Differentiation Domains of the Erythropoietin Receptor (43761)

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Abstract. **Ectopic expression of the erythropoietin receptor (Epo-R) in Ba/F3, an interleukin-3 (IL-3)-dependent progenitor cell line, confers both Epo-dependent cell growth and Epo-dependent induction of β-globin mRNA. We have used this system of limited erythroid differentiation to characterize the role of the Epo-R in differentiation. In particular, we have been interested in identifying a differentiation domain of the Epo-R. We have studied three chimeras encoding regions of the extracellular region of the Epo-R and the intracellular region of the IL-3Rβ$_{IL3}$. After transfection into Ba/F3 cells, all three chimeras conferred Epo-dependent growth and induced the expression of β-globin, suggesting that the extracellular region of the Epo-R plays a critical role in differentiation. However, a truncated Epo-R containing only the extracellular region of the Epo-R and a 15 amino acid cytoplasmic tail does not induce β-globin expression, although it is processed to the cell surface and binds Epo. These experiments show that the extracytoplasmic region of the Epo-R is necessary but not sufficient to induce erythroid-specific differentiation in this system.** [P.S.E.B.M. 1994, Vol 206]

The growth and differentiation of hematopoietic cells is regulated by cytokines which act through specific cell-surface receptors (reviewed in 1). Most hematopoietic growth factor receptors, including the receptor for erythropoietin (Epo-R), are members of the cytokine receptor family (2). Although the receptors have an accepted role in stimulating cell growth in hematopoiesis, two central questions about hematopoietic growth factor receptors remain unresolved. Does each hematopoietic growth factor receptor also generate a differentiation-specific signal and what is the nature of that signal?

Only a few studies have addressed these questions in detail, in part, because of a lack of appropriate cell systems (3, 4). However, we have begun to study these questions for the Epo-R expressed in Ba/F3 cells. Ba/F3 are a hematopoietic precursor cell line with an absolute dependence on interleukin-3 (IL-3) for growth. Transfection of the cDNA encoding the Epo-R confers Epo-dependent growth on Ba/F3-Epo-R cells. As we have recently shown, growth in Epo stimulates the expression of β-globin mRNA, a specific erythroid marker, after three days (5). This demonstrates that, in this cell system, Epo-R confers an erythroid-specific signal. We have used the Ba/F3 cell system to study erythroid-specific signaling through the Epo-R. We have shown that Ba/F3 parental cells express erythroid transcription factors which may provide the necessary cellular "environment" for the Epo-R to induce β-globin expression. In addition, we have begun to determine if there is a specific differentiation domain within the structure of the Epo-R. Initial studies suggest that the extracytoplasmic domain of the receptor has a critical role in signaling differentiation, although it appears not to be sufficient to transmit an erythroid-specific signal when expressed independently as a truncated Epo-R.

Materials and Methods

Cells, Electroporation, Transfection and Infection. *Cell culture.* Ba/F3 cells and DA-3 cells (a kind gift of Dr. James Ihle) were maintained in RPMI complete medium (containing antibiotics [ciprofloxacin], 50 μ*M* 2-mercaptoethanol, 10% fetal calf serum [FCS]), supplemented with 10% WEHI conditioned medium. CTLL-2 cells were grown in RPMI complete supplemented with recombinant IL-2 (20 U/ml). Ba/

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F3-EPO-R, DA-3-EPO-R, and CTLL-EPO-R cells were grown in RPMI complete supplemented with recombinant Epo (0.5 U/ml). Cells were grown in a 5% CO_2 incubator at 37°C. E86 cells were maintained in DMEM complete medium (containing streptomycin, penicillin, and 10% FCS) and grown in a 5% CO_2 incubator at 37°C.

