Rapid Induction of Mouse Virus-Like (VL30) Element Transcripts by Erythropoietin in Murine Erythroid Progenitor Cells

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We examined the activation of genes induced by erythropoietin (Epo) in erythroid progenitor cells that were isolated from the spleens of mice infected with anemia-inducing strain of the Friend virus. These erythroid progenitor cells, termed FVA cells, undergo in vitro differentiation to erythrocytes under the influence of Epo within 2 to 3 days. We used a differential hybridization procedure to screen a cDNA library constructed from FVA cells that were treated with Epo 2U/mL in the absence of serum for 2 hours. Of 20,000 recombinant phages, 47 plaques hybridized preferentially to cDNA probe prepared from Epo-stimulated cells. We found at least three different Epo-responsive genes (ERGs) and one of them corresponds to the mouse virus-like (VL30) element, similar to already reported BVL-1. The induction of VL30, which was evident within 30 minutes after Epo exposure, reached a maximum by 1 hour and remained stable for up to 4 hours. The treatment of FVA cells with cycloheximide (CHX) 10 μg/mL, which in itself activates the expression of VL30 caused a superinduction of the Epo signal. Changes in intracellular Ca2+ concentrations, either raised by ionomycin or depleted by EGTA, had no effect on the Epo-induced VL30 expression. In addition, protein kinase C (PKC) inhibitors such as staurosporine (3 μmol/L) or H7 (20 μmol/L) and a tyrosine kinase inhibitor, genistein (200 μmol/L), did not inhibit the Epo-induced expression of VL30. TPA (100 ng/mL), a PKC agonist, did not induce VL30 expression. Although the physiologic role of VL30 in the differentiation of erythroid progenitor cells is not known, our findings demonstrate that VL30 is an early ERG, and may be a useful indicator of the initial molecular actions of Epo.

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ERYTHROPOIETIN (Epo) plays an indispensable role in the complex program of erythropoiesis leading to the evolution of erythrocytes. The recent cloning of the human and simian Epo genes1,2 heralded a new era in which recombinant human Epo is readily available for the study of erythropoiesis. Recently, specific receptors for Epo with molecular weights of approximately 85 to 105 Kd have been identified and characterized in a variety of cells.3,4 Moreover, Epo receptors have been cloned from several sources, including a murine erythroleukemia (MEL) cell line,5 the human erythroleukemia cell line, OC1M, and fetal liver.6 However, the mechanism by which Epo directs the process of erythroid differentiation is still poorly understood.

One of the earliest observed effects of Epo on target cells is a stimulation of RNA synthesis, which precedes DNA synthesis, mitosis activation, and protein synthesis in the erythroid progenitor cells.7 In rat bone marrow cultures, the RNA species, which are produced preferentially in response to Epo, have sedimentation rates of 150, 55 to 65, 45, 28, 18, 9, 6, and 4 Svedberg units.8 Similarly, Epo induces the synthesis of 4Ss ribosomal precursor RNA, 28Ss and 18S ribosomal RNA, and transfer RNA in fetal liver cultures.9 RNA polymerase II, responsible for the synthesis of mRNA, is stimulated in splenic erythroblasts within 30 minutes of exposure to Epo. Shortly thereafter the activation of RNA polymerase I ensues.10 Spangler et al have shown that the proto-oncogene c-myc is an early Epo-responsive gene in Rauscher erythroleukemia cells14 and mouse erythroid progenitor cells that were produced in response to phencyclidine challenge.15 In light of the wide range of RNA species and multiple forms of RNA polymerases stimulated by Epo, it is evident that the repertoire of Epo-responsive genes (ERGs) is by no means simple.

We sought to identify ERGs in murine erythroid progenitor cells that were isolated from the spleens of mice infected with the anemia-inducing strain of Friend virus. These cells, termed FVA cells, have not yet initiated hemoglobin synthesis, but are poised to undergo in vitro differentiation by a process involving cell division and expulsion of their nuclei to form hemoglobin-rich reticulocytes within 48 to 72 hours of exposure to Epo and fetal calf serum.16 A detailed sequence of Epo-driven developmental changes of FVA cells has been elucidated, including increased expression of specific membrane proteins,17 anion transporters,18 globins,19 transferrin receptors,20 and alterations in cellular energetics.21 An increase in RNA synthesis has also been seen in FVA cells after Epo supplement.19 Here we report the results of our findings, which suggest that the mouse virus-like (VL30) element is rapidly induced by Epo in FVA cells.

