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Genetics

A 24-base-pair sequence 3′ to the human erythropoietin gene contains a hypoxia-responsive transcriptional enhancer

(transcriptional regulation/oxygen/transfection/Hep 3B cells/luciferase gene)

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ABSTRACT **Erythropoietin (Epo) synthesis increases in response to hypoxia. The hepatoma cell line Hep 3B produces low basal levels of Epo mRNA which increase markedly with hypoxia. To define the sequences necessary for this response, we linked fragments of the human Epo gene to a luciferase vector, introduced these plasmids into Hep 3B cells and assayed for luciferase activity after growth in 1% or 21% oxygen. A 621-bp Epo promoter fragment resulted in a 2.4-fold increase in luciferase activity with hypoxia. We tested several Epo gene fragments upstream of this Epo promoter fragment and found that a 613-bp *Bgl* II–*Pvu* II 3′ fragment had a 10-fold increase in activity with hypoxia regardless of orientation. This fragment had a similar level of activity when linked to a simian virus 40 promoter. Portions of this fragment retained activity, including a 38-bp *Apa* I–*Taq* I fragment that had a 17-fold increase in activity with hypoxia. Deletion of nt 4–13 or 19–28 from this 38-bp fragment resulted in a loss of activity. The 24-bp upstream portion of the 38-bp fragment showed an 8-fold increase in activity with hypoxia. However, deletion of nt 19–24 or mutagenesis of nt 21 or 22 in this 24-bp fragment resulted in loss of activity. Our studies indicate that the transcriptional response of the human Epo gene to hypoxia is mediated in part by promoter sequences and to a greater degree by an enhancer element located in a 24-bp portion of the 3′ flanking sequence of the gene.**

Erythropoietin (Epo) is a glycoprotein hormone that is the primary regulator of red blood cell production (1). It acts by binding to a specific cell surface receptor on erythroid progenitors in the bone marrow to stimulate their growth and proliferation (2, 3). Epo is synthesized principally in the peritubular interstitial cells of the kidney and under conditions of erythropoietic stress in the liver (4, 5). Epo production and secretion are increased in response to anemia or hypoxia (6). The Epo-producing cells respond to a decrease in local tissue oxygen tension by increasing Epo secretion into the bloodstream, which results in enhanced red blood cell production. The feedback loop is completed when the Epo-producing cells recognize the change in oxygen tension due to the increase in the number of circulating red blood cells and decrease Epo secretion.

The mechanisms by which Epo-producing cells in the kidney and liver sense a change in tissue oxygen tension and the pathways by which this signal causes an increase in Epo synthesis are poorly understood. Goldberg *et al.* (7) found that two human hepatoma cell lines, Hep 3B and Hep G2, regulated Epo production in a physiologic manner. They demonstrated that Epo production in Hep 3B cells was increased >50-fold by hypoxia or cobalt and that the response to hypoxia was blocked by either carbon monoxide or inhibitors of heme synthesis. Based on these studies they proposed that the oxygen sensor is a heme protein (8). However, this heme protein has not been identified nor has its role in Epo gene regulation been demonstrated.

Several lines of evidence indicate that the regulation of Epo gene expression is primarily at the level of transcription. First, in mice made anemic or subjected to hypoxia there is a marked increase in Epo mRNA levels in the kidney. Renal Epo mRNA is present 1 hr after the onset of hypoxia and reaches a peak in 4–8 hr, suggesting that Epo synthesis is regulated by the level of its specific mRNA (4, 9). Second, nuclear run-off experiments show active Epo gene transcription in kidney nuclei from anemic–hypoxic mice but not in nuclei from control mice (10). However, additional experiments have suggested that posttranscriptional events also influence Epo production by altering the stability of Epo mRNA in response to changes in oxygen tension (11).

Several groups of investigators have performed transient transfection studies in either Hep 3B or Hep G2 cells in order to identify the cis DNA sequences that participate in Epo gene response to hypoxia. Using a growth hormone reporter system, Imagawa *et al.* (12) showed that both a 1.2-kb 5′ fragment of the human Epo gene and a 256-bp portion of the 3′ untranslated region resulted in an ≈2-fold induction with hypoxia. Semenza *et al.* (13) used a chloramphenicol acetyltransferase (CAT) reporter system and found hypoxia-responsive enhancer activity in a different 256-bp fragment in the 3′ flanking region of the gene. Beck *et al.* (14) also used a CAT assay system and localized hypoxia-responsive enhancer activity to a 150-bp portion of the latter 256-bp fragment. Pugh *et al.* (15) found a similar enhancer element in a 70-bp fragment in the 3′ flanking region of the murine Epo gene.

