Enforced Expression of Hlx Homeobox Gene Prompts Myeloid Cell Maturation and Altered Adherence Properties of T Cells

By John D. Allen and Jerry M. Adams

Within the hematopoietic compartment, the murine Hlx homeobox gene is expressed in myeloid cells, most prominently in macrophages and granulocytes, and in immature B-lymphoid cells but not in erythroid, mast, or T-lymphoid cells. The level of Hlx mRNA increased with induced differentiation of the promyelocytic lines WEHI-3B and HL-60. To address its biologic action more directly, Hlx expression vectors were introduced into seven mouse hematopoietic cell lines representing several lineages. Although four lines did not tolerate stable Hlx expression, high-level expression was achieved in the early myeloid line FDC-P1 and in the immature T-cell lines Tikaut and WEHI-707. Overexpression of Hlx in FDC-P1 cells downregulated two markers of myeloid immaturity, Thy-1 and CD34, and also promoted changes in cellular and colony morphology, indicative of limited differentiation. Ectopic Hlx expression in the T-cell lines induced changes in cellular and colony morphology and adhesiveness, concomitant with decreased expression of adhesion molecules ICAM-1 (CD54) and Pgp-1 (CD44) and increased expression of heat-stable antigen. These results implicate Hlx in the control of myeloid maturation and the regulation of lymphoid adhesion processes.

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The intrinsic regulation of hematopoietic differentiation by specific growth factors has been increasingly well defined, but the genetic regulatory mechanisms that orchestrate lineage commitment and cellular maturation to generate at least eight types of mature blood cells remain unknown. Lineage-stage-specific transcription factors are likely to play pivotal roles, acting together with ubiquitous factors to selectively activate or repress expression of the arrays of genes responsible for the diverse differentiated phenotypes. Attractive candidates for regulators of differentiation include the homeobox genes. In addition to the critical roles of certain homeobox genes in morphogenesis, a number of vertebrate proteins with more divergent homeodomain sequences have been implicated recently in the control of cell fate or the expression of tissue-specific genes. There is increasing evidence of homeobox gene expression within the hematopoietic compartment, as recently reviewed. The best studied example is oct-2, which is important for Ig expression in B lymphocytes.

Homeobox genes with expression patterns restricted to certain hematopoietic lineages or stages of maturation are of particular interest. We have described a novel murine gene, Hlx, that bears a divergent homeobox and is not linked to the well-characterized homeobox gene clusters. Its expression within the hematopoietic compartment exhibits both lineage and stage specificity. Analysis of 67 murine hematopoietic cell lines and a range of normal cell populations indicated that Hlx was expressed in the myelomonocytic lineage, at highest levels in mature macrophages and granulocytes, and also in B-lymphoid cells, particularly at the pre-B stage, but not in the T-lymphoid, mast, or erythroid lineages. However, we have not yet been able to closely correlate Hlx expression with that of any particular hematopoietic marker.

One way to show the biologic role of a putative regulatory gene is to examine the consequences of enforced expression in different cell types. For instance, expression of the human HOX2B homeobox gene (HOXB6 by the most recently proposed nomenclature) within the erythroid lineage declines with maturation and its enforced expression in the K562 erythroleukemia cell line reduced the expression of erythroid-specific genes. The human homolog of Hlx, HB24, was reported to be expressed in activated human B and T lymphocytes, as well as in the primitive CD34+ population of cells in the bone marrow. Enforced expression of HB24 in the Jurkat human T-cell line was reported to increase growth rate and elicit changes in gene expression reminiscent of T-cell activation, but some of these results are at variance with our findings in the mouse (see below).

If Hlx expression influences differentiation, its effects should be most evident in cell lines representing early stages of hematopoiesis, in which the mature phenotype has not yet been fixed. We show here that Hlx expression in myelomonocytic cells increases with progression to mature macrophages and granulocytes, so it was also of interest to determine whether its overexpression in an immature myeloid cell line promotes differentiation. Similarly, insight into the role of Hlx in immature B-lymphoid cells was sought by ectopic expression of the gene in immature T-cell lines, which share many features with their B-lineage counterparts but do not express Hlx. Transfection experiments indicated that Hlx can promote limited myeloid maturation and evoke phenotypic changes in T-cell lines, implicating it in the regulation of cell adhesion.

