The Journal of Experimental Biology 201, 1153–1162 (1998)
Printed in Great Britain © The Company of Biologists Limited 1998
JEB1358

OXYGEN SENSING, HYPOXIA-INDUCIBLE FACTOR-1 AND THE REGULATION OF MAMMALIAN GENE EXPRESSION

PETER J. RATCLIFFE*, JOHN F. O'ROURKE, PATRICK H. MAXWELL AND CHRISTOPHER W. PUGH
Erythropoietin Group, Room 425, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DS, UK
*e-mail: peter.ratcliffe@hammer.imm.ox.ac.uk

Accepted 15 October 1997; published on WWW 24 March 1998

Summary

A great many aspects of the anatomy and physiology of large animals are constrained by the need to match oxygen supply to cellular metabolism and appear likely to involve the regulation of gene expression by oxygen. Some insight into possible underlying mechanisms has been provided by studies of erythropoietin, a haemopoietic growth factor which stimulates red cell production in response to hypoxia. Studies of hypoxia-inducible *cis*-acting sequences from the erythropoietin gene have led to the recognition of a widespread transcriptional response to hypoxia based on the activation of a DNA-binding complex termed hypoxia-inducible factor-1 (HIF-1). Perturbation of the transcriptional response by particular transition metal ions, iron chelators and certain redox-active agents have suggested a specific oxygen sensing mechanism, perhaps involving a haem protein in a flavoprotein/cytochrome system. In addition to erythropoietin, HIF-1-responsive genes include examples with functions in cellular energy metabolism, iron metabolism, catecholamine metabolism, vasomotor control and angiogenesis, suggesting an important role in the coordination of oxygen supply and cellular metabolism. In support of this, we have demonstrated an important role for HIF-1 in tumour angiogenesis. HIF-1 itself consists of a heterodimer of two basic-helix–loop–helix proteins of the PAS family, termed HIF-1α and HIF-1β, although other closely related members of this family may also contribute to the response to hypoxia. We have fused domains of HIF-1 genes to heterologous transcription factors to assay for regulatory function. These experiments have defined several domains in HIF-1α which can independently confer the hypoxia-inducible property, and they suggest a mechanism of HIF-1 activation in which post-translational activation/derepression of HIF-1α is amplified by changes in HIF-1α abundance most probably arising from suppression of proteolytic breakdown. Pursuit of the mechanism(s) underlying these processes should ultimately lead to better definition of the oxygen-sensing process.

Key words: erythropoietin, hypoxia-inducible factor-1, transcription, oxygen, redox.

Introduction

Reduction of oxygen provides one of the most important sources of metabolic energy for living organisms, and the full (four electron) reduction of dioxygen (O_2) to water has unusual properties which are well suited to this role. First, the energy available from reduction by biological substrates is large and, second, energy barriers to the activation of dioxygen mean that the reduction can be enzymatically controlled and that relatively high concentrations of oxygen can be tolerated. Following a major increase in oxygen production by photosynthesis 1–2 billion years ago, atmospheric oxygen concentrations have been very much greater than those required for maximal rates of mitochondrial electron transfer, and this has enabled multicellular organisms to utilize large oxygen gradients for transfer purposes. Even bigger organisms have evolved special transport systems (e.g. lungs, heart and blood) to augment the potential of these gradients to support oxygen transfer over large distances. The intricacy of the anatomical organization underlying oxygen transfer by these organs is striking, as is the microscopic organization of the oxygen-consuming tissues. This is seen, for instance, in analyses of blood capillary density, in the zonation of metabolic enzyme activities and in the intracellular disposition of organelles such as mitochondria (Weibel *et al.* 1992; Jungermann, 1995). In fact, the whole of the macro- and micro-anatomy of a large animal have to be constrained by the need for adequate provision of oxygen.

