Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 7972–7976, November 1987
Cell Biology

The regulated expression of erythropoietin by two human hepatoma cell lines

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Communicated by Irving M. London, August 3, 1987

ABSTRACT **The development of a cell culture system that produces erythropoietin (Epo) in a regulated manner has been the focus of much effort. We have screened multiple renal and hepatic cell lines (including MDCK, LLC-PK$_1$, BHK, WRL 68, CLCL, A704, CRFK, A498, ACHN, TCMK-1, LLC-MK$_2$, CaKi-2, HepG2, and Hep3B) for either constitutive or regulated expression of Epo. Only the human hepatoma cell lines, Hep3B and HepG2, made significant amounts of Epo as measured both by radioimmunoassay and *in vitro* bioassay (as much as 330 milliunits per 10^6 cells in 24 hr). The constitutive production of Epo increased dramatically as a function of cell density in both cell lines. At cell densities $< 3.3 \times 10^5$ cells per cm^2, there was little constitutive release of Epo in the medium (<30 milliunits per 10^6 cells in 24 hr). With Hep3B cells grown at low cell densities, a mean 18-fold increase in Epo expression was seen in response to hypoxia and a 6-fold increase was observed in response to incubation in medium containing 50 μM cobalt(II) chloride. At similar low cell densities, Epo production in HepG2 cells could be enhanced an average of about 3-fold by stimulation with either hypoxia or cobalt(II) chloride. Upon such stimulation, both cell lines demonstrated markedly elevated levels of Epo mRNA. Hence, both Hep3B and HepG2 cell lines provide an excellent *in vitro* system in which to study the physiological regulation of Epo expression.**

Erythropoietin (Epo) is a glycoprotein hormone (1) elaborated in the fetal liver (2) and the adult kidney (3) in response to hypoxia. (For a review of the subject, see ref. 4.) The hormone travels to hematopoietic tissues where it stimulates the proliferation and differentiation of erythroid progenitor cells. Little is known about the site of Epo production in the kidney and liver. In addition, little is understood about the intracellular pathway by which hypoxia leads to the increased expression of the Epo gene.

Over the past two decades many investigators have attempted to develop an *in vitro* cell culture system that produces significant amounts of Epo in a regulated manner. Early attempts to develop such a system included the demonstration of erythroid stimulating activity following hypoxic stress in adult animal kidney tissue (5–8) and mouse fetal liver tissue (9) grown in organ culture. Erythroid stimulating activity was encountered in isolated goat kidney glomeruli (10); however, production of this activity required a minimum of 30 days between changes of culture medium. More recently, Kurtz and co-workers (11) have shown low levels of stimulated Epo production in cultured rat kidney mesangial cells in response to hypoxia or to cobalt(II) chloride, a known inducer of Epo secretion *in vivo* (12), and in unfractionated fetal mouse liver cells (13) in response to hypoxia and adenylate cyclase stimulation. Caro and co-workers (14) reported low levels of Epo production in response to hypoxia and cobalt stimulation in an established kidney proximal tubular cell line. They also showed that the human hepatoblastoma cell line HepG2 made Epo (15); however, they did not show regulated expression under their culture conditions. Others have shown Epo release from cells derived from human renal cell carcinomas (16–20), hepatic carcinomas (19), and a mixed germ-cell tumor (21) grown in tissue culture. Epo production also has been demonstrated in several cloned erythroleukemia cell lines (22, 23), in mouse spleen macrophages grown in primary culture (24), and in isolated rat Kupffer cells (25).

It has been difficult to establish a cell culture system in which biologically active and immunologically identifiable Epo is produced in a regulated fashion by a single cell type. Many of the investigations cited above utilized tissues containing mixed cell types (5–9, 13). In other studies the demonstration of Epo production depended solely upon bioassays and/or inhibition of bioactivity by anti-Epo antibodies of questionable specificity (5–11, 13, 16, 19, 24). Some of the previously reported cell culture systems demonstrated only low levels of Epo production following stimulation by hypoxia or cobalt (II) (11, 13, 14, 25), while still others possessed large amounts of constitutive Epo production (15–23) with little regulated production.

In this report we demonstrate that, when grown at low cell density, two established human hepatoma cell lines can be induced to produce large amounts of biologically active and immunologically identifiable Epo in response to hypoxia or cobalt(II) chloride. Furthermore, upon such stimulation markedly increased levels of Epo mRNA were observed.