MATERIALS AND METHODS

Hlx expression constructs. The Hlx insert in both constructs was extended from the BamHI site 13 bp 5' of the initiator ATG to the EcoRI site 216 bp 3' of the termination codon (nucleotides 456-2020). The fragment was cloned into pGEM3Zf(+), excised as a HincII-XmnI fragment (XmnI 1 overlaps the EcoRI site in the

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cDNA) and blunt ligated into the Xho I site of the MPZen vector and the Xba I site of the pEF vector. Plasmids were banded in cesium chloride and linearized at the Nde I site in the backbone before electroporation. Helper-free retroviruses were produced by electroporation of the $2$ fibroblast packaging line with the MPZen vector constructs. Retroviral producer clones with titers greater than $10^6$ cfu/mL were selected by assays on FDC-P1 cells and judged by the proportion of G4 18-resistant colonies. Cells were harvested immediately after cocultivation in $24$ hours of cocultivation with irradiated $2$ fibroblast lines.

Growth, electroporation, and infection of cell lines. WEHI-3B$^{11}$ and Tikaut cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing $10$% fetal calf serum (FCS). Medium for WEHI-707, WEHI-401, and $70Z/3$ cells$^{25}$ also contained $50 \mu$mol/L $2$-mercaptoethanol, whereas FDC-P1$^{25}$ and LyH7 cells$^{26}$ were grown in the presence of $1,000$ U/mL interleukin-3 (IL-3), obtained as conditioned medium from the recombinant hybridoma X63/0 mIL3.$^{27}$ HL-60 cells$^{28}$ were cultured in human toxicity RPMI 1640 containing $10$% FCS. Granulocytic differentiation was induced in the promyelocytic lines by culturing as above for $5$ days in the presence of $1,000$ U/mL recombinant human granulocyte colony-stimulating factor (G-CSF) for WEHI-3B or $1.25$% dimethyl sulfoxide (DMSO) for HL-60. Macrophage differentiation was induced in HL-60 cells by culture for $2$ days in the presence of $16$ mmol/L tetradecanoyl phorbol acetate (TPA).

For electroporation, $10^7$ log phase cells were resuspended in $0.5$ mL of HEPES-buffered RPMI-1640 (without serum) and transfected to $0.4$-cm cuvettes containing $10 \mu$g of linearized plasmid. The cells were shocked in a Gene Pulser (BioRad, Richmond, CA), with capacitance set at $960$ $\mu$F, at a voltage selected to achieve an $80$% kill (in the range of $240$ to $290$ V). Time constants were approximately $20$ milliseconds. Shocked cells were left at room temperature for $5$ minutes before dilution to $3 \times 10^5$/mL in culture medium. After $12$ to $16$ hours at $37^\circ$C, G418 selection was applied at $800 \mu$g/mL for the WEHI-3B and WEHI-707 lines, $1,200 \mu$g/mL for the $70Z/3$, Tikaut, and LyH7 lines, and $1,600 \mu$g/mL for WEHI-401.

FDC-P1 cells were infected with the MPZen-Hlx and control retroviruses by $24$ hours of cocultivation with irradiated $2$ fibroblast producer line monolayers; $10$% to $20$% of cells were infected, judged by the proportion of G418-resistant colonies. Cells were selected immediately after cocultivation in $800 \mu$g/mL G418. Infected pools were also obtained as a byproduct of the retroviral titration procedure (see above).

Growth rates of clones were assayed by plating $5 \times 10^5$ cells/mL in $24$-well culture dishes and counting in triplicate on a Coulter counter (Coulter, Hialeah, FL) at $24$-hour intervals over a $5$-day period. Doubling times were calculated by regression analysis, confined to the exponential regions of the growth curves. Standard errors of the estimates for doubling times of individual clones were all less than $0.2$ hours.

Phagocytosis was assessed by incubation overnight at $37^\circ$C with sufficient $0.4-\mu$m latex beads to carpet the culture wells. After careful washing, cells were transferred to microscope slides by cytocentrifugation (at reduced speed to minimize bursting of engorged cells), fixed, and stained. Slides were washed with D-limonene or xylene to remove nonengulfed beads, coverslipped, and scored blind.

Agar cultures. Cells were resuspended in fresh medium at $37^\circ$C, containing $0.3$% bacto-agar (Difco, Detroit, MI) and $G418$, distributed promptly to $1$-mL plates, and allowed to set at room temperature for $15$ minutes before return to $37^\circ$C in a $10$% CO$_2$ incubator. After $7$ days, duplicate plates were fixed with $2.5$% glutaraldehyde in buffered saline and scored; colonies were classified as either compact, compact with halo, or diffuse. Platings covered a range of cell densities, but scoring was restricted to plates containing $50$ to $150$ colonies of greater than $50$ cells.