Transfection. pLXSN-EPO-R DNA (15 μg) was transfected by the calcium-phosphate precipitate method into subconfluent E86 cells. Transfected cells were incubated overnight with the precipitate, washed, and replated at low density. After 24 hr, cells were selected in RPMI 1640 complete containing G418 (1 mg/ml). Colonies which expressed high neo-resistance titers were pooled and are referred to as E86-Epo-R cells.

Electroporation. 10^7 Ba/F3, DA-3 or CTLL-2 cells were electroporated with the pXM-EPO-R (25 μg) DNA vector in a BIORad Electroporator set at 960 μF, 350 V. Cells were plated at limiting dilution in a 96-well microtiter plate, and cell clones were obtained after selection in RPMI complete supplemented with Epo. Infection: Ba/F3 cells were plated at low density and infected by a 48-hr co-cultivation with subconfluent E86-Epo-R cells grown in DMEM complete supplemented with 10% WEHI and 5 mg/ml polybrene. After 48-hr co-cultivation, Ba/F3 cells were gently resuspended, collected, and replated in RPMI complete containing 10% WEHI conditioned medium and G418 (1 mg/ml). After 24 hr, cells were replated at limiting dilution in a 96-well microtiter plate in the same medium. Cell clones were expanded in the same growth medium and either maintained under these conditions or switched to Epo growth (see Results).

DNA and RNA. *Plasmids.* pXM-Epo-R has been described (6). Chimeric receptors and truncation mutants of Epo-R have been previously described (6). pLXSN-Epo-R was derived by inserting the Epo-R cDNA into the EcoR1 cloning site of pLXSN (7).

RNA. Total RNA was isolated according to the guanidium isothiocyanate/cesium chloride method. Northern blot analysis was carried out according to manufacturer's recommendations: 15 μg of total RNA was fractionated over a 1% agarose-formaldehyde gel, transferred to a nylon membrane, and hybridized with a random primed labeled cDNA probe at 68°C for 2 hr in Quickhyb (Stratagene). Blots were washed at high stringency, and exposed to XAR-Kodak films. Mouse GATA-1 cDNA and mouse NF-E2 (p45) cDNA (8) were a kind gift of Dr. N. Andrews (Children's Hospital, Boston). Human GAPDH was obtained from Dr. R. Moreland (Boston University, Boston).

Results

β-globin Induction in Ba/F3 Cells. Stimulation of the Epo-R in Ba/F3 cells bearing the wild-type Epo-R (Ba/F3-Epo-R) induces the expression of β-globin mRNA, a specific marker of erythroid differentiation (Fig. 1; compare Lane 1 and 3 vs. 2 and 4). This induction occurs within 3 days of cells being switched to Epo (5). The level of β-globin mRNA continues to increase until Day 8 in Epo, is maintained as long as cells are grown in Epo media (data not shown), and is comparable to that seen in uninduced murine erythroleukemia cells (MEL) (cf., Fig. 1, Lane 5).

Erythroid Transcription Factors in Ba/F3-Epo-R Cells and Other Epo-Dependent Cell Lines. In order to characterize the extent of differentiation in Ba/F3-Epo-R cells, we have studied other markers of the erythroid lineage. Late stage markers such as the presence of hemoglobin (by benzidine staining) or of Band 3 mRNA (by Northern blot) were not induced (data not shown). However, three known erythroid transcription factors GATA-1, NF-E2 p45 (8), and EKLF (9) are all present in the parental Ba/F3 cells and in the Ba/F3-Epo-R cells (Fig. 1). Levels of mRNA for GATA-1 and EKLF are slightly increased when Ba/F3-Epo-R cells are grown in Epo whereas levels of NF-E2 do not change significantly (Fig. 1). In contrast, we have also examined two other factor-dependent cell lines, CTLL, a murine IL-2-dependent lymphocyte line, and DA-3, a murine myeloid cell line dependent on IL-3 for growth. Both lines can be transfected with the Epo-R and grown in Epo, but neither produce β-globin mRNA (Fig. 1, Lane 6–10). Our studies show that CTLL cells lack all three erythroid transcription factors (but contain GATA-3 which cross-hybridizes to the GATA-1 probe as seen in Fig. 1, Lane 7–8), whereas DA-3 cells have equivalent levels of GATA-1 and NF-E2 but either no or little EKLF (Fig. 1, Lanes 9 and 10). We cannot determine if the small amount of hybridization seen in Fig. 1, Lanes 9 and 10 is background hybridization to 18S rRNA. Thus, Ba/F3-Epo-R cells appear to be a unique cell line, capable of limited erythroid differentiation and expressing erythroid-specific transcription factors. In contrast, CTLL and DA-3 cells appear to be "nonpermissive" for erythroid differentiation.