MATERIALS AND METHODS

Isolation of murine erythroid progenitor cells. Female BALB/c mice were infected with FVA through tail vein or intraperitoneal injection as previously described.7 Two to three weeks later, mice were anesthetized by cervical dislocation. The enlarged spleens were removed, cut into small pieces, and strained through a nylon mesh. The filtrate was pipetted repeatedly to obtain a fine suspension of splenic nucleated cells. The erythroid progenitor cells were purified by velocity sedimentation at unit gravity through a continuous gradient of bovine serum albumin by the procedure of Sawyer et al.22 Approximately 1 X 106 cells were routinely obtained from a single mouse. These FVA cells were incubated at 37°C in a 5% CO2 humidified incubator at 1 X 106 cell/mL of Iscove's modified Dulbecco media (Sigma, St Louis, MO) supplemented with 0.5% albumin (Armour Pharmaceutical, Kankakee, IL), ampicillin (100 U/…
RESULTS

Identification of cDNA clones containing ERGs. We constructed a cDNA library from poly(A') RNA isolated from FVA cells that were exposed for 2 hours to Epo 2 U/mL and CHX 10 μg/mL. Although long-term in vitro culture of FVA cells ordinarily requires fetal calf serum, we did not use serum in short-term initial experiments in order to restrict the gene induction to those stimulated by Epo alone. However, we included CHX in this series of experiments to limit the detection of only genes that are induced directly by Epo in the absence of intervening protein synthesis. We screened approximately 20,000 plaques at a low density of 4,000 to 5,000 plaque-forming units per 150-mm plate. Two replicate nitrocellulose filters were lifted from each plate. One was probed with 32P-labeled single-stranded cDNA probes (107 cpm/mL) made by using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), [32P]dCTP (3,000 Ci/mmol; NEN, Dupont, Boston, MA), and poly(A') RNA from either Epo-stimulated FVA cells (Epo probe) or nonstimulated FVA cells (control probe). These probes were used for screening the cDNA library of Epo-stimulated FVA cells. Plaques that preferentially hybridized to the Epo probe were picked and re-screened twice. Plasmids were generated from consistently positive plaques by in vivo rescue procedure following the manufacturer's protocol (Stratagene). Plasmid DNA was isolated for sequence analysis and for the preparation of a radioactive DNA probe by random priming reaction, which was used for the Northern analysis.

Northern blot analysis. FVA cells at 1 × 10⁶ cells/mL were stimulated with Epo for a period up to 4 hours. Epo-induced FVA cells were washed twice with phosphate-buffered saline before RNA extraction. Total RNA (20 μg/sample) was separated by electrophoresis in 1.5% agarose gels containing formaldehyde. RNA was blotted onto nitrocellulose filters and hybridized to radioactive probes prepared from the plasmid DNA by random priming reaction. Radioactive Chinese hamster ovary cDNA clone B (CHO B) probe was used as an internal control in all experiments. Hybridizations were performed at 42°C in 50% formamide, 1 × Denhardt's solution, 20 mM NaPO₄, pH 6.5, 10% dextran sulfate, 100 μg salmon sperm DNA/mL, and radioactive probe of 1 × 10⁶ cpm/mL for 18 to 24 hours and washed in 0.1× SSC (1× SSC = 150 mmol/L NaCl, 15 mmol/L Na citrate, pH 7.0) and 0.1% sodium dodecyl sulfate (SDS) at 55°C for 30 minutes. The blots were exposed to intensifying screens (XAR-5 film; Eastman Kodak, Rochester, NY) at -70°C.

Effects of CHX and serum on VL30 induction. FVA cells were stimulated with Epo (1 U/mL) for 2 hours (Fig 1A) either with or without CHX (10 μg/mL). It is evident that either CHX or Epo alone increased transcripts of VL30. The combination of Epo and CHX resulted in a superinduction of VL30 expression. To determine whether Epo can elicit VL30 induction in the presence of serum, FVA cells were stimulated with Epo 1 U/mL in the presence of 20% fetal calf serum. As shown in Fig 1B, Epo enhanced the VL30 expression in the presence of serum as well. However, serum itself has little effect on the VL30 expression. In all subsequent experiments, both CHX and serum were withheld, since Epo alone was sufficient for the gene activation.

Time course of VL30 expression. Figure 2 depicts the time course of VL30 expression induced by Epo. FVA cells were stimulated with Epo (1 U/mL) in the absence of serum
and CHX for up to 4 hours. The expression of VL30 was evident within 30 minutes and reached a maximum by 1 hour.

Effect of Epo concentrations on VL30 expression. Figure 3 shows VL30 expression by FVA cells that were stimulated for 1 hour with various concentrations of Epo. The level of VL30 transcripts progressively increased as the Epo concentration was increased reaching a maximum at 1 U/mL or greater.

In the following series of experiments, we tested several agents that have been implicated in the signal transduction pathway mediating Epo action.

