

Regulation of Expression of the Human Erythropoietin Receptor Gene

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ABSTRACT: Interactions between erythropoietin (Epo) and its receptor (EpoR) are critical for the normal proliferation and differentiation of erythroid progenitor cells. EpoR is expressed in low numbers during early stages of erythroid maturation, while higher levels are expressed during later stages, suggesting that the expression of the EpoR gene is tightly regulated throughout erythropoiesis. We used the TF-1 erythroleukemia cell line to analyze the effects of various cytokines and reagents on the regulation of human EpoR gene expression. Human EpoR gene expression was significantly upregulated by IL-1 α and the protein inhibitor cycloheximide, but significantly downregulated by the calcium ionophore ionomycin and the phorbol ester PMA. These effects on EpoR gene expression were not due to changes in EpoR mRNA stability, suggesting that these agents directly affected EpoR gene transcription. The selective *in vitro* modification of EpoR expression by these agents highlights the complexity of human EpoR gene expression, and provides clues to its *in vivo* regulation.

Keywords: Erythropoietin receptor, erythropoiesis, gene expression, TF-1, cytokines, IL-1 α , ionomycin, phorbol ester

INTRODUCTION

The growth and differentiation of erythroid cells is regulated primarily by the interaction of erythropoietin (Epo) with its specific protein receptor (EpoR) on the surface of bone marrow erythroid progenitor cells. The binding of Epo to EpoR induces both specific proliferation and maturation signals: stage-specific effects include a proliferative signal to the erythroid burst-forming unit (BFU-E) and a differentiation signal to the erythroid colony forming unit (CFU-E) (1,2). Recent evidence suggests that these proliferative and differentiative signals through EpoR can be uncoupled (3). Although EpoR is a member of the hematopoietin receptor superfamily and therefore lacks intrinsic tyrosine kinase activity (4), signal transduction through EpoR leads to tyrosine phosphorylation of many intracellular proteins (5,6)

primarily through the activity of Jak2 (7). In addition, phospholipases A2 and C are activated (8) and levels of intracellular calcium increase (9,10). Epo binding to EpoR also prevents programmed cell death (apoptosis) in erythroid progenitor cells and thereby maintains cell viability (11,12).

EpoR is a member of the cytokine receptor superfamily that contains a W-S-X-W-S motif and four cysteine residues in the extracellular domain (13,14). EpoR is expressed in high or low affinity forms (15), but the high-affinity receptor is likely the more physiologically relevant class (16,17). A peak in EpoR expression occurs on erythroid progenitor cells at the CFU-E/proerythroblast stage, but subsequently decreases following maturation and differentiation (18); EpoR cannot be detected on reticulocytes (17). The number of EpoR sites on human bone marrow CFU-E is typically below

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1000 receptors per cell, and falls to approximately 200 receptors per erythroblast (17). Furthermore, EpoR mRNA has limited intracellular stability, with a mRNA half-life of only 90 minutes in human erythroid progenitor cells (18). Taken together, these data suggest that the expression of the human EpoR gene is tightly regulated and controlled during erythropoiesis.

To date, however, little is known about the regulation of the expression of the human EpoR gene. Using the TF-1 erythroleukemia cell line with phenotypic features of BFU-E, high constitutive expression of EpoR, and growth dependency on either IL-3, GM-CSF or Epo (19), IL-1 α was found to increase the number of surface EpoR receptors as measured by Epo binding studies (20), but no analysis of EpoR gene expression was performed. The erythroid-specific nuclear factor GATA-1, which regulates globin gene expression, also transactivated the human EpoR gene in vitro by binding to the EpoR gene promoter (21). Conversely, megakaryocytic differentiation of the human myeloid leukemia cell line UT-7 by phorbol myristate acetate (PMA) down-regulated EpoR surface expression by decreasing EpoR mRNA levels (22,23).

To investigate the regulation of the human EpoR gene, we analyzed the effects of various cytokines and reagents on EpoR gene expression. Expression of the human EpoR gene was upregulated by IL-1 α and the protein inhibitor cycloheximide, but downregulated by PMA and the calcium ionophore ionomycin. These effects on EpoR expression were not due to changes in EpoR mRNA stability, suggesting that these agents directly affected EpoR gene transcription.

MATERIALS AND METHODS

Cytokines, Reagents, and Monoclonal Antibodies

IL-1 α , IL-3, IL-6, IL-7, IL-9, and GM-CSF were kind gifts from Genetics Institute, Cambridge, MA. IL-2 was kindly provided by Chiron

Corporation, Emeryville, CA. Human recombinant erythropoietin (Epo) was purchased from Amgen, Thousand Oaks, CA. Other reagents included ionomycin (Calbiochem, La Jolla, CA), dimethylsulfoxide (DMSO, Sigma, St. Louis, MO), hemin (Sigma), delta-aminolevulinic acid (δ -ALA, Sigma), phorbol myristate acetate (PMA, Sigma), and cycloheximide (Sigma).