Flow cytometry and cytotoxic staining. Cell surface markers were analyzed by staining with monoclonal antibodies and conjugated fluorochromes as previously described.$^{29,30}$ Most antibodies are cited therein or included in the American Type Culture Collection catalog.$^{31}$ They included monoclonals against AA-4, B220 (RA3-6B2), BP-2 (G10), class I major histocompatability complex (MHC) (anti-H-2$^{K}$,$^{D}$,28.8.6S), class II MHC (anti-I-A,E: M5/114), heat-stable antigen (HSA) (M1/69), intercellular adhesion molecule-1 (ICAM-1) (YN1/1.7), IgM (anti-Ca.5.1), IL-2 receptor a (IL-2Rα) (PC 61), IL-2 receptor β (IL-2Rβ) (TMPI), lymphocyte function-associated antigen-1 (LFA-1) (ICD/7.7), Mac-2 (M3/38), Mac-3 (M3/84), MEL14, PB76 (G-5-2), and Sca-1 (E13-161.7). Briefly, cells were incubated at a concentration of $10^7$/mL in a volume of $50 \mu$L with saturating quantities of antibodies conjugated to fluorescein isothiocyanate, or to biotin with streptavidin-phycocerythrin as a secondary reagent. Nonspecific binding was blocked by preincubation of the cells with an anti-Fc receptor monoclonal antibody. Analyses were performed on a FACSscan (Becton Dickinson, Mountain View, CA).

Cytocentrifuged cell preparations were stained for α-naphthyl acetate esterase using a commercial kit (Sigma, St Louis, MO) and for myeloperoxidase by standard procedures. Cytotoxic staining for cell morphology included modified Wright’s and May-Grunwald-Giemsa.

Preparation and analysis of RNA and DNA. Poly(A)$^+$ RNA was isolated by proteinase K/sodium dodecyl sulfate (SDS) digestion and oligo(T)-cellulose chromatography. RNA (3 $\mu$g/lane) was fractionated on $0.9$% agarose gels containing $0.22$ mol/L formaldehyde, $20$ mmol/L MOPS, $pH$ 7. $1$ mmol/L EDTA, $5$ mmol/L sodium acetate, and $0.1$ $\mu$g/mL ethidium bromide. Gels were de-stained in water, photographed under UV illumination to check RNA quantity and integrity and the position of RNA size markers, and transferred to neutral nylon membranes (Hybond-N: Amer sham, Arlington Heights, IL) by capillary blotting. Genomic DNA was isolated by a guanidine hydrochloride method. Restriction endonuclease-digested DNA ($10$ $\mu$g per lane) was analyzed on Tris-acetate-buffered $0.9$% agarose gels, denatured, and transferred to Hybond-N by alkaline capillary blotting. Hybridization to DNA or RNA blots was performed at $42^\circ$C overnight with $1$ to $2 \times 10^8$ cpm/mL of denatured probe in $50$% formamide, $5$X SSC, $5$X Denhard’s solution, $0.1$% SDS, $100$ $\mu$g/mL denatured sheared herring sperm DNA, and $40$ $\mu$g/mL tRNA. Final washes were at $65^\circ$C in $0.1$ or $0.2X$ SSC, $0.1$% SDS.

RESULTS

Hlx expression increases with myelomonocytic differentiation. In our previous survey of hematopoietic cell lines, the highest levels of Hlx mRNA were observed in macrophage cell lines and, although no mature granulocytic lines were available, the level was at least as high in normal granulocytes sorted from bone marrow. It was therefore of interest to determine whether myelomonocytic differentiation was accompanied by an elevation in Hlx expression. G-CSF induces granulocyte differentiation in the D$^+$ subline of murine promyelocytic tumor WEHI-3B.$^{32}$ Figure 1 shows that granulocytic differentiation of WEHI-3B D$^+$ cells was accompanied by a severalfold increase in Hlx mRNA and comparable increases were observed in two other murine promyelocytic lines induced to granulocytic differentiation, BA34.6My and 4-38-113$^{33}$ (not shown). The D$^+$ subline of WEHI-3B, which does not respond to G-CSF, showed no
increase in \( Hlx \) mRNA. Because the sequences of \( Hlx \) and its human homolog, \( HB24,34 \) are well conserved (90% of amino acids, allowing for frameshift discrepancies and conservative changes\(^{8,30} \)), it was also feasible to analyze differentiation of the well-studied human promyelocytic cell line HL-60 by hybridization at relatively high stringency with the \( Hlx \) probe. Induction of either granulocytic differentiation in HL-60 cells by treatment with DMSO, or macrophage differentiation with phorbol ester, was accompanied by an elevation of the full retroviral genome as well as that of the internal neo cassette (Fig 2). Although transcripts of both types were evident in pools electroporated with the control vector, no LTR-derived transcripts were detectable in pools of cells from these four lines containing the \( Hlx \) construct. This anomaly was resolved when Southern analysis showed that the integrated vector in most \( Hlx \)-neo clones had undergone structural alterations, whereas that in most control clones had not (Table 1). Even clones that seemed to have an intact \( Hlx-neo \) provirus gave low or undetectable \( Hlx \) expression (data not shown). We infer that there was strong selection against stable expression of \( Hlx \) in these four lines (see Discussion).

