Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 6496–6500, July 1994
Genetics

Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: Similarities with the erythropoietin 3′ enhancer

J. D. FIRTH*, B. L. EBERT, C. W. PUGH, AND P. J. RATCLIFFE

Erythropoietin Group, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, England

Communicated by David J. Weatherall, March 11, 1994 (received for review January 13, 1994)

ABSTRACT **Production of the glycoprotein hormone erythropoietin (Epo) in response to hypoxic stimuli is almost entirely restricted to particular cells within liver and kidney, yet the transcriptional enhancer lying 3′ to the Epo gene shows activity inducible by hypoxia after transfection into a wide variety of cultured cells. The implication of this finding is that many cells which do not produce Epo contain a similar, if not identical, oxygen-regulated control system, suggesting that the same system is involved in the regulation of other genes. We report that the human phosphoglycerate kinase 1 and mouse lactate dehydrogenase A genes are induced by hypoxia with characteristics which resemble induction of the Epo gene. In each case expression is induced by cobalt, but not by cyanide, and hypoxic induction is blocked by the protein-synthesis inhibitor cycloheximide. We show that the relevant cis-acting control sequences are located in the 5′ flanking regions of the two genes, and we define an 18-bp element in the 5′ flanking sequence of the phosphoglycerate kinase 1 gene which is both necessary and sufficient for the hypoxic response, and which has sequence and protein-binding similarities to the hypoxia-inducible factor 1 binding site within the Epo 3′ enhancer.**

Erythropoietin (Epo) is the hormone which regulates the oxygen-carrying capacity of the blood by controlling erythropoiesis: hypoxia stimulates production by a mechanism which involves increased Epo gene transcription (1). Transient-transfection studies in cultured cells have defined cis-acting control sequences responsible for this effect, the most powerful of which is an oxygen-regulated transcriptional enhancer located 3′ to the poly(A)-addition site of the gene (2–6). Extensive screening of tissue culture cell lines has revealed only two (the hepatoma lines Hep 3B and Hep G2) that produce Epo in an oxygen-regulated manner (7), yet the Epo 3′ enhancer, when coupled to broadly active promoters, confers responsiveness to hypoxia in most (possibly all) cultured cells (8, 9). Furthermore, hypoxia-inducible factor 1 (HIF-1), which binds to a functionally critical region of the Epo 3′ enhancer, has been found both in cells which produce Epo and in those that do not (10, 11). These findings suggest that oxygen sensing, signal transduction, and gene activation mechanisms very similar, if not identical, to those present in Epo-producing cells must be widespread. The implication is that these mechanisms are involved in the regulation of other genes in cells which do not produce Epo.

We report here that the human phosphoglycerate kinase 1 (PGK-1) and mouse lactate dehydrogenase A (LDH-A) genes are regulated by hypoxia in a manner which is strikingly similar to Epo gene regulation. Using transient transfection, we have located cis-acting control sequences responsible for hypoxia-inducible expression in the 5′ flanking region of each gene. For the PGK-1 gene we have characterized an 18-bp element which is necessary for hypoxia-inducible expression and which has sequence similarity to a region within the Epo 3′ enhancer. Oligonucleotides containing the functionally active PGK-1 and Epo sequences cross-compete for a hypoxia-inducible factor (or factors) in electrophoretic mobility-shift assays.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. The cell lines used were Hep G2 (human hepatoma), HeLa (human cervical carcinoma), and L cells (mouse fibroblast) grown to ≈70% confluence. The medium was then replaced, and cells were subjected to the following conditions for 14–16 hr: (*i*) normoxia (20% O_2/5% CO_2/75% N_2); (*ii*) hypoxia (1% O_2/5% CO_2/94% N_2 in a Napco 7100 incubator); (*iii*) hypoxia with cycloheximide (100 μM); (*iv*) normoxia with cobaltous chloride (50 μM); (*v*) normoxia with cyanide (100 μM); or (*vi*) hypoxia with cyanide (100 μM).

