

Hematopoietic Expression of HOXB4 Is Regulated in Normal and Leukemic Stem Cells through Transcriptional Activation of the HOXB4 Promoter by Upstream Stimulating Factor (USF)-1 and USF-2

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Abstract

The homeobox genes encode a family of transcription factors that regulate development and postnatal tissue homeostasis. Since HOXB4 plays a key role in regulating the balance between hematopoietic stem cell renewal and differentiation, we studied the molecular regulation of HOXB4 expression in human hematopoietic stem cells. HOXB4 expression in K562 cells is regulated at the level of transcription, and transient transfection defines primary HOXB4 regulatory sequences within a 99-bp 5' promoter. Culture of highly purified human CD34⁺ bone marrow cells in thrombopoietin/Flt-3 ligand/stem cell factor induced HOXB4 3–10-fold, whereas culture in granulocyte/macrophage colony-stimulating factor, only increased HOXB4/luciferase expression 20–50%. Mutations within the HOXB4 promoter identified a potential E box binding site (HOX response element [HXRE]-2) as the most critical regulatory sequence, and yeast one hybrid assays evaluating bone marrow and K562 libraries for HXRE-2 interaction identified upstream stimulating factor (USF)-2 and microphthalmia transcription factor (MITF). Electrophoretic mobility shift assay with K562 extracts confirmed that these proteins, along with USF-1, bind to the HOXB4 promoter in vitro. Cotransfection assays in both K562 and CD34⁺ cells showed that USF-1 and USF-2, but not MITF, induce the HOXB4 promoter in response to signals stimulating stem cell self-renewal, through activation of the mitogen-activated protein kinase pathway. Thus hematopoietic expression of the human HOXB4 gene is regulated by the binding of USF-1 and USF-2, and this process may be favored by cytokines promoting stem cell self-renewal versus differentiation.

Key words: homeobox genes • self-renewal • stem cells • USF • transcription

Introduction

The homeobox genes comprise a family of genes that play a pivotal role in embryonic pattern formation and organogenesis. Over the past several decades, studies in species ranging from *Drosophila* to humans have identified genes in these families as critical to such processes as limb formation, vertebral orientation and sequencing, and formations of whole organs such as the pancreas and spleen. In addition to their critical role in embryonic development, homeobox

genes also appear to direct tissue-specific homeostasis between cellular compartments. In this role homeobox transcription factors, rather than dictating pattern formation which evolves over time, serve to maintain the balance of cellular compartmentalization and/or geometric orientation critical to maintaining tissue homeostasis during cell turnover (1–4).

In hematopoietic stem cell physiology, HOX genes maintain the critical ratio of stem cells to specifically differentiated blood cells throughout life. Studies in several laboratories demonstrated that the expression of several homeobox genes varies with stem cell differentiation (5–8). Of particular interest are the HOXA10, HOXB3, and

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HOXB4 genes, which are abundantly expressed in primitive hematopoietic cells, but then decline with lineage-specific terminal differentiation. Subsequent experiments have confirmed that expression of these genes is directly linked to maintenance of primitive stem cell phenotypes (9, 10). Enforced expression of HOXA10 and B3 in retrovirally transduced mouse hematopoietic cells resulted in a cellular phenotype resembling acute leukemia (11, 12). On the other hand, enforced expression of HOXB4 produced stem cells that have amplified competitive repopulating ability in cotransplant models, although producing more intermediate level progenitor cells per stem cell transplanted (13). These studies suggest that the molecular regulation of homeobox gene expression is a critical nodal point in stem cell self-renewal decisions.

Therefore, we have analyzed the molecular basis of HOXB4 expression in human hematopoietic cells, using both the K562 cell line and normal CD34⁺ human bone marrow cells. Our results demonstrate that HOXB4 expression is strongly regulated by a 99-bp promoter embedded within a larger 5' flanking sequence which is highly conserved among species. In contrast to their regulation in neural and mesodermal development, intronic and 3' sequences appear to have a less dominant role. HOXB4 transcription is induced by the binding of upstream stimulatory factor (USF)¹-1 and USF-2 to these sequences, while binding of microphthalmia transcription factor (MITF) binds but does not induce HOXB4 transcription. These results suggest a model in which transcription factor competition for binding to the HOXB4 promoter influences the self-renewal status of hematopoietic stem cells.

Materials and Methods

HOXB4 Genomic Cloning. A genomic clone containing the human HOXB4 gene was isolated from a human genomic placental library (CLONTECH Laboratories, Inc.) by hybridization with a 256-bp HOXB4 probe provided by Jeffrey Lawrence, M.D. (University of California at San Francisco, San Francisco, CA). The clone was restriction mapped, sequenced, and regions corresponding to the coding sequence and 5' and 3' noncoding sequences were identified. The complete genomic nucleotide sequence was submitted to EMBL/GenBank/DBJ, and is available under accession no. AF307160. Digested subclones were cloned into a luciferase reporter vector, PGL3 (Promega).

Mutagenesis. Mutations were introduced into specific clone sequences by site-directed mutagenesis using the Altered Sites II Mutagenesis system (Promega). All mutations were confirmed by DNA sequence analysis. For the experiments described, the NF1 site in the transcriptionally active HOXB4 promoter was mutated

from ATGGCT to AACCCT, and the E box was mutated from CACGTG to CATATG.

Cell Preparation and Culture. K562, U937, and KG1a cells (American Type Culture Collection) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (GIBCO BRL), 10 U/ml penicillin, and 10 µg/ml streptomycin.

CD34⁺ cells from human bone marrow were harvested from healthy donors, according to a protocol approved by the University of Pennsylvania Medical School Institutional Review Board. Mononuclear cells were isolated by centrifugation over Ficoll-Hypaque, and CD34⁺ cells were isolated by adherence depletion followed by immunoselection using an MS⁺/RS⁺ Minima column (Miltenyi Biotec). The selected cells were >95% CD34⁺ upon reanalysis by flow cytometry. For the analysis of HOXB4 constructs after electroporation, these enriched cells were incubated for 48 h in IMDM supplemented with 10% human umbilical cord serum (HUCS) plus 10 U/ml penicillin and 10 µg/ml streptomycin. Cells were cultured in 100 ng/ml Flt-3 ligand (Flt-3l), 100 ng/ml stem cell factor (SCF), and 100 ng/ml thrombopoietin (TPO) to promote proliferation with stem cell self-renewal, or in 20 ng/ml GM-CSF to promote proliferation with terminal myeloid maturation. Cultured cells were stained with PE-anti-CD34 and PE-anti-CD11b to assay for the persistence of primitive progenitor cells and the appearance of differentiated myeloid cells.

Northern Analysis. K562 cell cultures were supplemented with 10⁻⁷ M PMA (Sigma-Aldrich). PolyA⁺ RNA was isolated with Trizol (GIBCO BRL) followed by affinity purification using an Oligotex mRNA kit (QIAGEN). 2.5 µg of PolyA⁺ RNA was electrophoresed on a formaldehyde-formamide agarose gel and transferred onto nylon membrane. The mRNA was analyzed for levels of HOXB4 expression by hybridization with a 1,262-bp HOXB4 probe radiolabeled with [α -³²P]dCTP (Amersham Pharmacia Biotech) via random priming. The radiolabeled probe and the mRNA blot were hybridized in 50% formamide, 0.12 M Na₂HPO₄, pH 7.2, 0.25 M NaCl, and 7% SDS (Bio-Rad Laboratories) at 42°C overnight.