We have performed transient transfection studies in Hep 3B cells, using a luciferase reporter gene, to determine which portions of the human Epo gene contain hypoxia-responsive activity. Our studies indicate that the Epo promoter contains sequences which respond to a modest degree to hypoxia and that an enhancer element present in a 24-bp portion of the 3′ flanking region contributes to a greater degree to the transcriptional response to hypoxia.

MATERIALS AND METHODS

Cell Culture. Hep 3B cells were obtained from the American Type Culture Collection and cultured in Eagle's medium (with Earle's salts) supplemented with 10% fetal bovine serum (HyClone), penicillin (100 units/ml), streptomycin sulfate (100 μg/ml), and Fungi-Bact (25 μg/ml). Cells were maintained in a humidified 95% air/5% CO_2 incubator at 37°C. To maintain cells in a hypoxic environment, culture plates were placed in a modular incubator chamber (Billups–

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Abbreviations: Epo, Erythropoietin; CAT, chloramphenicol acetyltransferase; SV40, simian virus 40.

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Rothenburg, Del Mar, CA) which was flushed with a gas mixture containing 1% O_2, 94% N_2, and 5% CO_2.

Plasmid Constructions. We used a 4-kb *Hin*dIII–*Eco*RI genomic human Epo gene fragment in pUC8 (provided by Charles Shoemaker of the Genetics Institute, Cambridge, MA) to isolate a variety of fragments for ligation into the luciferase vector. The reporter plasmid pMG3 (provided by Paolo Moi) (16) contains a multiple cloning site upstream of an intronless firefly luciferase gene (nt +30 to +1747; ref. 17) with a simian virus 40 (SV40) small tumor antigen intron and polyadenylylation signal downstream. Two SV40 polyadenylylation signals are located immediately upstream of the multiple cloning site to prevent aberrant transcription initiation.

Initially we linked the 621-bp *Hin*dIII–*Apa*LI Epo gene fragment (Fig. 1), encompassing 5′ flanking sequence and the promoter and extending through the first exon to the ATG start site, upstream of the luciferase gene in pMG3. To prevent false initiation of transcription, the Epo ATG codon was changed to TTG by site-specific mutagenesis (18). Subsequent Epo gene fragments were subcloned by blunt-end ligation into the *Hin*dIII site of the 621-bp fragment. Restriction and sequence analysis was performed to determine the orientation and copy number of subcloned fragments. Only plasmids that contained a single copy of the fragment to be tested were used.

Epo gene fragments linked upstream of the 621-bp promoter fragment are shown in Fig. 1 and included a 555-bp *Sca* I–*Xba* I 5′ fragment containing the first intron, a 3.4-kb *Bst*EII–*Eco*RI fragment containing the entire gene except for the 621-bp promoter fragment, a 256-bp *Acc* I–*Bgl* II portion of the 3′ untranslated region, and an adjacent, downstream 613-bp *Bgl* II–*Pvu* II fragment. To localize hypoxia-responsive activity found in the 613-bp *Bgl* II–*Pvu* II fragment, portions of it were linked to the promoter fragment; these included a 283-bp *Ava* II–*Pvu* II fragment, a 227-bp *Hin*fI–*Pvu* II fragment, a 170-bp *Hin*fI–*Alu* I fragment, a 109-bp *Hin*fI–*Apa* I fragment, and a 38-bp *Apa* I–*Taq* I