Transient Transfection and RNA Analysis. In all experiments the test plasmid (10–100 μg), encoding either human α_1-globin or human growth hormone (GH) as a reporter, was cotransfected with a control plasmid (10–50 μg) by electroporation (4). After electroporation, transfected cells were split equally and incubated in parallel for 14–16 hr under normoxic or hypoxic conditions or were exposed to chemical agents, as described above. Details of test-plasmid design are shown in Fig. 1 and Table 1. RNA was extracted, analyzed by RNase protection, and quantitated as described (4). For assay of gene expression in transiently transfected cells, 3–10 μg of RNA was subjected to double hybridization with probes which protected 120 bp of GH mRNA and either 132 bp (α132) or 97 bp (α97) of α-globin mRNA, depending on whether the test or control plasmid encoded α-globin. For assay of endogenous PGK-1 gene expression, 50 μg of RNA extracted from Hep G2 cells was hybridized with an RNA probe which consisted of 121 bases from the 5′ end of the exon 3 of the human PGK-1 gene, together with 68 bases of adjacent intron. For assay of endogenous LDH-A gene expression, 25 μg of RNA obtained from L cells was hybridized with an RNA probe which consisted of 47 bases from the 5′ end of exon 1 of the mouse LDH-A gene, together with adjacent 5′ flanking sequence. In these experiments a small quantity (0.5 μg) of RNA extracted from K562 cells (a human erythroleukemic cell line which expresses α-globin mRNA abundantly) was added to each sample before hybridization, and the samples were probed concurrently with the α97 probe in addition to the PGK-1 or LDH-A probe. This provided a

Abbreviations: Epo, erythropoietin; GH, growth hormone; HIF-1, hypoxia-inducible factor 1; LDH, lactate dehydrogenase; PGK, phosphoglycerate kinase.

*To whom reprint requests should be addressed at: Room 420, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford, OX3 9DU, England.

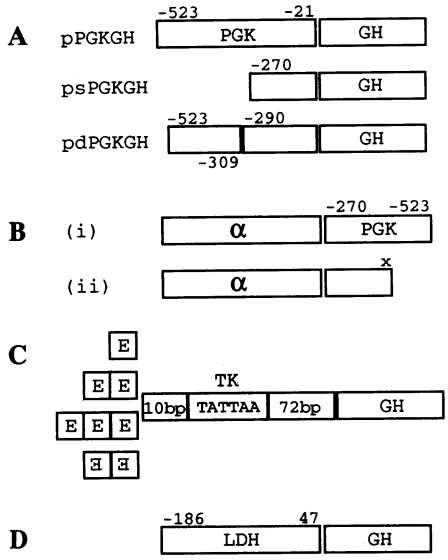

FIG. 1. (*A*) Plasmids used for deletion analysis of the promoter/enhancer region of the mouse PGK-1 gene. In pPGKGH, the *Eco*RI (−523)–*Taq* I (−21) fragment of the PGK-1 gene derived from pDEneo (12) (where the A of the ATG translational initiation codon is numbered +1) is fused to human GH reporter gene (1503 bp extending from the *Bam*HI site in the 5′ untranslated region). In psPGKGH, the 5′ region of construct pPGKGH was deleted by excision of the *Eco*RI (−523)–*Spe* I (−270) fragment. pdPGKGH is as pPGKGH except that 18 bp from the region between −308 and −291 have been deleted and replaced by the sequence ACTAGA, formed by ligation of *Xba* I- and *Spe* I-compatible ends. (*B*) Plasmids used for deletion analysis of the PGK-1 enhancer region. (*i*) *Eco*RI (−523)–*Spe* I (−270) fragment of PGK-1 inserted 3′ to the human α_1-globin reporter gene (α), which comprised a 2571-bp *Bgl* II–*Ppu*MI restriction fragment including 1.5 kb of 5′ flanking sequence and 0.5 kb of 3′ sequence. (*ii*) Series of deletions from the 5′ end of the PGK-1 enhancer made by exonuclease III/mung bean nuclease digestion. (*C*) Plasmid (pTKGH) used for assay of short sequence elements (E). Oligonucleotides (Table 1) synthesized with 5′ *Xba* I- and 3′ *Spe* I-compatible ends were cloned, either singly or as concatamers, in forward or reverse orientation, into an *Xba* I site 10 bp 5′ to the TATA box (TATTAA) of the herpes simplex thymidine kinase (TK) gene promoter (13), fused to GH as reporter. (*D*) Plasmid (pLDHGH) used for analysis of the mouse LDH-A gene. A 233-bp fragment from the promoter extending from position −186 to 47 (where 1 is the transcriptional start site) was obtained by PCR using oligonucleotides derived from published sequence (14) and cloned into the *Bam*HI site in the 5′ untranslated region of the GH reporter gene.

means of determining that sample processing and gel loading were comparable between specimens.

Nuclear Extract and Electrophoretic Mobility-Shift Assay. A modification of the protocol of Dignam *et al.* (16) was used to prepare nuclear extract. Oligonucleotides used as probes or competitors were purified by polyacrylamide gel electrophoresis. Labeling at the positions indicated (Table 1) was performed with [γ-^{32}P]ATP (3000 Ci/mmol; 1 Ci = 37 GBq) and phage T4 polynucleotide kinase. Labeled oligonucleotides were annealed with 4-fold molar excess of the complementary strand. Unlabeled oligonucleotides were annealed in molar equivalent quantities.