Nuclear Run-on. Nuclei were isolated from 25 × 10⁶ K562 cells that were induced to proliferate and differentiate towards monocytes by addition of 10⁻⁷ M PMA. The nuclei were transcribed with 100 µCi [α -³²P]UTP (Dupont/NEN Life Science Products) for 1 h at 30°C with shaking. To retrieve the newly transcribed radiolabeled RNA, the nuclei were treated with 40 µg of RNase-free DNase I (Roche) and 200 µg of proteinase K. The RNA was extracted with phenol/chloroform/isoamyl alcohol and then separated on a G-50 Sephadex column. The radiolabeled RNA was hybridized in 50% formamide, 0.12 M Na₂HPO₄, pH 7.2, 0.25 M NaCl and 7% SDS to 10 µg of DNA encoding the 3' untranslated HOXB4 cloned into the PCI vector, or 10 µg of control PCI vector alone, which were cross-linked to a Zeta Probe membrane (Bio-Rad Laboratories) for 24 h at 42°C.

5' Rapid Amplification of cDNA Ends. A modification of 5' rapid amplification of cDNA ends (RACE) (Takara Biomedicals) was performed using HOXB4-specific primers for reverse transcription, followed by a circularization by RNA ligase. Amplification was performed in two rounds using specifically designed oligonucleotide primers. Fragments were cloned and sequenced.

Plasmids. USF-1 cDNA was obtained as described previously (14). Dominant negative (D/N) USF and D/N MITF clones were constructed as described previously (15). The D/N USF contains a deletion of amino acids 132–212 in the USF basic region, whereas the D/N MITF consists of a TFE3 encoding plas-

¹Abbreviations used in this paper: DMSO, dimethyl sulfoxide; D/N, dominant negative; EGFP, enhanced green fluorescent protein; EMSA, electrophoretic mobility shift assay; HOX, homeobox; HLH, helix loop helix; HUCS, human umbilical cord serum; HXRE, HOX response element; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; MITF, microphthalmia transcription factor; PI3K, phosphatidylinositol 3-kinase; RACE, rapid amplification of cDNA ends; SCF, stem cell factor; TPO, thrombopoietin; USF, upstream stimulatory factor.

mid containing a deletion in amino acids 89–107, also in the basic region. These deletions destroy the DNA binding activities of these proteins without impairing their ability to dimerize with their cognate cellular proteins (USF-1 or USF-2 for D/N USF, MITF or TFE3 for D/N MITF). The clone encoding constitutively active RAS V12, in pCDNA3, was a gift from Dr. Len Stephens (Babraham Institute, Cambridge, UK). A D/N mitogen-activated protein kinase (MAPK) kinase (MEK)1 K97A construct was subcloned into pcDNA3zeo, and D/N phosphatidylinositol 3-kinase (PI3K) (p85 Δ 478–513) was cloned into pCMV5, as described previously (16). The soluble MEK inhibitor PD98059 (Parke Davis) was added at 100 μ M in DMSO as described previously (17).

Transient Transfection. K562 cells were grown to $0.5\text{--}1 \times 10^6$ cells/ml, washed, and then transfected with 10–20 μ g of DNA by the DEAE-dextran method as described previously (18) or by electroporation. Aliquots of 10^7 cells were washed once in complete medium (RPMI 1640 plus 10% fetal bovine serum plus penicillin/streptomycin), resuspended to 10^7 cells/300 μ l in complete medium and then transferred to a 0.4-cm electroporation cuvette (Bio-Rad Laboratories). A total of 10–20 μ g of DNA was added and the cuvette was electroporated at 200 V, 950 μ F (Gene Pulser; Bio-Rad Laboratories). After 10 min, the cells were plated in complete medium at 0.5×10^6 cells/ml. 2 μ g of either pSVBgal or PCH110, encoding β -galactosidase, was included in each transfection to normalize transfection efficiency. 36–48 h after transfection, K562 cells were lysed using Reporter Lysis buffer (Promega) and the protein concentrations were measured (DC Protein Assay; Bio-Rad Laboratories). Luciferase assays with equal amounts of protein were performed using Luciferase substrate (Promega) and a luminometer (Monolight 2010; Analytical Luminescence Laboratories), with β -galactosidase assays performed on all samples to control for transfection efficiency.

CD34⁺ cells were stimulated with Flt-3l (100 ng/ml)/SCF (100 ng/ml)/TPO (100 ng/ml) (R & D Systems) for 72 h before electroporation. For each sample, 2×10^6 cells were washed, resuspended in 400 μ l of electroporation media, and then transferred to a 0.4-cm electroporation cuvette (Bio-Rad Laboratories). 30 μ g of experimental DNA and 1 μ g of a renilla luciferase control vector (Promega) were added and the sample was electroporated at 240 V, 950 μ FD (Gene Pulsar; Bio-Rad Laboratories). After 10 min, the electroporated cells were plated at $1\text{--}2 \times 10^6$ cells/ml and cultured for 48 h in Flt-3l/SCF/TPO, or in GM-CSF. The cytokine-stimulated CD34⁺ cells were then lysed (Promega) and luciferase activity was measured. To control for electroporation efficiency within each sample, the renilla luciferase control was measured by quenching the luciferase luminescence and simultaneously activating the sea pansy renilla activity. For immunofluorescence studies, promoter constructs were subcloned into Pgreen Lantern (GIBCO BRL) in place of the CMV promoter. Promoter activation was detected by immunofluorescence 48 h after electroporation.

Stable HOXB4 Promoter K562 Transforms. K562 cells were grown to $0.5\text{--}1.0 \times 10^6$ cells/ml, washed, and then transfected with 20 μ g of either the native 99-bp HOX promoter cloned into PGL3 (Promega), the HOX response element (HXRE)-1 mutated HOXB4 promoter/PGL3, HXRE-2 mutated HOXB4 promoter/PGL3, or PGL3 alone, along with 1 μ g PCMV-script-neo by electroporation as stated above. 48 h after transfection, the neomycin-resistant K562 cells were selected with 1 mg/ml G418 (GIBCO BRL) for 7 d. Luciferase assays with equal amounts of protein were performed as per the manufacturer's instructions us-

ing luciferase substrate and a Monolight 2010 Luminometer. To control for position effects within individual clones, all experiments were performed with pooled, selected cells.

Electrophoretic Mobility Shift Assays. A 99-bp (extending from –153 to –55 from the ATG) double-stranded fragment with a 5' overhang was radiolabeled with [α -³²P]dCTP and Klenow fragment (New England Biolabs, Inc.). Nuclear extracts were prepared from K562 cells. In a typical electrophoretic mobility shift assay (EMSA), 2.5 μ g of nuclear extract was incubated at room temperature for 10 min in binding buffer (4% glycerol, 1 mM MgCl₂, 0.5 mM EDTA, 0.5 mM dithiothreitol, 50 mM NaCl, 10 mM Tris, pH 7.5, 2.5 μ g dIdC). Radiolabeled probe (50,000 disintegrations/s) was added to the tube, and the reaction was allowed to continue for 20 min at room temperature. Samples were loaded onto a 4% polyacrylamide nondenaturing gel and electrophoresed at room temperature for 4 h in 0.5% Tris-boric acid-EDTA buffer.

To identify specific DNA binding proteins in K562 nuclear extracts, supershift assays were performed. A 20-bp fragment (HOXB4 –149 to –129) was radiolabeled and incubated with nuclear extracts as above in the presence or absence of 2.5 μ g anti-MITF antibody (gift from David Fisher and Timothy Hemesath, Dana-Farber Cancer Institute, Boston, MA), 1 μ g anti-USF-1 (Santa Cruz Biotechnology, Inc.), 1 μ g anti-USF-2 (Santa Cruz Biotechnology, Inc.), or 2.5 μ g normal mouse IgG. The antibody–nuclear extract–DNA complexes were then electrophoresed as above and retarded bands were detected after transfer and autoradiography.

