Transcriptional and Posttranscriptional Regulation of the Expression of the Erythropoietin Receptor Gene in Human Erythropoietin-Responsive Cell Lines


With erythroid differentiation, committed progenitor cells acquire the ability to respond to erythropoietin (Epo). Epo interacts with target cells through the Epo receptor (Epo-R), whose expression is tightly regulated in a lineage-specific fashion. Epo-R expression is presumed to be progressively activated or repressed as cells progress along the erythroid or the myeloid pathway, respectively. Little is known of the mechanisms that underlie the erythroid-specific expression of the Epo-R gene. GATA-1, the major known transcription factor involved in Epo-R gene regulation, is not erythroid-specific. We have studied the regulation of the expression of the Epo-R gene in two related human Epo-responsive cell lines, UT-7 and UT-7 Epo. These lines express Epo-R at high levels because of amplification of the endogenous gene, which is apparently not rearranged. Treatment for 6 to 24 hours with the tumor promoter, phorbol myristate acetate (PMA), or 24 hours of growth factor starvation (Epo or granulocyte/macrophage colony-stimulating factor [GM-CSF]) decreased or increased the levels of Epo-R mRNA, respectively. In the case of growth factor starvation, the increase (3–4 fold) in the level of Epo-R mRNA correlated directly with an increase in the rate of Epo-R gene transcription as measured by run-off assay. Both increases were observed as early as 3 hours after the growth factor was withdrawn and were reversible; levels of mRNA and transcription rates returned to baseline 3 hours after the cells were reexposed to growth factors. The changes in Epo-R expression after growth factor starvation were coordinated with changes in the level of expression of GATA-1 that were detected both at the mRNA and at the gene transcription level under these conditions (suggesting that GATA-1 was responsible for this upregulation). During PMA treatment, after a transient increase in Epo-R mRNA at 1 hour, a progressive decline in the level of Epo-R mRNA was observed; the level of Epo-R mRNA decreased by 50%, and fell below the level of detection by 6 and 24 hours, respectively. This decrement was explained in part by a fourfold reduction in the rate of gene transcription as well as a reduction (measured as levels of Epo-R mRNA in the presence of actinomycin D) in mRNA stability. The changes in transcription rate occurred in the absence of changes in the level of GATA-1 binding activity. The decrement in Epo-R mRNA stability (from 2.5 hours to 30 minutes) was observed after 2 hours of incubation with PMA, and was dependent on de novo gene activation. These results indicate that multiple levels of Epo-R gene regulation exist, not all of them GATA-1 dependent. In particular, a GATA-1-independent mechanism appears to be involved in the repression of Epo-R gene expression during PMA treatment. This mechanism could be important in myeloid-specific suppression of Epo-R gene expression.

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DURING THE COMMITMENT process, stem cells progressively lose their capacity to self-replicate and acquire the potential to progress only along one of the several possible differentiation pathways. Central to the commitment process is the regulation of the expression of the receptors for lineage-restricted growth factors, which, in the case of erythroid differentiation, is erythropoietin (Epo). Both the murine and the human Epo-receptor (Epo-R) genes have been cloned, thus permitting studies of how the erythroid specificity of Epo-R gene expression is achieved. The Epo-R promoter is peculiar because it does not contain TATA sequences; however, it does contain functional SP-1 and GATA-1 binding sequences. SP-1 is a ubiquitous transcription factor unlikely to drive the erythroid-specific expression of Epo-R. GATA-1, a zinc-finger protein whose consensus sequences are present in the vast majority of erythroid genes in all vertebrate species examined so far, was originally believed to be sufficient for the erythroid-specific activation of the expression of Epo-R. In fact, reporter genes driven by sequences of the Epo-R promoter are transactivated in fibroblasts when cotransfected with an expression vector containing GATA-1. Subsequent experiments have shown that GATA-1 expression is not restricted to erythroid cells but it is also present in megakaryocytes, mast cells, and myelomonocytic cells. Therefore, it is not surprising that the expression of Epo-R is not restricted to erythroid cells. As the expression of both GATA-1 and Epo-R decreases as cells differentiate along the myelomonocytic pathway, the erythroid specificity of the expression of these genes must be achieved by their progressive inactivation in nonerythroid cells. Because normal progenitors are rare, heterogeneous, and asynchronous in their differentiation, studies designed to unravel the molecular basis of erythroid-specific Epo-R and GATA-1 expression rely on the availability of well-characterized, factor-dependent cell lines. Phorbol myristate acetate (PMA), a general inducer of myeloid differentiation, decreases the number of Epo-R binding sites in
Fig 1. (A) Northern analysis of Epo-R and GATA-1 mRNA in UT-7 Epo cells grown in Epo (lane 1), growth factor-starved for 24 hours (lane 2), or starved for 24 hours and reexposed to Epo for different periods of time (3 to 24 hours) (lanes 3 through 5). Each lane contains 15 μg of total RNA. The level of GA3PD mRNA is also presented for comparison. (B) The quantification of the data (mean of three experiments).