Directly conjugated monoclonal antibodies (mAb) included CD33-PE, CD34-PE, CD41-PE, CD42-FITC, CD71-FITC, Class II MHC-FITC, and control mAbs IgG-FITC and IgG-PE (Dako, Carpinteria, CA). Unconjugated mAbs included the anti-EpoR mAb 16.5.1, a gift from Dr. Alan D'Andrea (24); a sialoglycoprotein- β -specific mAb E5, a gift from Dr. Barton Haynes (25), and the control mAb P3 used as previously described (26). Goat-anti-mouse IgG (H + L) conjugated to phycoerythrin was purchased from Kirkegaard & Perry Laboratories, Gaithersburg MD.

Cells

TF-1 cells were a kind gift from Toshio Kitamura, DNAX Research Institute of Molecular Biology, Palo Alto, California. TF-1 cells were grown in RPMI 1640 (Gibco, Grand Island, NY) with 10% fetal calf serum (Gibco) and 5 U/ml Epo at $0.5\text{--}2 \times 10^6$ cells/ml using Falcon 1001 petri dishes (Becton Dickinson, Lincoln Park, NJ) at 37C in a 5% CO₂ humidified incubator.

Blastogenesis Assay

Proliferation of the TF-1 cells was measured by tritiated thymidine incorporation as described (27). Briefly, 100 μ l of TF-1 cells (10^6 cells/ml) were placed in triplicate in a 96-well U-bottom plate (Costar, Cambridge, MA) for 24 or 96 hours with 100 μ l of stimuli at the following final concentrations: IL-1 α (0.1 to 10 U/ml), IL-2 (10-100 U/ml), IL-3 (0.001 to 10 U/ml), IL-6 (1:1000 supernatant), IL-7 (1:1000 supernatant), IL-9 (1:1000 supernatant), GM-CSF (0.001 to 10

U/ml, Epo (0.001 to 10 U/ml), ionomycin (0.1 μ M to 20 μ M in DMSO), DMSO (0.001 to 5%), δ -ALA (0.5 mM), hemin (40 μ M), and PMA (0.1 to 20 ng/ml). Following a 24 or 96 hour incubation, the cells were pulsed with 20 μ l of tritiated thymidine (0.4 μ Ci, 6.7 Ci/mmol, New England Nuclear, Boston, MA) for 4 hours prior to harvest on a cell harvester (Cambridge Technologies, Watertown, MA). Samples were measured on a beta scintillation counter.

Immunophenotyping

Surface antigen expression was analyzed using a one or two-step immunophenotype as previously described (27). For the one-step assay, 0.5×10^6 TF-1 cells were incubated with saturating amounts of one or two directly-labeled monoclonal antibodies (mAb) for 30 minutes at 4C. The cells were then washed with PBS with 2% albumin (Sigma), fixed in paraformaldehyde and stored in the dark until analysis. For the two-step assay, cells were incubated with an unlabeled mAb for 30 minutes, washed, then incubated with goat-anti-mouse IgG-PE. Samples were analyzed on a FACSCAN (Becton Dickinson) flow cytometer using forward and side scatter gating and color compensation.

Northern Blot Analysis

TF-1 cells (2×10^6 cells/ml) were grown in media containing either Epo (final concentration 5 U/ml), IL-1 α (1 U/ml), cycloheximide (80 μ M), ionomycin (0.5 μ M) or PMA (2 ng/ml) for 16 hours. The cells were then centrifuged at 1500 rpm, washed twice in cold PBS, and lysed in GIT buffer consisting of 4.0 M guanidine isothiocyanate (International Biotechnologies, Inc., New Haven, CT) with 0.5% Sarkosyl (IBI) as previously described (28). Total RNA was pelleted by centrifugation for 20 hrs at 36,000 rpm through a 5.7 M cesium chloride cushion (IBI). The purified RNA was resuspended in ddH₂O, and 8.0 μ g were

