Erythropoietin

By Sanford B. Krantz

ERYTHROPOIETIN (EP) is a glycoprotein produced primarily by the kidney, and is the principal factor regulating red blood cell (RBC) production. Unlike most of the hematopoietic growth factors, EP is principally made by a single organ, the kidney, and it participates in a classic feedback control system. Hypoxia increases the production of the hormone, which enhances the manufacture of new RBCs, whereas hyperoxia reduces the amount of EP and, consequently, decreases RBC production. Also, in contrast to many other hematopoietic growth factors, EP is remarkably specific for the erythroid progenitor cells and has little effect on other cells.

EP has been assayed for biologic activity in the polycythemic mouse. This is a cumbersome and expensive procedure and a radioimmunoassay is now commercially available with a normal range of 5 to 25 mU/mL for human EP. A lyophilized crude preparation of human urinary EP, with a specific activity of 1 U per 1.48 mg of protein, became the first International Reference Preparation. This was replaced by a second similar preparation in which 0.2 mg is 1 U of EP. Purification of EP was prolonged and difficult, and the natural EP obtained was never in sufficient supply for extensive use. Nevertheless, the purification and sequencing of this material led to cloning and expression of EP in mammalian cells, which has produced an abundance of this material. The first International Reference Preparation was fully active in vitro. Recombinant hormone made in Escherichia coli also lacks carbohydrate and has greatly reduced activity in vivo, with preserved activity in vitro, possibly due to the same reason.

While the human EP gene has been localized on chromosome 7 (pter-q22), in the mouse it has been assigned to the D region of chromosome 5, which is partially homologous to the long arm of human chromosome 7. The gene encoding human EP is contained as a single copy in a 27-amino acid peptide. A 27-amino acid peptide

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leader sequence at the amino-terminal is cleaved during secretion of the hormone, which leaves a peptide of 166 amino acids with a molecular weight of 18,398.26 In addition, the carboxy-terminal arginine is lost in both urinary EP and rEP, possibly from posttranslational processing by an intracellular carboxypeptidase.27 rEP expressed in CHO cells has been compared with human urinary EP by circular dichroism, UV light absorbance, and fluorescence spectroscopy, and no significant difference has been noted.28 The molecular weight of rEP was 30,400 by the sedimentation equilibrium method and the carbohydrate content was 39%,28 which is significantly higher than that reported for urinary EP. However, a corrected carbohydrate content for urinary EP, based on a molecular weight of 30,400, would be 35%, which is not much different than the value for rEP.29

Because of the unavailability of natural urinary EP and the relative abundance of rEP, most of the structure studies have been performed with the recombinant hormone. The amino acid sequences of EP from humans,24-26 old-world monkeys,33 and mice31-32 are known. The latter two have 192 amino acids and the leader sequence in monkey rEP is 27 while in mice it is 26 amino acids. A very high degree of conservation is evident with no less than 151 identical residues, and 177 between monkeys and humans.31 Human EP has four cysteines linked by disulfide bonds between cysteine 7-161 and 29-33,34,35 These are required because their reduction leads to reduced biologic activity, which can inhibit biologic function or block receptor binding.36-38 But deletion of residues 9-21, 48-64, 83-98, 142-150, 151-157, or 151-165 led to a complete loss of biologic activity, but deletion of 111-119 had no effect on this activity. This deletion did not include a glycosylation site and should not have affected disulfide-bond formation.

EP has been predicted to have a tertiary conformation analogous to that of growth hormone (GRH) despite little similarity in amino acid sequence.39-47 The crystallographically determined GRH fold features an antiparallel four-a-helix bundle core with a unique loop topology.39 Equivalently, the globular EP structure is proposed to consist of four antiparallel a-helices with two long and one short loop connections (Fig 1),47 in accord with circular dichroic spectra that indicate a preponderance of helical structure in EP.26 The GRH-like EP model is also in agreement with experimental work because mutations within each of the four predicted helical segments resulted in reduced biologic activity, but a deletion mutant (aa. 111-119) on a long loop outside the predicted helical core had no effect.45 Other cytokines that are proposed to share the GRH-like helical fold and loop topology with EP include prolactin (PRL), interleukin-6 (IL-6), and granulocyte colony-stimulating factor (G-CSF).46,47,48 These structural links between cytokine molecules suggest analogous functional ties: eg, the receptor-binding epitope of GRH appears to be the exposed surface of the C-terminal helix; the EP model has an analogous helical segment, suggesting that selected residues arrayed on helix D (Fig 1) form the primary receptor-binding structure for EP.49 This prediction is consistent with neutralizing antibody studies for EP.46 A precise understanding of the relationship between form and function in EP awaits the resolution of the three-dimensional structure of EP by X-ray crystallography.