Such processes must be dependent on mechanisms for the sensing of oxygen and the regulation of gene expression. One potential mechanism of sensing is through the adequacy of high-energy phosphate supply from mitochrondrial oxidative phosphorylation. Many processes are probably responsive to such 'metabolic' oxygen sensing (Adair *et al.* 1990), but it has long been clear that there are difficulties in proposing the operation of such a system in isolation. First, it would require a level of continuing metabolic compromise to generate an 'error' signal. Second, the multiple processes involved in

oxygen delivery appear to be tightly coordinated so that capacity at different points is well matched, and it is difficult to understand how sensing at a single distal point could provide the necessary control. Third, analyses of two systems long known to be involved in the regulation of mammalian oxygen supply (the control of ventilation by the carotid body and the regulation of red cell production by erythropoietin) have strongly suggested the operation of other processes. In the case of erythropoietin production, the response to hypoxia cannot be mimicked by inhibitors of oxidative phosphorylation (Necas and Neuwirt, 1972). For the carotid body, it is clear that, although some response to cyanide exposure is observed, the operating P_{O_2} is very much higher than that limiting mitochondrial respiration (Gonzalez *et al.* 1994).

The rapid dynamic response and extreme sensitivity of these systems has attracted great interest in the underlying mechanism(s) of oxygen sensing. Though each had traditionally been regarded as involving the operation of a highly specialized and tissue-restricted 'sensor', recent evidence indicates that at least some of the underlying processes are much more widely distributed. Oxygen-sensitive K^+ channels, first recognized in the carotid body, have now been described in a number of different vascular cells, and oxygen sensitivity has been recognized for a variety of different ion channels (Weir and Archer, 1995). Studies of erythropoietin gene regulation have provided even more powerful evidence of a general oxygen-sensing system with a widely operative role in the regulation of gene expression (Maxwell *et al.* 1993; Bunn and Poyton, 1996).

Oxygen sensing and the regulation of the erythropoietin gene

Erythropoietin is the major regulator of erythropoiesis. Production by the kidneys and liver is increased in response to a reduction in haematocrit, arterial hypoxaemia or an increased haemoglobin oxygen-affinity. In this way, a feedback loop is completed in which a reduction in blood oxygen availability is sensed and corrected by stimulating red cell production in the bone marrow. Increased erythropoietin production parallels increases in erythropoietin mRNA production, and both are induced more than 100-fold by severe stimulation (Ratcliffe, 1993). In addition to this dramatic response to severe perturbations of oxygen delivery, small increases in erythropoietin production are induced by much more minor stimuli, such as the donation of a unit of blood (Lorentz *et al.* 1991). Furthermore, the basal plasma level is suppressed in cases of primary polycythaemia, demonstrating that regulation is operative in the normal physiological range of oxygen delivery (Cotes *et al.* 1986).

In enabling a molecular approach to the underlying mechanism, an important step was made by Goldberg *et al.* (1987), who demonstrated oxygen-regulated gene expression and erythropoietin production by certain human hepatoma cells. As had been observed *in vivo*, production was also induced by particular transition metal ions (cobalt II, nickel II and manganese II). When cells were exposed to carbon monoxide, stimulation of erythropoietin production by hypoxia, but not by cobaltous ions, was much reduced. This led the authors to propose the existence a haemoprotein-sensing molecule which (like haemoglobin) would respond to the liganding of oxygen (or carbon monoxide) with a conformational change. Arguing from knowledge that cobaltous ions are a substrate for ferrochelatase and that cobalt protoporphyrin does not bind oxygen, it was proposed that cobalt could substitute for iron in such a sensor, leading to a constitutive deoxy form which would stimulate gene expression even in normoxic cells (Goldberg *et al.* 1988).

Other data have suggested the operation of a redox process in the oxygen-sensing mechanism. For instance, induction of erythropoietin gene expression is very sensitive to hydrogen peroxide. Hydrogen peroxide production is itself oxygen-dependent, being reduced during hypoxia. Fandrey *et al.* (1994) have therefore proposed that this molecule is a signal pathway intermediate and have provided supporting evidence from the action of compounds with known effects on hydrogen peroxide production. However, it is not yet known whether these compounds act by perturbing hydrogen peroxide level within the concentration range observed in the normal normoxic to hypoxic transition, so that the evidence for hydrogen peroxide level as a sensor is incomplete. Another interesting characteristic is sensitivity to iodonium compounds (Gleadle *et al.* 1995*b*). These compounds are believed to operate as redox-activated flavoprotein inhibitors (O'Donnell *et al.* 1993). Since flavoproteins are often linked to haem proteins in electron transport systems, it might be that the interaction of oxygen with such a system forms the basis of sensing. Though such a model might accommodate the haem protein hypothesis, a different mode of operation is required. If it is proposed that a reduced electron flow to oxygen signals hypoxia and somehow activates the system, why do iodonium compounds and carbon monoxide (which should also act to block electron flow) not activate the system? A possible explanation is that the interaction of oxygen with a haem group regulates or even diverts electron flow from a signalling redox couple by providing an alternative electron acceptor.

