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Physiology

Inducible operation of the erythropoietin 3′ enhancer in multiple cell lines: Evidence for a widespread oxygen-sensing mechanism

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ABSTRACT **Adaptive responses to hypoxia occur in many biological systems. A well-characterized example is the hypoxic induction of the synthesis of erythropoietin, a hormone which regulates erythropoiesis and hence blood oxygen content. The restricted expression of the erythropoietin gene in subsets of cells within kidney and liver has suggested that this specific oxygen-sensing mechanism is restricted to specialized cells in those organs. Using transient transfection of reporter genes coupled to a transcriptional enhancer lying 3′ to the erythropoietin gene, we show that an oxygen-sensing system similar, or identical, to that controlling erythropoietin expression is widespread in mammalian cells. The extensive distribution of this sensing mechanism contrasts with the restricted expression of erythropoietin, suggesting that it mediates other adaptive responses to hypoxia.**

Erythropoietin (Epo) synthesis is induced by reduction of blood oxygen availability (reviewed in ref. 1). The Epo gene is expressed in subsets of cells in kidney and liver (2–4), suggesting that the specific oxygen-sensing mechanism underlying this response might be confined to these specialized cells.

After extensive screening of tissue culture cell lines, the hepatoma lines Hep3B and HepG2 were found to produce Epo in response to reduced oxygenation (5, 6). This showed that both oxygen sensing and Epo production could occur in the same cell, and provided a model system for studying regulation of the Epo gene. Transfection studies in these hepatoma cells have defined cis-acting control sequences (7–10), the most powerful of which is an oxygen-regulated enhancer located 3′ to the gene (8–10).

To examine other cells for oxygen-sensing properties, we have coupled the Epo 3′ enhancer to broadly active promoters and tested for hypoxic induction following transient transfection. The results indicate that oxygen sensing and transduction mechanisms capable of acting on the Epo 3′ enhancer are much more widespread than has been previously recognized. Physiological characterization of the hypoxic response observed shows features strikingly similar to those previously established for the native Epo gene.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The cell lines used were Hep3B and HepG2 (human hepatoma), MRC5 (human fetal lung fibroblast), 1BR3 (human skin fibroblast), U937 (human monocyte/macrophage), COS-7 (monkey renal fibroblast), LLC-PK1 (pig renal epithelium), RAEC (rat aortic endothelium), a23 (Chinese hamster lung fibroblast), K1 (Chinese hamster ovary), RAG (mouse renal adenocarcinoma), and MEL (mouse erythroleukemia, clones 585 and 707). Most cells were obtained from the European Collection of Animal Cell Cultures and cultured as specified by the supplier. Cells from other sources were a23 (11), RAEC (29), and MEL (30). The only nonadherent cells were MEL and U937.

Plasmids and Transient Transfection. Most experiments used two test plasmids with the α_1-globin gene as reporter, which were identical except that one contained the Epo 3′ enhancer and the other did not (Fig. 1*A*). The cotransfected control plasmid (FGH) contained a fusion gene consisting of the mouse ferritin promoter fused with the human growth hormone gene. Cells were transfected by electroporation using a 1-mF capacitor array. The optimum voltage for each cell line (325–400 V) was established in preliminary experiments. Approximately 10^7 cells were transfected in a mixture of test plasmid (56 μg/ml) and control plasmid (20 μg/ml; 30 μg/ml for 1BR3). Cell density was measured with a hemacytometer.

For comparison of hypoxic stimulation of the Epo 3′ enhancer with the SV40 enhancer, and to examine action on the SV40 promoter, three plasmids were used which contained the human growth hormone gene as a reporter (Fig. 1*B*). To investigate operation of the Epo 3′ enhancer on the Epo promoter itself, three plasmids were used, based on a 6.1-kb *Xba* I–*Nco* I restriction fragment containing the mouse Epo gene (Fig. 1*C*). In these experiments, pBSα was used as a transfection control.

