Progressive inactivation of the expression of an erythroid transcriptional factor in GM- and G-CSF-dependent myeloid cell lines

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ABSTRACT

The transcriptional binding protein NFE-1 (also called GF-1 and Ery-f1) is thought to play a necessary, but not sufficient, role in the regulation of differentiation-related gene expression in a subset of hematopoietic lineages (erythroid, megakaryocytic, and basophil-mast cell). In order to clarify the mechanism which underlies the lineage-specificity of the NFE-1 expression, as well as the relationship between the expression of this factor and growth factor responsiveness, we have evaluated the capacity of erythropoietin (Epo)-, granulomonocytic (GM)-colony stimulating factor (CSF)-, and granulocyte (G)-CSF-dependent subclones derived from the interleukin 3 (IL-3)-dependent cell line 32D, to express 1) NFE-1 mRNA, 2) NFE-1-related nuclear proteins, and 3) chloramphenicol acetyl transferase (CAT) activity when transfected with a CAT gene under the control of NFE-1 cognate sequences. NFE-1 mRNA was found to be expressed not only in cells with mast cell (IL-3-dependent 32D) and erythroid (Epo-dependent 32D Epo1) phenotypes, but also in cells with predominantly granulocyte/macrophage properties, such as the GM-CSF- (early myelomonocytic) and G-CSF- (myelocytic) dependent subclones of 32D. However, a gradient of expression, correlating with the lineage, the stage of differentiation, and the growth factor responsiveness of the cell lines, was found among the different subclones: Epo $\geq$ IL-3 > GM-CSF > G-CSF. Binding experiments demonstrated NFE-1 activity in all cell lines except the G-CSF-dependent line. Function of the NFE-1 protein was assessed by the expression of the CAT gene linked to the SV40 promoter and a mutant (-175 T→C) HPFH γ -globin promoter. High level CAT expression was seen only in the Epo1 cells although low level expression was also seen in the parent 32D. These results demonstrate that the specificity of the expression of NFE-1 for the

erythroid—megakaryocytic—mast cell lineages is obtained by progressive inactivation of its expression in alternative lineages.

INTRODUCTION

Mature end-cells are generated from pluripotent stem cells through processes leading to an irreversible commitment of progenitor cells. The hematopoietic and mesenchymal (muscle, adipose tissue, endothelial cells) systems are among the best examples of such processes¹⁻³. In the hematopoietic system, a self-renewing stem cell generates progenitor cells which, in turn, proliferate and progressively differentiate into mature red and white cells. This process is regulated by a series of growth factors in the following sequence: interleukin 3 (IL-3), granulomonocytic colony stimulating factor (GM-CSF) and lineage specific growth factors, such as erythropoietin (Epo), macrophage (M)- and granulocyte (G)-CSF. The molecular basis for the commitment of progenitor cells to differentiate into distinct cell types is unknown. Signals delivered by different growth factors might regulate the expression of specific transcription factors, inducing a cascade of effects leading to distinct differentiation pathways; alternatively, the expression of growth factor receptors might itself be regulated by a subset of lineage-specific transcription factors necessary for establishing a specialized pattern of gene transcription. These hypotheses are not mutually exclusive. An experimental analysis of these problems has been hampered by the lack of adequate cell lines. In fact, most of the available hematopoietic cell lines are leukemic and do not respond to hematopoietic growth factors. Furthermore, a comparison of the results obtained from lines with different phenotypes is difficult because of their different genetic backgrounds. On the other hand, although normal progenitor cells can be purified in low numbers^{4,5}, gene expression in these cells can be measured only with the relatively insensitive technique of 'in situ' hybridization; moreover, these homogeneous cell populations give

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rise to a heterogeneous progeny after only one cell division⁶.