Yet another characteristic requiring explanation is the stimulation of erythropoietin gene expression by iron chelators (Wang and Semenza, 1993*a*; Gleadle *et al.* 1995*a*). Here again, two quite different explanations have been suggested. Haem iron is not chelatable, but iron chelation might interfere with the synthesis of a rapidly turning over haem protein or with the function of a ferroprotein with a chelatable iron moiety. Alternatively, iron chelation might interfere with the generation of signalling active oxygen species through the Haber–Weiss reaction.

To date, the complexity of redox systems and our incomplete understanding of their many potential interactions with oxygen in mammalian cells has made it difficult to prove or disprove these interesting hypotheses. Together with the definition of the hepatoma-based tissue culture system, molecular cloning of the erythropoietin gene provided another

new approach to the problem – from the gene outwards. Isolation of the DNA control sequences which mediate oxygen-regulated expression could be followed proximally from the DNA-binding transcription factors to the signal transduction molecules, and so on, to the regulator. In pursuit of this, sequences from the Epo (erythropoietin) locus were assayed for oxygen-regulated activity after transfection of the Epo-producing cell lines Hep3B or HepG2. These studies identified a powerful regulatory element lying 3′ to both the human and murine genes (the Epo 3′ enhancer) (Beck *et al.* 1991; Pugh *et al.* 1991; Semenza *et al.* 1991). Detailed studies of this enhancer defined several binding sites, one of which was critical for hypoxia-inducible function and bound a complex termed hypoxia-inducible factor-1 (HIF-1) (Semenza and Wang, 1992). In keeping with an important regulatory role, activation of HIF-1 was found to share several key physiological characteristics with erythropoietin regulation: progressive activation by hypoxia of graded severity, activation by cobaltous ions and sensitivity to the protein synthesis inhibitor cycloheximide. Subsequently, affinity-purification of the complex enabled Semenza and colleagues to identify encoding cDNAs and to demonstrate that the DNA-binding complex was a heterodimer of two basic-helix–loop–helix proteins of the PAS family, HIF-1α and HIF-1β (Wang *et al.* 1995). HIF-1α was a newly defined member of this family. However, HIF-1β had previously been identified as a dimerization partner for another basic-helix–loop–helix PAS protein, the aryl hydrocarbon receptor (AHR), where it is essential for the xenobiotic response and is termed the aryl hydrocarbon nuclear receptor translocator (ARNT) (Reyes *et al.* 1992). The term PAS is an acronym derived from the names of the first members of this gene family Per, AHR, ARNT and *Sim*; *Per* and *Sim* are *Drosophila* genes involved in the control of periodic and midline gene expression, respectively. The defining characteristic is the PAS domain, an imperfectly duplicated sequence of approximately 50 amino acids containing the characteristic motif His-*X*-*X*-Asp (Huang *et al.* 1993). Recent data base searches and cloning experiments have greatly expanded the family (Hogenesch *et al.* 1997; Tian *et al.* 1997; Zhou *et al.* 1997). At least one other member, endothelial PAS protein-1 (EPAS-1), appears to be directly involved in hypoxic gene regulation, whilst several others appear to be able to interact with the HIF-1 dimerization partners.

A general system of gene regulation by oxygen

What was not expected when the analysis of erythropoietin gene regulation began was that this would uncover a general mechanism of cellular oxygen sensing and transcriptional control. The first clear evidence that this system was operative outside the context of erythropoietin regulation was provided by transfection studies of the erythropoietin enhancer (Maxwell *et al.* 1993). In contrast with the tight tissue restriction of erythropoietin gene expression, transiently transfected reporter genes linked to the erythropoietin enhancer showed similar hypoxia-inducible activity in both the hepatoma cells and a wide variety of non-erythropoietin-producing cells. In keeping with this, HIF-1 was found to be widely expressed (Wang and Semenza, 1993*b*), and functionally critical HIF-1 sites were defined in other genes, the first to be recognized encoding the glycolytic enzymes phosphoglycerate kinase-1 and lactate dehydrogenase A (Firth *et al.* 1994). The definition of a common regulatory mechanism for genes with functions quite different from erythropoietin suggested that many other HIF-1-responsive genes might exist, and a large number have now been identified through functional similarities with the induction of erythropoietin gene expression, reduced expression in HIF-1-deficient cells or the functional definition of HIF-1-binding sites in *cis*-acting sequences. These include genes involved in various aspects of energy metabolism (glucose transporters, glycolytic and gluconeogenic enzymes), iron metabolism (transferrin), catecholamine metabolism (tyrosine hydroxylase), vasomotor control (nitric oxide synthases, endothelin-1) and angiogenesis (vascular endothelial growth factor, platelet-derived growth factor) (see Table 1 for references). Though these studies strongly suggested a major role for HIF-1 in many physiological and pathophysiological processes, this cannot be immediately deduced from studies of gene expression in tissue culture.