Binding reaction mixtures (20 μl) contained 50 mM KCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 5 mM dithiothreitol, 5% (vol/vol) glycerol, and 0.15–0.30 μg of sonicated poly(dI-dC). Nuclear extract (5 μg unless otherwise stated) was incubated with this mixture for 5 min at room temperature, before probe (≈0.1 ng) and specific competitors were added. Incubation was continued for a further 10 min. Reaction products were electrophoresed (12.5 V/cm) at 4°C in 5% polyacrylamide with 0.3× TBE (30 mM Tris/30 mM boric acid/0.06 mM EDTA, pH 7.3 at 20°C).

Table 1. Oligonucleotides used in enhancer and electrophoretic mobility-shift assays

Name	Sequence (5′ to 3′)
	M2 *M3* *M6*
E18F	GCCCTACGTGCTGCCTCG
E24F	GCCCTACGTGCTGCCTCGCATGGC
E24M3F	GCCCTAATGTCTGCCTCGCATGGC
	**** **** * *
P18R	TGTCACGTCCTGCACGAC
P18M1R	TGTCCATTCCTGCACGAC
P24R	ATTTGTCACGTCCTGCACGACGCG
P24M2R	ATTTGTACATTCCTGCACGACGCG

For functional assays the oligonucleotides cloned into the *Xba* I site of the pTKGH reporter were made with *Xba* I/*Spe* I-compatible ends. Abbreviations: E, erythropoietin 3′ enhancer sequence; P, PGK enhancer sequence; *M2*, *M3*, and *M6* refer to E mutants previously shown to ablate function of the mouse Epo enhancer (9). The HIF-1 binding site in Epo, as defined by Wang and Semenza (15), is underlined; differences between mutant and wild-type oligonucleotides are shown in bold type; *, sequence identity of P reverse strand with E forward strand. For mobility-shift assays the 5′ G of E24F and the 5′ A of P24R were labeled as described in *Materials and Methods*.

RESULTS

Expression of Endogenous PGK and LDH Genes. For the endogenous human PGK-1 gene in Hep G2 cells (Fig. 2*A*) and the endogenous mouse LDH-A gene in L cells (Fig. 2*B*), the pattern of response to hypoxia and to chemical agents was strikingly similar to that reported for the endogenous Epo gene in Hep G2 and Hep 3B cells (7, 17), although somewhat lower in amplitude. Both hypoxia and exposure to cobalt (50 μM) led to a 2- to 3-fold induction of gene expression. The protein-synthesis inhibitor cycloheximide (100 μM) abrogated the response to hypoxia. Cyanide (100 μM) did not affect the hypoxic response, neither inducing expression in normoxia nor preventing induction by hypoxia.

The 5′ Flanking Sequences from PGK-1 and LDH-A Genes Contain Hypoxia-Inducible Elements. To locate the sequences responsible for inducible expression of the endogenous PGK-1 and LDH-A genes, portions of the 5′ flanking sequence of each were linked to a GH reporter gene. Expression of these fusion genes in transfected Hep G2 cells reproduced the pattern of endogenous gene response, both with hypoxia and with chemical agents (Fig. 2 *C* and *D*). The inducible operation of these sequences contrasted with the simian virus 40 promoter, the α-globin promoter, the ferritin promoter, and the herpes simplex thymidine kinase promoter, none of which had this property. Responses were not specific for Hep G2 cells; similar behavior was observed in transfected HeLa cells (data not shown). Further experiments were performed to locate the precise element(s) responsible for hypoxic inducibility of one of these sequences: the 5′ flanking region of the PGK-1 gene.

Deletion Analysis of the PGK-1 Enhancer Region. Deletion of the 253-bp *Eco*RI–*Spe* I fragment from the 5′ end of the pPGKGH construct (Fig. 1*A*, psPGKGH) was found to virtually abolish induction by hypoxia, suggesting that it contained the responsible element. That this element could operate as an enhancer was confirmed when it was found that it could confer hypoxic inducibility (ratio of hypoxic to normoxic expression was 15.2 ± 2.5, mean ± SEM; n = 5) when placed 3′ to an α-globin reporter gene (Fig. 1*B*). A construct in which the 153 bp on the 5′ side of this PGK sequence had been removed by exonuclease III/mung bean