MATERIALS AND METHODS

Cell Culture. All cell lines studied were obtained through the American Type Culture Collection. Cells were cultured in 100 × 20 mm tissue culture dishes (Corning no. 25020) and 25-cm^2 tissue culture flasks (Corning no. 25100) using α minimal essential medium (GIBCO) supplemented with glutamine, penicillin (100 units per ml), streptomycin (100 μg/ml), and 10% heat-inactivated (56°C for 30 min) fetal calf serum (Hazelton Research Products, Denver, PA). Cells were maintained in a humidified 5% CO_2/95% air incubator at 37°C. The culture medium was changed daily for 1 or 2 days prior to all experiments. In experiments in which the oxygen tension was varied, the 25-cm^2 flasks were fitted with rubber septa. The gas inlet was provided through a 9 cm 18 g needle, fitted with a 0.2-μm sterile filter, and the gas outlet was provided through a 4 cm 18 g needle connected by Silastic tubing to a closed water trap. Needles, tubing, and septa were autoclaved prior to use, and assembly was performed under sterile conditions. Experimental gases containing 1%, 2%, 3%, 5%, or 21% O_2, 5% CO_2, and the balance N_2 (Yankee Oxygen) were hydrated at 37°C and flowed at a rate of 2.4–4.2 ml/min into the tissue culture flasks. The flasks were immersed in a 37°C constant-temperature water bath. Po_2 and

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Abbreviations: Epo, erythropoietin; PGE_2, prostaglandin E_2.

Pco_2 were monitored by collecting the outflow gas in a glass syringe and injecting the gas into an automated blood gas analyzer (Instrumentation Laboratory Model 1304). At the end of each experiment, the volume of medium was measured, the cells were removed with trypsin, and cell counts were determined with a hemocytometer.

Bioassay. The *in vitro* bioassay was performed by the method of Krystal (26) with several modifications (27). Spleens were taken from C57BL × C3H F_1 hybrid mice (The Jackson Laboratory) on day 3 after the second of two daily injections of phenylhydrazine (≈60 mg/kg of body weight per day), and spleen cell suspensions were prepared. An erythrocyte lysis step was included to deplete the suspension of mature erythrocytes (28). The spleen cells (final concentration, 4×10^6 cells per ml) were then incubated in 96-well U-bottom tissue culture plates (Costar no. 3799) with various standard doses of Epo (Toyobo Epo-301; specific activity, 65 units/mg) or unknown samples for 22 hr and then pulsed with tritiated thymidine (New England Nuclear) for 2 hr. The cells were harvested onto a glass filter by using a Skatron cell harvester (Skatron, Sterling, VA). The filters were dried at 80°C for 1 hr, cut out, and assayed for radioactivity in an LKB model 1219 scintillation counter.

Standard curves were performed with and without cobalt(II) chloride in the culture media, and all unknown samples were assayed in triplicate.

Radioimmunoassay. The RIA for Epo was performed with polyclonal rabbit anti-human Epo antiserum raised against the C-terminal region of human recombinant Epo. The antiserum was a generous gift of Genetics Institute (Cambridge, MA). ^{125}I-labeled recombinant Epo (^{125}I-Epo) was obtained from Amersham. Standards were prepared from partially purified Epo (Toyobo Epo-301) diluted in α minimal essential medium containing 10% (vol/vol) fetal calf serum. Aliquots (0.5 ml) of standard or sample were added to 1.5-ml Eppendorf tubes. To this was added 0.1 ml of antibody diluted 1:100 in phosphate-buffered saline (2.62 g of $Na_2HPO_4 \cdot 7H_2O$, 0.243 g of $NaH_2PO_4 \cdot H_2O$, and 8.0 g of NaCl per liter) containing 0.5% bovine serum albumin and 0.05 ml (≈5,000 cpm) of ^{125}I-Epo diluted with the same buffer. The assay mixture was incubated overnight at 4°C with constant shaking. The next day 0.1 ml of a *Staphylococcus aureus* protein A preparation (Pansorbin Cell, Calbiochem) that had been washed twice with phosphate-buffered saline was added to each tube, and the tubes were returned to 4°C with constant shaking for 1 hr. The Pansorbin was pelleted by centrifugation in a Microfuge for 4 min, washed once with phosphate-buffered saline, recentrifuged for 10 min, and assayed in an LKB model 1282 γ counter.