Table 1. Rate of Transcription of the Epo-R, myb, GATA-1 and GA3PD Genes in UT-7 Cells Grown in the Presence of Growth Factors or Starved for 24 Hours

<table>
<thead>
<tr>
<th>Gene</th>
<th>UT-7 +GM-CSF (24 h)</th>
<th>UT-7 -GM-CSF (24 h)</th>
<th>UT-7 +Epo (24 h)</th>
<th>UT-7 -Epo (24 h)</th>
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<tr>
<td>GA3PD</td>
<td>2.4 ± 0.8</td>
<td>2.4 ± 0.6</td>
<td>1.4 ± 0.8</td>
<td>1.0 ± 0.6</td>
</tr>
<tr>
<td>Epo-R</td>
<td>1.9 ± 0.3</td>
<td>5.7 ± 0.9*</td>
<td>1.2 ± 0.6</td>
<td>3.2 ± 0.9*</td>
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<tr>
<td>myb</td>
<td>0.4 ± 0.1</td>
<td>1.3 ± 0.3*</td>
<td>0.1 ± 0.08</td>
<td>0.4 ± 0.2</td>
</tr>
<tr>
<td>GATA-1</td>
<td>0.4 ± 0.1</td>
<td>1.0 ± 0.2*</td>
<td>0.6 ± 0.2</td>
<td>1.2 ± 0.3*</td>
</tr>
</tbody>
</table>

The results are expressed as the mean (± SD) of the absorbance per unit of area measured by densitometric scanning with the automated Beckman DU-70 spectrophotometer (Beckman Instruments, Irvine, CA) in four separate experiments performed in duplicate.

* Statistically, significantly different (P < .05) with respect to the values observed in the presence of growth factors when analyzed with the paired t-test.

Fig 2. Run-off analysis of Epo-R, c-myb, and GATA-1 gene expression in UT-7 and UT-7 Epo cells growing in GM-CSF and Epo, respectively (first lane of each panel), starved of growth factor for increasing lengths of time (3, 6, or 24 hours; lanes 2, 3, and 4, respectively) or starved for 24 hours and reexposed to the growth factor for 3 hours (lane 5). The transcription rates for GA3PD and for pGEM are presented as controls. An increased rate of Epo-R and GATA-1 gene transcription was observed after growth factor starvation in both cell lines. The rate of transcription returned to basal levels when the cells were reexposed for 3 hours to the growth factors. (Note the reduced levels of c-myb transcription in UT-7 Epo [erythroid] when compared with UT-7.) All the run-off membranes were normalized for the amount of cDNA absorbed by the filter by hybridization with constant amounts of labeled in vitro–transcribed RNA. Equivalent amounts of cDNA were absorbed in almost all the cases. Among the exceptions is GA3PD cDNA adsorbed in lane 5 of the first panel and in lanes 2 to 4 of the second panel, which are lower than in the other lanes.

several hematopoietic cell lines. However, the endogenous Epo-R is expressed in these lines at levels detectable only by very sensitive techniques, such as RNase protection analysis or by PCR. These levels of expression are too low to be suitable for study of gene regulation.