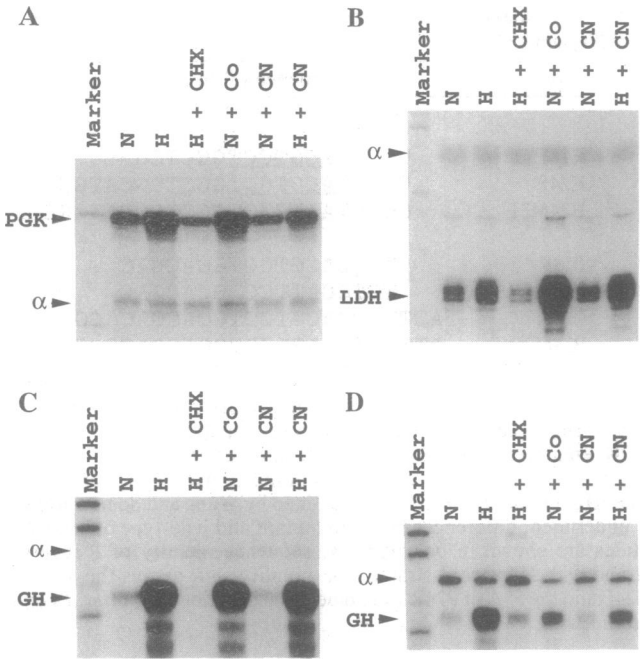

FIG. 2. RNase protection assays showing effect of hypoxia and of chemical agents on expression of endogenous PGK-1 and LDH-A genes in tissue culture and on expression of transiently transfected PGK–GH and LDH–GH fusion constructs. (*A*) Expression of the endogenous human PGK-1 gene in Hep G2 cells after exposure for 16 hr to normoxia (20% O_2; N), hypoxia (1% O_2; H), hypoxia and 100 μM cycloheximide (H + CHX), normoxia and 50 μM cobaltous chloride (N + Co), normoxia and 100 μM cyanide (N + CN), or hypoxia and 100 μM cyanide (H + CN). The protected α-globin band (α97 probe, α) is of comparable intensity in all lanes, indicating that differences in PGK expression were not attributable to variability of sample processing or gel loading. Marker, *Msp* I digest of pBR322. (*B*) Expression of endogenous mouse LDH-A gene in L cells. Details are as in *A*. (*C*) Expression of PGK–GH construct after transient transfection into Hep G2 cells. Details are as in *A* except that the α132 and GH probes were used to detect expression of the transfection control and test plasmids, respectively. (*D*) Expression of LDH–GH construct after transient transfection into Hep G2 cells. Details are as in *C*.

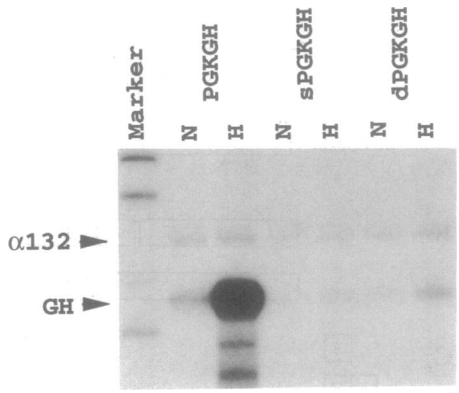

FIG. 3. Deletion analysis of PGK-1 enhancer/promoter region by RNase protection assay of reporter (GH) and control (α132) expression in Hep G2 cells transiently transfected with three different PGK–GH fusion constructs: PGKGH, full-length PGK promoter/enhancer (containing nucleotides −523 to −21 of the PGK gene); sPGKGH, shortened PGK promoter (containing nucleotides −270 to −21); and dPGKGH, a construct in which 18 bp have been deleted from PGKGH (containing nucleotides −523 to −309 and −290 to −21). Lanes: N, normoxic (20% O_2) incubation; H, hypoxic (1% O_2) incubation.

nuclease treatment was similarly active (16.2 ± 2.8-fold induction; $n = 6$), but deletions beyond this point led to progressive reduction in the amplitude of hypoxic induction, the most marked effect occurring with deletion of the region between 44 bp and 24 bp 5′ to the *Spe* I site (reduction from 8.0 ± 0.3-fold to 1.2 ± 0.1-fold hypoxic induction; $n = 4$ for each group). The functional importance of this region was confirmed by finding that a PGK–GH fusion gene from which 18 bp within this region had been removed (Fig. 1*A*, pdPGKGH) demonstrated vastly reduced hypoxic inducibility, the ratio of hypoxic to normoxic expression for PGKGH, sPGKGH, and dPGKGH being 15.4, 1.3, and 1.7, respectively (Fig. 3). In addition, these 18 bp may also play some role in the control of basal expression, since normoxic expression of the deletion construct was reduced by ≈50%.