electrophoresed through a 1.2% agarose gel with 2% formaldehyde/MOPS buffer (Stratagene, La Jolla, CA) and then transferred to a nylon membrane (Stratagene) using 20x SSC. The transferred RNA was immobilized by UV-illumination and immobilized by baking at 80C under vacuum for 90 minutes. Membranes were prehybridized in Quik Hyb solution (Stratagene) for 1 hr at 68C, then hybridized for 1 hr with α^{32} P-labeled human EpoR cDNA probe, a gift from Dr. Alan D'Andrea (29). The hybridized blots were washed for 30 min at RT with 2X SSC, 0.1% SDS, and 2 mM EDTA, then washed twice (15 min each) at 58C with 0.5X SSC, 0.1% SDS, and 2 mM EDTA. Autoradiograms were exposed for 2-7 d at -70C using Kodak Xomat AR-5 film with intensifying screens (Eastman Kodak Co., Rochester NY). Densitometry analysis of autoradiograms was conducted on an Ultrascan Laser Densitometer (Pharmacia LKB).

Analysis of EpoR mRNA Stability

After 16 hours incubation with the stimuli described above for Northern blot analysis, 20×10^6 TF-1 cells were removed for RNA isolation as a zero-hour timepoint. Actinomycin-D (Sigma) was then added at a final concentration of 10 μ g/ml to inhibit new RNA transcription; at four hour intervals additional aliquots of 20×10^6 cells were removed for RNA isolation. These RNA samples were probed for EpoR mRNA levels and mRNA stability was measured using densitometry values as previously described (28).

Statistical Analysis

The Primer of Biostatistics software package (McGraw-Hill, New York, NY) was used for all statistical analysis. Comparison of means was performed using the *t*-test to determine statistical significance.

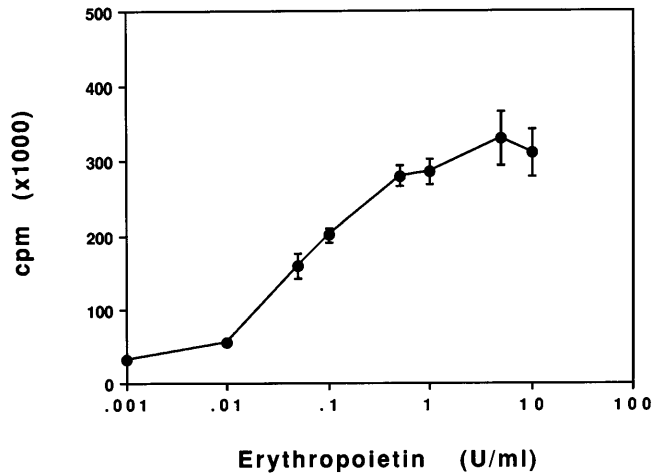


Figure 1. Proliferative response of TF-1 cells to erythropoietin (Epo). TF-1 cells were incubated in varying amounts of Epo for 96 hours as described in Methods, then assayed for tritiated thymidine incorporation. The dose response curve shows that TF-1 cells respond maximally to 5 U/ml Epo.

RESULTS

Epo-induced Proliferation of TF-1 Cells

The *in vitro* proliferative rate of TF-1 cells incubated in Epo was measured by blastogenesis assay, and a dose-response curve was generated (Figure 1). Maximal tritiated thymidine incorporation occurred with an Epo dose of 5 U/ml.

IL-1 α induced a weak, dose-dependent proliferative response in TF-1 cells when used as a single stimulus [$n = 3$, $P = 0.04$ (between highest and lowest concentrations), Figure 2A]. How-

ever, the addition of IL-1 α (2 U/ml) to cells suspended in Epo (5 U/ml) caused a significant increase in proliferation as compared to Epo alone (Figure 2B). This increase in Epo responsiveness occurred after 24 hours incubation ($190 \pm 12\%$, $P = 0.003$), and also at 96 hours ($156 \pm 9\%$, $P = 0.05$).

In contrast, the proliferative rate of TF-1 cells was decreased after incubation with the calcium ionophore ionomycin. Ionomycin inhibited TF-1 growth at 0.5 μ M (or higher) in the presence of 5 U/ml Epo (Figure 3A). The effect of ionomycin on TF-1 proliferation was more pronounced after a 24