Yeast One Hybrid Screen. Candidate cDNA encoding proteins that bind to the HXRE-2 site were isolated from K562 and human bone marrow cDNA libraries by fusion with Gal4 activation domain and yeast one hybrid selection using the Matchmaker system (CLONTECH Laboratories, Inc.). Three tandem copies of the HXRE-2 site (–129 to –149 bp) were cloned upstream of the His3 coding region in the pHis1 one hybrid vector, and this construct was integrated into the yeast (YM4271) genome. This reporter strain was then transformed with the Gal4 AD fusion library. 50 mM 3-amino-1,2,4-triazole (3-AT) was used to control for promiscuous HIS3 expression. Transformants were grown on defined medium lacking histidine at 30°C for 6–10 d. Gal4-cDNA fusions with activity were isolated from the yeast and their DNA sequences were determined.

Results

First we isolated and sequenced several overlapping genomic clones from a human bone marrow genomic library using a HOXB4 cDNA. The human HOXB4 gene contains two exons separated by a single intron. Comparison of the human and mouse sequences indicates that the human HOXB4 gene encodes a protein nearly identical to its murine homologue. The human and mouse homeobox genes also contain regions of significant homology in non-coding sequences. Most notably, the 240 bp immediately 5' to the ATG initiation codon in the human HOXB4 gene are 97% identical to the corresponding mouse sequences (Fig. 1) (19).

Northern analysis and reverse transcriptase PCR were used to survey several human leukemic cell lines to identify suitable candidates for the study of HOXB4 regulation. HOXB4 RNA was expressed at moderate levels in K562

Human	AAAGCCCAAGCTCTGTGCTTGGCCCCGTATGGTTCCTTTCGACACGGA	-559
Mouse	CAAGCTCAGCGCCCTATGACTAGTGGGTACCCATTCAAGAGAAGCGCTC	
Human	GCCCCAACCCAGTAACCTACTCCCATAGAGAGCAGGGTGCTCCCTC	-505
Mouse	CTGGTCGGCCAACTCTAAACCCACACAGACACCTAGTCGTCTCCCTC	
Human	CCGCCAGGGGAGGAGACTGGGGCTTXXGGAAGGTACAGAAXXXTGCTGGCG	-451
Mouse	TTATCTGGGGAGGAGAXTGGGGCCCTATGGAGTCAGAGAAAGGTGGGAG	
Human	AGAGGTGAGGCAGAACAGAGGGCTACACCGGAGCCCCAACTTCTAAGGGAT	-397
Mouse	ATTAGGTAGGGCAGGGGTGGGGCTACACCGCAGCCCCAACTTCTXGAGGGAT	
Human	CTAGGGTTTGCCTCTGCTAGCGCCCAACAGGCCCTCACTGCAACCTGCG	-343
Mouse	GCAGGGTTTGTCTCTGCTCAGCCCCCTACCCCCAAAAGCTCTCCAAATAA	
Human	CGCGCTCTCTCTCTGCTCTCTCTGATCCGGAGAGAGCGAGCXXXXXX	-289
Mouse	CTGCTCTACTCTTCTTAGTTCATACCCCTCTCTGTTGTGAGAAAGCAGGC	
Human	AAGGAGGGGGTCCGCCGAGGAGCCCTATGTAAATCCTGGTGTGGGTGGGTG	-235
Mouse	GAGGAGGGGGTCTCCCCCGGGAGCCCTATGTAAATCCTGGTGTXXXXXX	
Human	GGTGGGAGGGGGAAGGGAAGAGGGGAAATAAACCTCTTTGGCTGGAGTGGG	-181
Mouse	GGTGGGTGGGGAAGGCTAGAGAGGGGAAATAAACCTCTTTGGCTGGAGTGGG	
Human	TCCGGGTGAGCAGATTTCCTTATCCGGGAATCGCAGGCGGGCGGGCAATTGGCT	-127
Mouse	TCCGGGTGAGCAGATTTCCTTATCCGGGAATCGCAGGCGGGCTGGCCATTGGCT	
Human	CGGAGGATCACGTGGGCGCTTAACCTTTGTTCACTTGACAGTAAGTAGGAGGGCT	-73
Mouse	CGGAGGATCACGTGGGCGCTTAACCTTTGTTCACTTGACAGTAAGTAGGAGGGCT	
Human	TTCCGAAACAGGAAACGAGTCAGGGGTCGGAATAAATTTTAGTATATTTGTG	-19
Mouse	TTCCGAAACAGGAAACGAGTCAGGGGTCGGAATAAATTTTAGTATATTTGTG	
Human	GGCAATTCACAGAAATTAATGGCTATGAGTCTTTTGTGATCAACTCAAACATAT	36
Mouse	GGCAATTCACAGAAATTAATGGCTATGAGTCTTTTGTGATCAACTCAAACATAT	

M A M S S F L I N S N Y

Figure 1. DNA sequence of the human HOXB4 and mouse Hox-b4 5' regions. Identical bases are shown in gray, disparate bases in black, and missing bases as black X's. The coding sequence beginning with the ATG is set at +1. Transcription start sites identified by 5' RACE are shown with arrows. These sequence data, as well as the remainder of the complete HOXB4 genomic clone, are available from EMBL/GenBank/DBJ under accession no. AF307160.

cells, and at lower levels in U937 and KG1a cells. Nuclear run-on experiments in K562 cells demonstrated that HOXB4 transcription was induced during incubation of the cells in PMA (Fig. 2).

5' RACE analysis in K562 cell extracts identified two transcriptional start sites between 65 and 90 bp 5' to an ATG, initiating a long open reading frame similar to results found for the murine Hox-b4 gene (not shown) (19). These results suggested that transcription is initiated within a short distance of this candidate translation initiating ATG, and further suggested that these highly conserved proximal 5' sequences might be sites that govern transcriptional regulation.

Using transient transfection of luciferase reporter constructs into K562 cells, we found transcriptional stimulatory activity in the 688-bp 5' noncoding sequence, but not in the 3' sequence or in the intronic sequences. This transcriptional activity was localized by deletion analysis to a 99-bp fragment (Fig. 3 A, fragment 11) located -153 to -55 bp 5' to the ATG. Subdividing this fragment eliminated transcriptional activity from either fragment (Fig. 3 A, fragments 9 and 10). Thus, a region located between nucleotides -153 and -55 upstream of the translational initiation ATG appears to contain the core noncoding DNA sequences required for HOXB4 transcription in K562 cells. Similar results with this 99-bp sequence were also obtained in KG1a and U937 cells (data not shown).

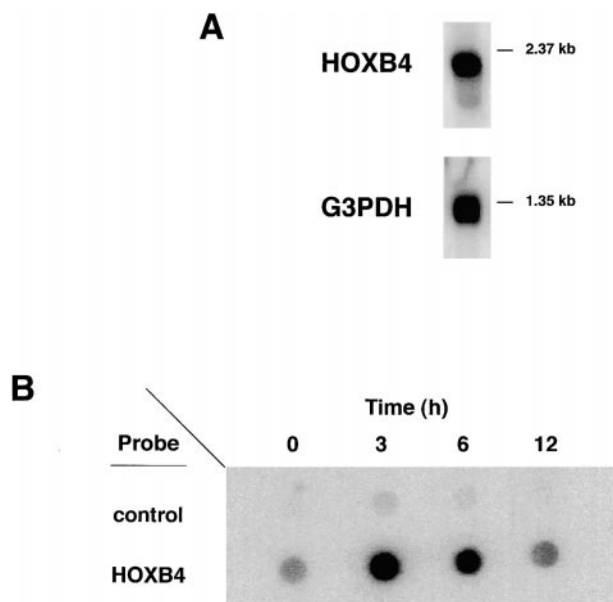


Figure 2. HOXB4 expression and transcription in human K562 cells. (A) Northern analysis. mRNA isolated from K562 cells analyzed with a HOXB4 3' untranslated region probe. Quantitation was performed by PhosphorImager, with glyceraldehyde 3-phosphate dehydrogenase (G3PDH) used as an internal control. (B) Nuclear run-on. Newly translated RNAs from nuclei isolated from K562 cells either before or after PMA induction were radiolabeled, extracted, and hybridized to HOXB4 or nonspecific probes.