Functional Analysis of the Oxygen-Regulated Element within the PGK Enhancer. For analysis of the functional characteristics of this isolated sequence, oligonucleotides (Table 1) were cloned into a site 10 bp 5′ to the TATA box of the herpes simplex thymidine kinase promoter (pTKGH, Fig. 1*C*). The capacities of monomers and/or concatamers to confer hypoxic inducibility are shown in Table 2. The 18-bp element (P18) whose deletion abrogated hypoxic induction of the PGK–GH construct was capable of conferring responsiveness to hypoxia when placed in either orientation. Concatamers operated more powerfully than monomers. Extension of P18 to a 24-bp element (P24) did not lead to increased activity. By contrast, a closely analogous sequence from the Epo 3′ enhancer (E18) was not active, indicating a difference in the function of the Epo and PGK hypoxic enhancer elements. Mutations within the PGK oligonucleotides were found to ablate function: P18M1 and P24M2 contained overlapping mutations within the area of closest homology between the PGK-1 and Epo sequences, and within the Epo HIF-1 binding site defined by Wang and Semenza (15) (Tables 1 and 2).

Binding of Hypoxia-Inducible Factors to the Oxygen-Regulated Element Within the PGK Enhancer. For analysis of the ability of this element to bind hypoxically inducible factors, double-stranded oligonucleotides (P24) were incubated with nuclear extract from normoxic and hypoxic Hep G2 cells. In electrophoretic mobility-shift assays, a pattern of sequence-specific inducible and constitutive retarded species was observed which closely resembled that obtained with an oligonucleotide from the Epo enhancer (E24) (Fig. 4 *A* and *B*). Nuclear extracts from normoxic and hypoxic HeLa cells produced essentially identical results, indicating that the

Table 2. Function of enhancer-containing TKGH constructs in transiently transfected Hep G2 cells

Enhancer		Ratio of hypoxic to normoxic expression
P18	+	2.5, 2.7
	++	11.9 ± 2.5 ($n = 4$)
	+++	18.0 ± 4.1 ($n = 5$)
	−−	11.1, 24.3
P18M1	++	1.0, 1.0
	++++	1.0, 0.9
P24	++	12.9, 17.8
P24M2	++	1.3, 1.2
	−−	1.4, 1.3
E18	++	1.1, 1.0
	−−	1.2, 1.2

Abbreviations for enhancer oligonucleotides are as in Table 1; +, oligonucleotide cloned in forward orientation; −, oligonucleotide cloned in reverse orientation; ++, +++, ++++, and −− refer to concatamers. Where two separate transfections were performed with a particular construct the hypoxic/normoxic ratio for each transfection is shown; where more than two transfections were performed the mean ± SEM is shown (n = number of separate transfections).