We recently obtained^{7,8} from the hematopoietic IL-3-dependent cell line 32D⁹ a number of lines with the same genotype immortalized at different stages of the differentiation process; these lines respond to each specific hematopoietic growth factor (Epo, GM-CSF and G-CSF) in a fashion similar to their normal counterpart, proliferating and differentiating along the appropriate lineage. In order to clarify the relationship between growth factor-responsiveness and expression of lineage-specific transcription factors, we studied the expression of the gene encoding the transcription factor NFE-1¹⁰ (also known as GF-1¹¹ or Ery-f1¹²) in these different subclones of 32D. This gene has been reported to be expressed, at the mRNA and/or protein level, in erythroid¹⁰⁻¹⁷, megakaryocytic^{16,17} and basophilic¹⁶ cells, but not in other hematopoietic cells¹⁰⁻¹⁷. NFE-1 binds to¹⁰⁻¹⁸, and possibly activates^{10,17-20}, promoter and enhancer regions of several erythroid and megakaryocytic specific genes, including globin and Epo-receptor²¹ genes, and might represent a 'master' gene regulating part of the differentiation program of these cells. In this paper, we show that mRNA encoding NFE-1 is not only transcribed in basophil/mast cells or in erythroblasts but also, at a lower level, in GM-CSF and G-CSF dependent cells committed to differentiate along the macrophagic and granulocytic pathway. Our data are compatible with the hypothesis that NFE-1 is first activated in an early pluripotential progenitor cell and that restriction of NFE-1 mRNA and functional protein to mature erythroid and mast cells occurs by extinction of NFE-1 gene expression in progressively differentiated granulo-monocytic cells. We discuss the possibility that GM-CSF and G-CSF are responsible for the reported granulo-monocytic extinction of NFE-1.

MATERIALS AND METHODS

Cell lines

The Friend erythroleukemia cell line was maintained by bi-weekly passage in IMDM supplemented with β -mercaptoethanol and 5% FBS. The 32D cl3 cell line⁹ and its Epo-, GM-CSF-, or G-CSF-dependent subclones⁷ were maintained by bi-weekly passage in McCoy's medium (Gibco) modified as described²² and supplemented with antibiotics, L-glutamine and pyruvic acid (1% vol/vol, Gibco Laboratories) and horse serum (10%, vol/vol, Hyclone Laboratories, Logan, UT) and the appropriate growth factor (see below). The cells were periodically tested for the presence of mycoplasma contamination with Mycoplasma T.C.II (Gen-probe, San Diego, CA) and were found to be mycoplasma free.

Growth factors

LBRM 33²³ cell-conditioned medium was the supernatant of a serum-free culture of phytohemagglutinin (PHA)-stimulated cells prepared as described²⁴. Pure recombinant murine IL-3²⁵ and GM-CSF²⁶ were provided by Dr. J.J. Mermod (Glaxo, Geneva, Switzerland). Pure recombinant human Epo²⁷ and G-CSF²⁸ were provided by Drs. J. Egrie and L. Souza (Amgen, Thousand Oaks, California), respectively. Each conditioned medium or growth factor was used at a concentration previously shown to be maximally effective in promoting colony formation by each growth factor dependent subclone of 32D⁷, as well as by purified murine progenitor cells^{6,24} under FBS-deprived conditions. These concentrations were 1% (v/v) for LBRM 33

CM, 4×10^{-10} M for GM-CSF, 6×10^{-10} M for IL-3, 5×10^{-10} M for G-CSF, or 1.5 U/ml for Epo.

Northern blot and RNA hybridization

RNA was prepared by phenol-chloroform extraction of acid guanidinium thiocyanate lysates²⁹. RNA, dissolved in MOPS buffer (20 mM MOPS, 5 mM EDTA, 5 mM sodium acetate, pH 7.0), containing 50% formamide and 1.9 M formaldehyde, was heated at 65°C for 15 min, supplemented with 1 μ g of ethidium bromide and loaded onto 1% agarose gel in MOPS buffer containing 0.66 M formaldehyde. The gel was run at 30 V for 18 hrs. After blotting to nitrocellulose membranes, the hybridization was carried out in $4.7 \times$ SSPE, 50% formamide, $0.47 \times$ Denhardt, 10% dextran sulphate, 0.1% SDS, 0.34% milk, 180 μ g/ml salmon sperm DNA, at 42°C for 18 hrs. Washing was down to $0.1 \times$ SSC, 0.1% SDS at 50°C. The filters were subsequently hybridized with a murine NFE-1 probe, mouse β -globin³⁰, myeloperoxidase³¹ or actin.