To determine the relationship of these findings in leukemic cell lines to normal stem cell physiology, the transcriptional activity of the 99-bp core promoter sequence (99bp5'HOXB4) was then tested in highly purified immature CD34⁺ bone marrow cells. TPO, SCF, and Flt-3L, alone and in combination, have been previously shown to stimulate human stem cell division while maintaining elements of stem cell self-renewal (20, 21). To test the response of the putative 99-bp HOXB4 promoter to growth factor stimulation supporting stem cell self-renewal, we cloned the 99-bp promoter 5' to enhanced green fluorescent protein (EGFP), or to luciferase, and transfected these constructs into CD34⁺ cells that were cultured in the presence of TPO, SCF, and Flt-3L, for 24–72 h. Under these culture conditions, expression of a 99bp5'/EGFP reporter was detectable by immunofluorescence in 2–5% of the electroporated CD34⁺ cells (Fig. 4 A). When the 99bp5'/luciferase construct was electroporated and the CD34⁺ cells were cultured for 24–72 h in TPO/SCF/Flt-3L, luciferase activity was stimulated 5–10-fold (Fig. 4 B). In contrast, culture of CD34⁺ cells in the presence of GM-CSF supported only minimal HOXB4 promoter-activated transcription and expression of reporter constructs (Fig. 4 C).

Inspecting the DNA sequence within the 99bp5'HOXB4 region, we found several consensus protein-binding sites, including an SP1 site, an NF1 site, a c-myc-like E box, and a c-myb-binding site (Fig. 5 A). We then determined the effects of mutations in these consensus sites and other sites not known to specify binding. Mutation of the NF1 site

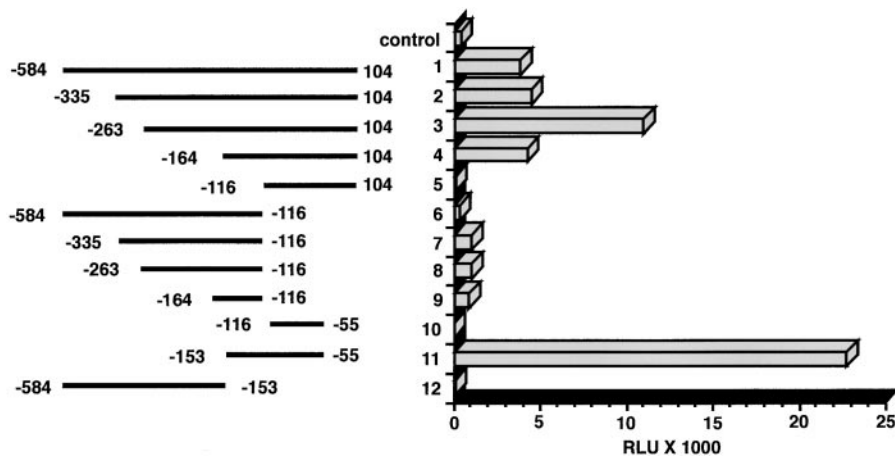


Figure 3. Transient transfection of K562 cells with 5' HOXB4/luc constructs. Fragments encompassing bases (–584 to 104) were tested for their ability to support luciferase transcription. Data shown are presented in relative luminescence units (RLU) normalized to β gal, and come from one of four similar experiments. The strongest promoter activity was detected in fragment 11 (–153 to –55); subfragments of 11 (fragments 9 and 10) had only baseline activity.

(ATTGGCT to AACGGCT) inhibited transcriptional activity in transient transfection assays 60–80%, mutation of the E box (CACGTG to CATATG) inhibited promoter activity 90–95%, while mutations of several other sites, including candidate SP1 and c-myb-binding sites, had no effect (Fig. 5 B).

To confirm these sites identified in transient transfection assays, stable cell pools of K562 cells transfected with the native or mutated 99-bp promoter were created. K562 cells were cotransfected with plasmids with resistance to G418 along with plasmids encoding either the native or mutated

promoter linked to luciferase. Stable transformant pools were selected in G418, and each pool was analyzed for luciferase production after 3 wk. As with the transient assays, luciferase activation via the HOXB4 promoter was inhibited by mutations at either site, and was essentially abolished by mutation of the E box site (Fig. 5 C). Based on these results, we have termed these two sites within the HOXB4 promoter HXRE-1 and HXRE-2.

These results, and in particular the strong effect of HXRE-2 mutation on transcriptional activation, suggested that transcription factors required for HOXB4 expression

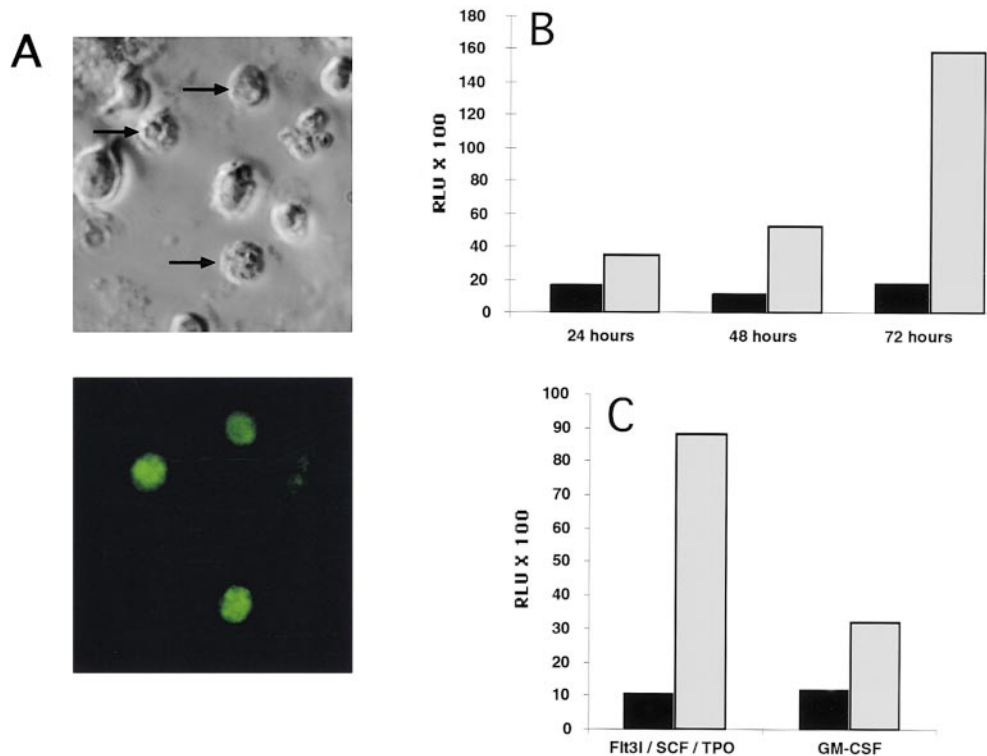


Figure 4. Activation of the HOXB4 promoter construct in normal CD34⁺ bone marrow cells. Immunopurified CD34⁺ bone marrow mononuclear cells were electroporated with HOXB4 promoter constructs linked either to GFP, for direct visualization, or to luciferase for relative quantitation of promoter activation. (A) Immunopurified (>97%) CD34⁺ bone marrow cells were electroporated with the 99bp5'HOXB4/EGFP after culture in SCF (100 ng/ml), TPO (100 ng/ml), and Flt-3l (100 ng/ml) for 48 h, and cells expressing EGFP were visualized by fluorescence microscopy (original magnification: $\times 20$). (B) CD34⁺ cells were cultured in SCF/TPO/Flt-3l for 24–72 h, and then electroporated with 99bp5'HOXB4/luc (gray bars) or with control PCI-luc (black bars). (C) 99bp5'HOXB4/luc transfected CD34⁺ cells cultured for 72 h in SCF/TPO/Flt-3l or in GM-CSF (20 ng/ml) before electroporation. The CD34⁺ cells cultured in SCF/TPO/Flt-3l for 72 h generated cultures in

which 12.2% of the cells remained CD34⁺ whereas 9.75% acquired CD11b. CD34⁺ expression on cells cultured in GM-CSF declined to 2.2%, and 32.3% acquired CD11b. Lysates were assayed in triplicate for luciferase activity, and results were normalized with renilla luciferase in each sample. Inter-sample variability was <15%.