Experimental Incubation Conditions. After electroporation, aliquots from a single pool of transfected cells were incubated in parallel for 16 hr in 100-mm Petri dishes in 8 ml of culture medium. Normoxic incubation was in humidified air with 5% CO_2. Hypoxic incubation, commencing 1 hr after electroporation, was in 1% O_2 (with 5% CO_2 and 94% N_2 in a Napco 7100 incubator) unless otherwise stated. In pharmacological studies, the following were added to the medium immediately after electroporation: cobaltous chloride (25–200 μM), cycloheximide (8–200 μM), and potassium cyanide (10–1000 μM). Heat shock was induced by incubating cells at 42°C in a normoxic atmosphere in medium supplemented with [^{35}S]methionine (Amersham) to permit detection of newly synthesized proteins. The 72-kDa heat shock protein (Hsp72) was detected by Western blotting using a mouse monoclonal antibody (RPN1197, Amersham) and immunoperoxidase.

RNA Analysis. RNA from transfected cells was extracted, analyzed by RNase protection, and quantitated essentially as described (10). In most assays, 3–15 μg of RNA was subjected to double hybridization with riboprobes from the α_1-globin cap region and either the cap region or the exon 3 boundary of FGH. In Epo mRNA assays, 8–45 μg of RNA was used and the expression of the cotransfected α_1-globin plasmid was analyzed separately on a 3-μg aliquot of RNA. For assessment of endogenous Epo expression, 100 μg of RNA from untransfected cells incubated under hypoxic conditions was assayed with complementary riboprobes for man, rat, and mouse. For the Chinese hamster a riboprobe was constructed using PCR and primers to exons 4 and 5 of the mouse gene.

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Abbreviations: Epo, erythropoietin; Hsp72, 72-kDa heat shock protein; SV40, simian virus 40.

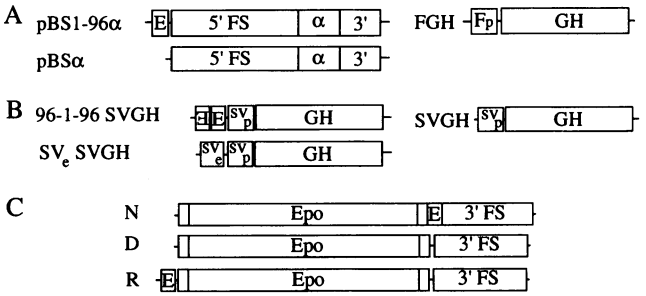

FIG. 1. (*A*) Plasmids used to test for hypoxic induction via the Epo 3′ enhancer. The two test plasmids, pBSα and pBS1-96/α, were identical except that pBS1-96/α included the Epo 3′ enhancer (E). The enhancer sequence consisted of 96 base pairs (bp) of DNA starting at an *Apa* I site ≈120 bp 3′ to the poly(A)-addition site of the mouse Epo gene and was cloned into the polylinker 1.5 kb 5′ to the α_1-globin promoter. The α_1-globin gene was contained in a 2571-bp *Bgl* II–*Ppu*MI restriction fragment which included 1.5 kilobases (kb) of 5′ flanking sequence (FS) and 0.5 kb of 3′ sequence. The cotransfected control plasmid (FGH) contained 290 bp of ferritin promoter (F_p) fused to the human growth hormone gene (GH). (*B*) Plasmids used to compare the effect of hypoxia on the simian virus 40 (SV40) enhancer with that on the Epo 3′ enhancer. In all three plasmids, 135 bp of the SV40 promoter (SV_p) was linked to the human growth hormone reporter gene. Plasmid 96-1-96 SVGH contained two copies of the Epo 3′ enhancer in opposite orientations (∃E), and SV_eSVGH contained 240 bp of the SV40 enhancer (SV_e). (*C*) Plasmid N contained the mouse Epo gene as a 6.1-kb *Xba* I–*Nco* I restriction fragment. In plasmid D, an *Apa* I–*Pvu* II fragment was removed, which included the 3′ enhancer (E). In plasmid R, a 96-bp segment containing the enhancer was inserted 5′ to the gene at the *Xba* I site.