SITE AND REGULATION OF EP PRODUCTION

EP Production by Kidney and Liver

In 1957, Jacobson et al.50 demonstrated that the kidney was the major organ responsible for increased serum EP levels. Rats with bilateral nephrectomies no longer increased EP production in response to anemia or the administration of cobalt. Serum Ep levels were also found to be extremely low in patients with end-stage renal failure and severe anemia, but after successful renal transplantation these levels reverted to normal.51 However, anephric rodents retained 10% of their capacity to produce Ep and anephic patients also had some EP in their plasma.52-54 The liver appears to be the primary site of extrarenal EP production because hepatectomy abolished the residual EP production of anephric rats52 and extrarenal EP production in response to anemia correlated with liver regeneration.
was noted by 1.5 hours with a rise of more than 200-fold by the exposed surface of proposed helix D forms the primary receptor during late gestation. Nevertheless, the cell of origin of EP was unknown and the development of genetic probes sheep and EP mRNA has been found in human fetal to be the major site of EP production during fetal life in addition, the liver appears to be the major site of EP production during fetal life in sheep, and EP mRNA has been found in human fetal liver[ and mouse fetal liver during mid-gestation, but not during late gestation. Nevertheless, the cell of origin of EP remained unknown and the development of genetic probes for EP mRNA provided a means for localizing EP production. Initial studies established the anemic mouse or rat as good models for studying EP production. RNA was isolated from various organs of normal and anemic mice, separated on agarose gels, transferred to nitrocellulose sheets, and hybridized with a radioactive EP mRNA specific probe. Trace amounts of EP mRNA were detected in normal kidneys when polyadenylated RNA was analyzed, and after anemia was produced an increase in EP mRNA paralleled the changes in EP mRNA levels, suggesting that EP production in response to hypoxia represents de novo synthesis. When hypoxia was discontinued, rat kidney EP mRNA decreased to undetectable levels by 3 hours, indicating a fairly short half-life. In both anemic kidneys and a human hepatoma cell line, Hep 3B cells, which respond to hypoxia with increased EP and EP mRNA, nuclei have been isolated and examined for transcription of the EP mRNA and both have a higher level of transcription of this specific gene after hypoxia. Stimulation of transcription generally began 2 to 4 hours after cobalt treatment of mice and was accompanied by an increase in EP mRNA and plasma EP concentration. Cycloheximide administration before hypoxia prevented the increase in mRNA, suggesting that protein synthesis was necessary. These nuclear run-off experiments indicate that the increase in EP mRNA can, at least partially, be attributed to an increased rate of transcription of the EP gene. In addition, recent experiments with the Hep 3B cells, where transcription was shut off by hyperoxia, indicate a 50% decrease in EP mRNA in 1.5 to 2 hours, like that seen in the rat kidneys. After actinomycin D was used to halt transcription, the overall stability of the EP mRNA greatly increased, with a half-life of 7 to 8 hours. Actinomycin D appeared to be preventing the transcription of a rapidly turning over RNA whose translation product destabilized EP mRNA, and this was supported by the fact that the addition of cycloheximide had the same effect on the stability of the EP mRNA. Thus, an additional mechanism affecting EP mRNA stability is also present in these cells, but whether this mechanism has a role in determining the life of this mRNA with alterations in hypoxia-induced transcription is not clear at the present time. Localization of EP-producing cells. The availability of labeled RNA or DNA probes for in situ hybridization of EP mRNA has provided a method for identifying the individual cells that make EP. Specific immunolocalization of EP in the kidney is very difficult because EP is present in the serum and urine. To be certain of the cellular source of the hormone the EP needs to be specifically localized within the Golgi or endoplasmic reticulum; thus far no one has provided such a study. However, EP mRNA should be localized to those cells that produce EP. Two groups using a radioactive probe for the EP mRNA, in situ hybridization, and autoradiography demonstrated that a peritubular interstitial cell, outside the tubular basement membrane, was the renal cell that had EP mRNA either under baseline conditions or after hypoxia (Fig 2). These cells were found in the cortex or outer medulla. The type of cell was not clear from the morphology, but because some interstitial cells have factor VIII-related antigen it was proposed that they may represent peritubular capillary endothelial cells. However, studies localizing both VIII-related antigen and mRNA to the same cell were not performed. When the number of interstitial cells with EP mRNA was measured in relation to increasing anemia, these cells increased
in an exponential manner in parallel with the exponential increase in total EP mRNA and serum EP.73 Thus, increased EP production was related to increased numbers of cells producing the EP mRNA rather than to increased production of the mRNA by the individual EP-producing cells. It was estimated that 20% to 30% of the total interstitial cell population of the inner cortex, but less than 10% of the interstitial cells in the subcapsular cortex, produced EP.73,74 Thus, EP-producing cells may represent a specialized cell type within the cortical interstitial rather than a generalized cell type, such as a peritubular capillary endothelial cell.72 Furthermore, the hypoxic stimulus does not appear to be uniform, but instead reaches a threshold for turning on EP-producing cells in focal areas.72,74 A similar threshold for EP production has been described in the HEP3B cells.75

While the precise identity of the EP-producing interstitial cells remains unknown, these cells are grouped in interstitial foci mostly adjacent to proximal convoluted tubules,76 which fits with the work of Eckardt et al,77 who found that EP production is directly related to proximal tubule function. It has been proposed that the focal areas of hypoxia could arise from a constriction of a small proximal artery to shunt arterial blood because of the anemia, or could arise from oxygen consumption by proximal renal tubule cells using a higher percentage of the available oxygen and producing increased hypoxia distally.73,74 Previous reports that macrophages manufacture EP78 could not be verified79 and await further confirmation, whereas a recent report of renal tubular cell production of EP was clouded by the use of a 32P-labeled probe for in situ hybridization that gave decreased resolution in the autoradiograms.80

The site of EP production in the liver is still unknown, but evidence has been presented for both the Kupfer cells and the hepatocytes.81 The adult liver’s contribution to total EP production with severe hypoxia is 10% to 15%.55,82 Because the liver is fourfold larger than two kidneys, the EP mRNA accumulation per producing cell might be much lower than in the kidney or there may be far fewer producing cells. For this reason, similar work to that reported for the kidney has been quite difficult and no decisive information is yet available.