UT-7, a human megakaryoblastic leukemia cell line,
depends for survival and proliferation on the presence of either granulocyte/macrophage colony-stimulating factor (GM-CSF) or interleukin-3 (IL-3). With GM-CSF, the cells are megakaryoblastic although cells with basophilic and eosinophilic characteristics are generated at low frequency. Epo prevents cell death but does not induce proliferation of this cell line. UT-7 Epo is a cell line cloned from the parental line on the basis of its ability to grow in Epo. It expresses high levels of erythroid-specific genes, such as the α and β-globin genes, and cannot respond any longer to GM-CSF because it is unable to bind the growth factor. After exposure to PMA, UT-7 cells differentiate by expressing megakaryocyte-specific genes such as platelet factor 4 and β-thromboglobulin; in PMA, UT-7 Epo dies without change in differentiation (this report). The number of Epo binding sites per cell decreases in both cell lines on exposure to PMA. Because these cell lines express high levels of Epo-R and GATA-1 mRNA, they represent a potentially useful model to investigate the regulation of Epo-R gene expression.

We have analyzed the effect of PMA and of growth factor starvation on the expression of Epo-R and GATA-1 in these cell lines. The results suggest complex regulation mechanisms of gene expression that include activation and repression of gene transcription as well as variations in the respective mRNA half-lives.

MATERIALS AND METHODS

Cell lines. UT-7 and UT-7 Epo cells (kindly provided by Dr N. Komatsu) were maintained in Iscove’s Modified Dulbecco’s Medium (IMDM; GIBCO, Grand Island, NY) supplemented with fetal bovine serum (FBS, 10% vol/vol; Hyclone Laboratories, Logan, UT), and purified recombinant human GM-CSF (1 ng/mL) or Epo (1 IU/mL) (K. Kaushansky, University of Washington, Seattle, WA, and J. Egie, Amgen, Thousand Oaks, CA, respectively). Some experiments were carried out in the presence of recombinant human IL-3 (10 ng/mL) (S. Clark, Genetics Institute, Cambridge, MA). Growth factor starvation was obtained by washing the cells twice with phosphate-buffered saline (PBS) and incubating them (2.5 to 5 × 10^5 cells/mL) for up to 24 hours with fresh medium without growth factors. Twenty-four hours later, Epo or GM-CSF was added at the usual concentrations. Alternatively, the cells (2.5 to 5 × 10^5 cells/mL) were incubated for up to 48 hours with PMA (0.1 µg/mL) in the presence of growth factors. In some experiments, cycloheximide (CHX; 20 µg/mL) or actinomycin D (Act D; 10 µg/mL) was also added. PMA, CHX, and Act D were from Sigma (St Louis, MO). All the cells were analyzed in the log phase of growth. K562 cells were also used as control.

RNA preparation and northern blot analysis. RNA was either extracted with phenol chloroform or precipitated by ultracentrifugation in a cesium chloride gradient (5.7 mol/L) from acid guanidinium-isothiocyanate cell lysates. RNA was size-fractionated by electrophoresis on agarose (1%) gel under denaturing conditions and blotted onto nylon membranes (Bio-Rad, Richmond, CA) to a specific activity of 4 to 8 × 10^6 dpm/mg. All the procedures were carried out according to standard protocols. After probing, the membranes were washed as recommended by the manufacturer and exposed for appropriate lengths of time with X-Omat film (Sigma) in cassettes for autoradiography (Amersham).