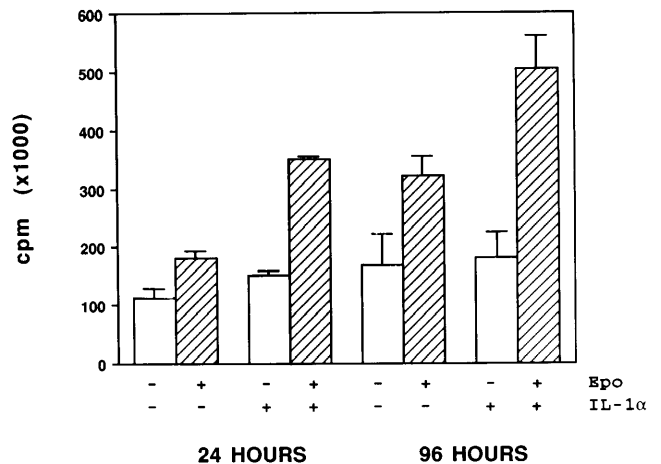
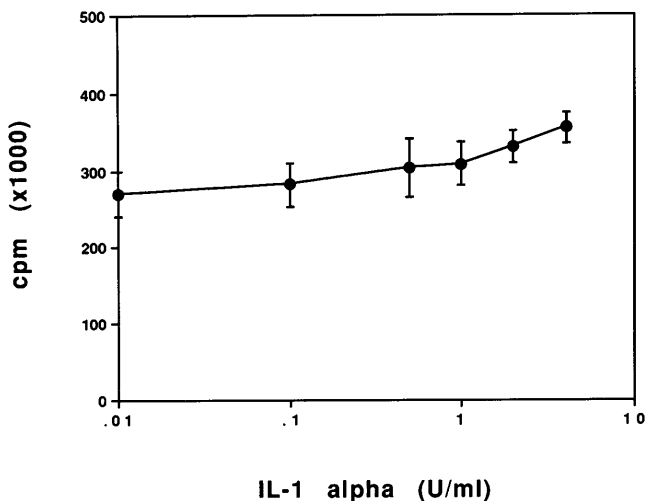


Figure 2. Proliferative response of TF-1 cells to IL-1 α . **Panel A** shows that as a single agent, IL-1 α induced a weak, dose-dependent response as measured by tritiated thymidine incorporation. **Panel B** illustrates that IL-1 α increased Epo-responsiveness of TF-1 cells, both at 24 and 96 hours of incubation. Stimuli included IL- α at 2 U/ml and Epo at 5 U/ml. These results demonstrated a direct effect of IL-1 α on Epo-EpoR interactions.

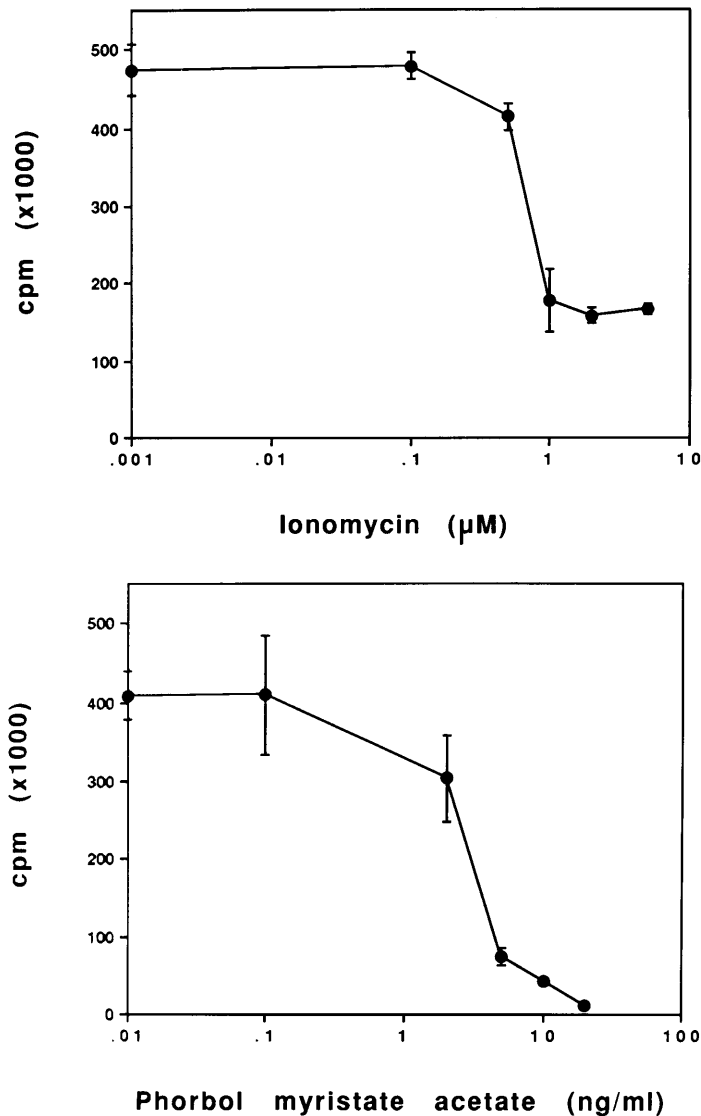


Figure 3. Proliferative response of TF-1 cells to Epo (5 U/ml) in the presence of ionomycin and phorbol ester. **Panel A** shows that ionomycin significantly decreased the Epo-responsiveness of TF-1 cells, at doses of 0.5 μM or higher. Similarly, **Panel B** shows that phorbol ester (PMA) decreased TF-1 proliferation at concentrations of 2.0 ng/ml or higher.