Although certain transcription factors, including many homeodomain proteins, are able to stimulate their own transcription,\(^{30} \) no evidence was obtained that \( Hlx \) can do so, because there was no change in the low levels of endogenous \( Hlx \) transcript in the FDC-P1 transfectants, nor any induction in the WEHI-707 transfectants (Fig 3A). The \( Hlx \)-expressing transfectants were analyzed for phenotypic changes. We examined at least four independent clones of FDC-P1, three of Tikaut, and two of WEHI-707, as well as an equivalent number of control neo clones from each line.

**Induction of limited differentiation in FDC-P1 cells.** Colony morphology in semisolid media is a sensitive index of myelomonocytic differentiation because the more mature cells are migratory. Like the parental line, FDC-P1 cells infected with the control neo retrovirus grew primarily as compact colonies in soft agar, with only occasional (<4%) diffuse colonies (like that arrowed in Fig 4A). However, a significantly greater proportion (12% to 23%) of cells in-
Hlx ALTERS PHENOTYPE OF HEMATOPOIETIC CELLS

Fig 2. Hlx expression constructs. The Hlx cDNA, identical in both constructs, spans the coding sequence (Materials and Methods). The homeobox is speckled. Constructs were linearized for electroporation at the unique NdeI site in the plasmid backbone. The pEF-MC1neo vector, which derives from pEF-BOS, uses the promoter from the human translation elongation factor 1α gene, including the first exon and part of the second, preceded by the SV40 origin. The polyadenylation sequence is from the human G-CSF gene and the neomycin resistance marker is transcribed from a polyoma enhancer/thymidine kinase promoter-driven cassette. The MPZenSVneo retroviral vector encodes Hlx in the genomic and subgenomic transcripts from the 5' LTR. Location and sizes of expected transcripts are shown (unspliced/spliced).

Table 1. Cell Lines Transfected With Hlx Expression Vectors

<table>
<thead>
<tr>
<th>Cell Line*</th>
<th>Vector</th>
<th>Expression in Pools</th>
<th>Intact Integrants†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early T</td>
<td>pEF</td>
<td>++</td>
<td>neo 3/3</td>
</tr>
<tr>
<td></td>
<td>MPZen</td>
<td>++</td>
<td>Hlx-neo 3/3</td>
</tr>
<tr>
<td>WEHI-707</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Early myeloid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WEHI-3B D†</td>
<td>MPZen</td>
<td>–</td>
<td>neo 3/3</td>
</tr>
<tr>
<td>FDC-P1</td>
<td>MPZen†</td>
<td>++</td>
<td>Hlx-neo 13/13</td>
</tr>
<tr>
<td>Pro-B/myeloid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LyH7</td>
<td>MPZen</td>
<td>–</td>
<td>neo 3/3</td>
</tr>
<tr>
<td>Pre-B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70Z/3</td>
<td>MPZen</td>
<td>–</td>
<td>neo 2/3</td>
</tr>
<tr>
<td>WEHI-401</td>
<td>MPZen</td>
<td>–</td>
<td>Hlx-neo 3/3</td>
</tr>
<tr>
<td>WEHI-401</td>
<td>pEF</td>
<td>–</td>
<td>Hlx-neo 3/3</td>
</tr>
</tbody>
</table>

* The lines are cited in Materials and Methods.
† Integraions were assessed by Southern analysis, using SacI digests for MPZen and HindIII for pEF; both cut near the start of the respective promoters and excise intact constructs (Fig 2).
‡ FDC-P1 cells were infected but all other cell lines were transfected by electroporation.

infected with the Hlx-neo virus gave rise to diffuse colonies (Table 2) and the majority of compact colonies had halos of migratory cells. The effect was consistent over five experiments using three independent retrovirus producer lines.

More than a dozen clones were established from colonies of Hlx virus-infected FDC-P1 cells, covering the range of colony morphologies. By appearance and phenotype, cells from diffuse colonies were more differentiated than the controls (see below). When replated, they generated only diffuse colonies, whereas cells from more compact colonies yielded a range of colony types, suggesting that the diffuse phenotype slowly became fixed. Most clones expressed similarly high levels of Hlx mRNA (Fig 3A), regardless of initial colony morphology. The data below pertain to Hlx clones derived from diffuse colonies; clones from more compact colonies exhibited similar but less pronounced changes. Cells infected with the control retrovirus behaved like the parental line regardless of the original colony type.