Run-off assay. Run-off assays were performed as described. The cells were washed three times with ice-cold PBS and resuspended (1 × 10^7 cells) in 2 mL NP40 lysis buffer (10 mmol/L Tris-HCl pH 7.4, 10 mmol/L NaCl, 3 mmol/L MgCl_2, 0.5% [vol/vol] NP40), incubated for 5 minutes on ice and centrifuged at 500g for 5 minutes. The nuclear pellet was washed once with 2 mL NP40 lysis buffer and resuspended in 100 µL NP40 lysis buffer (10 mmol/L Tris-HCl pH 8.3, 40% [vol/vol] glycerol, 5 mmol/L MgCl_2, 0.1 mmol/L EDTA, and frozen at -70°C. For the run-off transcription assay, the nuclei were thawed and mixed with 100 µL reaction buffer (10 mmol/L Tris-HCl pH 8.0, 5 mmol/L MgCl_2, 300 mmol/L KCl, 0.5 mmol/L...
each of adenosine triphosphate (ATP), cytidine triphosphate (CTP), and guanosine triphosphate (GTP) and 125 μCi of (α-phosphorus 32) UTP (760 Ci mmol⁻¹, Amersham) and incubated at 30°C for 30 minutes. The reaction mixture was treated twice at 30°C with DNase I (150 U/sample), 10 minutes each time, and with proteinase K (150 μg/sample) in 1% sodium dodecylsulfate (SDS) for 45 minutes at 42°C. The 32P-labeled RNA was extracted with phenol-chloroform and precipitated with ethanol and 2.5 mol/L ammonium acetate. The 32P-labeled RNA was then treated with a final concentration of 0.2 mol/L NaOH for 10 minutes on ice, neutralized by the addition of HEPES (to a final concentration of 0.24 mol/L) and precipitated with ethanol again. The 32P-labeled RNA pellet was resuspended in 10 mmol/L N-tris(hydroxymethyl)-methyl 2-aminoethane-sulfonic acid (TES), pH 7.4, 0.2% SDS, 0.15 mol/L NaCl, 0.0125 mol/L sodium citrate pH 7) plus 0.1 mmol/L EDTA, 600 mmol/L NaCl, and hybridized at 65°C for 60 hours to DNA immobilized on nitrocellulose. After the hybridization, the filters were washed several times with 0.1× SSC (1× SSC = 0.15 mol/L NaCl, 0.0125 mol/L sodium citrate pH 7) plus 0.1% SDS (twice for 15 minutes at room temperature, and twice for 15 minutes at 65°C). The filters were then air dried and exposed to Kodak SBS x-ray film (Eastman Kodak, Rochester, NY). For binding to nitrocellulose, each cDNA fragment was cut with the appropriate restriction enzyme, isolated, and denatured by incubation with 0.2 mol/L NaOH for 30 minutes at room temperature, followed by neutralization with 10 vol of 6× SSC. The DNA was spotted onto nitrocellulose using Hybrislot manifold apparatus (BRL, Gaithersburg, MD). One microgram of purified DNA fragment was applied to each slot.

Quantification of the levels of mRNA. Densitometric scanning (Model 620 Video Densitometer, Bio-Rad) of the radiograph was performed, and the optical density (OD) of each band considered to be a measure of the amount of mRNA. In Northern analysis, the OD of each band was normalized to the OD of the corresponding GA3PD band to correct for possible loading or transfer artifacts. To measure the mRNA half-life, the level of Epo-R or GATA-1 mRNA at each time point was expressed as a percent of time 0 (considered 100%) after being normalized to the level of expression of GA3PD in the corresponding lane. In the run-off experiments, no normalization with respect to GA3PD was done because each filter was internally controlled by hybridization with known amounts of in vitro–transcribed labeled mRNA.

Electrophoretic mobility shift assay. Nuclear extracts (25 to 50 μL) were prepared from 1 to 2 × 10⁶ cells growing in log phase according to Schreiber et al., in the presence of leupeptin, apro- tinin, and pepstatin. In vitro binding reactions and electrophoretic runs were performed as described.

RESULTS

Effect of growth factor starvation on the level of Epo-R and GATA-1 expression in UT-7 and UT-7 Epo cells. Growth factor starvation for 24 hours increased the level of Epo-R (≥fourfold) mRNA in UT-7 Epo (Fig 1A, B) and in UT-7 (Nicolis et al. and results not shown) cells. GATA-1 mRNA also slightly increased (≥twofold) after growth factor starvation (Fig 1B). If Epo was added to the cells, Epo-R and GATA-1 mRNA levels decreased by 50% after 1 hour (Fig 1B), and were back to the basal level by 6 to 24 hours.

Run-off experiments after growth factor starvation are presented in Fig 2 and Table 1. Growth factor starvation for 24 hours increased the rate of transcription of the Epo-R gene by approximately threefold. The rate of transcription of the Epo-R gene increased as early as 3 hours after growth factor starvation and was elevated during the whole period (24 hours) analyzed. Gene transcription rates decreased to basal levels 3 hours after the growth factors (GM-CSF for UT-7 and Epo for UT-7 Epo) were added back to the cultures. The transcription rate of GATA-1 also increased, although the increase was modest (≥twofold), and became statistically significant only when multiple experiments were performed (Table 1).