hour incubation than at 96 hours for equivalent drug concentrations (data not shown). Control experiments showed no effect from DMSO alone. Similarly, TF-1 incubated with the phorbol ester PMA showed decreased proliferation to Epo at concentrations of 2 ng/ml or higher (Figure 3B). There was no significant change in TF-1 proliferation to Epo following incubation with IL-2, IL-3, IL-6, IL-7, IL-9, δ -ALA, hemin or GM-CSF (data not shown). Taken together, these results suggested that changes in Epo-responsiveness of TF-1 cells was specific to certain stimuli; IL-1 α

significantly increased Epo-induced proliferation, while ionomycin and PMA significantly decreased the response of TF-1 cells to Epo.

Surface Antigen Immunophenotype

To investigate the mechanisms by which these stimuli affected the Epo-responsiveness of TF-1 cells, we performed immunophenotypic analysis by flow cytometry. At baseline, TF-1 cells were positive for surface expression of CD33, CD34, CD41, CD71, and E5 (Figure 4). TF-1 cells also

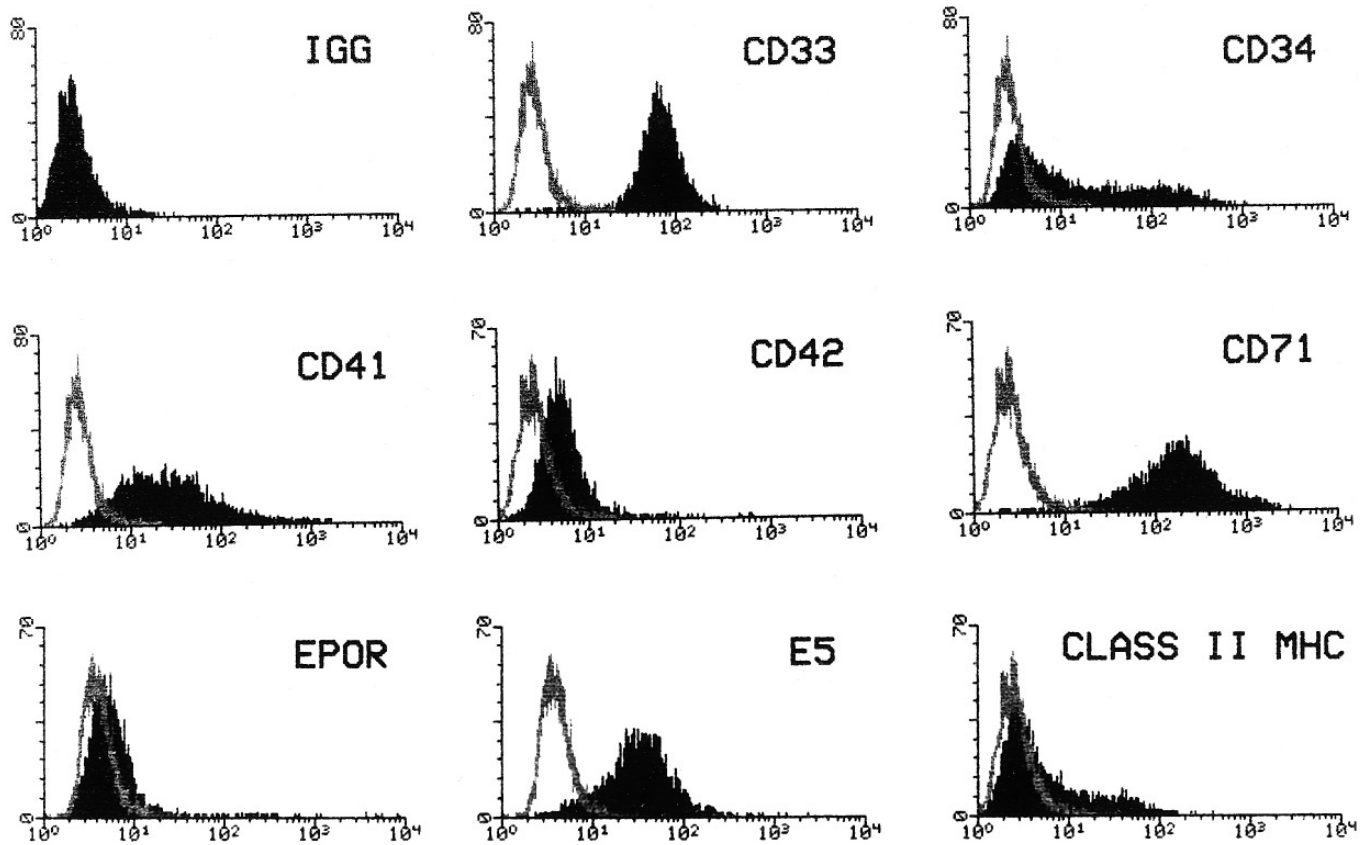


Figure 4. Baseline surface immunophenotype of TF-1 cells. For each histogram the isotype control (IgG) has been overlaid in gray. TF-1 cells had positive surface expression of CD33, CD34, CD41, CD71, and sialoglycoprotein- β (E5). TF-1 cells also express CD42 and Class II MHC, as well as faint expression of EpoR.