Cells of the FDC-P1 Hlx clones had an altered appearance. Whereas stained cytocentrifuge preparations of control clones comprised typical blast cells (Fig 4C), the Hlx clones contained a substantial proportion of larger cells with vacuolated cytoplasm (20% to 40%; see also Table 2), re-
Fig 4. Effects of Hlx overexpression on FDC-P1 cells. (A) Typical FDC-P1 colonies in soft agar. Rare diffuse colonies like that arrowed were more frequent in pools infected with Hlx retrovirus than controls. (B) May-Grünwald-Giemsa-stained cytocentrifuge cell preparations from a Hlx clone. Note the presence of larger, vacuolated cells compared with the neo-only control clone shown in (C).

Hlx induces changes in the behavior and surface phenotype of T-cell lines. Hlx provoked changes in the colony morphology, appearance, and aggregation of Tikaut cells. These cells normally produce dispersed colonies in soft agar, but 15% of the colonies from pools of Tikaut Hlx transfectants were compact (Fig 6A). This property was stable; cells from compact colonies yielded only compact colonies when replated. In liquid medium, cells of the parent line and control clones were semiadherent, pleomorphic in shape, and aggregated spontaneously at high density. In contrast, Hlx-expressing clones were nonadherent, more uniformly round, and, strikingly, did not aggregate (Fig 6B and C). Furthermore, the cells were smaller; mean cell volumes for three clones ranged from 630 to 7 μL compared with 740 to 760 μL for three control clones (measured on log phase cells at densities less than 10^5/mL). WEHI-707 cells would not grow in agar and aggregated in liquid culture even at low densities. Their Hlx derivatives had no discernible changes in these characteristics, nor in cell size or shape.

Flow cytometry showed that Hlx expression altered the surface phenotype of both T-cell lines (Table 3), as illustrated for representative clones in Fig 5. The level of Pgp-1 (CD44) and ICAM-1 (CD54) decreased, whereas that of HSA increased. As both Pgp-1 and ICAM-1 have roles in cell adhesion and aggregation, their downregulation may be relevant to the changes in cell shape, colony morphology, and reduced clumping of the Tikaut clones expressing Hlx (see Discussion). Decreased surface expression of Thy-1 and Ly-1 (CD5) was also noted in this line (Fig 5), but not in the WEHI-707-Hlx clones, which stained brightly for both markers. After several weeks passage, however, the WEHI-
Hlx ALTERS PHENOTYPE OF HEMATOPOIETIC CELLS

Table 2. Effects of Overexpression of Hlx in FDC-P1 Cells

<table>
<thead>
<tr>
<th>Cell behavior</th>
<th>neo</th>
<th>Hlx-neo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Colony morphology of infected pools*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Compact</td>
<td>53–73</td>
<td>15–36</td>
</tr>
<tr>
<td>% Compact + halo</td>
<td>24–44</td>
<td>42–73</td>
</tr>
<tr>
<td>% Diffuse</td>
<td>3–4</td>
<td>12–23</td>
</tr>
<tr>
<td>Cell morphology of derived clones:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Large, vacuolated cells (v blast)†</td>
<td>4.7 ± 2.9</td>
<td>27 ± 9</td>
</tr>
<tr>
<td>Phagocytosis of 4-μm beads</td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Cells containing beads†</td>
<td>2.8 ± 2.1</td>
<td>10.5 ± 2.8</td>
</tr>
<tr>
<td>Beads per cell†</td>
<td>1.3 ± 0.7</td>
<td>1.5 ± 0.8</td>
</tr>
<tr>
<td>Doubling time (h)§</td>
<td>10.9 ± 0.6</td>
<td>10.6 ± 0.4</td>
</tr>
<tr>
<td>Surface markers]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thy-1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Ly-1, Pgp-1, ICAM-1, LFA-1</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>HSA, Mac-1, -2, -3, GR-1</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>B220, AA-4, PB-76, BP-1, slgM</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Gene transcription]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34</td>
<td>++</td>
<td>+/–</td>
</tr>
<tr>
<td>Thy-1</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

* Cells infected with either the Hlx-neo retrovirus or neo control retrovirus were plated in soft agar in the presence of G418. The proportions of colonies with the indicated morphologies are shown as ranges obtained for three independent experiments. Multiple plates were scored blind, with more than 250 colonies per experiment. In all three experiments, $x^2 > 88$, calculated on the raw counts, yielding $P < .001$.
† Mean ± SD of six control clones and nine Hlx-neo clones, from blind scoring of 200 cells per clone on stained, cytocentrifuged preparations.
‡ Mean ± SD of three control clones and four Hlx-neo clones, 200 cells scored per clone, or 100 cells containing beads per clone (Materials and Methods).
§ Mean ± SD of three control clones and six Hlx-neo clones. Doubling times were less than those reported elsewhere for FDC-P1, probably because the high levels of IL-3 supplied in culture were not limiting.
[ ] Expression levels of surface markers are illustrated in Fig 5 and gene transcription levels in Fig 3. All FDC-P1 Hlx clones and controls were also class I MHC+, class II MHC+, F4/80+, Sca-1+, CD44-, MEL 14+, TER119-.