Proliferation in Response to Epo in Ba/F3-Epo-R Cells. The differentiation of cells in several systems has been linked with a decrease in their proliferative potential. Therefore, we studied the growth rate of Ba/F3-Epo-R cells grown in Epo and IL-3 as shown in Figure 2. After cells are washed and replated in media containing Epo as the sole growth factor, the rate of growth initially slows for approximately 8–10 days (Fig. 2A). During this period, the doubling time of the cells increases from approximately 12–14 hr to 18 hr. After Day 10, however, the cells again begin to grow at their usual rate with a doubling time, again between 12–14 hr. This suggests that there has been an adaptation to growth in Epo.

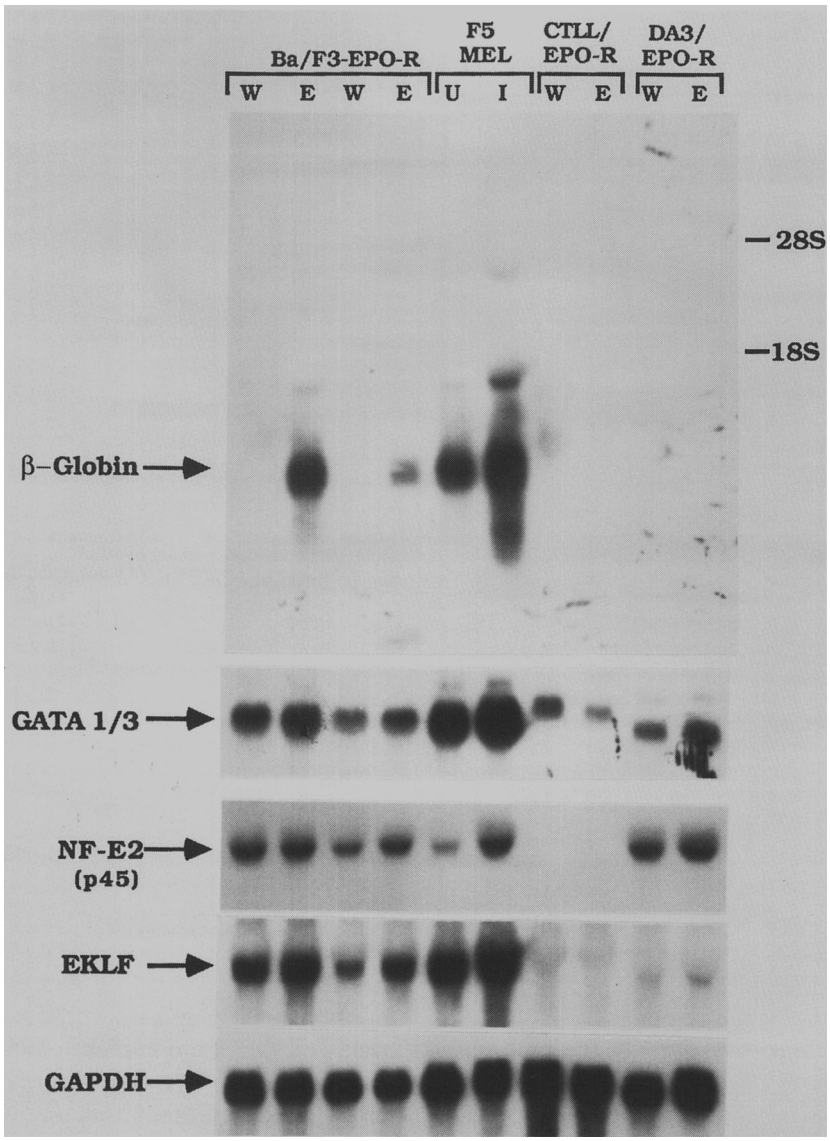


Figure 1. Expression of β-globin and erythroid transcription factors in hematopoietic cell lines. 15 μg of total RNA from indicated cell lines was fractionated on 1% agarose/formaldehyde gel, transferred to a nylon membrane and hybridized sequentially to the indicated probes. Cells were grown under indicated conditions: W, 5% WEHI-3 conditioned media; E, 0.5 units Epo; U, uninduced MEL conditions; I, MEL cells induced to differentiate with activin for 96 hr.

The Extracytoplasmic Region of Epo-R Contains a Differentiation Domain. We have performed further experiments using truncation mutants of the Epo-R cytoplasmic tail and chimeric receptors of the Epo-R and IL-3Rβ to define a differentiation domain of the Epo-R. The cDNAs encoding the truncated or chimeric receptors were co-transfected into Ba/F3 cells with the *neomycin* resistance gene. Cells were allowed to recover for 48 hr in WEHI conditioned media (WEHI CM) and then selected in G418-containing media. After selection, some of these cells were washed and switched to media containing Epo as a growth factor. Interestingly, as summarized in Table I, evaluation of chimeric receptors containing domains of the Epo-R with the IL-3Rβ_{IL3} shows that a critical differentiation function resides in the extracellular region of the Epo-R. Three chimeric receptors containing regions of the Epo-R and the IL3Rβ_{IL3} were examined. The first chimera (Epo-R/IL3R-A) contains the extracellular region of the Epo-R and the transmembrane and cytoplasmic regions of the IL-3Rβ_{IL3}. A second chimera (Epo-R/IL3R-B) contains the extracellular and transmembrane regions of the Epo-R and the cytoplasmic regions of the IL-3Rβ_{IL3}. The third chimera (Epo-R/IL3R-C) contains the extracellular region of the Epo-R extending to the WSxWS box. The remainder of the extracellular, transmembrane, and cytoplasmic regions are from IL-3Rβ_{IL3}. The cDNAs encoding the chimeric receptors were co-transfected into Ba/F3 cells with the *neomycin* resistance gene and selection was carried out as described above. All three chimeric receptors conferred Epo-