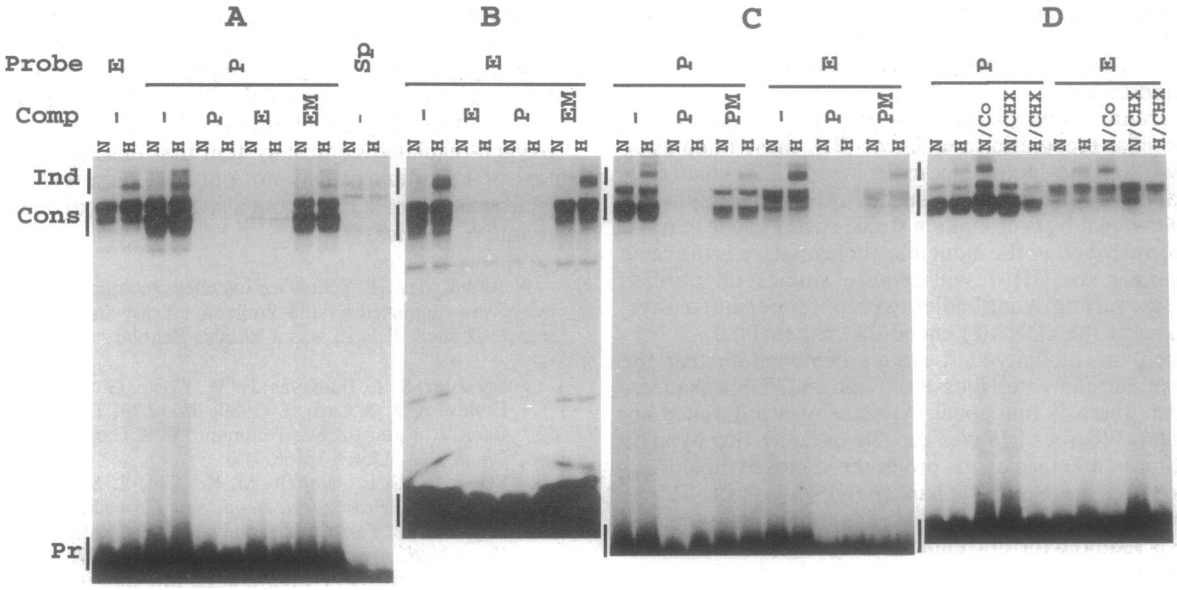

FIG. 4. Electrophoretic mobility-shift assays showing that oligonucleotides from the PGK-1 enhancer and Epo enhancer cross-compete for the binding of hypoxia-induced nuclear factors. The positions of inducible complexes (Ind), constitutive complexes (Cons), and free probe (Pr) are indicated by vertical bars. Labeled oligonucleotides used as probes are indicated (Probe), as are those lanes in which competition with a 200-fold molar excess of unlabeled oligonucleotide was employed (Comp). E and P refer to oligonucleotides E24 and P24, respectively; EM and PM refer to mutant oligonucleotides E24M3 and P24M2 (Table 1). Lanes show products of binding reactions with nuclear extract (5 μg) from normoxic (N) or hypoxic (H) Hep G2 (*A* and *B*) or HeLa (*C* and *D*) cells. (*A*) Similar inducible complexes bind labeled Epo oligonucleotides and labeled PGK-1 oligonucleotides. Unlabeled wild-type PGK and Epo oligonucleotides, but not mutant unlabeled Epo oligonucleotides, compete for formation of all specific complexes forming with the PGK-1 probe. Labeled Sp-1 (Sp) oligonucleotides bind equally to aliquots (1 μg) of these normoxic and hypoxic nuclear extracts, providing an internal control for extract quality. (*B*) Unlabeled PGK oligonucleotides compete with the complex formed with labeled Epo oligonucleotides. (*C*) PGK oligonucleotides bearing a 4-bp mutation are ineffective in competition for complexes with labeled PGK-1 or Epo oligonucleotides. (*D*) Effect of cobalt and cycloheximide on nuclear factors binding the PGK and Epo oligonucleotides. N/Co, cells exposed to 50 μM cobaltous chloride for 8 hr; N/CHX and H/CHX, cells exposed to 100 μM cycloheximide for 8 hr in either normoxia or hypoxia.

nuclear factors are common to both cell types (Fig. 4 *C* and *D*).

If the hypoxia-inducible factor binding the PGK enhancer sequence is the same as (or similar to) HIF-1, then it would be predicted that the PGK and Epo oligonucleotides would cross-compete. Fig. 4*A* shows competition of labeled PGK oligonucleotide with excess unlabeled Epo oligonucleotide; Fig. 4*B* shows competition of labeled Epo oligonucleotide with excess unlabeled PGK oligonucleotide. By contrast, functionally inactive mutant Epo and PGK oligonucleotides were almost completely ineffective as competitors (Fig. 4 *A–C*).

To test for further similarities in the binding of inducible nuclear factors by oligonucleotides from the PGK and Epo enhancers, nuclear extracts were prepared from cells which had been exposed to cycloheximide or cobaltous chloride. The results were entirely in keeping with the functional data for the PGK enhancer, in that cobaltous chloride induced the nuclear factor whereas cycloheximide treatment greatly reduced the hypoxic induction of this activity (Fig. 4*D*).

DISCUSSION

Epo production in response to hypoxia is restricted to particular cells within the liver and kidney (18). However, experiments outlined in the Introduction have shown that a very similar (if not identical) oxygen-regulated control system is present in most (possibly all) cell types. In cells which do not produce Epo the system must presumably be involved in the regulation of other genes, and we reasoned that it should therefore be possible to find oxygen-regulated control sequences in other genes which are similar both in terms of the functional characteristics of the response and in terms of binding to the same, or similar, nuclear factors.