707 Hlx clones declined in CD8 expression, perhaps reflecting gradual progression to the CD4+CD8- stage of development. As this change did not occur in the control clones, it may have been promoted by the ectopic Hlx expression. The reduced surface expression of both Pgp-1 and Thy-1 in the Tikaut clones reflected lower levels of the corresponding mRNAs (Fig 3C and D).

In contrast to the reported effects of enforced expression of the homologous human gene HB24 in the human Jurkat T-cell line,13 ectopic Hlx expression did not elicit changes indicative of activation in either of the mouse T-cell lines. There was no increase in the cell surface level of Ly-1, T-cell receptor chains, or class I MHC, and the IL-2Rα and β chains and class II MHC were not induced. The doubling times of Hlx clones did not differ from the controls (Table 3) and the levels of the growth associated fos mRNA did not change.

The possibility that the transfected T-cell lines might acquire characteristics of the hematopoietic lineages that normally express Hlx was also assessed. However, neither the B-lineage markers CD45R (B220), AA-4, BP-1, PB-76, and slgM, nor the myeloid markers Mac-1, Mac-2, Mac-3, GR-1, and F4/80 could be detected by flow cytometry.

DISCUSSION

We have previously shown that the Hlx homeobox gene is normally expressed in immature B-lymphoid cells and those of the myelomonocytic series, most prominently in mature granulocytes and macrophages.8 Consistent with those findings, Hlx expression increased severalfold when WEHI-3B D+ cells underwent granulocytic differentiation in response to G-CSF, as did that of its human counterpart HB24 when HL-60 promyelocytic cells were induced to monocytic differentiation with phorbol ester or to granulocytic differentiation with DMSO (Fig 1). To address more directly its biologic effects, we used enforced expression in hematopoietic cell lines. As sketched in Fig 7, overexpression in the myeloid cell line FDC-P1 promoted limited maturation, whereas ectopic expression in two immature T-cell lines induced changes in surface markers, including adhesion molecules.

The indicators of differentiation in FDC-P1 Hlx clones (Table 2) were an increased proportion of diffuse colonies, the emergence of larger, vacuolated cells (Fig 4), a modest increase in phagocytosis, and downregulation of two markers of immaturity, Thy-1 and CD34 (Figs 3 and 5). Because the cells did not acquire mature myeloid markers, the extent of maturation was limited, but it is pertinent that FDC-P1 cells normally exhibit a complete block to differentiation. Mature myeloid characteristics have been observed in FDC-P1 cell lines expressing high levels of Hlx.

Fig 5. Surface phenotype of cell lines expressing high levels of Hlx. Flow cytometry fluorescence profiles from representative Hlx clones (—) compared with neo control clones (– – –). Thin curves at the left of one panel for each cell line show background fluorescence. Additional data on surface phenotype are presented in Tables 2 and 3.
only in certain FDC-P1 subclones expressing an introduced tyrosine kinase growth factor receptor, such as that for M-CSF or fibroblast growth factor.\textsuperscript{37,38} Macrophage differentiation in one such derivative was inhibited by high levels of IL-3,\textsuperscript{37} but reduction of the IL-3 level (to 10 U/mL) did not promote further maturation of the FDC-P1 Hlx clones. Chemical inducers enhanced only their morphologic differentiation.

The evidence that Hlx can promote myeloid maturation is consistent with the observation that its mRNA levels increase with myelomonocytic differentiation (Allen et al\textsuperscript{8} and Fig 1). The effects of enforced Hlx expression seen in FDC-P1 cells are therefore likely to reflect its normal role in myeloid differentiation. Because FDC-P1 cells are normally refractory to differentiation, Hlx might have more potent differentiating effects in other myeloid cell lines or normal myeloid cells. Alternatively, Hlx may influence only certain aspects of myelomonocytic differentiation.