had weak expression of CD42 and Class II MHC, and demonstrated faint expression of EpoR. In contrast, there was no surface expression of CD14, CD16 or CD56 (data not shown). After incubation with IL-1 α , ionomycin or PMA for 16 hours, there was no significant change in surface expression of any markers tested, except for an increase in CD41 expression following PMA stimulation (data not shown). In addition, there were no changes in forward scatter or side scatter characteristics to suggest loss of cellular integrity or cell death.

Analysis of EpoR mRNA Levels

To determine whether changes in the proliferation of TF-1 cells to Epo resulted from changes in EpoR gene expression, we analyzed EpoR mRNA levels after stimulation. TF-1 cells were incubated

with IL-1 α , ionomycin, or PMA for 16 hours, then EpoR mRNA transcripts were measured by Northern blot analysis (Figure 5). As compared with Epo alone, TF-1 cells stimulated with IL-1 α had a significant increase in EpoR mRNA transcript levels ($142 \pm 13\%$, $n = 6$, $P = 0.009$). Conversely, incubation of TF-1 cells in ionomycin resulted in a significant down-regulation of EpoR mRNA levels ($71 \pm 8\%$, $n = 6$, $P = 0.005$). Similarly, PMA significantly decreased EpoR transcript levels to $67 \pm 7\%$ ($n = 6$, $P < 0.001$). Cycloheximide, an inhibitor of protein synthesis, induced an increase in EpoR mRNA transcript levels ($275 \pm 34\%$, $n = 6$, $P < 0.001$).

EpoR mRNA Stability

As changes in EpoR mRNA levels were detected in TF-1 cells after incubation with IL-1 α ,