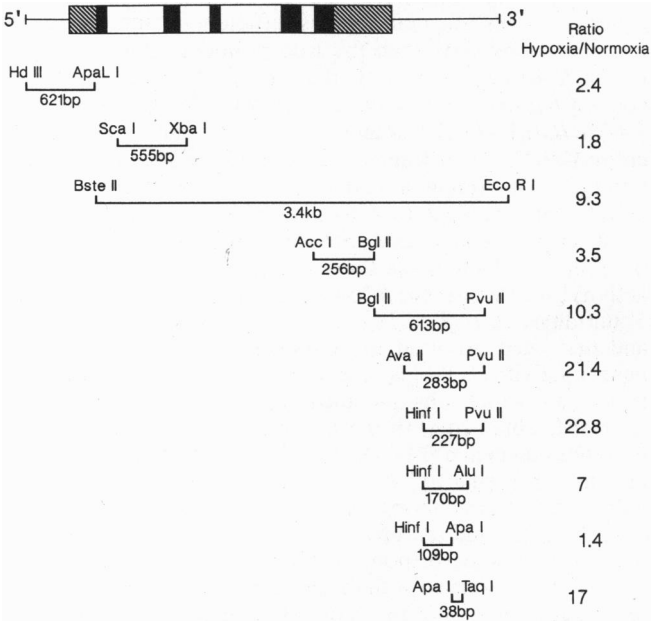

FIG. 1. The Epo gene and fragments tested for response to hypoxia. The gene is shown at the top with the coding sequence represented by black rectangles and the untranslated sequence by hatched rectangles. The restriction fragments tested in the transfection assay are indicated below the gene. The ratio of luciferase activity under hypoxic versus normoxic conditions is indicated.

fragment. Fragments larger than the 109-bp fragment were tested in both orientations relative to the promoter.

To localize activity present in the *Apa* I–*Taq* I fragment, three pairs of mutant oligonucleotides were designed bearing 10-bp deletions in the upstream (ΔL), middle (ΔM), and downstream (ΔR) portions, respectively, of that fragment (Fig. 2). The mutant oligonucleotides were based on the 42-bp sequence that included the entire recognition sequences for *Apa* I and *Taq* I. Relative to the first nucleotide of the *Apa* I site, nt 4–13 were deleted from ΔL, nt 19–28 were deleted from ΔM, and nt 30–39 were deleted from ΔR. Additional pairs of oligonucleotides were synthesized representing the upstream 24 bp (WT 24) and upstream 18 bp (WT 18) of the 38-bp fragment. Finally, two oligonucleotide pairs were synthesized with single point mutations near the end of the 24-bp fragment. The first of these had a C → T substitution at position 21 of the 24-bp sequence. The second had an A → G substitution at position 22 of the 24-bp sequence. Oligonucleotides were synthesized in an Applied Biosystems 380B DNA synthesizer. Homologous oligonucleotide pairs were annealed and linked by blunt-end ligation upstream of the 621-bp promoter in the luciferase plasmid. Sequence analysis was performed to confirm the presence of the desired insert, to determine orientation, and to ensure insertion of a single copy.

To determine whether the Epo promoter was required for the response of 3′ fragments to hypoxia, control transfections were performed with pMG3 bearing the SV40 promoter upstream of the luciferase gene either alone or linked to the 613-bp *Bgl* II–*Pvu* II 3′ Epo gene fragment.

Transfection. Plasmids were introduced into Hep 3B cells by lipofection following the manufacturer's protocol (Lipofectin Reagent, Bethesda Research Laboratories). Cells were grown in 100-mm culture plates to 80% confluency and each plate was transfected with 15 μg of test plasmid and, to control for transfection efficiency, 5 μg of pRSVCAT. Cells were then incubated for 12 hr in 95% air/5% CO_2 at 37°C. Medium was then adjusted to a final concentration of 10% fetal bovine serum and the plates were incubated for 60 hr in either 1% or 21% O_2 prior to harvest. Each experiment consisted of parallel transfection of four plates with a given test vector followed by incubation of two plates in hypoxia and two in normoxia.

Assays. Cells were lysed with a buffer containing 1% (vol/vol) Triton X-100, 25 mM glycylglycine, 15 mM $MgSO_4$, 4 mM EGTA, and 1 mM dithiothreitol and centrifuged at 4°C. The protein concentration of each supernatant was measured spectrophotometrically using a Bio-Rad kit (Bradford assay). Seventy-five micrograms of each protein lysate was added to an assay buffer containing 5 mM ATP. Each sample was then tested for luciferase activity by addition of 100 μl of 1 mM potassium luciferin in a Monolight 2001 luminometer (Analytical Luminescence Laboratories, San Diego).

Fifty micrograms of cell lysate from each transfection was assayed for CAT activity (19). After incubation with [^{14}C]chloramphenicol and acetyl-CoA, samples were subjected to thin-layer chromatography on silica gel plates and radioactivity was counted in a Molecular Dynamics PhosphorImager (Sunnyvale, CA). The percentage of acetylated chloramphenicol was determined for each extract and transfection efficiency was determined by comparison with results obtained after cotransfection of pRSVCAT with the promoterless luciferase vector. The luciferase activity obtained from each transfection was then corrected for transfection efficiency.