The RIA for prostaglandin E_2 (PGE_2) was performed by using a commercially available PGE_2 RIA kit from New England Nuclear.

RNA Blot-Hybridization Analysis. Total RNA was prepared from cultured cells by two different methods (29, 30). The RNA was denatured in formaldehyde, electrophoresed on a 1% agarose (Ultra Pure, Bethesda Research Laboratories) gel containing 2.2 M formaldehyde and a trace amount of ethidium bromide and was transferred to a GeneScreen*Plus* filter (New England Nuclear) with 1.5 M NaCl/0.15 M sodium citrate (31). Epo cDNA in a SP65 plasmid vector (clone E49F) was kindly provided by Genetics Institute. E49F was digested with the restriction enzyme *Eco*RI, and the Epo insert was identified by agarose gel electrophoresis, purified by electroelution (31), and ^{32}P-labeled to a specific activity of between 5×10^8 and 1.2×10^9 cpm/μg of cDNA (32). The radiolabeled cDNA was then mixed with carrier salmon sperm DNA, denatured by boiling for 10 min, and hybridized to the filter at 5×10^5 cpm/ml of hybridization solution (50% formamide/1 M NaCl/1% $NaDodSO_4$/10% dextran). Hybridization was performed at 42°C for 20 hr. The final washing was done in 0.075 M NaCl/0.0075 M sodium citrate at 65°C. Autoradiography was performed with intensifying screens at −80°C with Kodak X-Omat AR film.

Mouse β-actin cDNA was a gift from Bruce Spiegelman of the Dana–Farber Cancer Institute. It, too, was radiolabeled and hybridized to the same filters to provide an internal control for the efficiency of RNA transfer to the filters.

RESULTS

Epo Assays. Both the *in vitro* bioassay and the RIA gave standard curves that were reproducibly sensitive down to ≈5 milliunits per ml of medium. There was excellent agreement between replicates as well as between the bioassay and RIA as detailed in the data presented below.

Cell Culture. Multiple renal and hepatic cell lines (including MDCK, LLC-PK_1, BHK, WRL 68, CLCL, A704, CRFK, A498, ACHN, TCMK-1, LLC-MK_2, CaKi-2, HepG2, and Hep3B) were screened for either constitutive or hypoxia-induced production of Epo. All cell lines grew well under hypoxic conditions. The cell doubling times were similar in all of the cell lines at 1% and 21% oxygen. Only the human hepatoma cell lines, HepG2 and Hep3B, made readily measurable amounts of Epo as measured both by RIA and bioassay. The constitutive production of Epo increased dramatically as a function of cell density in both cell lines (Fig. 1). HepG2 cells grown to high cell densities and then exposed to the additional stress of hypoxia produced less Epo than did nonhypoxic cells at similar cell densities (data not shown). However, at cell densities of less than 3.3×10^5 cells