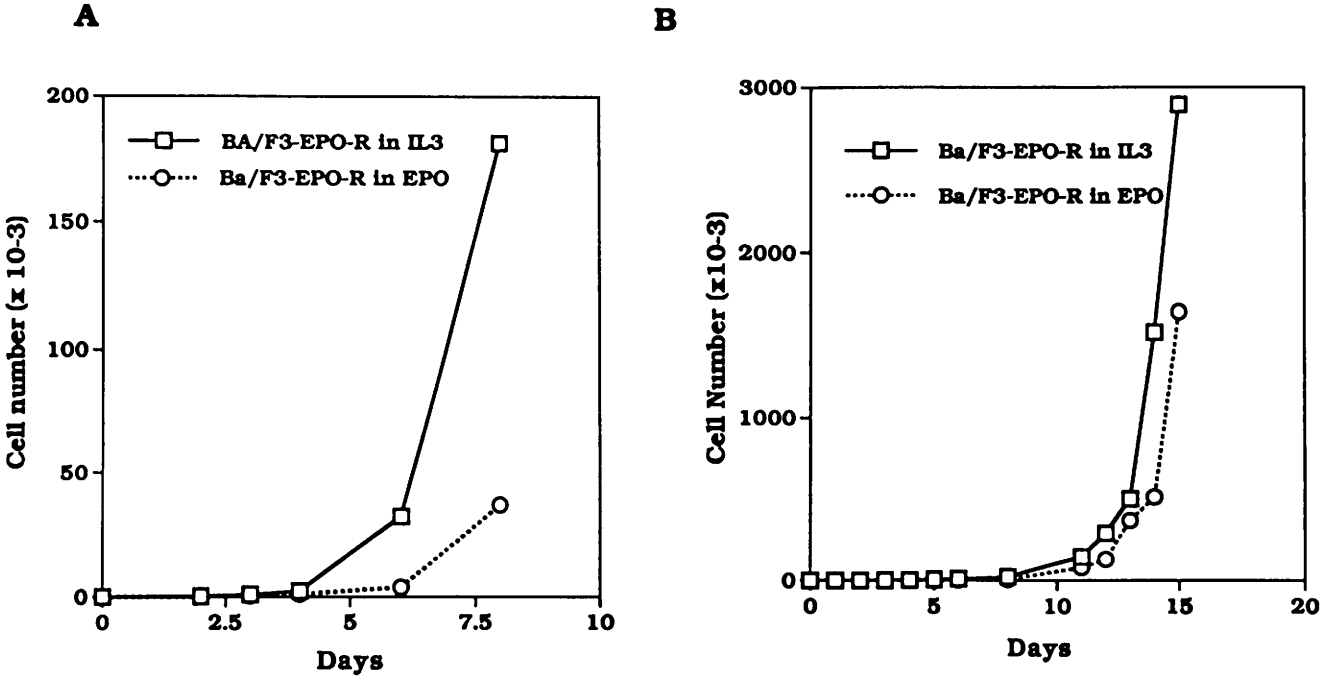


Figure 2. Growth kinetics in Epo and IL-3 for Ba/F3-Epo-R cells. Ba/F3-Epo-R cells were plated at an initial concentration of 2×10^4 cells/ml in RPMI 1640 media supplemented with either 5% WEHI conditioned media (open boxes) or 0.5 U Epo (open circles). Cells were split as needed to maintain density less than 1×10^6 cells/ml. Cell number was counted using a Coulter counter and adjusted for cell splitting. Panel A shows growth in the first eight days. Panel