The oxygen-regulated Epo control system has several well-defined characteristics. Cobalt induces Epo enhancer function and HIF-1, but neither are induced by heat shock (8, 10). Cyanide does not induce Epo expression, indicating that the sensing system is rather specific for hypoxia and not responsive to the metabolic consequences of interrupting mitochondrial electron transport (8). Abrogation of the hypoxic response by cycloheximide may indicate that synthesis of new protein is required (17). In this report we demonstrate that the widely expressed genes PGK-1 and LDH-A (which codes for the M subunit of LDH) respond to hypoxia, cobalt, cyanide, or cycloheximide in the same manner as Epo. This same pattern was reproduced in transient transfection of Hep G2 and HeLa cells with fusion genes comprising either 502 bp of the PGK enhancer/promoter or 233 bp of the LDH-A promoter coupled to a GH reporter. The greater amplitude of the responses observed in the transfection experiments, particularly in the case of PGK, may be explained by action on the endogenous gene of control elements not present in the transfected constructs, serving either to increase constitutive activity or to inhibit hypoxic induction. The crucial element in the PGK enhancer was found to be contained between positions −309 and −290 from the site of translational initiation, a region that is substantially conserved between mouse and man (19, 20) and that is known to possess constitutive enhancer activity (19) and to generate footprints in genomic DNA (20). There was sequence homology between this 18-bp PGK element (P18) and the Epo 3′ enhancer, including a region of 9 bp which was identical except for a G → C substitution. This 9-bp region overlapped with 7 of the 8 bp in the Epo enhancer indicated by Wang and Semenza (15) to be the Epo HIF-1 binding site. Electrophoretic mobility-shift analysis performed with 24-bp probes derived from Epo (E24) or PGK (P24) sequence showed that an inducible nuclear factor similar to, if

not identical with, HIF-1 binds to P24. The inducible complex formed with P24 had a mobility and characteristics similar to those of the complex formed with E24, and cross competition was demonstrated.

Since transcription factors may belong to families with closely similar recognition sequences (21), these findings do not prove that the factor binding to the PGK enhancer is HIF-1, and we have not been successful in determining its molecular weight by crosslinking studies using crude extract. Resolution of whether the inducible nuclear factor is the same as, or similar to, HIF-1 will require studies on purified extracts, the raising of antibodies to permit supershift assays, and cloning of the cDNA(s) encoding the gene(s).

Although the mobility-shift assays demonstrate that the protein-binding characteristics of the Epo and PGK enhancers are similar, there is functional evidence of a difference between them. Whereas P18 was capable of conferring hypoxic inducibility on a heterologous promoter, a closely analogous sequence from the Epo 3′ enhancer (E18) was not. This is consistent with previous work which has shown that sequence 3′ to E18 is required for Epo enhancer activity (5, 6, 9, 11, 22). There could be a number of explanations for this finding. It might be due to an ability of P18, but not E18, to recruit accessory factors necessary for functional activity of bound HIF-1. It might be due to P18 binding a species similar but not identical to HIF-1, with this related factor capable of hypoxically driving a promoter without the need for the same accessory factors as HIF-1.

What is the significance of the finding that an oxygen-regulated control system very similar, if not identical, to that which controls Epo is involved in the regulation of the PGK-1 and LDH-A genes? Although control of glycolysis is substantially effected by posttranslational modification of enzyme activity, there is evidence in muscle cells of coordinate regulation by oxygen of many metabolic enzymes at the level of mRNA (23, 24). Though some of these enzymes may have a secondary function—for instance, LDH-A binds single-stranded DNA (25)—the evidence of coordinate regulation strongly suggests that this is directed toward the primary metabolic function. Similar coordinated, reciprocal regulation of glycolytic and gluconeogenic enzymes by oxygen has been reported in rat hepatocytes, and it has been proposed that oxygen gradients in liver may thereby contribute to metabolic zonation (26). Interestingly, the reduction in glucagon-stimulated phospho*enol*pyruvate carboxykinase transcription in hypoxia could be mimicked by cobaltous ions and reduced by carbon monoxide (27, 28), features observed for the induction of Epo gene expression which led Goldberg *et al.* (17) to propose the operation of a heme protein sensor. It seems possible, therefore, that an oxygen-regulated control system similar to that controlling Epo may have a role in coordinating expression of many genes involved in glucose metabolism. Indeed, the system is not confined in its action to the genes examined in this report, since we have observed similar behavior with sequences from an enhancer at the human aldolase A locus. Regions within both the LDH-A and aldolase A control elements contain several sequences similar to HIF-1 sites: detailed deletional and mutational analysis will be required to prove or disprove which, if any, are critical to oxygen-regulated function.

Many enzymes of cellular metabolism, including PGK and LDH, show both structural and functional conservation in evolution (29), suggesting that induction of their mRNAs by hypoxia may be a phylogenetically ancient response. Substantial increase in PGK mRNA levels in an hypoxic environment has been found in *Penicillium* (30); levels of LDH mRNA increase in cereal roots in response to reduced oxygen tension (31); in bacteria, both glycolytic enzymes and the cytosolic form of LDH operative in fermentation can be induced under anaerobic conditions (32). Given that the ability to respond to changes in oxygen tension is fundamental and seems in the cases of the PGK and LDH genes to have been preserved in evolution, the possibility is raised that the oxygen-regulated control system first described in the context of Epo biology may not only act on a wide variety of genes in mammalian cells but also have an equivalent in more primitive species.

