano, T., Myint, Z., Yamada, K., Tanaka, T., Sato, A., Muramatsu, I., Okabe, M., Sato, M., and Noguchi, T. (2000) Homeobox gene *Hex* is essential for onset of mouse embryonic liver development and differentiation of the monocyte lineage. *Biochem. Biophys. Res. Commun.* **276**, 1155–1161; erratum: **279**, 739
14. Martinez Barbera, J.P., Clements, M., Thomas, P., Rodriguez, T., Meloy, D., Kioussis, D., and Beddington, R.S.P. (2000) The homeobox gene *Hex* is required in definitive endodermal tissues for normal forebrain, liver and thyroid formation. *Development* **127**, 2433–2445
15. Manfioletti, G., Gattei, V., Buratti, E., Rustighi, A., De Iuliis, A., Aldinucci, D., Goodwin, G.H., and Pinto, A. (1995) Differential expression of a novel proline-rich homeobox gene (*Prh*) in human hematolymphopoietic cells. *Blood* **85**, 1237–1245
16. Jayaraman, P.S., Frampton, J., and Goodwin, G. (2000) The homeodomain protein PRH influences the differentiation of haematopoietic cells. *Leuk. Res.* **24**, 1023–1031
17. Bogue, C.W., Zhang, P.X., McGrath, J., Jacobs, H.C., and Fuleihan, R.L. (2003) Impaired B cell development and function in mice with a targeted disruption of the homeobox gene *Hex*. *Proc. Natl Acad. Sci. USA* **100**, 556–561
18. Denson, L.A., McClure, M.H., Bogue, C.W., Karpen, S.J., and Jacobs, H.C. (2000) HNF3β and GATA-4 transactivate the liver-enriched homeobox gene, *Hex*. *Gene* **246**, 311–320
19. Kikkawa, E., Hinata, M., Keng, V.W., Myint, Z., Sato, A., Yamada, K., Tanaka, T., and Noguchi, T. (2001) Sp family members stimulate transcription of the *Hex* gene *via* interactions with GC boxes. *J. Biochem. (Tokyo)* **130**, 885–891
20. Rodriguez, T.A., Casey, E.S., Harland, R.M., Smith, J.C., and Beddington, R.S.P. (2001) Distinct enhancer elements control *Hex* expression during gastrulation and early organogenesis. *Dev. Biol.* **234**, 304–316
21. Künkel, T.A. (1985) Rapid and efficient site-specific mutagenesis without phenotypic selection. *Proc. Natl Acad. Sci. USA* **82**, 488–492
22. Igarashi, K., Kataoka, K., Itoh, K., Hayashi, N., Nishizawa, M., and Yamamoto, M. (1994) Regulation of transcription by dimerization of erythroid factor NF-E2 p45 with small Maf proteins. *Nature* **367**, 568–572
23. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, *2nd ed.* (Nolan, C., ed.), pp. 16.33–16.36, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York
24. Schreiber, E., Matthias, P., Muller, M.M., and Schaffner, W. (1989) Rapid detection of octamer binding proteins with 'mini-extracts', prepared from a small number of cells. *Nucleic Acids Res.* **17**, 6419
25. Zhang, X., Xing, G., Fraizer, G.C., and Saunders, G.F. (1997) Transactivation of an intronic hematopoietic-specific enhancer of the human Wilms' tumor 1 gene by GATA-1 and c-Myb. *J. Biol. Chem.* **272**, 29272–29280
26. Lozzio, B.B., Lozzio, C.B., Bamberger, E.G., and Feliu, A.S. (1981) A multipotential leukemia cell line (K-562) of human origin. *Proc. Soc. Exp. Biol. Med.* **166**, 546–550