RNAase Protection Experiments

Different fragments of the murine NFE-1 cDNA, obtained by polymerase chain amplification from mRNA of MEL cells, were cloned into the HindIII site of the pGEM7 plasmid. In the experiment shown, a labelled 1462 nt. long antisense RNA probe, spanning nucleotides 940 to 1312 of NFE-1 cDNA¹¹, was obtained by transcribing with SP6 polymerase³² a plasmid linearized by digestion with XmnI (the specific activity of the CTP used was 400 Ci/mmol). After RQ1 DNAase digestion and phenol-chloroform extraction, the probe was ethanol-precipitated and used without further purification. Total RNA from MEL, 32D, 32D subclones and NIH 3T3 fibroblasts was hybridized for 18 hrs to the RNA probe ($\approx 5 \times 10^6$ cpm) at 41°C in 30 μ l of 80% formamide, 0.4 M NaCl, 40 mM PIPES pH 6.4, 1 mM EDTA, and then digested for 30 mins at 30°C in 300 μ l with RNAases A and T1 (40 μ g/ml and 1 μ g/ml, final concentration, respectively), followed by proteinase K digestion and phenol-chloroform extraction. Gel electrophoresis was in standard 6% acrylamide, 8 M urea sequencing gels.

Electrophoretic mobility shift assay

Nuclear extracts (25–50 μ l) were prepared from $1-2 \times 10^6$ cells growing in the log phase according to³³, in the presence of leupeptin, aprotinin and pepstatin. In vitro binding reactions and electrophoretic runs are as in^{13,14}.

Plasmids for Cell Transfection

The plasmids used for cell transfection have been already described³⁴; briefly, they consist of a CAT vector (pSV0)³⁴ driven by the normal or a mutated (–175 TC HPFH) γ -globin promoter¹⁹. The promoterless pSV₀, the SV40 enhancer-driven pSV₂ CAT constructs, and the β -galactosidase reporter plasmid PCH111 have been described in refs. 34, and 35, respectively.

Cell transfection

Cells (20×10^6 /ml) were transfected by electroporation (Cell porator, BRL, Bethesda, MD; pulse field strength = 750 v/cm and capacitance = 800 μ F, temp = 4°C) with 10 μ g of β -galactosidase reporter plasmid (pCHIII)³⁵, and/or 25 μ g of a CAT construct. After 48 hours of incubation at 37°C in fresh culture medium supplemented with growth factors, the cells were harvested, counted, resuspended in lysis buffer (0.25M Tris. HCl

pH 7.8) at a concentration of $10^6/10 \mu\text{l}$ and lysed by repeated cycles of freezing and thawing. CAT activity was measured by ^{14}C -chloramphenicol butyrylation test^{19,36} and β -galactosidase activity according to ref. 35.

RESULTS

Characterization of the growth factor-dependent subclones of 32D

The 32D cell line and its subclones have been described elsewhere^{7,8}. During the course of this study, we have periodically checked the characteristics of these cells which are most relevant to this paper (Table I).

32D clone 3 is a basophil/mast cell line maintained in IL-3 (10 units/ml) which clones at low frequency in GM-CSF (1 ng/ml) (1 colony/ 10^3 cells), Epo (1 unit/ml), or G-CSF (10^3 units/ml) (1 colony/ 10^4 cells). Therefore, a minority of 32D clone 3 cells may be considered multipotent progenitor cells.

32D Ro is a version of 32D maintained for more than a year in conditioned medium containing IL-3 and GM-CSF³⁷. 32D Ro still differentiates into basophil/mast cells in the presence of IL-3 and gives rise to colonies at low frequency (1 colony/ 10^3 cells) in GM-CSF and G-CSF but has lost the capability to respond to Epo. In the presence of GM-CSF or G-CSF, 32D Ro differentiates into monocytes expressing mac1, mac2 and mac3 antigens and M-CSF receptors³⁸ or into neutrophils expressing myeloperoxidase and lactoferrin^{37,38}, respectively. A minority of 32D Ro cells may be considered as progenitor cells restricted to the mast cell and myelo-monocytic lineage.

32D Epo-1 and Epo-2, 32D GM-1 and 32D GM-2, 32D G-1 and 32D G-2 are independently isolated Epo-, GM-CSF or G-CSF-dependent subclones of 32D clone 3. The concentrations of Epo, GM-CSF or G-CSF which induce maximal response from these cell lines are similar to those which induce maximal response from normal progenitors under the same culture conditions^{6,7,24}. Epo-dependent cells show typical erythroid markers such as hemoglobin, erythrocyte-specific carbonic anhydrase II and Epo receptors (400 Epo receptors per cell with a Kd of 410 pM, values similar to those obtained for CFU-E³⁹). IL-3-responsive revertants (Epo1-1) of the Epo-1 and Epo-2 lines may be selected once again by growing the cells in IL-3.