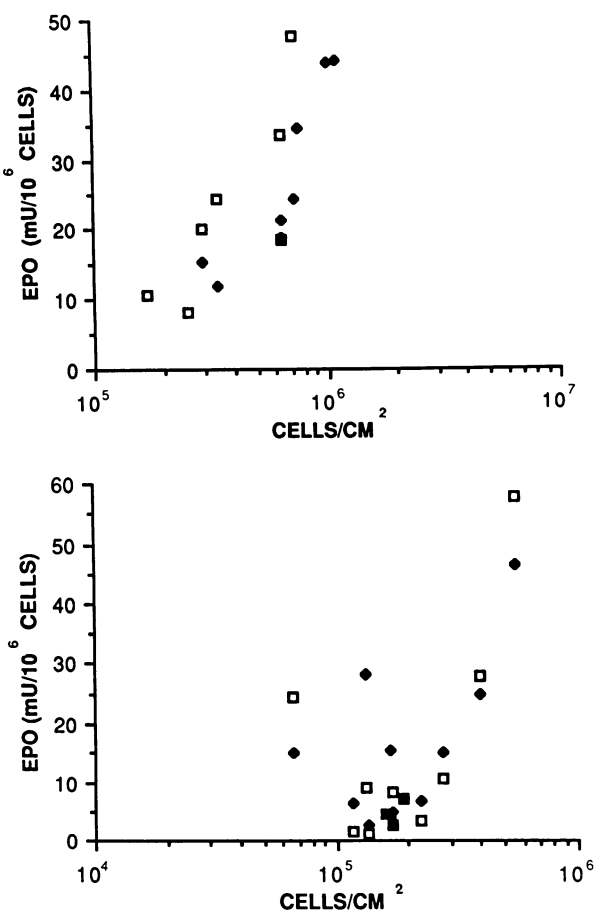

FIG. 1. Epo production over 24 hr by HepG2 cells (*Upper*) and Hep3B cells (*Lower*) as a function of cell density as measured both by bioassay (◆) and RIA (□). Cell counts were done at the end of the 24-hr period. Cells were grown in 25-cm^2 tissue culture flasks with 5 ml of medium.

Table 1. Effect of hypoxia on Epo production by HepG2 cells

Epo assay	Epo, milliunits per 10^6 cells (mean ± SD)		
	21% O_2	5% O_2	2% O_2
Bioassay	8.6 ± 3.0 (n = 3)	24.7 ± 2.5 (n = 3)	30.5 ± 6.2 (n = 4)
RIA	10.5 ± 2.2 (n = 2)	22.4 ± 14.8 (n = 3)	29.8 ± 9.9 (n = 4)

Cells were grown for a period of 20 hr at low cell densities (1.1–3.2 × 10^5 cells per cm^2) in an atmosphere containing 5% CO_2, the percentage of O_2 as indicated in the table, and the balance N_2. n, Number of assays performed in duplicate (RIA) or triplicate (bioassay).

per cm^2, there was little constitutive release of Epo into the culture media by both cell lines. At these lower cell densities, Epo production in HepG2 cells could be enhanced ≈3-fold by stimulation with either hypoxia, Table 1, or with 50 μM cobalt(II) chloride (Table 2). Increased Epo release into the medium was observed by hour 8 after stimulation with cobalt(II) and continued to increase for the duration of the experiment. Cell doubling times were not altered by cobalt(II) chloride at the concentration used in these experiments.

Hep3B cells exhibited an even greater enhancement in Epo production in response to hypoxia (Fig. 2) or cobalt(II) (Table 2). Although there was wide variation in absolute numbers between individual experiments, the mean erythropoietin production in a 24-hr period by Hep3B cells grown in an atmosphere of 1% oxygen/5% CO_2/94% N_2 was 18-fold greater than comparable cells grown in 21% O_2/5% CO_2/74% N_2 (Epo RIA; 115.9 ± 62.4 milliunits per 10^6 cells at 1% O_2 versus 3.9 ± 2.6 milliunits per 10^6 cells at 21% O_2; Epo bioassay: 134.9 ± 61.0 milliunits per 10^6 cells at 1% O_2 versus 9.5 ± 2.6 milliunits per 10^6 cells at 21% O_2. Eight experiments were performed at 1% O_2 and seven experiments at 21% O_2). Furthermore, when cells were grown in the presence of 50 μM cobalt(II) chloride for 24 hr, an average 6-fold increment in Epo production was observed (Table 2).

RNA Blot-Hybridization Analysis. Blot-hybridization analyses of mRNA from Hep3B cells and HepG2 cells are shown in Fig. 3. Hybridization with the ^{32}P-labeled Epo DNA probe was seen just below the 18S ribosomal RNA band, compatible with an mRNA size of slightly less than 1.8 kilobases. Epo mRNA levels were greatly increased in both Hep3B and HepG2 cells grown in an atmosphere containing 1% or 2% oxygen and 5% CO_2 for 24 hr compared to levels in comparable cells grown in an atmosphere containing 21% oxygen. These elevated levels of Epo mRNA correlated closely with increased release of Epo into the culture medium. Similarly, Hep3B cells showed a marked increase in the levels of Epo mRNA when the cells were grown in the presence of 50 μM cobalt chloride, while HepG2 cells did not. This corresponded to the relatively greater effect that cobalt(II) chloride had on Epo protein production in Hep3B cells as compared to HepG2 cells (Table 2). In addition, a second hybridizing band was noted on occasion just below the 28S ribosomal RNA band, particularly in the HepG2 cells (Fig. 3). At this time we do not know what this band represents. Hybridization of the filters with the radiolabeled mouse β-actin confirmed that similar amounts of total RNA were transferred to the filters in each lane of the paired experiments (Fig. 3).