Statistical Analysis. Each plasmid construction was tested in 6–10 independent experiments. To determine the fold increase in luciferase activity with hypoxia for each construction, the logarithm of the ratio of the luciferase activity in hypoxic versus normoxic cells for each experiment was

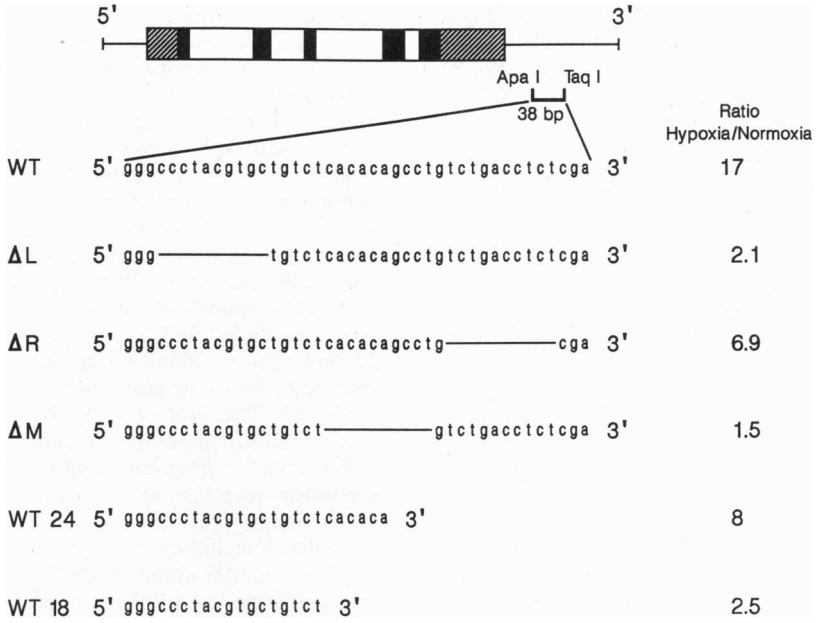

FIG. 2. Normal and mutant Epo gene 3′ flanking sequences tested for response to hypoxia. The location of the normal *Apa* I–*Taq* I fragment is indicated below the Epo gene. The normal sequence (wild type, WT) is shown above three fragments with 10-bp deletions in the upstream (ΔL), downstream (ΔR), and middle (ΔM) portions of the fragment. Only the top-strand sequence is shown for each fragment. Fragments containing the upstream 24 nt (WT 24) and upstream 18 nt (WT 18) of the *Apa* I–*Taq* I fragment are shown at the bottom. The ratio of luciferase activity under hypoxic versus normoxic conditions is indicated.

first determined. Then the mean logarithm for each construction tested was calculated. The antilogarithm is the true ratio of the activity in hypoxic versus normoxic cells and is the reported value for each fragment tested. To determine whether the luciferase activity of a given construction was significantly different in hypoxia versus normoxia we used a one-sample *t* test to test whether the mean logarithm was significantly different from zero ($P < 0.05$). To determine whether linkage of a given Epo gene fragment to the 621-bp Epo promoter fragment resulted in a significantly greater fold induction with hypoxia than that observed with the promoter alone, a two sample *t* test was performed comparing the ratio of activity under hypoxic versus normoxic conditions for the promoter fragment alone with that of the fragment linked to the promoter (20). With the exception of the Epo promoter fragment, all *P* values reported for a given fragment represent the result of a two-sample *t* test.

RESULTS

To localize the sequences underlying Epo gene response to hypoxia, various portions of the Epo gene and flanking sequence were linked to a luciferase vector, and these plasmids were transfected into Hep 3B cells. After growth in 21% and 1% O_2, cell lysates were prepared and assayed for luciferase activity. After correction for transfection efficiency, hypoxia-responsive activity for each construct was expressed as the ratio of luciferase activity for cells grown under hypoxic versus normoxic conditions. A one-sample *t* test was used to determine whether the luciferase activity for each construct was significantly greater under hypoxic conditions than under normoxic conditions.