However, these clones express erythroid, but not mast cell, markers. Conversely, GM-CSF-dependent lines (GM-1 and GM-2) only form colonies in response to GM-CSF or IL-3 but not in the presence of Epo or G-CSF. G-CSF dependent lines (G-1 and G-2) only form colonies in response to G-CSF. These cells divide few times in the presence of IL-3 and then die. Morphological and cytochemical analyses of these cell lines show that the GM-CSF-dependent cells have characteristics typical of myeloblasts, while G-CSF-dependent cells more closely resemble myelocytes or neutrophilic metamyelocytes. Therefore, the progenitor cells present in the Epo, GM-CSF and G-CSF-dependent subclones of 32D are lineage-restricted.

Presence of NFE-1 mRNA and binding activity in the subclones of 32D

To assess the relationship between the expression of this gene and commitment to erythropoiesis, we measured the levels of NFE-1 mRNA in all subclones of 32D (Fig. 1). Northern blot analysis showed a single) 2.2 Kb band hybridizing to NFE-1 cDNA that is present at relatively high levels not only in mouse erythroleukemia (MEL) cells, in the Epo-1 and Epo-2 lines and also in the parent 32D, but also in GM-1 and GM-2 cells. Based on normalization by hybridization to actin mRNA (not shown) or evaluation of rRNA, the level of NFE-1 mRNA in the subclones of 32D was 10–60% of that observed in MEL cells. In particular, the level of NFE-1 was higher in 32D and Epo-dependent lines and slightly lower in GM-1, GM-2 and 32D Ro. A very low level of NFE-1 mRNA was also found in the well-differentiated G-CSF-dependent lines, G-1 and G-2, upon overexposure of the same autoradiograph (not shown; but see Fig. 2). GM-1 and GM-2 cells, when switched from GM-CSF to IL-3, showed 2–3 fold increased levels of NFE-1 mRNA indicating that GM-CSF may inhibit NFE-1 gene expression (Fig. 1a, lanes 6 and 7). Control experiments (Fig. 1) using probes for globin and myeloperoxidase as erythroid and myeloid markers, respectively, and for ubiquitous α -actin mRNA (not shown), demonstrated expression of globin mRNA exclusively in the Epo-1 and Epo-2 lines, and of myeloperoxidase mRNA predominantly in G-1, G-2, GM-1 and 32D Ro cells and at lower levels in GM-2 and 32D cells. Expression of NFE-1 mRNA was also investigated by RNAase analysis; using various fragments

Table I. Growth characteristics of the 32D subclones used in this study*

	CELL LINES					
	32D	32D Ro	32D Epo (1 and 2)	32D Epo1.1	32D GM (1 and 2)	32D G (1 and 2)
Major growth factor responsiveness (>1 colony/ 10^3 cells)	IL-3	IL-3	Epo	IL-3/Epo	GM-CSF IL-3	G-CSF
Cell phenotype	basophil/ mast cell	basophil/ mast cell	erythroid	erythroid	early myelo- monocytic	myelo- cytic
Minor growth factor responsiveness (1 colony/ 10^4 – 10^6 cells)	Epo GM-CSF G-CSF	GM-CSF G-CSF	IL-3	—	—	IL-3

* All the cell lines investigated die within 24–48 hours in the absence of growth factors, have the diploid male karyotype of the original 32D cell line and do not induce tumors when injected into histocompatible recipients (7). Growth factor responsiveness has been evaluated on the basis of the capacity of the cells to give rise to colonies in semisolid cultures in the presence of pure recombinant growth factor under serum-deprived conditions. The cell phenotype has been determined on the basis of cytochemical analysis (7) or on the basis of the level of expression of lineage-specific markers (7 and Fig. 1). A family tree describing the origin and the characteristics of all the lines has been published in ref. 8.