The exact mechanism for the translation of hypoxia into a recruitment of renal EP-producing cells is still unknown. Administration of cyclic adenosine monophosphate (cAMP) to mice increases EP production and the RBC mass57,79; prostaglandin E1 (PGE1) raises cAMP and EP concentration in isolated perfused dog kidneys84 and PGE2 increases EP levels and erythropoiesis in mice85; renal artery constriction increases PGE and EP blood levels in dogs, and indomethacin, which inhibits PGE formation, prevents increased EP production after hypoxia.86 On the basis of these scattered experiments, many of which are in vivo with multiple variables, it has been proposed that renal hypoxia might result in the release of PGE, which in turn could increase renal cAMP and, thereby, the synthesis of EP. cAMP could act through a reduction in the intracellular concentration of Ca2+ because cobalt, which stimulates EP production, is a general inhibitor of calcium channels, low calcium medium enhances EP production by renal carcinoma cells in vitro, and verapamil and diltiazem, which block calcium entry, also enhance EP production.87,88 This mechanism might apply in the same cell that produces EP or in adjoining cells that trigger the EP-producing cell. In humans, however, agents that block PGE synthesis and calcium channels have not led to increased EP production.

Initiation of EP mRNA transcription by hypoxia. The chemical transducer for hypoxia is difficult to study in primary renal or hepatic cells because of the small percentage of EP-producing cells in these organs and the lack of purification of these cells. However, cell lines have been
screened for EP production in response to hypoxia and several have been found.66,67,75 The Hep3B cell line constitutively produces EP and appears to have one of the largest responses, about 30-fold, to both hypoxia, CoCl₂, NiCl₂, and MnCl₂.66,67 Barely detectable mRNA for EP at room air oxygen levels is greatly increased with exposure to 2% oxygen or 50 to 100 μmol/L CoCl₂. Because cobalt, nickel, or manganese can be substituted for iron in heme, and, in the case of hemoglobin, lock the protein into a deoxy conformation, like that of the ferroheme protein with low oxygen, it has been proposed that a heme protein may regulate EP production.66 Evidence for this proposal consists of: (1) a lack of an enhanced effect of cobalt or nickel with hypoxia, suggesting a common pathway; (2) carbon monoxide administration, which keeps hemoglobin in the oxy state, reduces the hypoxia-induced increase in EP production, but has no effect on cells treated with cobalt or nickel, which form heme proteins that do not bind carbon monoxide; (3) inhibition of heme synthesis with either desferrioxamine, which chelates iron,90 or 4,6-dioxohaptenoic acid, which inhibits aminolevulinate dehydratase,61 reduces the increase in EP synthesis that occurs with hypoxia without any change in overall protein synthesis.66,67

The Hep3B cells are an excellent system for analyzing the mechanism of EP production, because they are a homogeneous collection of cells that respond to hypoxia in vitro with a marked increase in EP production, and this work may provide the best insight available to a chemical mechanism for the control of EP production. Eventually, similar work will have to be applied to highly purified, normal, EP-producing kidney cells to determine if the mechanism that is worked out for a liver cell line is also physiologically active in the normal organ that principally manufactures EP. The proposal that a heme protein may be an intermediate regulator is intriguing and it remains to be determined if this protein is the messenger to the nucleus, or if other proteins are involved in a second messenger system.

Transcriptional regulation of the EP gene. A comparison of sequences between the human and mouse EP genes shows several areas of very high sequence conservation in noncoding regions: (1) the 140 bp upstream from the transcriptional start site; (2) two segments within the first intron; and (3) 100 to 220 bp downstream from the translation termination codon.31,32 One approach to studying EP transcription has been to put a genomic segment containing the entire human EP gene with 0.4 or 6 kb of 5′-flanking sequence and 0.7 kb of 3′-flanking sequence into mouse zygotes to generate transgenic mice.31,32 These mice had regulated hepatic EP transgene expression with increased hepatic expression after hypoxia or CoCl₂, but did not have a similar response of renal transgene EP. Thus, sequences responsible for liver expression map within 0.4 kb of 5′-flanking sequences and 0.7 kb of 3′-flanking sequences, and DNA sequences that control inducible kidney expression have not been found within 6 kb 5′ or 0.7 kb 3′. In addition, the smaller transgene had expression in diverse other tissues, which was not generally seen with the larger gene, suggesting that the EP gene might contain a negative regulatory element between 0.4 and 6 kb 5′ to the coding region that restricted expression to the liver and kidney. These studies indicate that control of EP expression is complex with oxygen-sensitive, tissue-specific, and negative regulatory sequences. Additionally, multiple RNA transcription initiation sites were also detected.49

Another approach is to make plasmid constructs containing selected potential transcription control sequences 5′ and 3′ to a reporter gene and examine the transcription efficiency of the reporter gene in expression assays after transfection of the plasmid constructs into appropriate mammalian cells. Goldberg et al49 have used GRH as the reporter gene. Their construct contained a 1,192-bp HindIII-XbaI fragment that extended from 378 bp 5′ to the cap site of the EP gene through the first intron attached 5′ to the GRH coding sequence. In addition, a 255-bp AccI-BglII fragment that extended 67 bp upstream from the EP termination codon and covered most of the 3′ noncoding region of homology was added 3′ to the GRH sequence. The plasmid DNAs were transfected by electroporation into Hep3B cells, which were then exposed to CoCl₂ or 1% oxygen, and measurement of GRH in the cell medium indicated a response with a reduction of the response in the presence of carbon monoxide as seen with endogenous EP synthesis of Hep3B cells. These results show oxygen regulatory sequences within the above regions of the human EP gene.