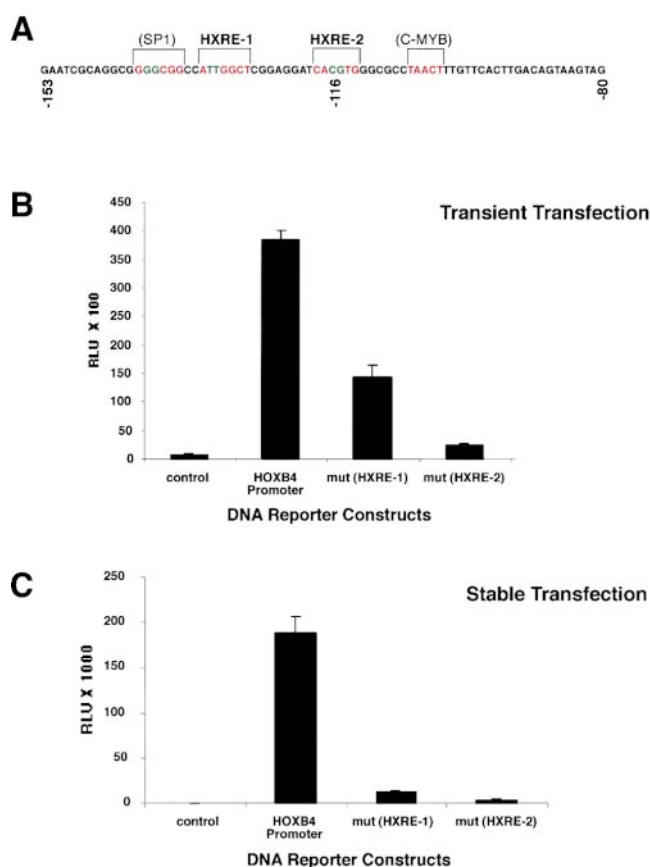


Figure 5. Identification of critical sequences within the HOXB4 core promoter via transient and stable transfection in K562 cells. (A) DNA sequence of the 99bp 5' HOXB4 promoter, with candidate protein-binding sites shown in red and the mutated sequences shown in green. (B) Transient transfection of luciferase reporter constructs including the native HOXB4 promoter, HOXB4 promoter mutated (mut) at the NF1 (HXRE-1) site (TT to AC), or mutated at the E box (HXRE-2) (CG to TA). (C) Luciferase production over 24 h in stable K562 cell pools cotransfected and selected with the same constructs. In transient transfection, mutation at HXRE-1 inhibited transcription via the promoter 60–80%, and mutation at HXRE-2 inhibited 90–95%. In stably transfected cells, HXRE-1 mutation inhibited transcription 85–90%, and HXRE-2 mutation 95–98%. Results are shown from one of three similar experiments for each set and are presented as means \pm SD of triplicate measurements. RLU, relative luminescence units.

bound to these sites. We next performed EMSAs using radiolabeled 20-bp probes containing the HXRE-2 site. Incubation of K562 nuclear extracts with this resulted in a clear and reproducible pattern of protein–DNA complexes. Several of these were significantly diminished by 50-fold molar excess of unlabeled nonspecific oligonucleotide (Fig. 6 A, lane 4). Others were not, but were specifically diminished or eliminated by incubation with competitor DNA corresponding to the 20-bp probe (Fig. 6 A, lane 3). These data suggested that the retarded bands were caused by specific interactions between K562 nuclear proteins and the promoter oligonucleotide.

To identify HXRE-2-binding transcription factors, we next performed a series of yeast one hybrid selection experiments (22). We used the core HXRE-2 sequences as the

binding target and the K562 and bone marrow cDNA libraries as the sources of potential transcription factors. Using this technique we isolated several clones for each of the two transcription factors, USF-2 and MITF. USF-2, a member of the helix-loop-helix (HLH) family of DNA binding is widely expressed (23–25) and often binds as a homodimer or as a heterodimer with USF-1 (26, 27). USF-1 and/or USF-2 control the transcription of many genes critical to basic cell metabolism, including pyruvate kinase (28), fatty acid synthase (29), and the mitochondrial ATP synthase complex α subunit (30). Surprisingly, the yeast one hybrid screens did not identify any cDNAs encoding human USF-1. On the other hand, the screen did identify MITF, another HLH transcription factor whose mutation causes microphthalmia, mast cell and skin pigmentation defects in mice (31, 32), and Waardenburg syndrome type 2 (autosomal dominant hearing loss and depigmentation) in humans (33). MITF appears to be a dominant transcription factor for melanocyte-specific genes, including tyrosinase (34), tyrosinase-related protein 1 (35), and mast cell proteases 5 and 6 (36–38). Of particular interest, MITF activates mast cell-specific genes in response to c-kit activation by SCF (39, 40).

To determine whether USF-2, MITF, and their respective heterodimeric binding partners USF-1 and TFE3 are, in fact, present in K562 nuclear extracts and capable of binding to HOXB4 HXRE-2 sites, we performed EMSAs in the presence of transcription factor-specific antibodies. Specific antibodies to TFE3, c-myc, and E47 neither eliminated EMSA bands nor generated higher mobility supershifted bands when preincubated with labeled HXRE-2 probe. In contrast, altered mobility shift patterns were created by coincubation of either anti-USF-1, anti-USF-2, or anti-MITF. Anti-USF-1 shifted the dominant band in the middle of the electrophoresis pattern, creating complexes of lower mobility. Anti-USF-2 decreased but did not eliminate the intensity of the major band, and created a new band of the same mobility as one of the anti-USF-1 shifted bands. Anti-MITF blocked the appearance of the fastest mobility band, creating a new band of slightly larger size (migrating slightly faster than the dominant band in the control incubation). When anti-USF-1 and anti-USF-2 were added together with nuclear extracts and labeled probes, additional shifts to larger complexes were found. When anti-MITF was added along with anti-USF-1, the effects were additive and no new bands were seen (Fig. 6 B). These supershift patterns show that USF-1, USF-2, and MITF are present in K562 nuclear extracts and bind to the HXRE-2 site.