RESULTS

Oxygen-Dependent Operation of the Epo 3′ Enhancer in Multiple Cell Lines. Twelve cell lines were transfected with plasmids containing the Epo 3′ enhancer coupled to the α_1-globin gene (Fig. 1*A*). To distinguish enhancer action from any other effect of hypoxia on transcript accumulation, transfections using plasmids with or without the Epo 3′ enhancer were performed on aliquots of cells from the same pool. Fig. 2 shows examples of individual transfection experiments for the human cell lines Hep3B, MRC5, 1BR3, and U937. Experiments on all cell lines are summarized in Tables 1 and 2. Hypoxic induction via the Epo 3′ enhancer was demonstrated in 11 of the 12 cell lines. The 3- to 10-fold induction in 1% O_2 was similar to that observed in the Epo-producing hepatoma cell lines Hep3B and HepG2. More

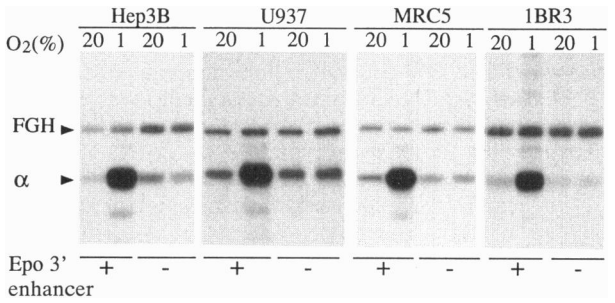

FIG. 2. RNase protection assays of reporter (α) and control (FGH) transcripts from transiently transfected human cell lines Hep3B (hepatoma), MRC5 (lung fibroblast), 1BR3 (skin fibroblast), and U937 (monocyte/macrophage). Alternate lanes are from parallel normoxic and hypoxic incubations. In each panel the first pair of lanes shows the result when the test plasmid contained the Epo 3′ enhancer coupled to the reporter gene. The enhancer was absent for the second pair of lanes. Induction of enhancer activity by hypoxia is observed in all these cell lines.

Table 1. Effect of hypoxia on reporter gene expression in various cell lines

Cells	Species and type	% O_2	Hypoxic induction
Hep3B	Human hepatoma	1	9.4 (0.9)
		1	6.9 (1.0)
HepG2	Human hepatoma	1	8.2 (1.0)
		0.1	8.8 (1.5)
MRC5	Human fetal lung	1	5.2 (0.7)
		1	4.5 (0.7)
		1	8.7 (1.3)
		1	9.5 (1.2)
1BR3	Human skin fibroblast	1	5.8 (0.9)
		1	7.9 (1.0)
		1	5.8 (1.0)
		1	3.2 (0.9)
COS-7	Monkey renal fibroblast	1	5.4 (1.8)
		1	6.5 (1.6)
		1	4.1 (1.6)
		0.5	10.8 (1.7)
LLC-PK1	Pig renal epithelial	1	3.7 (0.6)
		1	8.3 (1.2)
		1	6.4 (1.4)
		0.1	6.7 (1.0)
RAEC	Rat aortic endothelial	1	4.6 (1.7)
		1	7.6 (1.1)
		1	5.1 (1.8)
		0.5	5.0 (1.8)
a23	Chinese hamster lung fibroblast	1	20.9 (2.0)
		1	15.5 (1.4)
		1	7.5 (1.8)
		0.5	12.6 (1.2)
K1	Chinese hamster ovary	1	5.2 (1.6)
		1	5.6 (1.3)
		1	6.5 (1.7)
		1	6.8 (2.4)
RAG	Mouse renal adenocarcinoma	1	2.8 (0.8)
		1	4.5 (0.9)
		1	3.5 (1.0)
		1	3.8 (0.8)
MEL	Mouse erythroleukemia (clone 707)	1	1.1 (1.1)
		1	1.1 (1.0)
		0.1	0.9 (0.6)
		0.1	1.3 (1.1)

Each line summarizes the results of one experiment. Cells were transfected with the plasmids in Fig. 1*A* and incubated in parallel in normoxia and hypoxia. RNA was assayed by RNase protection. Reporter gene (α_1-globin) expression was corrected for expression of the cotransfected control gene (FGH). Hypoxic induction is the ratio of hypoxic to normoxic expression for the plasmid containing the Epo 3′ enhancer (pBS1-96α). Numbers in parentheses give the ratio obtained for the parallel transfections with the plasmid which did not contain the Epo 3′ enhancer (pBSα). Enhancer activity which was inducible by hypoxia was demonstrated in all cell lines except MEL.

severe hypoxia did not, in most cells, increase induction of enhancer activity.