Costa-Giomi et al68 and Caro et al64 have taken yet another approach and have prepared nuclear extracts from hypoxic Hep3B cells to determine if they enhance the transcription of the EP gene. These extracts were added to an in vitro transcription system using a DNA template construct consisting of a HindIII-EcoRI fragment extending from 380 bp 5′ to 600 bp 3′ of the EP gene, in a pUC8 vector. This fragment was transcribed to a greater extent in the presence of extracts from hypoxic nuclei compared with extracts from normal nuclei. The nuclear extracts from hypoxic Hep3B cells appear to contain functional transacting factors that specifically stimulate transcription of the EP gene, and these factors appear to interact with sequences in the above genomic fragment that confer responsiveness to hypoxia in Hep3B cells. EP mini genes were also constructed by deletion of 201 and 384 internal base pairs of the previously described human EP gene and were transfected into Hep3B cells by electroporation.69 These genes were expressed in the Hep3B cells after hypoxia, which confirmed that genomic sequences between 380 bp 5′ and 600 bp 3′ were sufficient to confer regulation of the EP gene by hypoxia in Hep3B cells.

Finally, Beru et al45 have taken a double-stranded deoxyoligonucleotide corresponding to region -61 to -45 relative to the start site of transcription of the mouse EP gene and have used this in DNA mobility shift assays. In kidney extracts they found one protein and one ribonucleoprotein that bind to this oligonucleotide, and the amounts of two RNA species from the ribonucleoprotein were reduced after stimulation of the kidneys by CoCl₂. Thus, the ribonucleoprotein may be a negative transcriptional factor which, when the RNA components are reduced in concentration, allows transcription of the EP gene to proceed. These
methodologies should provide a means for characterization of the controlling sequences and the trans-acting factors necessary for EP synthesis, but much more work is now necessary for precise determinations.

**SITE AND MECHANISM OF ACTION OF EP**

**Erythroid Progenitor Cells**

The pluripotential hematopoietic stem cell gives rise to specific erythroid progenitor cells that respond to EP. These have been termed the colony-forming unit-erythroid (CFU-E) and the burst-forming unit-erythroid (BFU-E) and have been identified by the plasma clot and methylocellulose culture techniques that are capable of supporting the growth of discrete erythroid colonies. The CFU-E is a rapidly dividing cell, as demonstrated by thymidine suicide experiments, which is highly responsive to small concentrations of EP and gives rise to erythroblast colonies of 8 to 49 cells in 7 days (human) or 8 to 64 cells in 2 days (mouse). The BFU-E is a much more immature cell that divides less frequently. This cell requires another growth factor such as IL-3 or granulocyte-macrophage CSF (GM-CSF) plus EP to develop into groups of cells in erythroblasts, or larger colonies of greater than 500 erythroblasts, in 15 days (human) or 8 days (mouse). These cells are part of a continuum of development from the earliest BFU-E to the latest CFU-E, with intermediate cells termed mature BFU-E, but the selection of particular cells in the sequence of events supported by EP stimulation and have been identified by the plasma clot and methylcellulose culture techniques that are capable of supporting the growth of discrete erythroid colonies.

**Murine CFU-E**

These cells have been purified through the use of the Friend virus that produces anemia (FVA) or the administration of thiamphenicol to anemic mice, followed by centrifugal elutriation and Percoll density gradient centrifugation. FVA consists of a replicating helper virus plus a defective spleen focus-forming virus. These cells have been used to study the biochemical sequence of events after EP stimulation and to characterize EP receptors.

The addition of EP to FVA cells in vitro leads to a progressive erythroid differentiation into erythroblasts and then reticulocytes by 48 to 60 hours of culture. These cells have allowed studies of the sequence of events supported by the hormone, before the development of RBCs and including the enucleation process. For example, it has recently been shown that vimentin filaments are absent during enucleation and that F actin is essential for this process. At 12 hours after addition of EP to FVA cells, hemoglobin synthesis is detected and at 6 hours the initiation of transcription of mRNA for the globin genes can be identified (Fig 3). With the thionemycin method initiation of mRNA for the globin genes occurs earlier, but this could be due to a different level of maturity of the CFU-E. Additional nuclear digestion experiments showed that the b-major globin gene is very sensitive to DNase I before the addition of EP and remains so afterwards. DNAase I hypersensitive sites in the b-major globin gene region also are unaffected by the addition of EP.

Thus, EP addition to FVA cells is soon followed by the specific transcription of the globin genes, but chromatin structural alterations have already occurred at an earlier time. Since initiation of transcription of the a- and b-globin genes on different chromosomes occurs at the same time, it is likely that a specific transcription initiation factor is synthesized, released, or activated, and that it can act in a trans fashion at multiple sites.

By 6 hours after the addition of EP to FVA cells, an increase in the number of transferrin binding sites also occurs, and by 24 hours the transferrin binding sites increase from 200,000/cell to 400,000/cell. During this period, the transferrin-transferrin receptor association constant of 3 to 5 nmol/L did not change and the rate of uptake of 55Fe from transferrin remained constant. It is possible that the increase in the number of transferrin binding sites results from a reduced availability of intra-cellular iron, due to iron sequestration for heme synthesis, because chelators of iron have been shown to increase messenger RNA for transferrin receptors.

While an effect of EP on DNA synthesis by unpurified narrow erythroid cells has been noted, no effect has been found with highly purified FVA cells. However, 3 hours after EP is added to the latter, an increase in RNA synthesis is seen as reported with narrow EP responsive cells. The precise composition of this RNA and its overall role in erythroid differentiation has never been clarified. Increased glucose uptake has been shown to

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**Fig 3. Time course of biochemical events after addition of EP to FVA cells.**
occur between 2 and 4 hours after the application of EP to FVA cells. A very early effect of the addition of $^{32}$P-EP to FVA cells is the internalization of EP. Presumably the receptor is also internalized at this time, but direct evidence for this has not yet been produced.