To assess the functional consequences of these transcription factors binding to HXRE-2 in hematopoietic cells, cotransfection experiments were performed measuring the effect of enforced expression of each of these proteins, or of their dominant negative mutants, on transcriptional activation of luciferase reporter constructs via the core HOXB4 promoter. Overexpression of either USF-1, USF-2, or both USF-1 and USF-2 in K562 cells activated HOXB4 promoter transcription two- to fourfold. In contrast, MITF

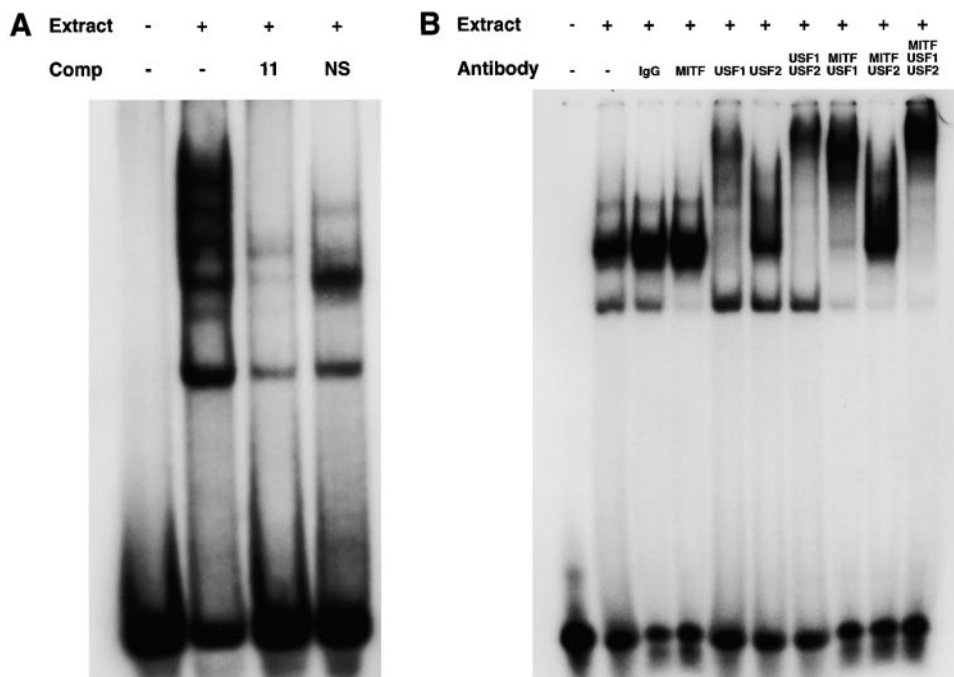


Figure 6. Detection of nuclear proteins specifically binding to the HOXB4 promoter by EMSA. (A) Radiolabeled 99-bp HOXB4 oligonucleotide probes (fragment 11 from Fig. 3) were incubated with K562 nuclear extracts, with or without cold competitor (Comp) oligonucleotides as shown, and electrophoresed through a 4% acrylamide gel. Competition with 50-molar excess probe (–153 to –55 bp fragment 11, Fig. 3) specifically blocked nuclear protein binding to this probe, eliminating bands which were not eliminated by competition with unrelated probe (NS, nonspecific probe). (B) Antibodies to USF-1, USF-2, and MITF were incubated with K562 nuclear extracts and labeled HXRE-2 probe, and electrophoresed through 4% acrylamide. Each antibody alone retards specific bands in the control EMSA, and combinations of two or three antibodies have additive effects. These supershifts show that USF-1, USF-2, and MITF are present in K562 nuclear extracts and bind to the HXRE-2 site in HOXB4.

overexpression did not activate transcription, but in some experiments actually depressed transcription somewhat (Fig. 7 A). Expression of the endogenous HOXB4 mRNA was similarly induced two- to threefold in K562 cells transiently transfected with USF-1 or USF-2 expression plasmids (Fig. 7 B). This level of HOXB4 mRNA induction may underrepresent the stimulatory effects of USF overexpression, as the efficiency of K562 cell electroporation is only 50–60%, whereas the Northern blot analysis measures

the (averaged) HOXB4 expression in transfected and untransfected cells. Overexpression of a dominant negative mutant USF (D/N USF) depressed basal HOXB4 transcription in K562 cells 30–60%, but D/N MITF had no measurable effect (Fig. 7 C). These data suggest that USF-1 and USF-2 independently can upregulate HOXB4 transcription, that USF-1 and/or USF-2 are responsible for HOXB4 basal transcription in K562 cells, and that MITF has neither a basal nor a stimulatory effect.

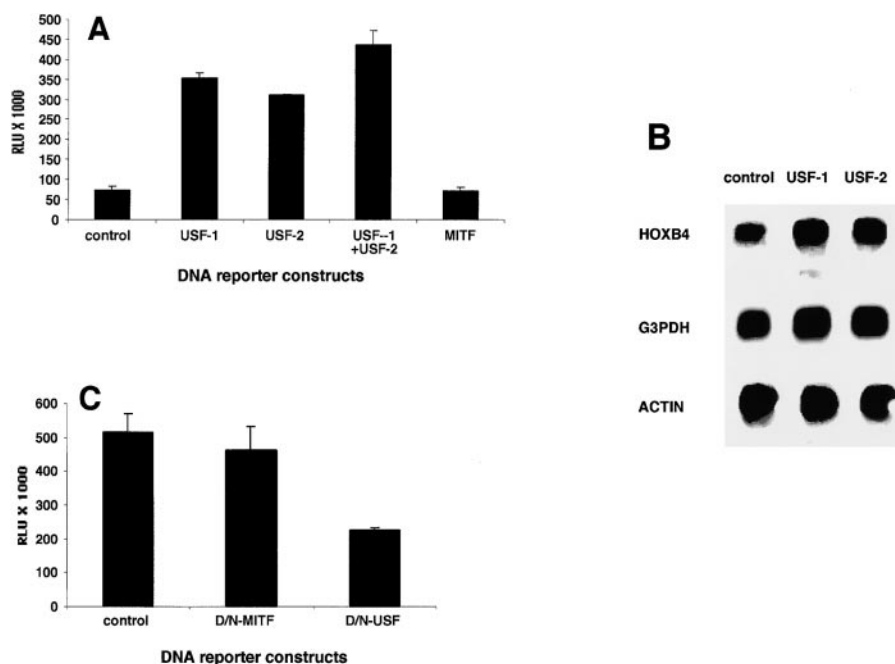


Figure 7. Effects of cotransfection of USF and MITF expression plasmids on transcriptional activation of HOXB4 promoter (HXRE-2) construct and on endogenous HOXB4 expression, in K562 cells. (A) HOXB4/luc transcription after cotransfection with expression plasmids encoding MITF, USF-1, or USF-2. Each bar indicates the mean \pm SD of quadruplicate measurements in a single experiment, from one of four similar experiments. (B) Northern analysis of the K562 cell HOXB4 mRNA expression after transient transfection with USF-1 and USF-2 expression plasmids. 5 μ g polyA⁺ RNA from 100 \times 10⁶ K562 cells was loaded in each lane, and both glyceraldehyde 3-phosphate dehydrogenase (G3PDH) and β -actin were used as internal controls. (C) 99bp5'HOXB4/luc transcription after cotransfection with D/N MITF or D/N USF. The data shows mean \pm SD of quadruplicate measurements in a single experiment, from one of three similar experiments. RLU, relative luminescence units.

Studies by Hemesath et al. have indicated that activation of melanocytic cell lines to upregulate MITF to induce tyrosinase transcription is mediated by the RAS/MAPK pathway (40). Although MITF does not activate the HOXB4 promoter, we hypothesized that MITF and USF-1/2 might play analogous roles in aspects of lineage-specific transcription and self-renewal favoring transcription, respectively. If this were true, perhaps the RAS pathway might be involved in activation of the HOXB4 promoter in cells capable of activating its transcription. To test this, a plasmid encoding constitutively activated (V12) RAS was cotransfected into K562 along with the HOXB4/luc construct. V12 RAS cotransfection induced HOXB4/luc expression fivefold. RAS activation of HOXB4/luc was blocked by cotransfection with plasmids encoding D/N MEK, or by incubation with the soluble pharmacologic MEK inhibitor PD98059, but not by cotransfection with a

plasmid encoding D/N (Δ p85) PI3K (Fig. 8 A). D/N MEK and PD98059, but not D/N PI3K, also inhibited basal HOXB4/luc expression, suggesting that the MAPK pathway was also involved in basal HOXB4 transcription (Fig. 8 B). This interpretation was supported by the findings that D/N USF blocked RAS activation of HOXB4/luc, and that cotransfection of V12 RAS and USF-1 led to synergistic activation of HOXB4/luc (Fig. 8 C). Finally, cotransfection of V12 RAS along with an HXRE-2 (E Box) mutated HOXB4/luc failed to activate the promoter, whereas V12 RAS transfection along with HXRE-1 mutant HOXB4/luc caused partial activation (Fig. 8 D). This suggests that the HXRE-2 (E box) site is required for RAS activation of the HOXB4 promoter.