Intracellular calcium. Intracellular Ca\(^{2+}\) levels of FVA cells were altered over a wide range to examine the calcium dependence of VL30 induction by Epo. To deplete intracellular Ca\(^{2+}\), FVA cells were treated with EGTA (2 mmol/L) for 1 hour. Under these conditions, intracellular Ca\(^{2+}\) was reduced from 250 nmol/L to 50 nmol/L as measured by fluorescence dye fura2. EGTA reduced the extracellular Ca\(^{2+}\) concentration to approximately 0.1 μmol/L as determined by Ca\(^{2+}\)-sensitive electrode. To increase the intracellular Ca\(^{2+}\) content, the FVA cells were incubated with 1 mmol/L calcium and 2 μmol/L ionomycin for 1 hour. The concentration of intracellular Ca\(^{2+}\) increased to greater than 3 μmol/L, but the exact value could not be estimated since the levels exceeded the linear detection range of the dye. As
shown in Fig 4A. Neither EGTA nor ionomycin had any effect on the VL30 expression, suggesting that neither augmented nor depleted intracellular Ca\(^{2+}\) levels affected the VL30 induction by Epo (Fig 4A).

Staurosporine, H7, and genistein. In light of the findings showing that Epo increases c-myc mRNA by a protein kinase C (PKC)-dependent pathway in Rauscher erythroleukemia cells\(^{14}\) and in mouse erythroid progenitor cells,\(^{15}\) it was of interest to determine whether inhibitors and activators of PKC affect the VL30 expression. The results depicted in Fig 4B illustrate that staurosporine (3 pmol/L) and H7 (20 pmol/L) did not inhibit expression of VL30. If anything, staurosporine tended to enhance the signal. A tyrosine kinase inhibitor, genistein (200 pmol/L), also did not inhibit but rather enhanced the Epo-induced VL30 expression. Tetradecanoyl phorbol acetate (TPA) by itself (up to 100 ng/mL) was without effect.

DISCUSSION

The recent availability of recombinant Epo and the development of a wide variety of model cell lines have stimulated mounting interest in erythropoiesis. However, the mechanism by which Epo directs the remarkably complex program of cellular differentiation and maturation still remains elusive. In this report, we describe the isolation and characterization of cDNA clones corresponding to genes that are rapidly induced by Epo in the FVA cells. At the time of their isolation, FVA cells were mostly in the colony-forming unit-erythroid (CFU-E) stage of erythroid development. Sawyer, Koury, Bondurant and their associates have extensively investigated a sequence of specific events accompanying Epo-mediated differentiation of FVA cells.\(^{19,20,29,30,44}\) Within 48 to 72 hours of culture with Epo, FVA cells undergo cell division with a fourfold to fivefold decrease in individual cell size, synthesize hemoglobin, remodel membrane transporters, discard Epo and transferrin receptors, adapt glycolytic machinery, extrude their nuclei, and emerge as reticulocytes or erythrocytes.

We used a differential hybridization procedure for the identification of ERG. From initial screening of 20,000 recombinant phages, we identified 47 potential ERGs. Interestingly, cDNA clones corresponding to a mouse VL30 element comprise more than half of the entire collection of cDNA clones induced by Epo. Insert from one of these clones, ERG4, hybridized to both ERG7, and ERG13, which bear striking sequence similarity to both the LTR and internal region, respectively, of the BVL-1 element. Thus all three clones were presumably derived from a single transcript that has a sequence very similar to BVL-1.\(^{28}\) It is not known whether ERG7/ERG13 is BVL-1 itself or another member of VL30. It is possible that the scattered differences between the sequence of BVL-1 and those of ERG7/ERG13 may have resulted from cloning artifact or sequencing errors.

The mouse VL30 element is a repeated gene family with structural features similar to those of retroviruses and transposable genetic elements.\(^{31,32}\) An elevated VL30 RNA level has been reported in transformed cells and in rapidly proliferating cells.\(^{33,34}\) VL30 is also induced in a number of cell systems by a variety of agents, including epidermal growth factor, insulin-like growth factor, anoxia, and TPA.\(^{35-38}\) In Friend erythroleukemia cells, which are distinct from the FVA cells, the expression of the intracisternal A-particle (IAP) LTR sequence, belonging to another repeated retrovirus-like gene family, is linked to the cell cycle.\(^{39}\) IAP is also abundantly expressed in mouse tumor cells\(^{40}\) and during the
early phase of embryogenesis. Thus, the transcription of repeated retrovirus-like gene families that are reminiscent of transposable elements appears to be regulated under general growth conditions, especially during cell proliferation and development. However, inasmuch as a VL30-encoded polypeptide has yet to be identified and the coding potential of the element is not known, the physiologic significance of VL30 induction by Epo remains to be explored.