We first tested the 621-bp *Hin*dIII–*Apa*LI 5′ Epo gene fragment that includes the promoter along with 5′ flanking sequence (Fig. 1). This fragment showed a modest but statistically significant increase in luciferase activity of 2.4-fold in response to hypoxia ($P < 0.025$). A 403-bp *Pst* I–*Apa*LI Epo promoter fragment had a similar, modest response to hypoxia (data not shown). This indicated that promoter sequences participated in the response to hypoxia but suggested that additional sequence elements were necessary for full response. We therefore linked a number of Epo gene fragments upstream of the 621-bp Epo promoter fragment in the luciferase vector and tested their activity in response to hypoxia. A two-sample *t* test was performed to determine whether linkage of each fragment to the promoter resulted in a significantly greater induction with hypoxia compared with that seen with the promoter alone. We first tested the 555-bp *Sca* I–*Xba* I fragment (Fig. 1) that contains the first intron, since this fragment has a high degree of sequence conservation between the human and murine Epo genes (21). This fragment was less efficient in stimulating the response to hypoxia than the Epo promoter alone (1.8-fold versus 2.4-fold), indicating that the first intron does not contain hypoxia-responsive sequences. We next tested the 3.4-kb *Bst*EII–*Eco*RI fragment (Fig. 1) that includes the entire 4-kb Epo gene fragment except for the 621-bp promoter fragment. This fragment had a 9.3-fold increase in luciferase activity in response to hypoxia, which was significantly greater than that obtained with the promoter fragment alone ($P < 0.001$). To localize the sequences responsible for this activity, we then tested a 256-bp *Acc* I–*Bgl* II portion of the 3′ untranslated region (Fig. 1) that also is highly conserved and had been reported to contain hypoxia-responsive enhancer activity (12). This fragment showed a 3.5-fold increase in activity, which was not statistically significantly different from that obtained with the promoter fragment alone ($P > 0.1$). The adjacent 613-bp *Bgl* II–*Pvu* II 3′ fragment was tested next and showed a 10.3-fold increase in response to hypoxia, which was significant ($P < 0.01$). This fragment encompasses the 256-bp (13) and 150-bp (14) 3′ fragments previously shown to contain hypoxia-responsive enhancer activity. Equivalent activity was observed with this 613-bp fragment regardless of orientation relative to the promoter. This fragment retained a similar level of activity when linked upstream of a SV40 promoter, rather than the Epo promoter, in the luciferase plasmid. The SV40 promoter alone showed no response to hypoxia (data not shown).

To further localize the active sequences, smaller portions of the 613-bp fragment were tested, including the 283-bp *Ava*

II–*Pvu* II, 227-bp *Hin*fI–*Pvu* II, and 170-bp *Hin*fI–*Alu* I fragments (Fig. 1). All three fragments retained enhancer activity, with 21.4-, 22.8-, and 7-fold increase in luciferase activity in response to hypoxia, respectively ($P < 0.05$ for each construction). The next fragment tested, the 109-bp *Hin*fI–*Apa* I fragment, showed a 1.4-fold increase in activity with hypoxia and thus lacked enhancer activity. This suggested that the active sequences were located downstream of the *Apa* I site. This was confirmed when we tested the 38-bp *Apa* I–*Taq* I 3′ fragment, which showed a 17-fold increase in activity in response to hypoxia ($P < 0.001$).

We next tested fragments bearing 10-bp deletions in the upstream, middle, and downstream portions of the *Apa* I–*Taq* I fragment (Fig. 2). Oligonucleotide pairs were designed based on the 42-bp sequence that included the complete *Apa* I and *Taq* I restriction sites. Deletion of nt 4–13 relative to the *Apa* I site (ΔL) reduced the hypoxia response to 2.1-fold. Deletion of nt 30–39 (ΔR) resulted in a 6.9-fold increase in activity with hypoxia ($P < 0.001$). Deletion of nt 19–28 (ΔM) reduced the response to hypoxia to 1.5-fold. These findings suggested that sequences in the upstream portion of the *Apa* I–*Taq* I fragment were necessary for activity.

We subsequently tested the activity of the upstream 24 bp of the *Apa* I–*Taq* I fragment (Fig. 2). This fragment (WT 24) resulted in an 8-fold increase in activity in response to hypoxia ($P < 0.05$). Further truncation to an 18-bp fragment (WT 18), resulted in a reduction in the response to hypoxia to 2.5-fold. This suggested that the 6 bp at the downstream end of the 24-bp fragment were essential for activity. Comparison of human and mouse sequence revealed that 3 of these 6 nt were identical (nt 19, 21, and 22 relative to the *Apa* I site). Mutation of nt 21 from C to T and mutation of nt 22 from A to G in the 24-bp fragment resulted in a reduction in the response to hypoxia to 2-fold and 1.5-fold, respectively.