To further test the role of USF transcription factors in activation of the HOXB4 promoter in stem cells, cotransfection experiments were then performed with HOXB4/

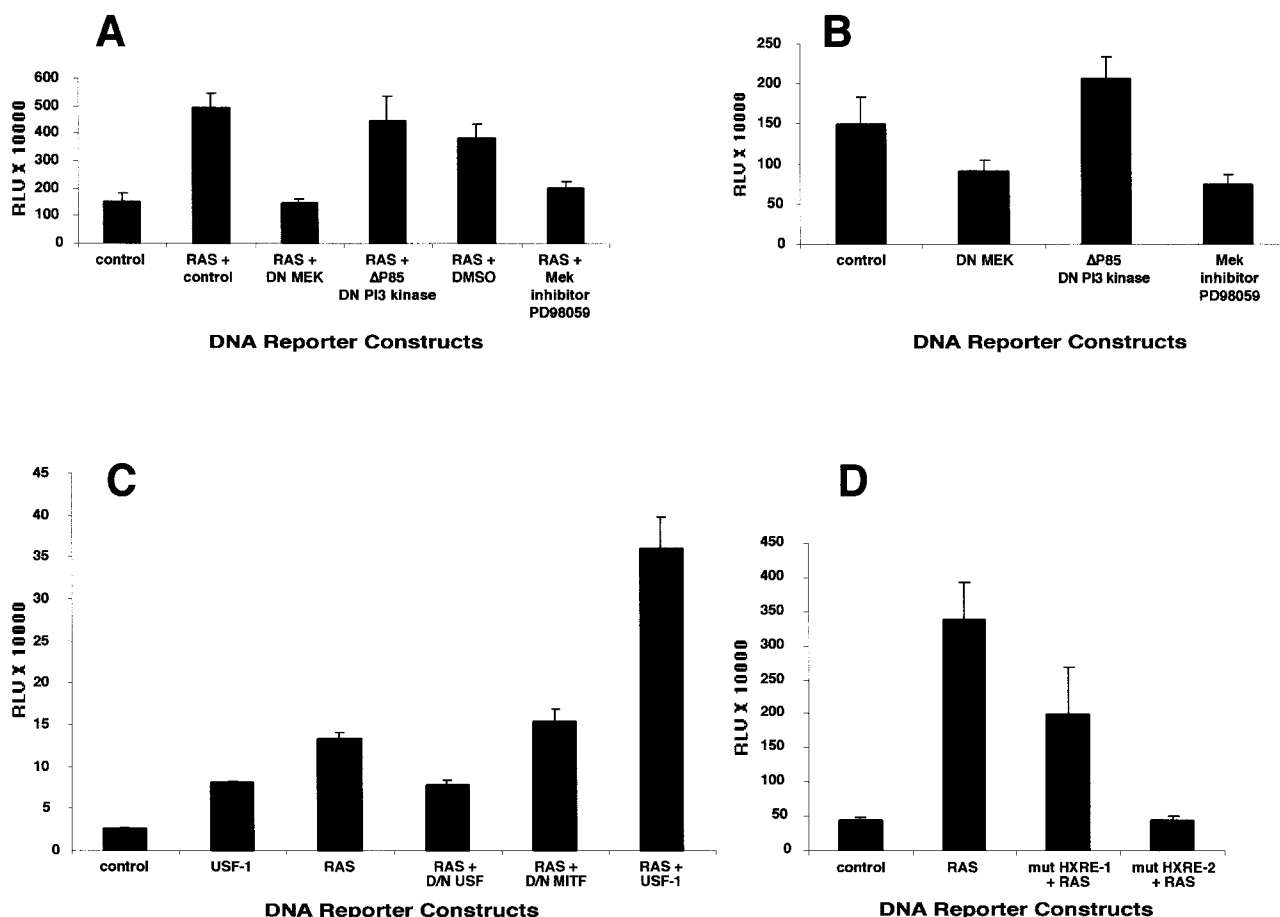


Figure 8. Interaction of the RAS/MAPK pathway and the HOXB4 promoter. K562 cells were transiently transfected with 3 μ g of the 99-bp HOXB4 promoter construct, along with the indicated expression plasmid constructs. (A) RAS activates the HOXB4 promoter. 5 ng plasmids encoding constitutively activated RAS, along with 10 μ g PCI vector (control), D/N MEK, D/N PI3K, the soluble MEK inhibitor 100 μ M PD98059 (in DMSO), or DMSO alone were cotransfected along with the HOXB4 promoter, and luciferase activity was measured at 48 h. (B) Basal activity of the HOXB4 promoter is transmitted via the RAS/MAPK pathway. Plasmids encoding D/N MEK, D/N PI3K, PCI, or 100 μ M PD98059 were cotransfected along with the HOXB4 promoter. (C) RAS activation of the HOXB4 promoter is transmitted via USF. HOXB4 promoter activation induced by cotransfection of plasmid encoding activated RAS is blocked by D/N USF, but not D/N MITF, and HOXB4 activation by cotransfected RAS plus USF-1 is higher than either alone. (D) RAS activation of the HOXB4 promoter requires an intact HXRE-2 (E box) site. Intact HOXB4 promoter/luc reporter construct, and HXRE-1-mutated HOXB4 promoter are activated by V12 RAS, but HXRE-2 (E box)-mutated HOXB4 promoter/luc reporter is not activated by RAS. Each bar indicates the mean \pm SD of quadruplicate measurements in a single experiment, from one of three to four similar experiments.

luc and USF-1, USF-2, or D/N USF in normal primary CD34⁺ bone marrow cells. In these assays, overexpression of either the USF-1 or USF-2 protein activated the HOXB4 promoter 5–20-fold, indicating that USF-1 and USF-2 are able to activate the promoter in normal cells. Consistent with this, expression of the D/N USF protein blocked HOXB4 promoter activity 40–80%, indicating that USF-1 and/or USF-2 bind to and activate the HOXB4 promoter in CD34⁺ cells stimulated to divide and self-renew in the presence of TPO, SCF, and Flt-3l (Fig. 9). Taken together, these data suggest that USF-1 and USF-2 mediate the activation of the HOXB4 gene in human stem cells undergoing proliferation accompanied by self-renewal.

Discussion

These studies identify and characterize the human HOXB4 promoter in an attempt to identify the regulatory proteins that help maintain normal hematopoietic stem cell self-renewal and homeostasis. The results of these studies include: (a) identification of the genomic regulatory sequences responsible for regulating human hematopoietic

stem cell HOXB4 expression, both in normal bone marrow cells and in leukemic cell lines; (b) demonstration that these sequences mediate HOXB4 transcription differentially in response to hematopoietic growth factors that stimulate stem cell self-renewal; and (c) identification of USF-1 and USF-2 as transcription factors that bind to and can activate the HOXB4 promoter in stem cells.

Comparison of the murine Hox-b4 and human HOXB4 genes showed areas of high homology, particularly within the proximal 5' sequences (19). The most proximal of these regions, located within 200 bp of the translation initiation ATG, was >97% homologous between species, and contained strong HOXB4 regulatory activity. Transient transfection assays in K562 cell lines first identified a 99-bp 5' regulatory region that was necessary and sufficient for HOXB4 expression. Expression driven by this construct declined with PMA-induced differentiation, suggesting that this construct might be sufficient to transmit downregulated expression with differentiation as well, a feature essential to HOXB4 function in vivo (41). This same construct was active in normal CD34⁺ bone marrow cells, comprising the most primitive 1% of hematopoietic cells.