PGE_2 Analysis. HepG2 cells did not release significant amounts of PGE_2 into the culture medium (<10 pg/ml of media) when grown in hypoxic or nonhypoxic conditions. Even at high cell densities, HepG2 cells that secreted >180 milliunits of Epo per ml of medium at 24 hr did not show any measurable increase in PGE_2 in the presence or absence of 50 μM cobalt(II) chloride. Furthermore, medium containing 100 μM indomethacin did not inhibit Epo secretion by Hep3B cells grown under hypoxic conditions. Finally, incubation of Hep3B cells for 24 hr in medium containing 0.1, 1, or 10 μM PGE_2 did not result in any increase in erythropoietin secretion.

DISCUSSION

The lack of a cell culture system that produces readily measurable amounts of Epo in a regulated manner has hindered efforts to better understand the intracellular and molecular events by which hypoxia induces increased production of Epo. Much of the difficulty in establishing such a system lies in the fact that the identity of the Epo-producing cells in the adult kidney and fetal liver is unclear. In the kidney there is evidence both in support of glomerular (10, 11, 33) and nonglomerular, or tubular (14, 34–36), sites of Epo production. In the fetus the liver is the primary source of Epo (2, 37), but the relative contribution of the Kupffer cell and parenchymal cell is unknown. Some evidence suggests that the Kupffer cell is a site of Epo production or storage or both (38–42). However, these studies did not directly quantitate the amount of Epo produced by Kupffer cells nor did they demonstrate regulated production of Epo by these cells. Epo production has been demonstrated by isolated Kupffer cells grown in primary culture (25). Production in these cells appeared to increase 2-fold upon stimulation by latex particle-induced phagocytosis. However, the level of Epo production in this system was much less than in the parenchymal liver cells described in our paper.

Our results suggest a physiologically important role for the fetal hepatocyte in the regulated production of Epo. Both the HepG2 and Hep3B cell lines, which were generated from human hepatic carcinomas (43), have been shown histologically and biochemically to possess characteristics of well-differentiated liver parenchymal cells (43, 44). They both have many of the biosynthetic capabilities of normal hepatocytes and have been shown to secrete 17 of the major

Table 2. Effect of $CoCl_2$ on Epo production by HepG2 and Hep3B cells

Cell line	Epo assay	Epo, milliunits per 10^6 cells (mean ± SD)	
		Without $CoCl_2$	With $CoCl_2$
HepG2	Bioassay	22.6 ± 3.8 (n = 5)	50.9 ± 4.4 (n = 5)
HepG2	RIA	24.5 ± 12.0 (n = 5)	72.1 ± 11.3 (n = 5)
Hep3B	Bioassay	11.9 ± 3.3 (n = 3)	68.4 ± 9.8 (n = 3)
Hep3B	RIA	6.7 ± 3.6 (n = 3)	82.7 ± 18.9 (n = 3)

Cells were grown at low cell densities (8.1 × 10^4 to 2.4 × 10^5 cells per cm^2) in the presence or absence of 50 μM $CoCl_2$ for 24 hr. n, Number of experiments performed.

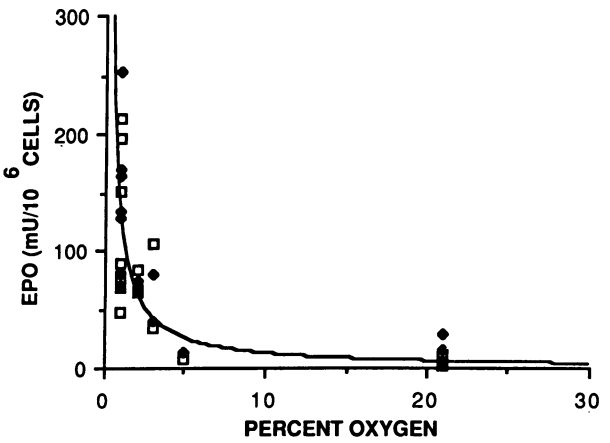

FIG. 2. Epo production by Hep3B cells over a 24-hr period in cells grown at various oxygen partial pressures. All experiments had cell densities between 8.8×10^4 cells per cm^2 and 2.2×10^5 cells per cm^2 at the end of the 24-hr incubation. Results of both bioassay (◆) and RIA (□) are shown.

plasma proteins into cell culture medium, including albumin and α-fetoprotein. Despite the similarities between these two cell lines and hepatocytes in intact liver, one must be careful in making analogies between gene expression in a tumor cell and that in a normal hepatocyte.