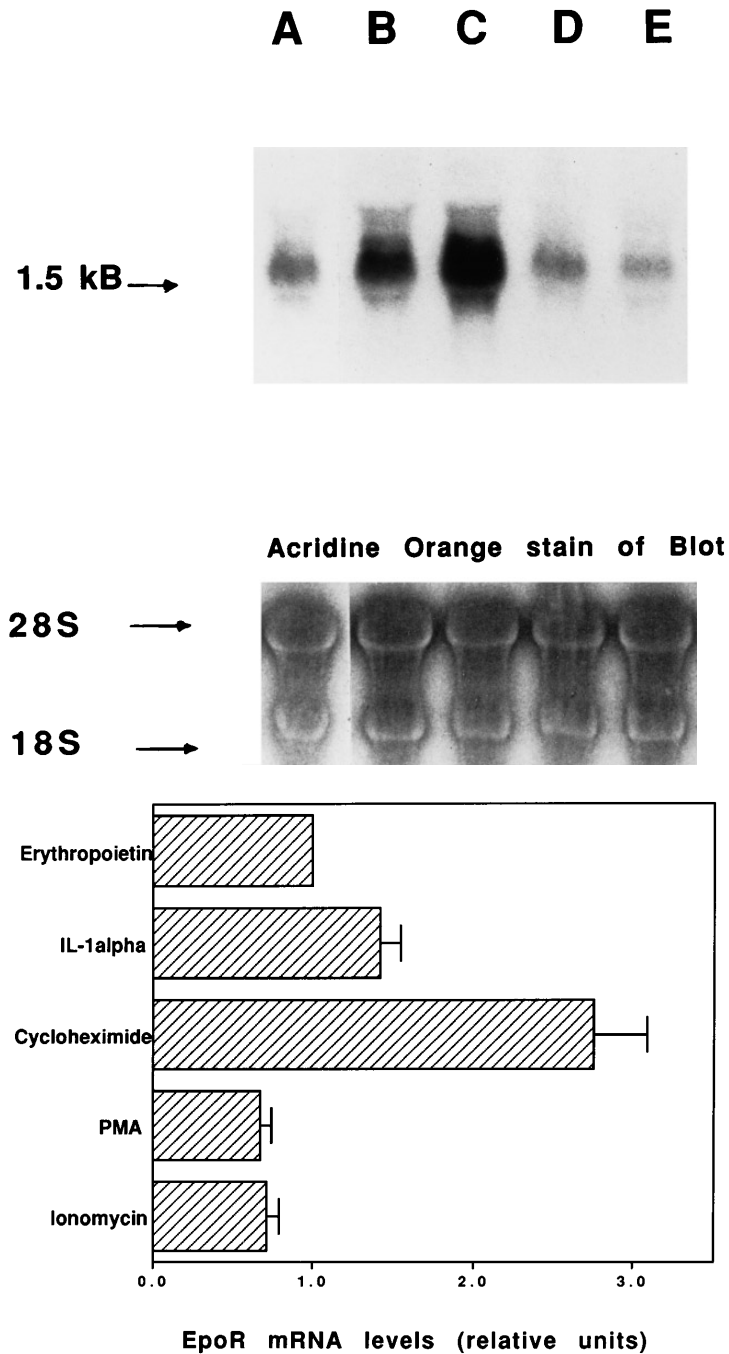


Figure 5. Analysis of TF-1 EpoR mRNA levels. TF-1 cells were incubated for 16 hours in Epo alone, or with the addition of IL-1 α , cycloheximide, ionomycin, or PMA. The upper panel shows increases in EpoR mRNA levels after treatment with IL-1 α (Lane B) or cycloheximide (Lane C), as compared with Epo alone (Lane A). In contrast, EpoR mRNA levels were decreased following exposure to ionomycin (Lane D) or PMA (Lane E). The middle panel illustrates that equivalent RNA loading is documented by acridine orange staining of total RNA. The lower panel shows these results in a graphic form.

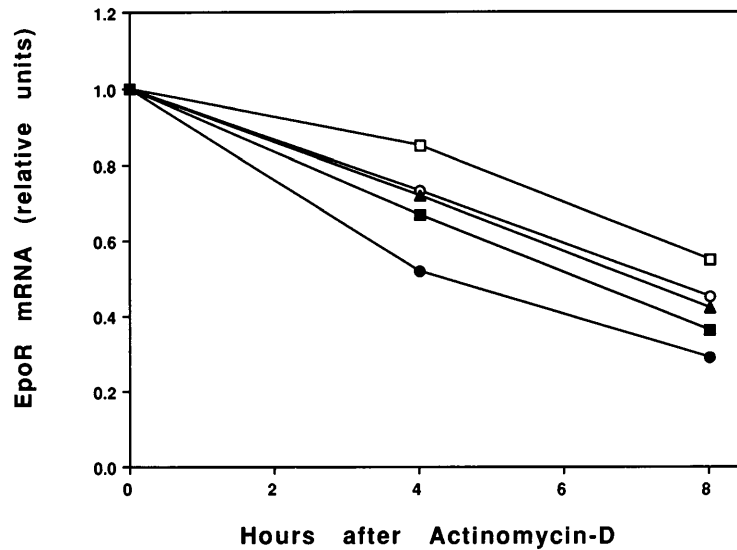


Figure 6. Analysis of TF-1 EpoR mRNA stability. TF-1 cells were stimulated for 16 hours, then Actinomycin-D was added to inhibit new RNA transcription. EpoR mRNA levels were measured at time = 0 hours, then at 4 and 8 hours following Actinomycin-D treatment. Densitometry values demonstrate that there was no significant change in EpoR mRNA stability following treatment with these stimuli.

ionomycin, PMA or cycloheximide, we next measured EpoR mRNA stability. After 16 hours of incubation with these stimuli, Actinomycin-D was added to inhibit new RNA transcription. EpoR mRNA levels were then measured at 0, 4, and 8 hours to allow the determination of EpoR mRNA transcript stability. Although the absolute amounts of EpoR mRNA varied for each stimulus, the relative rates of EpoR message stability were not significantly different following stimulation with IL-1 α , ionomycin, PMA or cycloheximide (Figure 6).