In the whole organism, hypoxia most commonly occurs in the more complex setting of ischaemia and, in assessing the importance of HIF-1, it is important to understand the circumstances under which activation occurs *in vivo*, the effects on gene expression in this setting and the pathophysiological consequences. Such studies will ultimately require the development of techniques for the assay of HIF-1 activation that can be applied to heterogeneous tissues in whole animals. In the meantime, we have addressed this issue (at least in the tumour setting) by analysis of tumour xenografts from tissue culture cells in nude mice (Maxwell *et al.* 1997). We examined a series of hepatoma (mouse hepa-1) cell lines which were originally selected for a defective xenobiotic response through resistance to benzo[a]pyrene (Hankinson, 1979). Cells from one complementation group of resistant cells are deficient in the common heterodimerization partner HIF-1β/ARNT (Hoffman *et al.* 1991) and, in addition to manifesting a deficient xenobiotic response, they are unable to form the HIF-1 complex and show defective oxygen-regulated gene expression in tissue culture (Wood *et al.* 1996). We therefore compared gene expression, vascularization and growth in tumours from wild-type cells, a HIF-1β/ARNT-deficient line (c4), a revertant selected from c4 which has regained wild-type levels of HIF-1β/ARNT (Rc4) and a line (c31) that has a different defect in the xenobiotic response and is functional for HIF-1.

Fig. 1A shows an *in situ* hybridization study for the glucose transporter Glut-3 and vascular endothelial growth factor (VEGF) mRNAs in tumours from wild-type and HIF-1β/ARNT-defective (c4) cells. In wild-type tumours, areas of intense focal expression of Glut-3 and VEGF mRNA were