These findings are notable in light of the elegant studies in mice and chickens by Krumlauf, Whiting, Gould, and colleagues (41–43). These investigators found that neural specific expression of HOXB4 is dictated, both in space and time, by enhancer elements located within the HOXB4 intron and 3' to the HOXB4 exons. These 3' regulatory elements include potential PBX-binding sites between the HOXB4 and HOXB3 genes which appear to be candidates for cross-regulation by HOX proteins (43). In addition, a more distal retinoic acid response element located within the HOXB3 gene itself (44) might regulate the induction of both these genes by retinoic acid (45, 46). These authors also found that sequences localized to the immediate 3' noncoding region were responsible for expression of HOXB4 within the developing mouse lung (42). In the present study, we found that proximal 5' sequences are sufficient to drive HOXB4 transcription in primitive normal and malignant hematopoietic stem cells, and to downregulate HOXB4 transcription in response to growth factors and biochemical inducers that stimulate loss of stem cell self-renewal and differentiation. However, it is possible that the 3' neural enhancers might have modulatory roles, interacting with core 5' promoter-driven transcription, in normal or malignant human hematopoiesis.

Transcriptional activation via the 5' HOXB4 promoter was induced in CD34⁺ hematopoietic cells by culture in the hematopoietic growth factors TPO, SCF, and Flt-3l. The CD34⁺ hematopoietic cell pool includes extremely primitive pluripotent stem cells capable of self-renewal, as well as lineage-restricted but phenotypically immature progenitor cells. HOXB4 transcription is found to be highest in the most primitive CD34⁺ stem cells, but is distinctly lower in more differentiated cell compartments (6). Moreover, overexpression of HOXB4 has been found to confer increased stem cell self-renewal, as measured in competitive repopulation assays (13). Using a variety of approaches, in-

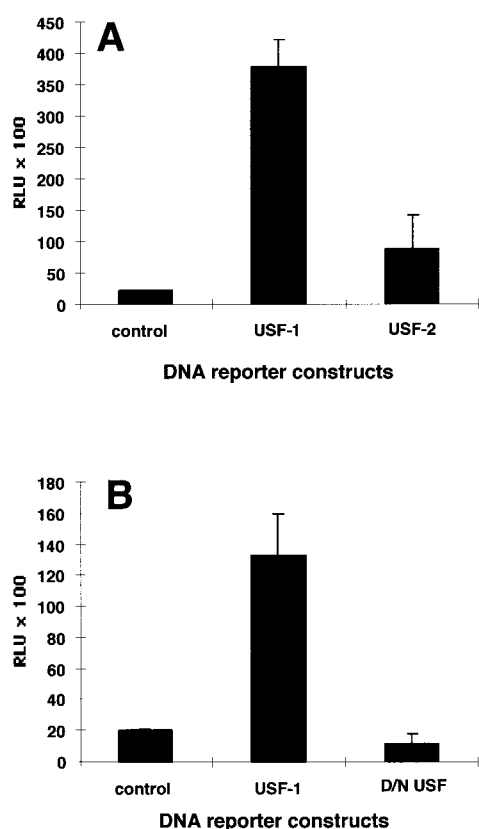


Figure 9. USF-1 and USF-2 activate the 99bp5'HOXB4 regulatory element in normal CD34⁺ bone marrow cells. Immunoselected CD34⁺ cells were electroporated with 99bp5'HOXB4/luc, with or without expression plasmids encoding USF-1 (A and B) USF-2 (A), or D/N USF (B). The data are expressed as mean ± SD of triplicate samples and are taken from one of three similar experiments.

cluding studies of mutant mouse strains, long-term bone marrow cultures, and competitive repopulation transplantation, investigators have implicated TPO, SCF, and Flt-3l as each supporting stem cell self-renewal, and the combination of the three hormones appears to be notably effective (20). The observation that HOXB4 transcription via the 99-bp 5' promoter was highly induced by the CD34⁺ culture in TPO/SCF/Flt-3l and far less so during culture in GM-CSF strongly suggests that these hormones, at least in part, trigger their maintenance of stem cell maintenance and self-renewal via these sequences. Of particular note, CD34⁺ cells proliferated to a comparable extent over 72 h in either TPO/SCF/Flt-3l or GM-CSF, indicating that the HOXB4 promoter induction was not simply indicative of cell cycling. However, CD34⁺ cells cultured in SCF/TPO/Flt-3l for 72 h generated cultures in which 12.2% of the cells remained CD34⁺ whereas 9.75% acquired CD11b, only 2.2% of CD34⁺ cells cultured in GM-CSF remained CD34⁺, and 32.3% acquired CD11b.

Yeast one hybrid screens identified USF-1, USF-2, and MITF as candidates for transcription factors mediating HOXB4 activation through HXRE-2. Supershift EMSAs showed that each of these proteins was present in K562 extracts and capable of specific binding to the HXRE-2 site. Cotransfection assays based on these results demonstrated that of these three transcription factors, USF-1 and USF-2 can activate HOXB4 promoter constructs. Overexpression of USF-1 and USF-2 can also upregulate endogenous HOXB4 expression, to an extent similar to that seen with the HOXB4 promoter constructs. These findings support the recent findings that USF-2 is upregulated in response to IL-3 in the IL-3-responsive leukemic cell line (38), and provide a molecular explanation for how this induction might promote leukemic cell proliferation. In addition, the findings that either USF-1 or USF-2 can independently activate the HOXB4 promoter, but MITF does not, may explain why the USF-1/USF-2 double knockout mice die early in embryogenesis (47).

MITF contrasts with USF-1 and USF-2 in its interaction with the HOXB4 promoter. On the one hand, anti-MITF antibodies clearly detect extractable nuclear MITF protein capable of binding to the HXRE-2 E box site within the HOXB4 promoter. However, cotransfection of K562 cells (or CD34⁺ cells) with plasmids encoding MITF (and capable of activating the tyrosinase promoter in melanocyte cell lines) does not transcriptionally activate HOXB4. Rather, overexpression of MITF results in no change, or in some experiments, slight diminution of HOXB4 activation. Thus, MITF appears to be a neutral potential occupant of the HOXB4 promoter. These findings are of note in light of the recent studies of Hemesath et al. (40), who demonstrated that MITF plays a central role in melanocyte differentiation. In these studies, stimulation of melanocytic cell lines by SCF causes activation of the RAS pathway, leading to the transcriptional activation of the tyrosinase gene via the phosphorylation of MITF. This is particularly interesting, inasmuch as SCF plays a role in both stem cell proliferation and melanocyte differentiation. In these studies, RAS

activation likewise resulted in the activation of the HOXB4 promoter; however, MITF expression does not induce this effect. These results suggest that the complex patterns of self-renewal and differentiation displayed by stem cells in response to individual and multiple growth factors might be explained by their activation of specific patterns of transcription factors, and that the basic HLH zipper proteins are likely included among such proteins.

In summary, the human homeobox gene HOXB4, which plays a pivotal role in stem cell self-renewal and homeostasis, is transcriptionally regulated in hematopoietic stem cells via a 99-bp core promoter sequence. This sequence contains a consensus E box site to which USF-1, USF-2, and MITF bind, and their the balanced activities can regulate the transcription of the HOXB4 gene. Further studies of human stem cell HOX gene regulation can now explore distinct models of transcriptionally regulated self-renewal versus differentiation-specific pathways. These studies should contribute directly to our understanding of the molecular mechanisms of normal cell and tissue homeostasis as well as dysplasia and transformation.

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