We thank Mrs. K. Yeates for excellent technical assistance. This work was supported by the Wellcome Trust and the Medical Research Council. B.L.E. was a Rhodes Scholar.

1. Schuster, S. J., Badiavas, E. V., Costa, G. P., Weinmann, R., Erslev, A. J. & Caro, J. (1989) *Blood* **73**, 13–16.
2. Beck, I., Ramirez, S., Weinmann, R. & Caro, J. (1991) *J. Biol. Chem.* **266**, 15563–15566.
3. Semenza, G. L., Nejfelt, M. K., Chi, S. M. & Antonarakis, S. E. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 5680–5684.
4. Pugh, C. W., Tan, C. C., Jones, R. W. & Ratcliffe, P. J. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 10553–10557.
5. Blanchard, K. L., Acquaviva, A. M., Galson, D. L. & Bunn, H. F. (1992) *Mol. Cell. Biol.* **12**, 5373–5385.
6. Madan, A. & Curtin, P. T. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 3928–3932.
7. Goldberg, M. A., Glass, G. A., Cunningham, J. M. & Bunn, H. F. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 7972–7976.
8. Maxwell, P. H., Pugh, C. W. & Ratcliffe, P. J. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2423–2427.
9. Pugh, C. W., Ebert, B. L., Ebrahim, O. & Ratcliffe, P. J. (1994) *Biochim. Biophys. Acta* **1217**, 297–306.
10. Wang, G. L. & Semenza, G. L. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 4304–4308.
11. Beck, I., Weinmann, R. & Caro, J. (1993) *Blood* **82**, 704–711.
12. Rathjen, P. D., Toth, S., Willis, A., Heath, J. K. & Smith, A. G. (1990) *Cell* **62**, 1105–1114.
13. McKnight, S. L. (1982) *Cell* **31**, 355–365.
14. Fukasawa, K. M. & Li, S. S. (1986) *Biochem. J.* **235**, 435–439.
15. Wang, G. L. & Semenza, G. L. (1993) *J. Biol. Chem.* **268**, 21513–21518.
16. Dignam, J. D., Lebovitz, R. M. & Roeder, R. G. (1983) *Nucleic Acids Res.* **11**, 1475–1489.
17. Goldberg, M. A., Dunning, S. P. & Bunn, H. F. (1988) *Science* **242**, 1412–1415.
18. Ratcliffe, P. J. (1993) *Kidney Int.* **44**, 887–904.
19. McBurney, M. W., Sutherland, L. C., Adra, C. N., Leclair, B., Rudnicki, M. A. & Jardine, K. (1991) *Nucleic Acids Res.* **19**, 5755–5761.
20. Pfeifer, G. P., Tanguay, R. L., Steigerwald, S. D. & Riggs, A. D. (1990) *Genes Dev.* **4**, 1277–1287.
21. Curran, T. & Franza, B. J. (1988) *Cell* **55**, 395–397.
22. Semenza, G. L. & Wang, G. L. (1992) *Mol. Cell. Biol.* **12**, 5447–5454.
23. Webster, K. A. (1987) *Mol. Cell. Biochem.* **77**, 19–28.
24. Webster, K. A., Gunning, P., Hardeman, E., Wallace, D. C. & Kedes, L. (1990) *J. Cell Physiol.* **142**, 566–573.
25. Sharief, F. S., Wilson, S. H. & Li, S. S. (1986) *Biochem. J.* **233**, 913–916.
26. Wolfle, D. & Jungermann, K. (1985) *Eur. J. Biochem.* **151**, 299–303.
27. Kietzmann, T., Schmidt, H., Probst, I. & Jungermann, K. (1992) *FEBS Lett.* **311**, 251–255.
28. Kietzmann, T., Schmidt, H., Unthan, F. K., Probst, I. & Jungermann, K. (1993) *Biochem. Biophys. Res. Commun.* **195**, 792–798.
29. Markert, C. L., Shaklee, J. B. & Whitt, G. S. (1975) *Science* **189**, 102–114.
30. Renno, D. V., Saunders, G., Bull, A. T. & Holt, G. (1992) *Curr. Genet.* **21**, 49–54.
31. Hondred, D. & Hanson, A. D. (1990) *Proc. Natl. Acad. Sci. USA* **87**, 7300–7304.
32. Mat, J. F., Alam, K. Y. & Clark, D. P. (1989) *J. Bacteriol.* **171**, 342–348.