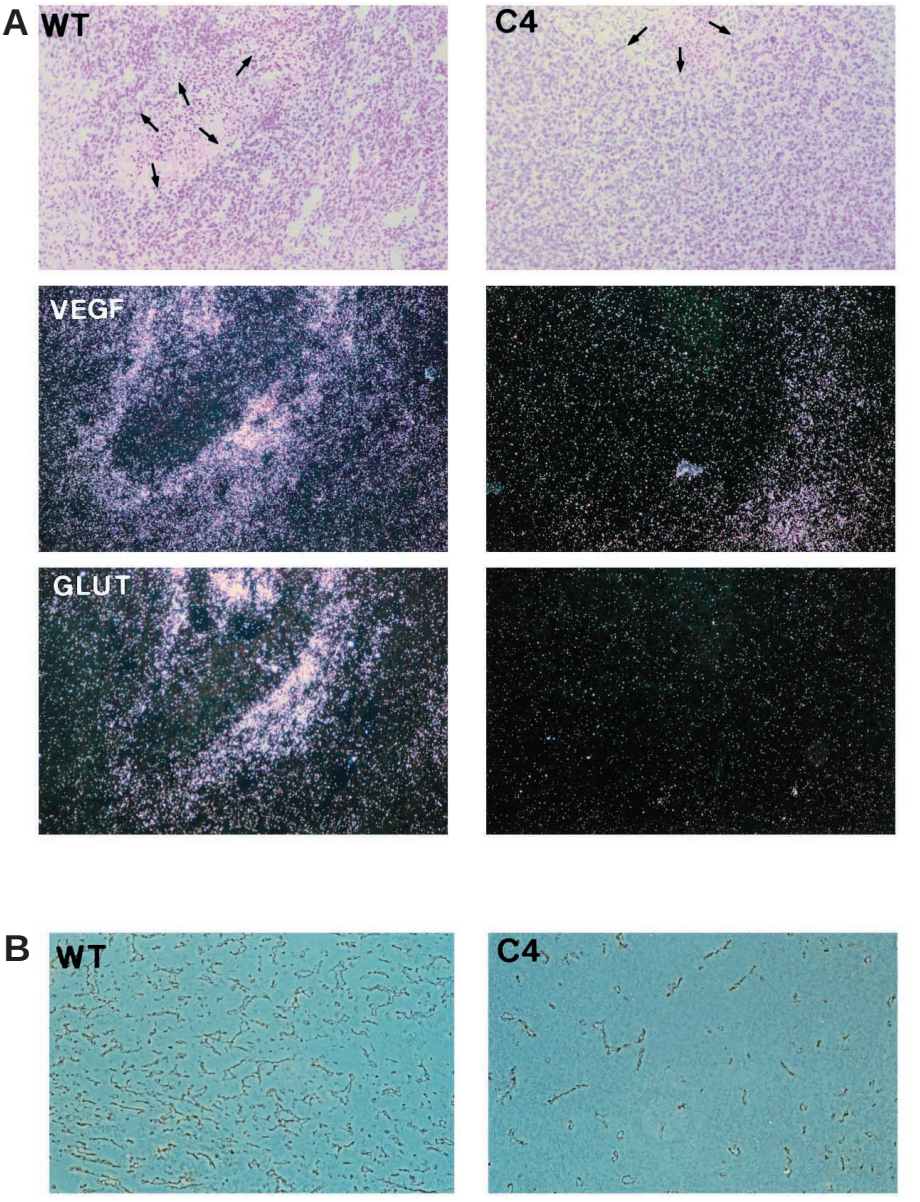

Fig. 1. Gene expression and vascularity in tumour grafts from wild-type (WT) hepa-1 and HIF-1β/ARNT-deficient c4 cells (C4). (A) *In situ* hybridisation for vascular endothelial growth factor (VEGF) and glucose transporter (Glut-3) mRNA in wild-type Hepa-1 (left) and c4 (right) tumours. Bright-field (upper) and dark-field (centre) views of sections hybridised to the antisense VEGF probe. Semi-serial sections hybridised for Glut-3 mRNA (GLUT) are also shown (lower panels). In the wild-type Hepa-1 tumour, high-level VEGF and Glut-3 expression borders on an area of necrosis (arrows). This focal expression is not seen or much reduced (arrows) in the c4 tumours. (B) Comparison of vascular development in tumour xenografts derived from wild-type Hepa-1 cells and the HIF-1β/ARNT derivative c4. Immunoperoxidase labelling for vascular endothelium using a monoclonal antibody to CD31/PECAM. More capillaries are present in the wild-type tumour (left-hand panel) than in the c4-derived tumour (right-hand panel). Data from Maxwell *et al.* (1997).

often observed, and these were often close to necrotic regions. This intense focal gene expression was not seen in tumours derived from the mutant (c4) cells, but was observed in the revertant line (Rc4) and in the c31 cells, both of which form HIF-1 normally. This pattern of VEGF expression around (presumably hypoxic) necrotic zones has been noted previously and has led to the suggestion that hypoxia is a major regulator of tumour angiogenesis (Plate *et al.* 1992; Shweiki *et al.* 1992). Our experiments show that, at least in the context of these hepatoma cells, activation of HIF-1 is largely responsible for this pattern of gene expression within the tumour. When vascularity and growth were examined, substantial differences were observed which correlated with the presence or absence of a functional HIF-1β/ARNT gene product. In particular, tumours derived from the HIF-1β/ARNT-deficient (c4) cells showed a striking reduction in vascularity (Fig. 1B).

These experiments strongly suggest that HIF-1 is activated within solid tumours and provide evidence for an important pathophysiological role in this setting. Although the evidence linking hypoxia and erythropoietin regulation, through HIF-1, to hypoxia and tumour angiogenesis is rather strong, it is necessary to recognize the importance of other factors in shaping the pattern of gene expression. The genes listed in Table 1 are apparently linked to a common mechanism of regulation by oxygen, yet the pattern of inducible expression is in each case quite different. For instance, the VEGF gene is also expressed in normal tissues – yet in many organs, including kidney, it is induced modestly, if at all, by anaemic stimulation sufficient to generate a massive increase in erythropoietin production (Sandner *et al.* 1996). These differences cannot easily be explained by cell-specific gene expression and heterogeneous organ oxygenation. Rather,