27. Heinemeyer, T., Wingender, E., Reuter, I., Hermjakob, H., Kel, A.E., Kel, O.V., Ignatieva, E.V., Ananko, E.A., Podkolodnaya, O.A., Kolpakov, F.A., Podkolodny, N.L., and Kolchanov, N.A. (1998) Databases on transcriptional regulation: TRANSFAC, TRRD and COMPEL. *Nucleic Acids Res.* **26**, 362–367
28. Mucenski, M.L., McLain, K., Kier, A.B., Swerdlow, S.H., Schreiner, C.M., Miller, T.A., Pietryga, D.W., Scott, W.J., Jr., and Potter, S.S. (1991) A functional c-*myb* gene is required for normal murine fetal hepatic hematopoiesis. *Cell* **65**, 677–689
29. Merika, M. and Orkin, S.H. (1993) DNA-binding specificity of GATA family transcription factors. *Mol. Cell. Biol.* **13**, 3999–4010
30. Ko, L.J. and Engel, J.D. (1993) DNA-binding specificities of the GATA transcription factor family. *Mol. Cell. Biol.* **13**, 4011–4022
31. Evans, T. and Felsenfeld, G. (1989) The erythroid-specific transcription factor Eryf1: a new finger protein. *Cell* **58**, 877–885
32. Yamamoto, M., Ko, L.J., Leonard, M.W., Beug, H., Orkin, S.H., and Engel, J.D. (1990) Activity and tissue-specific expression of the transcription factor NF-E1 multigene family. *Genes Dev.* **4**, 1650–1662
33. Pevny, L., Simon, M.C., Robertson, E., Klein, W.H., Tsai, S.F., D'Agati, V., Orkin, S.H., and Costantini, F. (1991) Erythroid differentiation in chimaeric mice blocked by a targeted muta-

tion in the gene for transcription factor GATA-1. *Nature* **349**, 257–260
34. Takahashi, S., Onodera, K., Motohashi, H., Suwabe, N., Hayashi, N., Yanai, N., Nabesima, Y., and Yamamoto, M. (1997) Arrest in primitive erythroid cell development caused by promoter-specific disruption of the GATA-1 gene. *J. Biol. Chem.* **272**, 12611–12615
35. Tsai, F.Y., Keller, G., Kuo, F.C., Weiss, M., Chen, J., Rosenblatt, M., Alt, F.W., and Orkin, S.H. (1994) An early haematopoietic defect in mice lacking the transcription factor GATA-2. *Nature* **371**, 221–226
36. Pandolfi, P.P., Roth, M.E., Karis, A., Leonard, M.W., Dzierzak, E., Grosveld, F.G., Engel, J.D., and Lindenbaum, M.H. (1995) Targeted disruption of the *GATA3* gene causes severe abnormalities in the nervous system and in fetal liver haematopoiesis. *Nat. Genet.* **11**, 40–44
37. Ting, C.N., Olson, M.C., Barton, K.P., and Leiden, J.M. (1996) Transcription factor GATA-3 is required for development of the T-cell lineage. *Nature* **384**, 474–478
38. Mack, D.L., Leibowitz, D.S., Cooper, S., Ramsey, H., Broxmeyer, H.E., and Hromas, R. (2002) Down-regulation of the myeloid homeobox protein Hex is essential for normal T-cell development. *Immunology* **107**, 444–451
39. Scott, E.W., Simon, M.C., Anastasi, J., and Singh, H. (1994) Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. *Science* **265**, 1573–1577
40. Cheng, T., Shen, H., Giokas, D., Gere, J., Tenen, D.G., and Scadden, D.T. (1996) Temporal mapping of gene expression levels during the differentiation of individual primary hematopoietic cells. *Proc. Natl Acad. Sci. USA* **93**, 13158–13163
41. Guo, Y., Chan, R., Ramsey, H., Li, W., Xie, X., Shelley, W.C., Martinez-Barbera, J.P., Bort, B., Zaret, K., Yoder, M., and Hromas, R. (2003) The homeoprotein Hex is required for hemangioblast differentiation. *Blood* **102**, 2428–2435