Figure 1. Northern blot analysis of NFE-1 (top panel), mouse β -maj globin (middle panel) and myeloperoxidase (bottom panel) in growth factor-dependent cell lines. Ten μ g of total RNA (from cells growing in the log phase) was loaded for each cell line and the level of hybridization to a mouse NFE-1 cDNA clone was compared to the levels of hybridization with increasing concentrations (5–30 μ g) of total RNA from uninduced MEL cells. Controls for the level of RNA loaded in each lane have been represented by the intensity of the ribosomal RNA bands and by the level of expression of the actin gene (not shown). For NFE-1 detection, filters were exposed for 24 hrs; a 15-day exposure revealed a similar NFE-1 band in the G-1 line (not shown).

of NFE-1 as riboprobes, we found the expected bands in 32D, Epo, GM and, at a much lower level, G cells (Fig. 2 and data not shown). The decreased level of NFE-1 mRNA in GM cells treated with GM-CSF versus IL-3 was also confirmed (Fig. 2, lanes 8 and 9). No hybridization was detected with RNA from non-hemopoietic cells such as NIH 3T3 (lane 16).

To assess whether NFE-1 mRNA is translated into protein, we determined the ability of nuclear extracts from the various cell lines to bind in vitro to NFE-1 cognate sequences on appropriate oligonucleotides. Figure 3 shows that binding can be clearly detected with extracts from 32D, Epo- and GM-CSF-dependent lines. An oligonucleotide^{13,14} comprising a sequence of the γ -globin promoter capable of binding either NFE-1 or the ubiquitous OTF-1 factor was used. The NFE-1 band comigrated with that generated with MEL extracts, and was selectively abolished in the presence of a competing unlabelled mouse α -globin promoter oligonucleotide¹⁴ carrying a NFE-1 (Fig. 3B, lanes 5 and 6), but not an OTF-1 binding site; neither the NFE-1

Figure 2. RNAase protection assay of NFE-1 mRNA expression. Lanes 1 and 2: labelled size markers; lanes 3–6: MEL RNA (10, 5, 1 and 0.1 μ g); lanes 7–16: RNAs from the cell lines, as described in Fig. 1 (20 μ g, except Epo-1: 7 μ g). GM-2 cells were grown in IL-3; when grown in GM-CSF, NFE-1 level was approximately 2-fold lower (not shown). Epo-1 cells were grown in Epo, and 32D in IL-3. G-1 and G-2 cells were cultured in the presence of G-CSF or IL-3; in the latter condition, these cells only survive for a few days.

Figure 3. In vitro binding to a 32P-labelled q-globin promoter oligonucleotide containing NFE-1 and OTF-1 binding sites with nuclear proteins from 32D cells and subclones. The labelled oligonucleotide spans nucleotides –201 to –156 of the human γ -globin promoter, and exhibits overlapping OTF-1 and NFE-1 binding sites^{13,14}. Three μ l of nuclear extract were incubated with labelled oligonucleotide ($\approx 4 \times 10^4$ cpm) and unlabelled competitor where indicated. The amount of oligonucleotide was 0.05–0.1 ngs in A and C, and 0.5 ngs in B. Unlabelled mouse α -globin promoter oligonucleotide was added to specifically compete for NFE-1 binding¹⁴ in a 50-fold molar excess relative to the labelled oligonucleotide (A, compare lanes 1 and 2; B, compare lanes 4 and 5) or in a 200-fold excess (B, lane 6). Unspecific competitor (γ -globin CCAAT box region oligonucleotide, ref. 14) was also added at 50–200 fold molar excess (B, lanes 7 and 8). 32D, Epo-2, and GM-1/GM-2 cells were grown in IL-3, Epo, and GM-CSF, respectively where not otherwise indicated.

nor the OTF-1 band was competed by an unrelated oligonucleotide from the γ -globin CCAAT box region¹⁴ (Fig. 3B, lanes 7 and 8). The ability to bind OTF-1 was used as an internal marker, and allowed us to demonstrate NFE-1 binding in different lines at relative levels roughly consistent with those observed by RNA analysis (that is, higher in Epo and slightly lower in GM and 32D lines). Significantly, growth of GM cells in IL-3, rather than in GM-CSF, correlated with a 2–3 fold higher NFE-1/OTF-1 ratio (Fig. 3C), as expected from the corresponding mRNA levels of NFE-1. Additionally, 32D Ro had a much lower NFE-1 level when treated with G-CSF than when grown in IL-3 (not shown). A very faint band migrating in the NFE-1 position was also seen in the G-1 line (consistent with the very low mRNA levels) but the occurrence of some degradation of these extracts prevented conclusive confirmation of its nature (not shown).