Effect of PMA on the level of Epo-R and GATA-1 mRNA in UT-7 and UT-7 Epo cells. If UT-7 or UT-7 Epo cells are incubated with PMA, a time-dependent reduction in the levels of Epo-R mRNA is observed (Figs 3 and 4), preceded by a rapid (at 1 hour) increase (Fig 4B). Epo-R mRNA is reduced by 50% at 6 hours and almost no Epo-R mRNA is detectable after 48 hours of incubation with PMA.

After 48 hours of PMA treatment, other changes are also seen in the two cell lines. UT-7 cells ceased to proliferate and differentiated toward megakaryocytes by increasing the number of polyploid cells, the mean ploidy number per cell and the level of platelet factor 4 and β-thromboglobulin expressed per cell (Miura et al. and N. Komatsu, personal communication, September 1991). In contrast, more than 60% of UT-7 Epo cells are trypan blue–positive after 48 hours of PMA treatment (data not shown). Because UT-7 Epo cells are strictly dependent on the presence of Epo, it is not surprising that they die if they do not express Epo-R. No changes in the level of expression of γ-globin were observed in UT-7 Epo cells in the first 6 hours of treatment with PMA (Fig 4B).

PMA treatment reduced GATA-1 mRNA in both UT-7 and UT-7 Epo cells (Figs 3 and 4). However, in this case, the level of mRNA decreased by 50% at 6 hours and remained constant thereafter.

Incubation with CHX partially blocked the reduction of both Epo-R and GATA-1 mRNA. A 50% reduction was observed—in this case at 24 hours—or was not seen at all (GATA-1 in UT-7 Epo), indicating that the molecular mechanism responsible for the reduction of GATA-1 and Epo-R mRNA after PMA treatment was complex and partially dependent on de novo protein synthesis.

To determine if the effect of PMA was affected by the growth factor in which the cells were maintained, we have analyzed the level of Epo-R and GATA-1 mRNA in cells incubated for 24 hours with PMA in the presence of Epo, GM-CSF, or IL-3 (Fig 5). PMA decreased the level of Epo-R and GATA-1 mRNA to the same extent, independent of the growth factor.

Effect of PMA on the transcription rate of the Epo-R and GATA-1 genes in UT-7 and UT-7 Epo cells. To understand the molecular mechanisms underlying the reduction of Epo-R and GATA-1 mRNA during PMA treatment, we measured the rate of transcription of these genes by run-off analysis.

One hour after PMA treatment, the rate of transcription of the Epo-R and GATA-1 genes had decreased by more than 50% (Fig 6). The rate of transcription of GATA-1, but not of Epo-R, was back at the basal level after 24 hours of
incubation with PMA (Fig 6B). Similar results were obtained with UT-7 Epo cells (not shown).

Because PMA decreased the transcription rate of the Epo-R gene, it became of interest to measure the level of GATA-1 (Fig 7) and SP-1 (data not shown) binding activity in UT-7 and UT-7 Epo cells during 24 hours of PMA treatment. The levels of the ubiquitous factor OTF-1 were also measured to normalize for the amount of nuclear extract used (Fig 7). No changes in the levels of GATA-1 were detected after 6 hours of PMA treatment. At 24 hours, GATA-1 binding activity decreased in UT-7 cells, but not in UT-7 Epo cells. We have not found evidence of decreased levels of SP-1 mRNA or binding activity during PMA treatment of these cells (results not shown).

Effect of PMA on the stability of Epo-R and GATA-1 mRNA in UT-7 and UT-7 Epo cells. The Epo-R and GATA-1 mRNA half-lives were measured in cells coincubated with PMA and/or Act D for increasing lengths of time. The experiments with CHX suggested that part of the effects of PMA were dependent on de novo protein synthesis and, therefore, on gene activation. Therefore, the mRNA half-life was measured either by adding PMA and Act D together, or preincubating the cells for 1 to 2 hours with PMA before adding Act D. In preliminary experiments, we measured the ability of Act D to reduce gene transcription by run-off experiments. Already, after 30 minutes of Act D incubation, the overall transcription rate decreased by more than 90% and no GA3PD signal could be detected by run-off (data not shown).