The earliest effect of EP that has been shown is a stimulation of $^{45}$Ca$^{2+}$ uptake within 1 minute after its addition to FVA cells generated in vitro. The rate of $^{45}$Ca$^{2+}$ uptake in low calcium medium was linear for 8 minutes and was clearly increased by EP, but EP also stimulated the efflux of $^{45}$Ca$^{2+}$ from FVA cells. Subsequently, work with the fluorescent chelators Quin-2 and Fura-2 indicated that the addition of EP to human marrow mononuclear cells, or BFU-E generated CFU-E, produced a dose-related rise in intracellular free Ca$^{2+}$ and, in one study, a twofold to threefold higher Ca$^{2+}$ concentration was found in the nucleus compared with the cytoplasm. However, other investigators have not found an increase in Ca$^{2+}$ in erythroid progenitors from human fetal liver or mouse CFU-E after EP treatment and, in FVA cells, the intracellular levels of free Ca$^{2+}$ and the production of inositol phosphates (IP$_1$, IP$_2$, and IP$_3$) in cells labeled with [H]$\text{H} \text{myoinositol}$ were not changed by EP. In the presence of an analogue of cAMP, exposure to EP lowered the intracellular calcium concentration. This probably indicates an activation of a calcium pump by EP and suggests a mechanism for the increased exchange of $^{45}$Ca$^{2+}$ in these cells that was observed previously. The effect of EP on the calcium flux across the membrane directly correlates with the binding of EP to its receptors, but requires binding of much more EP than that required for EP maturation. In addition, neither phorbol esters, which activate protein kinase C, nor cAMP or cyclic guanosine monophosphate (cGMP) had any influence on erythroid maturation of FVA cells, nor did EP increase cAMP or cGMP in rat fetal liver cells or in an EP-dependent cell line. Calcium ionophores enhanced the effect of EP on mouse marrow CFU-E in vitro, but could not substitute for the hormone. The identity of the second messenger system for EP is completely unknown at the present time. No tyrosine-specific protein kinase activity or reproducible change in intracellular cyclic nucleotide levels has been identified as a part of the mechanism of action of EP.

Human CFU-E

Human erythroid progenitor cells have also recently been highly purified. CFU-E and mature BFU-E are almost completely lacking in blood, but immature BFU-E are present. Partially purified blood BFU-E were cultured in methylcellulose for 7 days to give rise to CFU-E. From 400 mL of blood, $10^7$ CFU-E with a purity of 70% ± 18% were generated. All of these cells had EP receptors, but EP binding continuously declined with increased erythroid maturation. The growth requirements for these highly purified human CFU-E were studied in a serum-free medium and it was shown that the cells require a small concentration of deionized and delipidated crystalline albumin, transferrin, oleic acid, L-α-phosphatidyl choline, cholesterol, EP, and physiologic concentrations of insulin-like growth factor (IGF)-I or pharmacologic concentrations of insulin. Limiting dilution studies excluded any contribution of contaminating accessory cells, and a wide variety of hormones such as hydrocortisone, thyroid hormone, testosterone, platelet-derived growth factor, growth hormone, IL-3, and GM-CSF had no additional effect on colony growth. Previous reports of an effect of these hormones on CFU-E used mixed marrow cell populations and the effects observed might have been on BFU-E or through the stimulation of accessory cells. Growth hormone has been shown to stimulate CFU-E through an effect on accessory cells that produces a release of IGF-I and an enhancing effect of triiodothyronine on BFU-E growth has been shown to occur through the release of erythropoietic growth factors by accessory cells. Thus, the use of highly purified progenitor cells is just as important in the study of erythropoiesis as other pure soluble reagents and these cells have been quite useful for the characterization of human EP receptors.

Human BFU-E

While CFU-E require IGF-I in addition to EP for further erythroid development, BFU-E require a factor that was initially termed burst-promoting activity (BPA). In cultures of unpurified marrow cells, immature BFU-E proliferated, initially, in the absence of added EP if a source of BPA, extracted from human urine, was present. However, a specific BPA was never completely purified, and with the advent of IL-3 and GM-CSF it was found that these growth factors supported the development of immature BFU-E like BPA, but also acted on other cell lineages. Exposure of animals to changes in EP levels through the production of anemia and thrombocythemia, or the administration of impure EP, produced clear increases or decreases in the number of CFU-E, but only variable effects were noted on the number of BFU-E. However, the administration of large amounts of EP to patients led to a significant increase in the number of BFU-E in the bone marrow and the percentage that were in DNA synthesis increased from 9% to 38%. In addition, the nonerythroid progenitors also increased in number and percentage in DNA synthesis, although there were no significant increases in blood granulocytes and platelets. Whether this was a direct effect of EP or occurred through the stimulation of erythropoiesis by accompanying growth factor production, or through nonspecific stimulation of accessory cells is not known. When EP was added to human marrow BFU-E in vitro, it doubled the percentage of BFU-E in DNA synthesis within 24 hours, as measured by tritiated thymidine suicide.

Purification of human BFU-E has been achieved with a purity of 18% to 55% from fetal liver, 5% to 18% from bone marrow cells, and 17% to 23% from the blood of particular individuals with high concentrations of BFU-E. Recently, a high percentage of purified blood BFU-E has been obtained from normal adults using MoAbs and immunomagnetic microspheres that allow benchtop separation without the need for a cell sorter. From 400 mL of blood, 50,000 BFU-E with a purity of 57% ± 14% (range of
granulocytic series with IL-3 or GM-CSF, or into erythroblasts with EP (reviewed by Sawyer\textsuperscript{36}). Many of the previously mentioned cell lines have been used to characterize EP receptors and to study the secondary biochemical events after treatment with EP.