Expression of the erythroid-specific γ -globin promoter in different subclones of the 32D cell line

Finally, we evaluated the ability of NFE-1 to support transcriptional activity using erythroid-specific γ -globin promoter-driven chloramphenicol acetyltransferase reporter (CAT) plasmids¹⁹. It has been reported^{18,19} that a nucleotide substitution (–175 T→C) in the γ -globin promoter (which is one mutation leading to Hereditary Persistence of Fetal Hemoglobin (HPFH)), results in erythroid-specific overexpression of a reporter HPFH, relative to the normal γ -globin CAT plasmid; the mutation quantitatively and/or qualitatively improves NFE-1 binding to the promoter, and increased expression is dependent on this effect^{14,18,19}. Using these plasmids, we found (Fig. 4) that expression of the γ -globin promoter and the effect of the mutation were seen only in the Epo-dependent cell lines, whether grown in Epo or IL-3. It is noteworthy that, although the cells with the potential to express an erythroid phenotype have been selected with Epo, the events required for erythroid specific expression are inherited in a dominant fashion by the progeny independently from the growth factor in which they grow. In fact, IL-3-dependent revertants of the Epo-dependent subclones are benzidine-positive and have the capacity to express high levels of CAT activity when driven by the γ -175 γ -globin promoter (not shown).

DISCUSSION

In this paper, we present evidence that NFE-1 is not only expressed in the subclones of the 32D cell lines with basophil/mast cell (the original 32D cell line) or erythroid (32D Epo1) phenotype, but also in subclones with early myelomonocytic (32D GM-1, GM-2) or myelocytic (32D G-1, G-2) phenotype, which depend for growth on GM-CSF or G-CSF, respectively, and which have irreversibly lost the capacity to give rise to erythroid (or mast cell) clones. The gradient of expression found among the different subclones correlated with the lineage, the stage of differentiation, and the growth factor responsiveness of the cell lines (Epo IL-3 $\geq$ GM-CSF > G-CSF).

The observation that, among mature blood cells, NFE-1 mRNA or protein can be detected in erythroid, megakaryocytic and mast cells only, led to the hypothesis that the NFE-1 gene may be first activated in a progenitor restricted to the erythroid-megakaryocytic-mast cell lineage^{16,17}. However, our data indicate that NFE-1 is also expressed in cells developing into

Figure 4. Expression of the erythroid-specific γ -globin promoter in different subclones of the 32D cell lines. A: plasmid pSV γ -175¹⁹ was cotransfected with a β -galactosidase reporter plasmid (PCH 111)³⁵ into the indicated cell lines (each one grown in the factor for which it is dependent). The level of β -galactosidase activity observed in mock transfected cells was usually less than 0.050 O.D. and was subtracted from the levels reported. A representative experiment is shown: similar results were obtained in three separate experiments. B: expression of the normal (pSV γ) and HPFH (pSV γ -175) γ -globin promoter in Epo-1 (hatched bars) and GM-1 (open bars) cell lines. The promoterless (pSVo) and SV40 enhancer-promoter driven (pSV2)³⁴ CAT constructs were also transfected as negative and positive controls, respectively. Background activities obtained with pSVo have been subtracted from the activities presented in the figure. The results represent the mean (SD) of four separate experiments.

early granulocytes and monocytes, suggesting that the NFE-1 gene might be first activated in a common progenitor to both the erythroid-megakaryocytic-basophilic and myeloid lineages. These data imply that NFE-1 expression is not sufficient (though it might be necessary) for commitment to the erythroid-megakaryocytic-basophilic lineage.

We propose that following activation in a common progenitor, NFE-1 mRNA and protein are produced in cells developing into a variety of lineages, including the myeloid one. Cells developing into erythroblasts or basophils maintain (or possibly increase) their NFE-1 level; however, as myeloid precursors further differentiate, the expression of NFE-1 progressively declines and, eventually, is extinguished. Thus, the observed lineage-specific expression of NFE-1 could be mediated by selective repression of the previously activated gene, in the course of myeloblast differentiation, rather than by selective and lineage-specific activation of the gene during early phases of the commitment process^{16,17}. We are aware that this model is based on cell lines

which, although non-transformed and with an apparent normal karyotype, might differ significantly from normal hematopoietic cells. We are currently investigating the expression of NFE-1 during the process of normal myeloid differentiation *in vitro* using the polymerase chain reaction technique.