In untreated UT-7 and UT-7 Epo cells, the half-life of Epo-R mRNA was approximately 2.5 hours (Fig 4A, B), consistent with that reported in mouse erythroleukemic cells and in human progenitor cells. The half-life of GATA-1 was surprisingly long for a transcription factor (>70% of the mRNA was still present after 6 hours of incubation with Act D) and was very similar to the half-life of γ-globin mRNA (Fig 4B). If Act D and PMA were added together, the half-life of both Epo-R and GATA-1 mRNA were increased (Epo-R mRNA half-life greater than 6 hours; GATA-1 mRNA half-life ≥24 hours) (Fig 4A and results not shown). This indicates that PMA initially induced a stabilization of both mRNAs, probably mediated by phosphorylation of an already existing protein. Such stabilization explains why the level of Epo-R and GATA-1 mRNA actually increased during the first hour of PMA treatment (Fig 4A), although PMA already decreases the transcription rate after 30 minutes of incubation. If PMA was added 1 hour before Act D, the half-lives of both mRNAs were back to the basal level; if PMA was added 2 hours before Act D, the half-lives of both mRNAs were reduced (less than 30 minutes for Epo-R; 3 hours for GATA-1), indicating that PMA had activated the transcription of a new gene, possibly responsible for the increased degradation of both mRNAs. Similar results were obtained with UT-7 Epo cells (results not shown).

**DISCUSSION**

We have studied transcriptional and posttranscriptional regulation of the Epo-R and GATA-1 genes in a human pluripotent hematopoietic cell line, UT-7. Transcriptional activation of both genes was observed during growth factor starvation. GM-CSF or Epo-starvation of this line increased the level of Epo-R and GATA-1 mRNA by fourfold and twofold, respectively. This increase was stoichiometrically explained by corresponding and rapid changes in the rate of gene transcription. The increase in gene transcription rates and mRNA levels was reversible: reexposing the cells to growth factor reduced the rate of transcription to basal levels within 3 hours and the amount of mRNA within 3 to 6 hours.

Transcriptional repression and posttranscriptional regulation of the Epo-R and GATA-1 genes were observed during PMA treatment of the cells. PMA treatment resulted in a brief increase in the levels of Epo-R and GATA-1 mRNA that was then followed by a progressive reduction in their levels: a 50% reduction was seen at 6 hours of incubation and Epo-R mRNA was barely detectable by 24 hours. This reduction was partially sensitive to treatment with CHX, suggesting that the reduction should involve both de novo protein synthesis-dependent and independent mechanisms.

Three hours of PMA treatment reduced the transcription rate of Epo-R by greater than fourfold and GATA-1 by twofold. Because the half-life of Epo-R is about 2.5 hours,
the reduction in transcription rate should have resulted in reduced Epo-R mRNA levels much earlier than was detected (a 50% reduction was observed by 6 hours). This apparent discrepancy is explained by the fact that, during the first hour of treatment, PMA stabilizes Epo-R mRNA by a mechanism that is not affected by Act D—possibly phosphorylation of an already existing protein. However, prolonged treatment (2 hours) with PMA results in destabilization of Epo-R mRNA (the half-life decreased from 6 hours to 30 minutes) by a process that is sensitive to Act D and, therefore, dependent on de novo gene activation. This destabilization explains why, although the transcription rate decreased by only 50%, almost no Epo-R mRNA was detectable after 24 hours of PMA treatment.

GATA-1 mRNA was very stable for a transcription factor (half-life greater than 6 hours), and almost as stable as γ-globin mRNA or the housekeeping gene, GA3PD. During PMA treatment, the half-life of GATA-1 mRNA changed with a pattern similar to that of Epo-R, but the magnitude was less. GATA-1 mRNA half-life increased from 6 to 24 hours during the first hour of PMA treatment and decreased to 3 hours subsequently. Overall, the GATA-1 mRNA half-life was greater than the half-life of Epo-R, which explains why the levels of GATA-1 mRNA decreased by no more than 50%.

No changes in γ-globin mRNA half-life were observed during the first 6 hours of PMA treatment. Because these cells are Epo-dependent and die if they do not express Epo-R (ie, after 24 hours of PMA treatment), we cannot confirm whether longer exposure of the cells to PMA would have decreased the γ-globin mRNA half-life as described for K562 cells.34

Although the rate of Epo-R gene transcription decreased by 50% after 6 hours of PMA treatment, the level of GATA-1 binding activity was not similarly affected. GATA-1 is a phosphoprotein and, although changes in GATA-1 phosphorylation apparently do not affect in vitro binding activity (S. Orkin, personal communication, May 1992), such changes could affect its binding activity within the cell. However, the data suggest a GATA-1-independent mechanism of Epo-R gene regulation that, in this case, exerts a negative effect.