Initial experiments on U937 and MEL did not demonstrate induction by 1% O_2. However, when more severe hypoxia and higher cell densities were tested, U937 cells exhibited a similar level of hypoxia-inducible enhancer activity to the adherent cell lines. Table 2 summarizes the results of four experiments in which serial dilutions of U937 cells were made following transfection. In each experiment, lower cell density reduced the magnitude of the response. In the two experi-

Table 2. Hypoxic induction in U937 varies with cell density

Index density, no. × 10^{-6}/ml	% O_2	Hypoxic induction —	1:3	1:9	1:18
5.7	1	4.7 (0.8)	3.0 (0.9)	1.5 (1.0)	1.4 (0.9)
2.2	1	1.3 (0.8)	1.3 (0.6)	1.2 (0.8)	1.0 (0.7)
2.9	0.1	9.8 (0.7)	5.4 (0.6)	4.4 (0.7)	4.5 (0.7)
2.3	0.1	4.7 (0.6)	4.3 (0.7)	3.0 (0.8)	3.0 (0.8)

In four experiments, U937 cells were cultured at various cell densities in normoxia and two levels of hypoxia. In each experiment the dilutions (1:3, 1:9, and 1:18) were made from a single pool of transfected cells. Hypoxic induction was calculated as in Table 1. Oxygen-regulated enhancer activity was greater at higher cell density and with more severe hypoxia.

ments at 0.1% O_2, inducibility was greater than that seen at 1% O_2. In contrast, MEL cells (both 585 and 707) did not show hypoxia-inducible enhancer activity when studied over a similar range of cell densities and at O_2 concentrations ranging from 0.1% to 3%. They were, thus, the only cell type tested which did not show this property.

Operation of this element was not confined to interaction with the α_1-globin promoter. Constructs containing two copies of the Epo 3′ enhancer placed immediately adjacent to a 135-bp SV40 promoter showed 20-fold and 3-fold induction by hypoxia in a23 cells and COS-7 cells, respectively, but did not show inducibility in MEL cells. In contrast, neither this SV40 promoter nor the SV40 enhancer showed hypoxic inducibility when tested in a23 or COS-7 cells. When Epo sequences other than the 3′ enhancer were coupled to the α_1-globin gene and tested in these cells, no hypoxic induction was seen.

Expression of the Transfected Mouse Epo Gene. To examine the interaction of the Epo 3′ enhancer with the Epo promoter, the different cell types were transfected with plasmids containing the mouse Epo gene with and without the 3′ enhancer (Fig. 1*C*). Transcripts were assayed using a riboprobe which crossed the Epo cap site. As reported previously (10), enhancer action was observed in the hepatoma cell lines Hep3B and HepG2. In both, some incorrectly initiated transcripts were seen, but these were more evident in HepG2. In contrast, in all the other cell types the Epo promoter functioned very weakly and the majority or all of the transcripts were aberrantly initiated (Fig. 3). Enhancer action was consequently difficult to assess but was nevertheless discernible on aberrantly initiated transcripts.

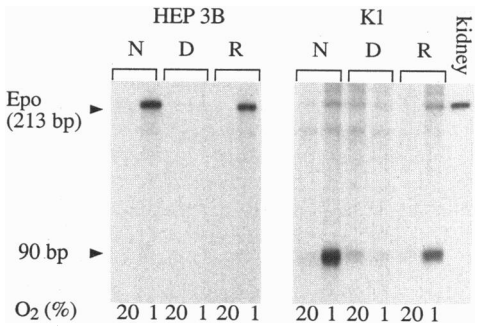

FIG. 3. RNase protection assay showing expression of transfected mouse Epo genes in Hep3B and Chinese hamster ovary K1 cells. Alternate lanes are from parallel normoxic and hypoxic incubations. In lanes N the entire Epo gene was used, in lanes D the 3′ enhancer has been excised, and in lanes R a 96-bp segment containing the enhancer was reinserted at the 5′ end of the Epo gene. In Hep3B, transcripts were predominantly correctly initiated, producing a fragment of the same length (213 bp) as is seen in stimulated mouse kidney. Inducible enhancer action is evident. In K1 cells, almost all transcription was aberrantly initiated. The band at 90 bp probably arises from splicing of upstream aberrantly initiated transcripts, as this is a consensus splice acceptor (12). Inducible enhancer action is evident on aberrantly initiated transcription.