**EP Receptors**

Quantitation and relation to erythroid maturation. Initial attempts to radioiodinate purified urinary EP resulted in a complete loss of biologic activity,\textsuperscript{14} but a low specific activity [HJEP was made that retained biologic activity and specific binding was demonstrated with FVA cells.\textsuperscript{66} This report underestimated the number of receptors and binding affinity because of the low specific activity. Subsequently, it was shown that EP could be radioiodinated with full retention of biologic activity as long as there were 1 or less atoms of \textsuperscript{125}I per molecule of EP.\textsuperscript{86} Self-displacement analysis showed that the \textsuperscript{125}I-rEP had the same affinity for the EP receptors as unlabeled rEP.\textsuperscript{87} With this reagent, FVA cells were shown to have 800 to 1,000 cell surface EP receptors with 300 high-affinity receptors having a kd of 0.09 nmol/L and the remaining having a lesser affinity with a kd of 0.57 nmol/L.\textsuperscript{12} FVA cell membranes specifically bound \textsuperscript{125}I-rEP with a similar pattern.\textsuperscript{8} The bound \textsuperscript{125}I-rEP was rapidly internalized with the subsequent release of degradation products into the medium.\textsuperscript{125} The degradation of \textsuperscript{125}I-rEP was twofold higher, suggesting internalization of the ligand.\textsuperscript{129} After \textsuperscript{125}I-rEP binding the high-affinity receptors were downregulated and \textsuperscript{125}I-rEP was rapidly internalized with the subsequent release of degradation products into the medium.\textsuperscript{125} The degradation of \textsuperscript{125}I-rEP was greatly inhibited by chloroquine and NH\textsubscript{4}Cl, which suggested that this took place in the lysosomal compartment.\textsuperscript{125}

Uninfected mouse fetal liver cells\textsuperscript{80} and splenic CFU-E\textsuperscript{73} have similar \textsuperscript{125}I-rEP binding parameters and purified human CFU-E\textsuperscript{82} also have approximately 1,000 EP receptors, with 200 that have a kd of 0.10 nmol/L and 800 with a lower affinity and kd of 0.57 nmol/L. In contrast, the Friend MEL cell line, clone 745, which is not responsive to EP, nevertheless has 500 to 750 receptors with a lower affinity and a kd of 1.3 nmol/L.\textsuperscript{125} Many cell lines have now been studied that either have both classes of receptors or only those with a lower affinity and the latter range widely in concentration, but have not exceeded 4,000/cell (reviewed by Sawyer\textsuperscript{36}). While most cells that have erythroid maturation in response to EP have both classes of receptors, CFU-E generated from patients with polycythemia vera have only low-affinity receptors, but still maintain differentiation into erythroblasts in response to EP.\textsuperscript{171} Murine CFU-E with all of the high-affinity receptors downregulated also continue to respond to the hormone.\textsuperscript{70} In these cases, either downregula-
tion for diverse reasons may not allow adequate measurement of high-affinity receptors by equilibrium binding, or low-affinity receptors, which can also internalize the hormone, may participate in the differentiation process. An effect of EP through the lower affinity receptors is also shown with the HCD33 and HCD57 cells, which need EP for survival and enhanced proliferation and only have the lower affinity EP receptors.172 Autoradiographic analysis of binding of 125I-rEP to mouse bone marrow cells also showed specific binding to megakaryocytes and equilibrium binding studies to partially purified rat megakaryocytes (20% to 40%), where the megakaryocytes appeared to be the only significant EP binding cells, showed a single class of 6,500 binding sites per megakaryocyte with a kd of 0.29 nmol/L.164

Nonhematopoietic cells such as liver, brain, lung, and skeletal muscle do not have EP receptors,122 and nonerythroid hematopoietic cells such as monocytes and lymphocytes also lack EP receptors.138 However, mouse and rat placenta have low-affinity EP receptors that might be involved in the transfer of EP from maternal to fetal plasma in these species.173 This is supported by the observation that 125I-rEP can be transferred to the fetus from the mother.174 Because Zanjani et al174 have not found a similar transfer of EP in sheep and monkeys and because sheep placentas did not appear to have EP receptors (Sawyer ST: unpublished observations, September 1989), this relationship may be limited to rodents.

The presence of EP receptors correlates very well with response to the hormone in normal BFU-E and CFU-E. With the development of EP receptors in human BFU-E, EP dependency begins. After the peak receptor number is reached at the CFU-E stage, EP receptors decline in human and murine CFU-E parallel to a reduced dependency on the hormone.137,146,170,175,176 The late basophilic erythroblasts and their descendants no longer appear to need EP for continued erythroid maturation.1,10,175

Whether EP receptors are internalized and degraded or recycled to the cell membrane is not known. However, after HCD33 and HCD57 cells are treated with EP, the receptor number is downregulated to 10% to 24% and the recovery of EP receptors after removal of EP is dependent on protein synthesis.172 This suggests that downregulation of EP receptors in this cell line is associated with their degradation and that the subsequent upregulation of receptors when EP is removed occurs through the synthesis of new receptors.

Structure. Cross-linking of 125I-rEP bound to FVA cell membranes using disuccinimidyl suberate (DSS) identified two bands of radioactivity with apparent molecular masses of 100 Kd and 85 Kd when the mass of EP was subtracted.104,173 These are not disulfide-bridged subunits of a larger complex because the results were the same in the presence and absence of a reducing agent. A similar structure has been reported using mouse fetal liver erythroid cells,172 and a variety of murine and human cell lines,9 normal human CFU-E,113 CFU-E from patients with polycythemia vera,173 and mouse and rat placenta.138 This has been a repeated and reproducible observation with all cells or membranes that specifically bind EP, irrespective of subsequent erythroid differentiation (reviewed by Sawyer164). While it is possible that DSS was artificially cross-linking a nearest neighbor protein to the hormone, the diversity of these tissues would make that possibility somewhat unlikely.