DISCUSSION

Interactions between Epo and EpoR on human erythroid cells are regulated primarily at the level of EpoR expression, but in several different ways. First, only erythroid progenitor cells at a specific stage of maturation and differentiation express measurable numbers of EpoR sites. Human BFU-E are very sensitive to the effects of Epo, but lose responsiveness to Epo as EpoR mRNA levels decline, and eventually become insensitive to Epo in the final stages of erythroid differentiation (18). Additionally, there is control of Epo-EpoR interac-

tion by the relatively low number of EpoR sites on erythroid cells. Broudy et al. (17) have shown that primary human erythroblasts express only 135 EpoR sites/cell, with 1100 EpoR sites/cell in the Day 9 BFU-E but only 300 sites/cell in Day 14 BFU-E, and no detectable EpoR sites on reticulocytes. Such stringent regulation of EpoR expression implies that Epo-EpoR interactions must be precisely controlled during normal erythropoiesis.

The regulation of the human EpoR gene, however, is not well understood at this time. Specifically, agents that alter EpoR gene expression, and the mechanisms by which they act, have not been identified. As the bone marrow microenvironment is a rich source of soluble growth factors, we hypothesized that cytokines might affect EpoR expression. Kitamura et al. (20) reported that treatment with IL-1 α led to increased numbers of surface EpoR on TF-1 cells. Our data confirmed this observation, by demonstrating that IL-1 α led to increased Epo-responsiveness (Figure 2A). The mechanism of this effect by IL-1 α was at the level of new EpoR gene transcription, as IL-1 α induced a significant increase in EpoR mRNA levels after overnight stimulation (Figure 5) without altering EpoR mRNA stability (Figure

6). In contrast, IL-3 or GM-CSF, as well as IL-2, IL-6, IL-7, or IL-9 had no effect on EpoR expression and Epo-responsiveness. These findings suggest that regulation of EpoR gene expression by cytokines is a restricted process, rather than a redundant one as has been suggested for the majority of hematopoietic regulation (30).

We also examined the effect of an ionomycin-induced transmembrane calcium flux on EpoR expression. Previous studies documented that Epo stimulation of erythroid progenitor cells transiently increased intracellular calcium levels, depending upon the differentiation stage of the cell (9). Furthermore, this Epo-induced rise in intracellular calcium derived from extracellular sources rather than from release of intracellular calcium stores (31), and the calcium ions localized preferentially to the nucleus (10). Our data demonstrated that ionomycin led to a reduction in Epo-responsiveness and a significant decrease in EpoR mRNA levels, without affecting other surface markers or cell integrity. We hypothesize that the increase in intracellular calcium that results from Epo binding to EpoR on erythroid progenitor cells leads to a direct inhibition of EpoR gene transcription. Such a negative feedback loop would allow tighter control on EpoR gene expression and limit Epo-EpoR interactions.

The phorbol ester PMA has been shown previously to reduce EpoR mRNA levels and induce megakaryocytic differentiation in the UT-7 cell line (22,23). TF-1 cells, in contrast, predominantly acquire macrophage morphology rather than a megakaryocytic surface phenotype following exposure to PMA (19,20). However, we observed that low doses of PMA also led to a loss of Epo-responsiveness in TF-1 cells and a significant diminution in EpoR mRNA levels. These findings suggest that decreased EpoR gene expression plays a key role in the inhibition of erythroid-specific signals, which is a critical step for non-erythroid differentiation.

Finally, we observed that treatment of TF-1 cells with cycloheximide, an inhibitor of protein synthesis, led to a significant increase in EpoR mRNA levels. Such “super-induction” of mRNA

was originally described for steroid-inducible genes, but has been described for several other genes including cytokines IL-1 (32), IL-2 (33), GM-CSF (30), c-myc (34), and others. To our knowledge, EpoR is the first cytokine receptor for which super-induction has been described. We hypothesize that erythroid progenitor cells normally have short-lived cytoplasmic nucleases which degrade EpoR mRNA; cycloheximide inhibits this cellular “shut-off” mechanism and leads to an accumulation of EpoR mRNA.

Each of the agents that increased or decreased TF-1 EpoR mRNA levels had no effect on EpoR mRNA stability, suggesting a direct effect on EpoR gene transcription. In the UT-7 cell line, 100 ng/ml PMA has been reported to have both transcriptional and posttranscriptional effects on EpoR gene expression (23). However, we used PMA at a dose of 2.0 ng/ml, and found no change in EpoR mRNA stability at this lower dose. Further investigation will be required to understand these dose-dependent effects of PMA on EpoR gene expression and non-erythroid differentiation.

In conclusion, we provide *in vitro* evidence that the regulation of human EpoR gene expression occurs primarily at the transcriptional level in TF-1 cells. Our data also suggest that human EpoR gene expression is tightly regulated during erythropoiesis by a limited number of cytokine stimuli. Future experiments using human bone marrow will be helpful in determining the *in vivo* relevance of these findings.

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