Expression of the Native Epo Gene. Endogenous Epo gene expression was examined by RNase protection. Total RNA from the hypoxically stimulated cell lines of human, rat, Chinese hamster, and mouse origin was analyzed with fully complementary riboprobes. Expression of the native Epo gene was observed in the hepatoma cells but not in any other cell type. RNA from COS-7 cells (monkey) and LLC-PK1 cells (pig) was hybridized with a human riboprobe. No crossreacting fragments were protected by RNA from COS-7, but a very low level of hypoxically induced fragments was protected by RNA from LLC-PK1.

Physiological Characteristics of Epo Enhancer Action in a23 Cells. To characterize the oxygen-sensing system interacting with the transfected Epo enhancer in cells that did not produce Epo, additional experiments were performed on the Chinese hamster lung fibroblastoid line a23.

Cobalt induces native Epo gene expression both *in vivo* (13) and in hepatoma cells (5). In a23 cells, the transfected Epo enhancer was activated ≈10-fold by cobaltous ions in the range 25–200 μM (Fig. 4*A*). In contrast, but in keeping with the physiological regulation of the Epo gene (14, 15), hypoxic stimulation in a23 cells was not mimicked by exposure to cyanide. Thus, cyanide (10–1000 μM) did not induce enhancer activity, nor did it abrogate the response to concurrent hypoxic stimulation (Fig. 4*B*), although total RNA yield was reduced by a factor of 6 at the highest dose. The protein synthesis inhibitor cycloheximide blocked induction of enhancer activity by hypoxia in a23 cells but did not affect unenhanced transcription of the α_1-globin reporter gene (Fig. 4*C*). This is consistent with the observation that cycloheximide blocks induction of the endogenous Epo gene in hepatoma cells (6).

Finally, the response to heat shock was assessed. Exposure of a23 cells to 42°C for 16 hr did not induce enhancer activity (Fig. 5*A*). To be sure that a heat shock response was induced by these conditions, newly synthesized protein was labeled with [^{35}S]methionine and analyzed by PAGE. The cells exposed to heat shock showed two discrete bands at approximately 70 and 110 kDa. Induction of Hsp72 was proved by Western blotting (Fig. 5*B*). Because anoxic stress has been shown to induce heat shock (16), we determined whether the hypoxic conditions used to induce Epo enhancer activity produced a heat shock response. After exposure of a23 cells to 1% O_2 for 16 hr, no changes were detected in labeled proteins by electrophoresis, nor was Hsp72 detectable on the Western blot (Fig. 5*B*).

DISCUSSION

Regulated induction of Epo gene expression by hypoxic stimuli is a central component of the feedback system controlling blood red-cell content (1). Hypoxia is a fundamental physiological stimulus to which many other adaptive responses are made (17–19). We therefore considered the possibility that, despite tightly restricted expression of the Epo gene itself, the oxygen-sensing mechanism might be widespread, and that tissue-specific restriction of Epo gene expression was achieved by other mechanisms. To assess this, we transiently transfected cell lines with recombinant plasmids containing the Epo 3′ enhancer coupled to an α_1-globin reporter gene. This reporter was chosen because it contains a promoter which, under conditions of transient transfection, operates powerfully in multiple cell types and gives rise to a stable mRNA product. That nucleoprotein interactions with transiently transfected DNA allow promiscuous expression of tissue-specific genes, such as α-globin, is well known. However, our demonstration that transfected Epo enhancer activity was inducible by hypoxia strongly