B shows the same curves with the vertical axis expanded to show growth from Day 8–15. All values are the average of three experiments.

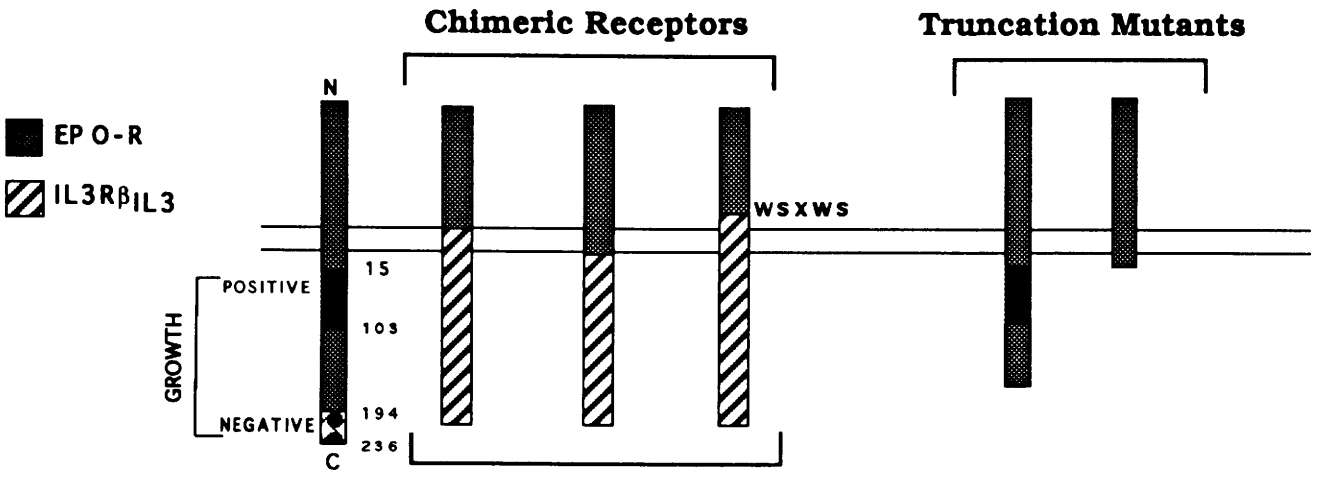
dependent growth, as previously reported (6), and also induced the expression of β-globin mRNA in response to Epo. These results suggest that a critical differentiation function of the Epo-R is actually a function of the extracellular region of the receptor, independent of the transmembrane or cytoplasmic regions.