A potential AP-2 binding site has been recognized recently located between SP-1 and GATA-1 binding sites in the Epo-R promoter.35 The consensus sequences of this putative AP-2 binding site exert a negative effect on the tran-
scription of a reporter gene under the control of the Epo-R promoter. Furthermore, a repetitive element upstream (-1, -1.7 kb) of the murine Epo-R promoter region also apparently inhibits the expression of the Epo-R gene. Either or both the AP-2 binding site and a human physiologic equivalent of the murine repetitive element (there is no evidence at the DNA sequence level of repetitive sequences in the human Epo-R gene) could be involved in the reduction of the transcription rate observed during PMA treatment of UT-7 cells.

<table>
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<tr>
<th>K562</th>
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<th>UT-7 Epo</th>
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Fig 5. Northern analysis of Epo-R and GATA-1 mRNA in UT-7 and UT-7 Epo cells maintained for 24 hours in Epo, IL-3, or GM-CSF with (+) or without (-) PMA. Each lane contains 15 μg of total RNA. The level of expression of GA3PD is also presented for comparison. Twenty-four hours of incubation with PMA decreased the level of GATA-1 by 50% and of Epo-R by 70% to 80% in both cell lines, independent of the growth factor in which the cells were kept.

Fig 6. (A) Run-off analysis of Epo-R, c-myb, and GATA-1 gene expression in UT-7 cells not exposed to PMA (lanes 1 to 4) or treated with PMA for 6 and 24 hrs (lanes 5 to 8). The rate of Epo-R transcription decreased by fourfold after 6 hours of PMA treatment. A representative experiment is presented. Controls were run in quadruplicate and PMA-treated samples in duplicate. (B) Quantitation of the transcription rate of (○) GA3PD, (□) Epo-R, (□) c-myb, and (□) GATA-1 mRNA in UT-7 and UT-7 Epo cells treated for increasing periods of time (0.5 to 24 hours) with PMA. The data represent the mean (± SD) of three experiments performed in duplicate. All the run-off membranes were normalized for the amount of cDNA absorbed by the filter by hybridization with constant amounts of labeled in vitro–transcribed RNA.
REGULATION OF THE EXPRESSION OF THE EpOR GENE

Table: Levels of GATA-1 binding activity in nuclear extracts from UT-7 and UT-7 Epo cells treated for several hours with PMA. Nuclear extracts were incubated with GATA-1-specific oligos plus oligos for the ubiquitous factor, OTF-1, as internal control. The levels of activity in extracts from K562 cells are also reported. Some of the extracts (lanes 1 and 3) were incubated with an excess of cold oligos, which specifically competes with GATA-1. Reduced levels of GATA-1 binding activity were found only after 24 hours of PMA treatment in UT-7 whereas the levels of GATA-1 binding activity never decreased in UT-7 Epo cells.

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Fig 7. Levels of GATA-1 binding activity in nuclear extracts from UT-7 and UT-7 Epo cells treated for several hours with PMA. Nuclear extracts were incubated with GATA-1-specific oligos plus oligos for the ubiquitous factor, OTF-1, as internal control. The levels of activity in extracts from K562 cells are also reported. Some of the extracts (lanes 1 and 3) were incubated with an excess of cold oligos, which specifically competes with GATA-1. Reduced levels of GATA-1 binding activity were found only after 24 hours of PMA treatment in UT-7 whereas the levels of GATA-1 binding activity never decreased in UT-7 Epo cells.