DISCUSSION

We have used transient transfection studies in Hep 3B cells to identify the DNA sequences that participate in the transcriptional response of the human Epo gene to hypoxia. These studies indicated that a 621-bp 5′ portion of the Epo gene, including the promoter, was sufficient for a modest but statistically significant response to hypoxia. A significantly greater response to hypoxia was observed when various 3′ portions of the Epo gene were linked, in either orientation, upstream of the Epo promoter fragment. These results demonstrated that a hypoxia-responsive enhancer element was present in the 3′ portion of the Epo gene. This enhancer retained a similar level of activity when linked to an SV40 promoter, indicating that Epo promoter sequences are not required for enhancer function. Experiments employing progressively smaller 3′ Epo gene fragments localized the hypoxia-responsive enhancer to a 24-bp portion of the 3′ flanking region of the gene. Deletion and mutagenesis experiments indicated that nt 4–13 and 19–24 were essential for enhancer activity.

Imagawa *et al.* (12) performed transfection experiments in Hep 3B cells by using a growth hormone reporter gene and found that a 1.2-kb Epo gene fragment extending from the 5′ flanking sequence through the first intron had a 1.7-fold increase in response to hypoxia. We have found a similar modest activity in a 621-bp fragment that contains the Epo gene promoter but no activity in a 555-bp fragment spanning most of the first intron. Thus, the Epo promoter appears to contain sequences sufficient for part of the transcriptional response of the human Epo gene to hypoxia. Imagawa *et al.* also linked a 256-bp portion of the 3′ untranslated region, in either orientation, downstream of the 1.2-kb 5′ Epo fragment and growth hormone gene and found a 2.3-fold increase with hypoxia. We have tested the same fragment, in both orientations, upstream of the 621-bp Epo promoter fragment and found no significant difference in response to hypoxia compared with the response obtained with the promoter fragment alone. Pugh *et al.* (15) tested the same human Epo gene fragment and the homologous murine Epo gene fragment in transfection studies in Hep 3B and Hep G2 cells and did not find hypoxia-responsive enhancer activity. Taken together, these results suggest that this fragment contains little or no hypoxia-responsive activity.

Beck *et al.* (14) performed transfection experiments in Hep 3B cells with a CAT reporter gene and localized a hypoxia-responsive enhancer to a 150-bp *Apa* I–*Pst* I fragment in the 3′ flanking region of the human Epo gene. All of the 3′ fragments that had hypoxia-responsive enhancer activity in our studies either spanned this fragment or were a portion of it. The 24-bp fragment that retained activity in our experiments represents the upstream end of the 150-bp fragment. In similar transfection experiments, Semenza *et al.* (13) localized hypoxia-responsive enhancer activity to a 256-bp fragment which encompassed the 150-bp fragment. In addition, they performed DNase I protection studies using nuclear extracts from mouse liver cells and demonstrated binding of nuclear protein to four different portions of the 256-bp fragment. Binding to two of the four protected regions was observed only in extracts from anemic mice. One of these two inducible footprints overlaps the 24-bp enhancer fragment identified in our studies. The other inducible footprint is located ≈20 bp upstream in a fragment that had no activity in our transfection studies.

Pugh *et al.* (15) used transfection experiments in Hep G2 cells to localize a hypoxia-responsive enhancer element to a 70-bp 3′ flanking portion of the murine Epo gene. Comparison of the mouse sequence to the human Epo 3′ flanking sequence revealed 80% conservation. The 24-bp enhancer fragment identified in our studies is homologous with the upstream end of the 70-bp murine sequence. In fact, the first 17 nt and 20 of 24 nt in the human enhancer fragment are identical to the mouse sequence. However, in contrast to our studies with the human enhancer, Pugh *et al.* found that shortening the murine enhancer to <70 bp resulted in a loss of full activity. These results may be due to an intrinsic difference in murine and human Epo gene response to hypoxia or may result from use of a different cell line and reporter system.