To examine the effect of regions of the cytoplasmic domain of the Epo-R on β-globin induction, two truncated receptors have been examined. Epo-R(-99) lacks the distal 99 amino acids (aa) of the cytoplasmic region of the Epo-R. This receptor is hypersensitive to Epo and, as shown in Table I, induces the expression of β-globin mRNA when Ba/F3-Epo-R(-99) cells are grown in Epo. Epo-R(-221) retains only 15 aa of the cytoplasmic region of the Epo-R. This receptor is transported to the cell surface and binds Epo (data not shown), however, it does not confer Epo-dependent growth. Therefore, cells were maintained in a combination of Epo and IL-3. To examine the effects of Epo plus IL-3 on cells containing full-length Epo-R, we first assayed for the expression of β-globin mRNA in Ba/F3-Epo-R cells. Under these conditions, cells also express β-globin mRNA after three days but the level of expression is reduced compared with growth in Epo alone (data not shown). In contrast, in the Ba/F3-Epo-R(-221) cells no β-globin mRNA is induced. These experiments suggest several conclusions. First, it is apparent that the presence of IL-3 in the media suppresses the expression of β-globin. Second, the distal 99 aa of the cytoplasmic tail are not required for differentiation signaling. Finally, the combination of the data from the chimeric receptors and the Epo-R(-221) truncation mutant together suggest that the extracytoplasmic region of the Epo-R is necessary but not sufficient to induce β-globin expression. Perhaps a second nonspecific domain is provided by the IL-3R cytoplasmic tail of our chimeric receptors. In this regard, it is interesting that Fukunaga *et al.* (3) describe two important differentiation domains in the G-CSF-R, although the function of these two separate domains is unknown.

Discussion

We have previously shown that the Epo-R is necessary but not sufficient for the induction of β-globin mRNA in a hematopoietic cell system (5). The results described herein extend our previous results and further characterize the Ba/F3 cell system as a model system for limited erythroid differentiation. Ba/F3-Epo-R cells express β-globin mRNA in response to Epo in contrast to CTLL or DA-3 cells transfected with the Epo-R and grown in Epo. The data shown in Figure 1 suggest that this may correlate with the expression of known erythroid transcription factors in Ba/F3 cells. In many systems, differentiation and cell growth are intimately linked. In general, the induction of cellular differentiation leads to an arrest of cell growth. Ba/F3 cells appear to be capable only of limited differentiation, but continue to grow after induction of β-globin mRNA. Although this makes the sys-

Table I. Growth in Epo and β-Globin Expression Induced by Chimeric and Truncated Receptors[a]



	wt	A	B	C	-99	-221
GROWTH in EPO	+	+	+	+	+	-
DIFFERENTIATION (β-globin)	+	+	+	+	+-	-

[a] Structure of chimeric receptors of Epo-R and IL3Rb$_{IL3}$ and truncated forms of Epo-R are shown. The table summarizes induction of Epo-dependent growth and β-globin induction in various subclones.

tem limited in some respects, it makes it a useful system in analysis of the differentiation functions of the Epo-R.