### Table 3. Effects of Hlx Expression in T-Cell Lines

<table>
<thead>
<tr>
<th>Cell behavior</th>
<th>Tikaut</th>
<th>WEHI-707</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Hlx-ne0</td>
</tr>
<tr>
<td>Colony morphology</td>
<td>Dispersed, Pleomorphic, semi-adherent</td>
<td>Compact, Round, smaller, nonadherent</td>
</tr>
<tr>
<td>Clumping</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Doubling times (h)\textsuperscript{†}</td>
<td>11.1, 11.7</td>
<td>10.9, 11.4</td>
</tr>
<tr>
<td>Surface markers*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thy-1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Ly-1</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Pgp-1</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HSA</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>CD8</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>LFA-1</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Mac-1, -2, -3, GR-1</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>B220, PB-76, PB-1, slgM</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

\textsuperscript{*} WEHI-707 did not grow in soft agar.

\textsuperscript{†} Determined for two Hlx-neo clones and two control clones (Materials and Methods).

\textsuperscript{†} Expression levels are illustrated in Fig 5. The three Tikaut Hlx clones analyzed, two control clones, and the parent line were Sca-1\textsuperscript{−}, AA4\textsuperscript{−}, CD2\textsuperscript{+}, CD3\textsuperscript{+}, CD4\textsuperscript{+}, TCR\alpha\beta\textsuperscript{−}, class I MHC\textsuperscript{−}, class II MHC\textsuperscript{−}, IL-2R\alpha\textsuperscript{−}, MEL14\textsuperscript{+}, TER119\textsuperscript{−}. The two WEHI-707 Hlx clones analyzed, two control clones, and the parent line were Sca-1\textsuperscript{−}, AA4\textsuperscript{−}, CD2\textsuperscript{−}, CD3\textsuperscript{−}, CD4\textsuperscript{−}, TCR\alpha\beta\textsuperscript{+}, class I MHC\textsuperscript{+}, class II MHC\textsuperscript{−}, IL-2R\alpha\textsuperscript{+}, IL-2R\β\textsuperscript{−}, MEL14\textsuperscript{−}, and TER119\textsuperscript{+}.  

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**Fig 6. Effects of ectopic Hlx expression on Tikaut cells.** Panels compare representative Hlx and neo control clones. (A) Colony morphology in soft agar, illustrating dispersed and compact (arrowed) colonies. (B) Cell shape in culture. (C) Reduced aggregation of Hlx clones.
Several of the surface markers affected by ectopic Hlx expression in the T-cell lines participate in adhesion processes. Pgp-1 (CD44) contributes to homing of recirculating lymphocytes to the high endothelial venules of the lymphoid organs\(^6\) (expression of MEL14, a molecule of related function, was unaffected). Although the function of Thy-1 in the immune system remains unclear, it may convey stimulatory signals and promote adherence to thymic stroma.\(^{41,42}\) ICAM-1, another member of the Ig superfamily, is the principal ligand of the leukocyte-specific molecule LFA-1. This ligand-receptor pair has major roles in adherence of lymphocytes to inflamed tissue, homing to the high endothelial venules, and homotypic lymphocyte aggregation.\(^43\) ICAM-1 expression is usually absent or low on resting lymphocytes, but is readily induced by stimulation of various surface molecules,\(^46\) including Pgp-1 and Thy-1, which also participate in lymphocyte aggregation.\(^45,46\) The reduced clumping in the Tikaut Hlx clones may thus result from downregulation of Pgp-1 and Thy-1 as well as ICAM-1 (LFA-1 expression was not altered). Downregulation of such molecules may also explain the reduced adherence to culture dishes, rounder cell shape, and loss of migration in agar, reflected in compact colonies. The WEHI-707-Hlx clones, which lacked obvious changes in shape or aggregation, did not downregulate Thy-1, and some ICAM-1 expression remained (Fig 5).

The changes in surface markers observed in the Tikaut line may be related to their normal modulation in the early stages of B-lymphoid ontogeny in the bone marrow, during which Hlx is expressed. Thy-1 is found at low levels on progenitor cells in the marrow, including the earliest B progenitors, but not on pre-B cells.\(^46\) Similarly, Pgp-1 is downregulated during pre-B-cell development but reappears on recirculating B cells, where it provides a homing function\(^47\) at a stage where Hlx expression has been extinguished. HSA, a molecule of unknown function, becomes abundant on pre-B cells at the time Ig gene rearrangement commences, but later declines in maturing B cells.\(^48\) Ly-1, which was downregulated by Hlx expression in the Tikaut T-cell line, is not normally found on bone marrow-derived B-lineage cells; the Ly-1\(^+\) B1 subset is thought to have a separate ontogeny.\(^49\)

The effects of Hlx expression clearly depended on cellular context. Although Ly-1 and Thy-1 levels decreased in Tikaut cells, the high levels of these markers in WEHI-707, as illustrated for Thy-1 mRNA in Fig 3C, were unaffected. Maturation stage may account for this difference, because both markers are normally strongly upregulated during the CD4\(^+\)CD8\(^+\) stage of T-cell maturation, represented by WEHI-707.\(^50\) A decrease in Thy-1 was also observed in the FDC-P1 line, but other changes seen in the T-cell lines were not paralleled. An indication that the Pgp-1 gene is likely to be regulated differently in myeloid and lymphoid cells is the distinct transcript pattern (Fig 3D). It is not surprising that a transcription factor would function differently in different cell types, or at different stages of maturation, because transcription factors act combinatorially, so that the effects of any particular factor will depend on the others present in the cell.