Further studies have shown that both bands have less than 5% glycosylation, but the receptor was cross-linked to EP at the time of treatment with multiple glycosidases, and this could have prevented full activity of these enzymes.179 Protease digestion demonstrated identical fragments from the 100- and 85-Kd proteins, which strongly suggests that the primary amino acid sequence of these two proteins is similar if not identical.180 In addition, increasing the number of protease inhibitors during the preparation of membranes and the binding and cross-linking steps increased the ratio of radioactive 100-Kd protein to 85-Kd protein.179 The 85-Kd protein could be an artifact resulting from protease activity, or it could result from a posttranslational modification of the 100-Kd protein. While one might suppose that the 100- and 85-Kd proteins could each be related to a single class of binding sites on the FVA cell membranes, both were labeled in the same ratio, as the concentration of 125I-rEP was increased for binding and cross-linking.174 The ratio was the same at low concentrations of 125I-rEP where mainly the higher affinity receptors were bound, and at high concentrations where predominantly the lower affinity receptors were occupied.

D’Andrea et al180 have cloned a cDNA encoding an EP receptor protein from an expression library of MEL cell clone 745. Positive clones were identified by the presence of a high concentration of functional receptors for 125I-rEP on COS cell transfectants. While the cDNA came from a cell line that expressed only lower affinity receptors, the COS cells expressed higher and lower affinity EP receptors with binding constants like the FVA cells. Cross-linking of 125I-rEP to the surface of these transfected COS cells resulted in cross-linked bands of 140 and 100 Kd. Correction for the molecular mass of EP led D’Andrea et al to conclude that EP receptor proteins of 105 and 65 Kd were present in these cells.180 The absence of the 85-Kd protein is unexplained, but the COS cells may provide an environment that does not lead to the apparent modification or proteolysis of the 105-Kd protein. The 55-Kd protein coded by the cloned cDNA is apparently glycosylated to form a 65-Kd functional receptor in the transfected cells.181 Immunoprecipitation of [35S]methionine-labeled proteins from an NIH-3T3 fibroblast cell line transfected with the EP receptor, using an antiserum to the N-terminal peptide of the receptor protein, or Western blot analysis, showed a doublet of approximately 66 Kd confirming this molecular mass.102,183 The 66-Kd protein was detected in FVA cells with a similar antibody, but was not detected with 125I-rEP cross-linking.102,184 The reason for this lack of detection is still not apparent, but may relate to its concentration or location. Transfection of the IL-3-dependent Ba/F3 cell line with a plasmid containing the EP receptor gene converted the line to EP-dependence, whereas coinfection with the spleen focus-forming virus (SFFV) released the cells from EP dependence.12 In fibroblast cells, immunopre-
cipitation with antibody to the SFFV membrane glycoprotein, gp55, or antibody to the EP receptor showed that antibody against one protein coprecipitated the other, indicating an association between these two proteins. Therefore, the SFFV might be conferring EP independence through binding of its gp55 envelope protein to the EP receptor.

A principle question is the relation of the 100- to 105-Kd EP-binding protein to the 66-Kd receptor. D'Andrea et al\(^{190}\) initially proposed that the former might be either a highly glycospalylated form of the latter, or it might represent the cross-linking of two 55-Kd receptor proteins by DSS, which is a homobifunctional cross-linker. However, the latter possibility has been studied using a radiolabeled heterobifunctional cross linker, the Denny-Jaffe (DJ) reagent.\(^{190}\) rEP was chemically attached to one end of the 121-DJ reagent and then, after 125I-DJ-rEP binding to the FVA cells, the other end with a labile reactive group was activated by UV light to covalently attach rEP to the receptor. Because 125I-DJ-rEP is a monofunctional reagent, rEP was cross-linked to a single protein and bands of 140 and 125 Kd were identified similar to the bands identified previously. After treatment with sodium dithionite to break the azo bond in the 121-DJ-rEP and remove the rEP, molecular masses of 100 and 90 Kd were directly delineated for these cross-linked proteins free of the hormone, corresponding very well to the predicted sizes after subtraction of the molecular mass of rEP. These experiments also show that the 100- and 90-Kd EP-binding proteins do not arise from artificial cross-linking of two 55-Kd proteins, and suggest that the larger bands are either different proteins not related to the cloned gene or that the cloned gene product may be modified to form a new protein, either by covalent coupling of two altered 55-Kd subunits or by the addition of another gene product.

The cDNA sequence for the EP receptor has several interesting features.\(^{190}\) It encodes a 507 amino acid polypeptide that appears to have a single 23 amino acid membrane-spanning a-helical segment between amino acids 250 and 272, with an amino terminus, extracellular domain, and a carboxy terminus cytoplasmic domain typical for a type I transmembrane protein. It has two potential sites of N-linked glycosylation and a large number of cysteines, but shows no evidence for tyrosine kinase activity. Recently, cDNA clones encoding the human EP receptor were isolated using the murine EP receptor gene or synthetic oligonucleotides derived from the known composition of the murine EP receptor.\(^{186,197}\) The derived 508-amino acid sequence was 82% identical to the murine EP receptor and was without any major structural differences. The principle structural features of these receptors are conserved, except for an amino acid insertion between amino acids 71 and 76 and the absence of one N-linked glycosylation site for the human EP receptor.\(^{197}\) Both the murine and human EP receptors have a high frequency of serine and threonine residues, which are potential sites for O-linked glycosylation and phosphorylation.\(^{186}\) Two distinct short 3' untranslated regional homologies between the human and murine receptors were observed that could be involved in EP receptor gene expression.\(^{198}\) The human EP receptor gene was localized to human chromosome 19p by in situ hybridization and the assignment was confirmed by hybridization to a panel of sorted human chromosomes,\(^{186}\) but no inherited disorders of human erythropoiesis have been mapped to 19p.\(^{198}\)