In transfection experiments that included different fragments containing the 3′ enhancer, we found a 6- to 23-fold increase of luciferase activity in response to hypoxia. This level of induction is significantly less than the <50-fold increase in Epo mRNA found in Hep 3B cells in response to hypoxia (8). However, our results are similar to those of transfection studies by other groups using different reporter systems, who found 4-fold (13), 10-fold (15), and 15-fold (14) increases in activity in response to hypoxia. Epo mRNA stability in Hep 3B cells is altered by changes in oxygen tension (11). This would amplify the effect of hypoxia on Epo gene transcription and lead to a greater fold induction of Epo mRNA than is observed in transfection studies.

Two recent papers have examined in detail the DNA elements and protein factors responsible for Epo gene response to hypoxia (22, 23). Semenza and Wang (22) linked portions of Epo 3′ flanking sequence downstream of a SV40 promoter–CAT reporter gene and localized a hypoxia-inducible enhancer to a 50-bp fragment. This fragment begins 3 nt downstream from the *Apa* I restriction site which is the upstream end of the enhancer fragment identified in our studies. They also found that a 33-bp portion of this fragment (nt 3–35 relative to the *Apa* I site) retained approximately half the hypoxia-responsive activity of the 50-bp fragment. Mutagenesis experiments revealed that nt 6–14 and 21–25, relative to the *Apa* I site, were essential for activity. These

findings are in agreement with our studies, which indicate that nt 4–13 and 19–24 are essential for activity. Using electrophoretic mobility-shift assays, Semenza and Wang (22) have identified a nuclear protein factor which is inducible by hypoxia and which binds to nt 6–14 relative to the *Apa* I site.

Blanchard *et al.* (23) have used transfection experiments in Hep 3B cells stimulated by a combination of hypoxia and cobaltous chloride to identify Epo promoter and enhancer elements responsible for induction. They defined a 117-bp fragment which contains the minimal promoter sequences required for inducibility and localized a 53-bp portion of this fragment which mediates inducibility. In addition, they localized enhancer activity to a 43-bp *Apa* I–*Hpa* II fragment in the 3′ flanking sequence of the Epo gene. This fragment encompasses the *Apa* I–*Taq* I enhancer fragment identified in our studies. They noted the presence in both the 53-bp promoter fragment and the 43-bp enhancer fragment of two copies of a sequence, YGACCY, which is homologous to the consensus steroid/thyroid hormone response element (24). Using mobility-shift and footprinting studies, they demonstrated specific binding to these sequences in the enhancer fragment by using extracts from normoxic and hypoxic Hep 3B cells and extracts from a number of other cell lines. Interestingly, they showed that mutation of both copies of this motif in a previously active enhancer fragment resulted in complete loss of enhancer activity. Our 38-bp *Apa* I–*Taq* I enhancer fragment contains only one of these two motifs, yet it retains activity. Additionally, the 24-bp fragment enhancer fragment contains neither motif, yet it still retains hypoxia-inducible activity. It should be noted, however, that these fragments were tested in our studies upstream of the Epo gene promoter fragment. It is possible that the steroid/thyroid hormone consensus sequences in the Epo promoter fragment are able to substitute for those normally present in the enhancer. However, Semenza and Wang (22) found that a 33-bp fragment which lacked both motifs was able to function as a hypoxia-inducible enhancer in a SV40 promoter–CAT reporter gene plasmid. Thus, the role of these sequence motifs in the function of the Epo gene enhancer deserves further investigation.

The mechanisms by which Epo-producing cells sense hypoxia and the signal pathways that lead to increased Epo production are not understood. Goldberg *et al.* (8) have suggested that the oxygen sensor is a heme protein and proposed that conformational changes in response to hypoxia might lead to increased Epo production. Such a heme-based oxygen-sensing system participates in the regulation of a number of genes in lower organisms, including the nitrogen-fixation genes of *Rhizobium meliloti* (25) and the *COX5* genes of yeast (26). We have not found homology between the 24-bp human Epo gene enhancer sequence and these and other genes from lower organisms that respond to hypoxia. A number of human genes, including those encoding platelet-derived growth factor B (27), endothelin 1 (28), and transforming growth factor β (29), have been shown to respond to hypoxia with an increase in gene transcription. No homology was found between these genes and the Epo enhancer sequence. We hope that identification of this 24-bp enhancer fragment will facilitate the isolation of the genes encoding protein factors that direct the transcriptional response of the human Epo gene to hypoxia. Characterization of these protein factors would shed light not only on the pathways underlying Epo gene response to hypoxia but also more generally on oxygen-regulated gene expression.

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