There appeared to be selection against stable overexpression of Hlx in some cell lines, because G418-resistant cells isolated from the Hlx-transfected WEHI-3B, LyH7,702/3, and WEHI-401 lines did not express a significant level of Hlx mRNA (Table 1). This is unlikely to represent nonspecific toxicity, because Hlx was expressed abundantly in the two T-cell lines and FDC-P1 and neither their growth rates nor their cloning efficiency in agar were reduced. However, overexpression of Hlx in certain refractory lines may have provoked terminal differentiation and thereby prevented isolation of stable transfectants. WEHI-3B D\(^+\) cells, for example, are known to lose clonogenicity on differentiation.\(^21\) Resolution of this issue would require the use of an induc-
ible vector. It may be relevant that the Hox-2.4 homeobox gene (Hoxb-8 by newest nomenclature) is expressed ectopically in WEHI-3B, due to a proviral insertion near its 5' end, and is also expressed in 70Z/3 for undetermined reason.51 Because Hox-2.4 appears to inhibit terminal differentiation in such lines,53 the Hlx protein might counteract the effect of Hox-2.4 by competing for the same target sites. The likelihood that Hlx and Hox-2.4 share target sites follows from their near identity in the homeodomain third helix, which determines site-specific binding to DNA.

There are notable differences between the T-cell results obtained with Hlx and those reported for its human homolog HB24. Deguchi et al12,15 reported HB24 expression in human T-cell lines and normal T cells stimulated with phytohemagglutinin and phorbol ester, and suggested that it played a role in T-cell activation. Enforced constitutive expression of HB24 in Jurkat cells was found to increase the growth rate and the expression of growth-associated genes in such lines,33 the their near identity in the homeodomain third helix, which because of lipopolysaccharide or T-cell blasts generated with concanavalin A and IL-2. Moreover, treatment of the B- and T-cell blasts with phorbol ester did not elevate Hlx mRNA levels (J. Allen, unpublished results). In the present study, enforced Hlx expression in two murine T-cell lines did not increase growth rates or fos mRNA levels in any of the clones, nor elevate levels of surface activation markers, including the IL-2 receptor chains, class II MHC antigens, or ICAM-1 (Table 3). Thus, no evidence has been found to support a role for Hlx in mouse T-cell activation. Whether all these discrepancies reflect different behavior of the mouse Hlx gene and its human homolog is presently unclear.

The data reported here indicate that Hlx can contribute to the regulation of several surface markers, including adhesion molecules. The surface changes reflected altered message levels in the cases we were able to check (Pgp-1 and Thy-1), although it remains to be determined whether Hlx directly regulates the activity of their promoters. A role for Hlx in control of adhesion processes might account for its expression in a number of nonhematopoietic tissues (Allen et al10 and T. Lints, personal communication). It appears noteworthy that the promoter activity (in fibroblast lines) of the gene for the neuronal cell adhesion molecule N-CAM has recently been shown to be stimulated by a cotransfected Hox-2.3 (Hoxb-9) homeobox gene and depressed by delivery of the Hox-2.4 gene.52 If all these studies with cell lines prove to be valid models for in vivo behavior, they may help to clarify one important way in which homeobox genes regulate cellular differentiation.

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REFERENCES


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27. Karasuayama H, Melchers F: Establishment of mouse cell lines which constitutively secrete large quantities of interleukin 2, 3, 4 or 5, using modified cDNA expression vectors. Eur J Immunol 18:97, 1988


30. Elefanty AG, Cory S: bcr-abl-induced cell lines can switch from mast cell to erythroid or myeloid differentiation in vitro. Blood 79:1271, 1992


39. Li M, Bernard O: FDC-P1 cells engineered to express fibroblast growth factor receptor 1 proliferate and differentiate in the presence of fibroblast growth factor and heparin. Proc Natl Acad Sci USA 89:3315, 1992


52. Jones FS, Prediger EA, Bittner DA, deRobertis EM, Edelman GM: Cell adhesion molecules as targets for Hox genes: Neural cell adhesion molecule promoter activity is modulated by co-transfection with Hox-2.5 and -2.4. Proc Natl Acad Sci USA 89:2086, 1992

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