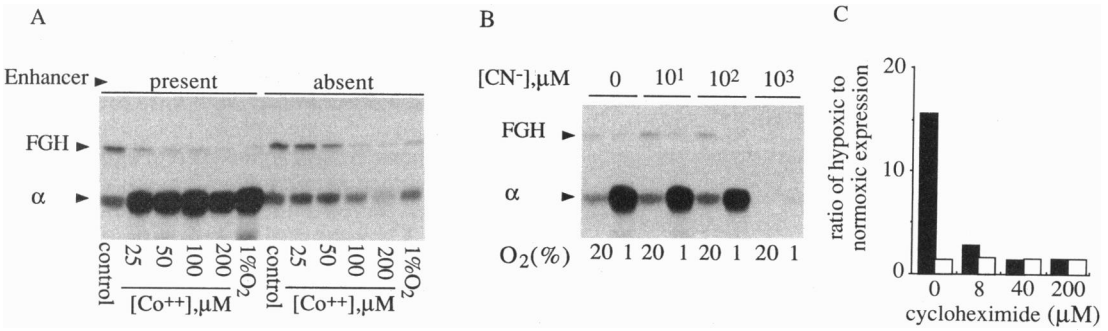

FIG. 4. The physiological features of Epo regulation are observed in transfected a23 lung fibroblastoid cells. (*A*) RNase protection assay showing increased expression of the reporter plasmid containing the Epo enhancer after exposure to cobaltous ions in the range 25–200 μM. The effect is similar to that induced by hypoxia. No response was observed when the reporter plasmid did not contain the Epo enhancer. (*B*) RNase protection assay showing that exposure to cyanide does not mimic hypoxia. Alternate lanes are from normoxic and hypoxic incubations in the presence of increasing concentrations of cyanide. In the range 10–1000 μM, cyanide did not increase expression of the enhancer-linked gene. Except at the highest concentration, which caused near total cell death, cyanide did not substantially interfere with the response to hypoxia. (*C*) Histogram showing that cycloheximide blocks the hypoxic response. Transfected cells were incubated in normoxic and hypoxic conditions in the presence (8–200 μM) of cycloheximide. Solid bars show the ratio of hypoxic to normoxic expression (as calculated in Table 1) in the presence of the enhancer. Open bars show the ratio in the absence of the enhancer. At the lowest dose (8 μM), cycloheximide substantially reduced hypoxic induction. Normoxic expression was not reduced even at the highest concentration used (200 μM).

suggests that a physiologically relevant component of the oxygen-sensing mechanism responsible for regulation of Epo operates widely.

Our further studies on the lung fibroblastoid cell line a23 indicate that in three important respects the response is identical to that governing physiological Epo expression in liver and kidney. Thus, it is activated by cobalt, but not by cyanide, and is abrogated by cycloheximide (5, 6, 13–15). The dose range of cyanide was chosen to include concentrations which cause minimal, partial, and total inhibition of cellular respiration (15, 20). Oxygen consumption was not measured directly in the current experiments, but the expected toxicity of 1000 μM cyanide was apparent from a reduced yield of viable cells and RNA. The lack of effect of cyanide demonstrates that the hypoxic induction of Epo 3′ enhancer action is distinct from the other cellular stresses attendant on the compromise of oxidative metabolism. Heat shock induction operates widely and anoxia has recently been reported to activate heat shock transcription factor (16). We therefore sought to determine whether the hypoxic sensing mechanism might be a manifestation of a heat shock response.

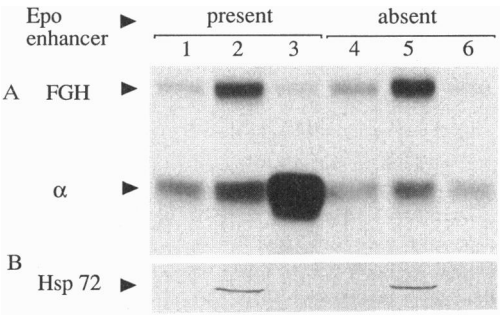

FIG. 5. Comparison of the effects of heat shock and hypoxia in a23 cells. (*A*) RNase protection assay showing that heat shock does not induce a response similar to that induced by hypoxia. Transfected cells were incubated in parallel under normoxic conditions at 37°C (lanes 1 and 4) and 42°C (lanes 2 and 5) and with 1% O_2 at 37°C (lanes 3 and 6). A slight increase in reporter gene expression (α) relative to expression of the transfection control (FGH) was seen in normoxia in the presence of the enhancer. This is similar to that seen in the cells exposed to heat shock. In contrast, a large effect of the enhancer is evident in hypoxia. (*B*) Western blot showing that 1% O_2 does not induce Hsp72. Protein was analyzed from aliquots of the transfected cells. Hsp72 was detected only in cells exposed to heat shock (lanes 2 and 5).