Having identified an Epo-inducible VL30 element, it was of interest to examine the influence of various signal transduction pathways that have been implicated as mediating Epo action on VL30 expression. The intracellular Ca²⁺ concentration, which has been explored in relation to Epo action, was without effect in the VL30 activation by Epo. Moreover, we found that Epo does not alter intracellular Ca²⁺ levels in FVA cells (data not shown), in agreement with the results reported by Kelley et al.

Several studies have suggested that PKC is involved in the regulation of erythropoiesis. Fibach et al. reported that TPA, a nonhydrolyzable agonist of PKC, enhanced erythroid colony formation in normal mouse bone marrow-derived CFU-E and burst-forming unit-erythroid (BFU-E). Jenis et al. showed that PKC inhibitors H7 and staurosporine produced dose-dependent inhibition of erythroid differentiation induced by cytarabine, thymidine, or hemin. Spangler et al. corroborated the findings of Todokoro and Ikawa that c-myc is an early-response gene in Epo-sensitive Rauscher murine erythroleukemia cells, and found that Epo increases c-myc transcripts, which can be blocked by PKC inhibitors including staurosporine, sphenogosine, and sangivamycin. Moreover, treatment of the virus-transformed Rauscher cells with TPA resulted in the phosphorylation of a major PKC substrate, an 80-Kd protein. Since Epo increased the phosphorylation of the same protein, it was suggested that PKC is required for the activation of c-myc expression by Epo. That PKC inhibitors attenuate the Epo-induced c-myc expression was also confirmed in erythroid progenitor cells produced in response to phenylhydrazine (PHZ) challenge in mice. Unlike the results with PKC inhibitors, the effect of PKC activator, TPA, is more complex. For example, in PHZ-stimulated erythroid progenitor cells, TPA alone was sufficient for increasing c-myc transcripts levels, as opposed to the finding with the Rauscher cells, in which TPA was without effect. In addition, the activation of PKC by TPA failed to induce terminal differentiation of PHZ-stimulated erythroid progenitor cells.

In FVA cells, TPA did not induce the activation of VL30 expression (Fig 4B). H7 and staurosporine did not inhibit the VL30 expression. Instead, staurosporine at 3 μmol/L concentration enhanced the VL30 expression by Epo. These findings imply that the activation of PKC per se is not likely required for the VL30 expression.

In addition to PKC, tyrosine kinase also has been implicated in Epo action. We showed that the Epo receptor isolated from FVA cells was phosphorylated at a tyrosine residue after Epo exposure. Dusant-Fourt et al. confirmed the tyrosine phosphorylation of the Epo receptor in the human Epo-responsive hematopoietic cell line UT-7. Moreover, it was observed that Epo increases tyrosine phosphoprotein in B6SU1 cells and in Epo receptor cDNA transfected DA-3 cells. These findings imply that an inhibition of a tyrosine kinase may lead to a blockade of differentiation. Contrary to the expectation, herbimycin, which inhibits tyrosine kinase, did not affect the differentiation of the MEL cell line. Other tyrosine kinase inhibitors, ST638 and genistein, were reported to induce in vitro erythroid differentiation of MEL cells in a synergistic manner with a DNA replication blocker. We found that although genistein alone cannot activate VL30 expression (data not shown), genistein augments Epo-activated VL30 expression. Taken together, these findings implicate tyrosine kinase in VL30 expression, although the exact mechanism remains to be determined. In this regard, it is noteworthy that staurosporine was found to inhibit both soluble and membrane-bound protein tyrosine kinases in human neutrophils. Thus, it is possible that the enhancement of VL30 transcripts seen with staurosporine also may reflect an inhibition of tyrosine kinase in FVA cells.

At present, the physiologic role for VL30 induced by Epo is not known. Nevertheless, our findings suggest that a VL30 element represents a primary response gene activated by Epo in FVA cells. Induction of VL30 may be useful as a molecular marker to help unravel the early steps of Epo action. We are currently in the process of characterizing the remainder of the cDNA clones corresponding to Epo-regulated genes in FVA cells.

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REFERENCES

32. Foster DN, Schmidt LJ, Hodgson CP, Moses HL, Getz MJ: Polyadenylated RNA complementary to a mouse retrovirus-like multigene family is rapidly and specifically induced by epidermal growth factor stimulation of quiescent cells. Proc Natl Acad Sci USA 79:7317, 1982
48. Fibach E, Marks PA, Rifkind RA: Tumor promoters enhance myeloid and erythroid colony formation by normal mouse hemopoietic cells. Proc Natl Acad Sci USA 77:4152, 1980


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