By sequence and structural pattern matching techniques D'Andrea et al\(^{109}\) and Bazan\(^{69,70}\) have shown that the EP receptor has many features in common with several other receptors: the IL-2 receptor β-chain, and the IL-3, -4, -6, and -7 receptors and the GM-CSF, G-CSF, growth hormone, and prolactin receptors. These belong to a novel class of receptors termed the hematopoietin receptor superfamily,\(^{47,48,109}\) which shares a common, ~200 amino acid cytokine binding domain with two distinctive features: an N-terminal set of four conserved cysteines that form extra-cytoplasmic disulfide bonds, and a C-terminal (membrane proximal) Gly-X-Trp-Ser-X-Trp-Ser-X-Pro motif termed the “WS X WS” box. It has been proposed that the generic cytokine binding domain is composed of two discrete, ~100 amino acid modules that share a common β-rich fold and are distantly related to more primitive structural repeats in fibronectin.\(^{37,49}\) An equivalent structural and functional architecture is expected for the cytokine-binding domains of interferon receptors.\(^{47,48}\) A likely cytokine binding site in the diverse receptors of the extended superfamily is indicated by a trough between packed, tandemly linked modules (Fig 4). In this model the characteristically spaced tryptophan residues of the WS X WS box lie on a loop deep in the binding crevice of the receptor and are available for interaction with a complementary aromatic sidechain on the surface of helix D in a docked EP molecule (Fig 4).\(^{47,49}\) The back side of the bound hormone forms a potential binding surface for secondary proteins, accessory receptor subunits that aid in forming a high-affinity binding complex on the cell surface.\(^{47,48}\)

Several of the hematopoietin receptors appear to have well-defined subunits\(^{101,102}\) and are without tyrosine kinase activity,\(^{101,103}\) although tyrosine phosphorylation of the receptor is evident, in some, after binding to the appropriate ligand.\(^{101,104,105}\) By analogy, and considering the previously mentioned binding protein, the EP receptor probably consists of more than one protein and it is possible that tyrosine kinase activity, or another signal transducer, may yet be found on an additional subunit. Thus, even though the EP receptor has been cloned, and extensive cross-linking studies have provided information on the size of the EP receptor binding proteins, the precise organization of the receptor as well as the identity of the second messenger remain unknown.

**Apoptosis.** The addition of EP to immature blood BFU-E in cell culture for 24 hours leads to a twofold increase in the percentage of cells in DNA synthesis.\(^{152}\) No increase in hemoglobin synthesis occurs during this time.\(^{199}\) However, the addition of EP to CFU-E leads to a rapid initiation of mRNA for α- and β-globins and hemoglobin synthesis commences shortly thereafter.\(^{112,131,132}\) The mechanism by which one hormone could initiate two different processes

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Fig 4. Predicted configuration of a generic receptor binding segment on the cell surface. The linked, similarly folded β-barrel modules (arrows labeled A through G and A' through G' are β-strands; see reference 49) are drawn at an angle to emphasize the V-shaped trough with a buried WS X WS loop (one-letter amino acid code; X is a nonconserved residue) close to a hydrophobic hinge region. The receptor is shown docked to a cytokine (gray diamond with receptor recognition corner in black); the transmembrane helix (black square) links the binding domain with a nonspecific cytoplasmic segment. The receptor/cytokine complex may interact with a secondary binding molecule that recognizes the free surface of the bound cytokine. Reprinted with permission (Proc Natl Acad Sci USA 87:6934, 1990).

has not been clear. However, it is conceivable that EP simply maintains the cellular viability of the erythroid progenitor cells, which allows these cells to proceed with mitosis and terminal differentiation. CFU-E have been known to require EP for survival irrespective of any effect on mitosis or differentiation. Recently, Koury and Bondurant have shown that DNA cleavage is characteristic of CFU-E and that EP retards this cleavage. FVA cells were prelabeled with [3H]thymidine and the size of the DNA was measured on agarose gels after subsequent incubation with or without EP. The labeled cells, cultured without EP, showed net breakdown of DNA, with a loss of labeled, high molecular weight DNA and an accumulation of low molecular weight DNA fragments of 5,000 bases or less. A slight breakdown of DNA occurred in cells that were cultured with EP, but the majority of the high molecular weight DNA remained intact. When DNA synthesis was inhibited with 500 µmol/L thymidine the DNA breakdown was faster, but was still greatly retarded by EP, indicating that the effect of EP was on the cleavage rate and not on the rate of DNA repair. An analysis of the [3H]thymidine-labeled DNA breakdown on neutral, nondenaturing agarose gels showed fragments of 100 to 5,000 bp that were unchanged after digestion with S1 nuclease, which specifically cleaves single-stranded DNA, indicating that the small DNA fragments were double stranded. A similar fragmentation was also seen with normal CFU-E generated after phenylhydrazine treatment of mice.

The mechanism by which EP retards the rate of DNA cleavage in erythroid progenitor cells is not clear, but this process is characteristic of cells undergoing programmed cell death (apoptosis). Without EP the process is rapid and the cells die, while with the hormone the process is reduced and the CFU-E survive and develop into RBCs. Although EP could have additional effects on mitosis or terminal differentiation, this model offers a unifying hypothesis that can explain diverse physiologic events that occur after EP administration to early and late erythroid progenitor cells. The model predicts that in normal human beings, the majority of CFU-E are not surviving, but with anemia and increased EP levels many of the CFU-E that would ordinarily die are now rescued and can progress to RBC differentiation. This would provide a very sensitive switch for a quick and large increase in erythropoiesis and an equally rapid switch for turning off this process.

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