The results indicate that this is not the case. First, hypoxia of the severity we used did not induce the major heat shock proteins; second, heat shock did not activate the Epo enhancer; and third, cycloheximide abrogated the Epo enhancer-mediated response but is known to induce heat shock mRNA accumulation and thermotolerance (21). Furthermore, the Epo 3′ enhancer sequence does not contain a heat shock element.

Kidney and liver are the major organs producing Epo. In rodents, very low levels of hypoxically inducible Epo mRNA are detectable in some other organs (22). The cellular source within those organs was not determined, and it is, thus, difficult to relate them to our current findings. In kidney and liver, Epo expression is limited to a small subset of cells (2–4). The range of cells chosen in the current experiments includes several which are not known to make Epo and several which are derived from organs which do not contribute importantly to Epo production *in vivo* (1, 22). To be sure of this, we assayed for endogenous Epo mRNA by RNase protection. Apart from the hepatoma cell lines, no Epo expression was detectable with the possible exception of LLC-PK1. Since we did not use a fully complementary probe for LLC-PK1, this finding is not secure but might be consistent with a previous report of low-level Epo production by LLC-PK1 (23).

Although clear evidence of hypoxia-inducible enhancer activity was observed in 11 of the 12 cell lines tested, activity did vary somewhat among these cell lines. Since the partial pressure of O_2 in the microenvironment of the sensor could bear a different relationship to the incubator partial pressure of O_2 in different cells, it is unlikely that the degree of hypoxia was optimized for enhancer activity in every cell. Thus, apparent differences in inducibility may be intrinsic or may arise from differences in effective oxygenation. In U937 cells, despite some variability, an interaction between severity of hypoxia and cell density was seen, suggesting that oxygen consumption by respiring cells contributes to cellular hypoxia. Other explanations, such as interaction with cell growth and division, are possible. Increased hypoxic induction at high cell density was also seen in some adherent cell lines. Interestingly, the reverse has been noted for Epo expression in Hep3B and HepG2 cells, where greater induction was observed at lower cell density (5). Other experimental conditions also affect the magnitude of induction; for instance, in some cell lines, transfection at higher voltage reduced the hypoxic response.

Despite the difficulties in drawing a firm negative conclusion, the response we describe does not appear to be universal. After extensive study of different conditions, we were unable to show inducible enhancer action in two different MEL clones. It is interesting that Beck *et al.* (8) did not find hypoxic induction of a human Epo minigene containing the human Epo 3′ enhancer in HeLa cells. Our previous report (10) that oxygen sensing was not seen in Chinese hamster ovary cells appears to be in error and probably arose from the use of lower cell density. In subsequent experiments (at higher cell densities), oxygen sensing was reproducibly observed.

In transgenic mice bearing a human Epo gene containing the 3′ enhancer, induction by anemia was not observed in organs showing aberrant expression (24). This is not incompatible with our results. It is likely that chromatinization patterns would restrict operation of the enhancer/promoter interaction in transgenic animals. It is also possible that when anemia is induced *in vivo*, the operating range for the oxygen-sensing mechanism is reached only in certain tissues, whereas in cell culture experiments we achieved more uniform hypoxia.

Further transgenic studies indicate that tissue-specific expression of the Epo gene requires distant sequence (25). In our experiments, the Epo 3′ enhancer operated widely in the transfected cell lines whereas the Epo promoter did not, being barely active except in the Epo-producing hepatoma cells.

The operation of tissue-specific gene repression, superimposed on a widespread sensing and signaling mechanism, is well established for several responses such as the adenylate cyclase system (26). The widespread existence of this oxygen-sensing system indicates that it serves other purposes, rather than solely controlling Epo production. Candidates for interaction with such a system include adaptation of cell metabolism (18), tissue repair and neovascularization (27), and vasomotor control (28). Which, if any, of these responses involve genes controlled by similar cis-acting elements will require further investigation.

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