The use of chimeric receptors to study functional domains of the cytokine receptors is well established. We have used chimeric receptors of the Epo-R and IL-3Rβ$_{IL3}$ to study the control of β-globin mRNA induction in Ba/F3 cells. Interestingly, the extracellular region of the Epo-R is capable of inducing the expression of β-globin mRNA, but only in the context of a functional chimeric receptor. The truncation mutant of the Epo-R which contains the entire extracellular domain and a 15 aa cytoplasmic tail does not induce β-globin mRNA. In addition, the distal 99 aa of the cytoplasmic region does not appear to mediate the induction of β-globin, in contrast to the G-CSF-R. As with previous results regarding the pattern of tyrosine phosphorylated proteins seen in response to stimulation of Epo-R/IL-3R chimerae (10, 11), two models could explain these results. It is possible that there is an additional, unidentified transmembrane protein which binds to the extracellular region of the Epo-R after Epo binding and stimulates the induction of β-globin mRNA. On the other hand, the homodimerization of the chimera which occurs in the presence of Epo may create a unique configuration (homodimerization of the cytoplasmic region of the IL-3Rβ$_{IL3}$) which is capable of inducing β-globin expression. Future experiments will address these two models.

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1. Metcalf D. The molecular control of cell division, differentiation, commitment and maturation in hematopoietic cells. Nature **339**:27–30, 1989.
2. Miyajima A, Kitamura T, Harada N, Yokota T, Arai K-I. Cytokine receptors and signal transduction. Annu Rev Immunol **10**:295–331, 1992.
3. Fukunaga R, Ishizaka-Ikeda E, Nagata S. Growth and differentiation signals mediated by different regions in the cytoplasmic domain of granulocyte colony-stimulating factor. Cell **74**:1079–1087, 1993.
4. Otani H, Siegel J, Erdos M, Gnarra J, Toledano M, Sharon M, Mostowski H, Feinberg M, Pierce J, Leonard W. Interleukin (IL)-2 and IL-3 induce distinct but overlapping responses in murine IL-3 dependent 32D cells transduced with human IL-2 receptor b chain: Involvement of tyrosine kinases(s) other than p56^{lck}. Proc Natl Acad Sci **89**:2789–2793, 1992.
5. Liboi E, Carroll M, D'Andrea A, Mathey-Prevot B. The eryth-

ropoietin receptor signals both proliferation and erythroid-specific differentiation. Proc Natl Acad Sci **90:**11351–11355, 1993.

6. Zon LI, Moreau J-F, Mathey-Prevot B, D'Andrea AD. The erythropoietin transmembrane region is necessary but not sufficient for activation by the Friend virus gp55 glycoprotein. Mol Cell Biol **12**(7):2949–2957, 1992.
7. Miller AD, Rosman GJ. Improved retroviral vectors for gene transfer and expression. BioTechniques **7:**980–989, 1989.
8. Andrews N, Erdmont-Bromage H, Davidson M, Tempst P, Orkin S. Erythroid transcription factor NF-E2 is a hematopoietic specific base leucine zipper protein. Nature **362:**722–728, 1993.
9. Miller IJ, Bieker JJ. A novel erythroid cell-specific murine transcription factor that binds to the caccc element and is related to the kruppel family of nuclear proteins. Mol Cell Biol **13:**2776–2786, 1993.
10. Chiba T, Nagata Y, Machide M, Kishi A, Amanuma H, Sugiyama M, Todokoro K. Tyrosine kinase activation through the extracellular domains of cytokine receptors. Nature **362:**646–648, 1993.
11. Sakamaki K, Wong H, Miyajima I, Kitamura T, Todokoro K, Harada N, Miyajima A. Ligand-dependent activation of chimeric receptors with the cytoplasmic domain of the interleukin-3 receptor beta subunit (beta IL3). J Biol Chem **268:**15833–15839, 1993.