UT-7 is a leukemic cell line with a nearly tetraploid male karyotype. It has two copies of the X chromosome and three copies of chromosome 19, where the human genes for GATA-1 and EpR are located, respectively. These chromosomes are apparently not rearranged. However, an abnormal EpR gene has been observed in the human erythroleukemic cell line TF-1. Because of the high level of endogenous EpR expression in these cell lines, we analyzed the genomic organization of the GATA-1 and EpR genes in UT-7 cells. No gross rearrangements of the two genes were identified with the restriction enzymes used (HindIII and BamHI, data not shown). As expected, the intensity of the GATA-1 bands in UT-7 was equivalent to the intensity of the corresponding bands in diploid fibroblasts from a female donor. However, the intensity of the EpR bands was approximately sixfold to eightfold higher than the bands from diploid fibroblasts or from normal mononuclear cord blood cells. This indicates that the number of EpR genes per cell exceeds the three copies expected from the karyotype, and that the EpR gene must be amplified in this cell line.

Although results of studies of gene regulation using such cell lines should be interpreted cautiously, we would like to propose some general concepts from these data.

Stimulation of hematopoietic cells with growth factors activates a series of signal transduction pathways. For example, GM-CSF and Epo have been reported to activate (1) protein kinases of the src family, (2) the GTP-GDP exchange activity of the Ras protein, and (3) protein kinase C. It is now recognized that, not only could each of these pathways be involved in a specific signaling function of a receptor, but each pathway could also transduce a different signal according to its level of activation.

The most frequent effect reported for PMA and 12-O-tetradecanoylphorbol-13-acetate (TPA), another member of the phorbol ester family, in human, rodent, and avian cells, is the induction of granulo-monocytic differentiation, while repressing the erythroid differentiation potential.

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The most frequent effect reported for PMA and 12-O-tetradecanoylphorbol-13-acetate (TPA), another member of the phorbol ester family, in human, rodent, and avian cells, is the induction of granulo-monocytic differentiation, while repressing the erythroid differentiation potential. Believed to transduce its signal through the protein kinase C pathway, PMA could share some of the GM-CSF signal transduction components. In fact, PMA can substitute for GM-CSF in preventing the activation of the apoptotic program in a human GM-CSF–dependent cell line.

GM-CSF and Epo are two competitive growth factors in the sense that, in rodents, and also somewhat in humans, one is more restricted to the granulo-monocytic pathway and the other to the erythroid pathway. Treatment of the murine cell line 32D with GM-CSF selects for the survival of cells that have activated a program that switches off the potential for erythroid differentiation. Furthermore, both cell lines and enriched populations of normal progenitors, when stimulated with GM-CSF, progressively inactivate the expression of EpR and GATA-1. Conversely, transfection of the murine cell line FDCP-1 with the EpR gene suppresses the capacity of the cells to respond to GM-CSF.

Because activation or repression of protein kinase C is associated with myeloid or erythroid differentiation, respectively, it is possible that, in UT-7 cells and UT-7 Epo cells, we are analyzing a physiologic mechanism central to the problem of coupling the expression of a specific receptor with differentiation. A cell that has activated a differentiation program inappropriate for the environment in which it finds itself is in a condition equivalent to “growth factor starvation.” If the cell does not encounter the appropriate growth factor, the apoptotic program is activated. Therefore, it should overexpress the gene for the growth factor receptor appropriate for the cell’s internal “environment” to increase the likelihood of interacting with the appropriate growth factor. Because hematopoietic growth factors can exert their effect at very low levels of receptor occupancy, increasing the rate of transcription of the gene for the receptor achieves the purpose.

On the other hand, the differentiation signal is accompanied by downregulation of the expression of inappropriate...
growth factor receptors, which is achieved by decreasing both the gene transcription rate and mRNA stability. This is seen in UT-7 cells exposed to PMA but not in cells exposed to GM-CSF, probably because the number of cells that differentiate in GM-CSF is so low\(^{21}\) that their contribution to the total mRNA is irrelevant.

The effects observed after either growth factor starvation or PMA treatment could result from protein kinase C activation.\(^{3,4,5,6}\) Because of the transformed nature of these cell lines, GM-CSF and Epo could only be partially coupled with the protein kinase C pathway, and signal transduction could proceed only to a certain point (a level necessary to partially repress the expression of Epo-R). Signal transduction induced by PMA might progress further, to a point where greater repression of Epo-R and GATA-1 gene transcription are achieved and posttranscriptional regulation of both Epo-R and GATA-1 are seen. This complex mechanism of repression of Epo-R gene expression during PMA treatment appears to be GATA-1-independent and could be important in the myeloid-specific suppression of the expression of this gene.

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