Further evidence suggesting that this *in vitro* system accurately reflects the normal physiological situation *in vivo* is provided by the blot-hybridization analysis. The Epo mRNA size appears similar to that previously reported for RNA from normal human fetal liver (45) and from the kidneys of intact rodents exposed to various hypoxic stresses (46, 47). In addition, the finding that regulation occurs at the mRNA level is in agreement with previous *in vivo* data (46, 47).

It is not surprising that these cell lines do not constitutively produce large quantities of Epo when grown at low cell densities because unlike previous studies (18, 20) in which Epo-producing cell lines were derived from renal cell carcinomas of patients with erythrocytosis, the patients from

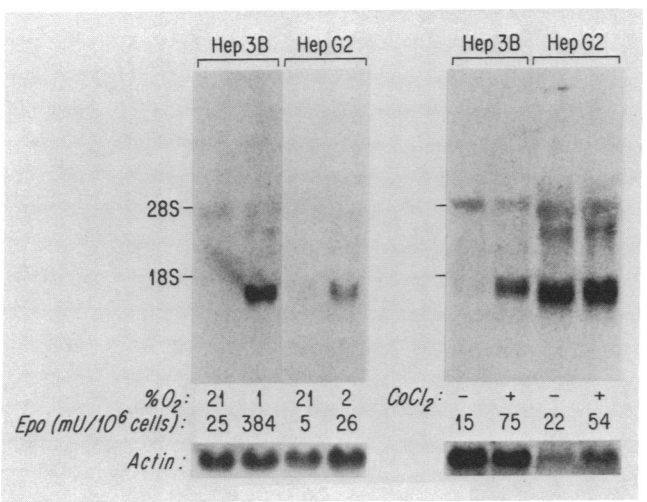

FIG. 3. Effect of hypoxia or $CoCl_2$ on Epo mRNA levels. RNA blot analyses were performed after growth of Hep3B and HepG2 cells for 24 hr in the presence or absence of hypoxia or 50 μM $CoCl_2$. RNA was hybridized with ^{32}P-labeled Epo cDNA; 30 μg of total RNA was loaded in all lanes except for the last two lanes (HepG2 with or without $CoCl_2$) in which 22 μg was loaded in each lane. The film was exposed for 6 to 7 days. The corresponding Epo protein production by these cells is depicted. Hybridization to radiolabeled mouse β-actin is shown at the bottom.

which the HepG2 and Hep3B cell lines were generated were not reported to have erythrocytosis. Nonetheless, Hep3B cells produce ≈10-fold more Epo after exposure to hypoxia than do the rat mesangial cell preparations of Kurtz *et al.* (11). This property makes them an excellent model system.

The finding that HepG2 cells produce large amounts of Epo at high cell densities is similar to results obtained by others (15). We also observed this with the Hep3B cell line as did Hagiwara *et al.* (18) with a renal cell carcinoma cell line. The reason for this relationship is unclear.

We have demonstrated that Epo production in this system does not appear to be dependent upon cyclooxygenase products, such as PGE_2, in contrast to the mechanism previously suggested in other systems (48–51). PGE_2 is not produced by either HepG2 or Hep3B cells, and Epo production is not significantly impaired when hypoxic Hep3B cells are grown in the presence of indomethacin, a potent cyclooxygenase inhibitor. Furthermore, Hep3B cells grown in medium containing various concentrations of PGE_2 did not express increased amounts of Epo.

In summary, we have demonstrated that the HepG2 and Hep3B cell lines produce biologically active and immunologically identifiable Epo in a regulated manner. In addition, we have shown a direct correlation between Epo production and the level of Epo mRNA. Hence, both the HepG2 and Hep3B cell lines provide an excellent *in vitro* system in which to study the physiological regulation of Epo production.

We thank Drs. Charles Shoemaker and Ed Fritsch of Genetics Institute (Cambridge, MA) for their advice and support during all phases of this work. In addition, we thank Dr. David Scadden for his advice and assistance in the development of our RNA blot analyses.

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