The discrepancy between the previous results and the present conclusions could be explained in several ways; the lack of expression of NFE-1 mRNA in the few mouse (2.12) and human (HL60, U937) myeloid lines^{11,40} previously studied may reflect the absence, in these leukemic cells, of sufficiently early precursors comparable to those maintaining the GM-CSF- and G-CSF-dependent lines. Alternatively, the differences could be due to the use, in the previous experiments, of the relatively insensitive Northern blot technique, which, in our hands, would detect NFE-1 mRNA only after very long exposure in the G1, but not in the G2 line; NFE-1 mRNA is, however, readily detected by RNAase protection (Fig. 2) in the same lines. Similarly, NFE-1 mRNA levels may be too low in morphologically recognizable myeloid cells to be detected by 'in situ' hybridization¹⁶.

We have shown an inverse correlation between levels of NFE-1 mRNA and the ability of the cell to differentiate along the myeloid lineage and to respond to either GM-CSF or G-CSF. We do not know whether decreased expression of NFE-1 mRNA in G1 and G2 cells is simply concurrent with differentiation or causally related to G-CSF treatment. Interestingly, the level of NFE-1 expression was regulated to some extent by switching the cells from one factor to another. In particular, the level of NFE-1 increased in 32D GM when switched from GM-CSF to IL-3, and decreased in 32D Ro, switched from IL-3 to GM-CSF or G-CSF. Since a potential NFE-1 binding site is present in the promoter of the erythropoietin-receptor gene, the down-regulation of NFE-1 observed in differentiating GM-CSF- and G-CSF-dependent myeloid cells might prevent the expression, in more mature cells, of a receptor (such as the Epo's), which is inappropriate to granulocytic differentiation. To test this hypothesis, we measured the level of Epo-receptor mRNA in the different 32D subclones (manuscript in preparation). In effect, the gradient of Epo receptor-mRNA expression found among the different lines correlates very well with the gradient of NFE-1 expression (Epo-receptor mRNA level being the highest in the Epo-dependent and in the original 32D cell line, detectable but low in the GM-CSF dependent but Epo unresponsive GM1 and GM2 lines, and undetectable in the G-CSF dependent lines). These results delineate a network of interactions between growth factor receptors and transcription factors which might be relevant to our understanding of the molecular basis for hematopoietic differentiation.

It is not clear whether NFE-1 plays any role in the early myeloid cells in which it is expressed; as NFE-1 regulates several genes in both erythroid and megakaryocytic cells, it is tempting to speculate that NFE-1 might also control a subset of genes expressed in early myeloid cells. This subset could be represented either by genes expressed in all hematopoietic lineages or by genes specific for myeloid cells.

Finally, although NFE-1 is the only known tissue-specific DNA binding protein able to interact with and activate the γ -globin promoter^{14,18,19}, the mere presence of NFE-1 is insufficient to activate either endogenous globin gene expression (as previously shown^{16,17}), or even exogenous globin transcription (as assayed using NFE-1-dependent γ -globin promoters) (Fig. 4). Since NFE-1 from cells such as 32D and GM, which show little or

no expression of the transfected γ -globin promoter, is able to bind γ -globin promoter oligonucleotides containing its cognate site (Fig. 3), it is possible that repression of negatively acting factors is needed to activate the γ -globin promoter in these cells. This point emphasizes the importance of the previous developmental history of cells for specific gene expression, in agreement with recent data on myo-D-induced muscle cell differentiation⁴¹. Alternatively, in order to provide its transcriptional activation function, NFE-1 may require post-translational modifications in cells expressing globin genes.

After submitting this paper, we learned that Perkins et al.⁴² reported the presence of very low amounts of a NFE1-like binding activity in several non-erythroid cells, including Hela. However, since mRNA protection studies were not reported, it is not yet clear whether the binding activity reported in this study is due to NFE-1 protein, or to other related proteins such as those recently detected in several laboratories.

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