Table 1. *Some processes involved in oxygen-regulated gene expression*

Process	References	Oxygen-regulated gene	Expression
Erythropoiesis	Goldberg *et al.* 1991	Erythropoietin	↑
Glycolysis	Firth *et al.* 1994, 1995; Marti *et al.* 1994; Semenza *et al.* 1994, 1996; Ebert *et al.* 1995	Lactate dehydrogenase A	↑
		Phosphoglycerate kinase-1	↑
		Aldolase A and C	↑
		Phosphofructokinase L and C	↑
		Pyruvate kinase M	↑
		Enolase A	↑
Gluconeogenesis	Hellkamp *et al.* 1991; Kietzmann *et al.* 1993	Phosphoenolpyruvate carboxykinase	↓
Glucose transporters	Ebert *et al.* 1995; O'Rourke *et al.* 1996	Glucose transporter 1	↑
		Glucose transporter 3	↑
		Glucose transporter 2	↓
Catecholamine synthesis	Czyzyk-Krzeska *et al.* 1992	Tyrosine hydroxylase	↑
Iron transport	Rolfs *et al.* 1997	Transferrin	↑
Growth factors	Kourembanas *et al.* 1990; Shweiki *et al.* 1992; Goldberg and Schneider, 1994; Gleadle *et al.* 1995*a*	Vascular endothelial growth factor	↑
		Transforming growth factor β	↑
		Platelet-derived growth factor B	↑
		Placental growth factor	↓
Nitric oxide synthesis	McQuillan *et al.* 1994; Melillo *et al.* 1995	Inducible nitric oxide synthase	↑
		Endothelial nitric oxide synthase	↓
Vasomotor regulator	Bodi *et al.* 1995	Endothelin-1	↑
High-energy phosphate metabolism	O'Rourke *et al.* 1996	Adenylate kinase-3	↑
Haem metabolism	Lee *et al.* 1997	Haem oxygenase 1	↑

Evidence for a common mechanism of regulation for the genes listed is provided by functional similarities in the response (e.g. induction by hypoxia, Co^{2+} ions and iron chelators) and/or the definition of functionally critical HIF-1 sites in *cis*-acting sequences and/or altered regulation in mutant cells which do not produce HIF-1.

An arrow indicates increased (↑) or reduced (↓) expression during hypoxia.

they suggest the operation of cooperative effects at the cellular level, in which transcriptional activation by HIF-1 requires ancillary factors that may be cell-type-specific or responsive to some other aspect of cellular physiology. For example, VEGF expression is also increased by a number of activated oncogenes, and it may be that an interaction with such molecules constrains the circumstances under which the gene is inducible by HIF-1 (Mazure *et al.* 1996). Other insights into the mechanisms underlying the selective responses to HIF-1 have come from studies of sequences close to HIF-1 binding sites. For instance, the function of the Epo enhancer is also dependent on at least two other sites adjacent to the HIF-1-binding site (Blanchard *et al.* 1992; Semenza and Wang, 1992; Pugh *et al.* 1994). One of these has been shown to bind the tissue-specific transcription factor hepatic nuclear factor (HNF-4) (Galson *et al.* 1995). In the lactate dehydrogenase A gene promoter, a functional interaction between an HIF-1 site and a cyclic AMP response element has been defined (Firth *et al.* 1995). Thus, the assembly of a functional transcriptional complex requires factors other than HIF-1, and it appears likely that this requirement for cooperative factors is used to define the individual features of an inducible response.

Activation of HIF-1

In pursuing the mechanism of oxygen sensing, a great deal of attention has been turned towards analysis of the mechanism of activation of HIF-1. In keeping with the demonstration of HIF-1 DNA-binding activity in a wide variety of cell types, mRNAs for HIF-1α and HIF-1β have been found in all cells and tissues examined (Wenger *et al.* 1996; Wiener *et al.* 1996). In the majority of studies, the mRNAs themselves were not found to be inducible by hypoxic stimulation, indicating that the activation of HIF-1 involves post-translational and/or translational mechanisms (Huang *et al.* 1996; Wenger *et al.* 1996; Wood *et al.* 1996). We were unable to observe translational regulation in fusions containing the HIF-1 promoter and 5′ untranslated region fused to a luciferase reporter and focused our attention on analyses likely to define post-translational mechanisms of regulation. As with other transcription factors, studies of regulatory mechanisms are

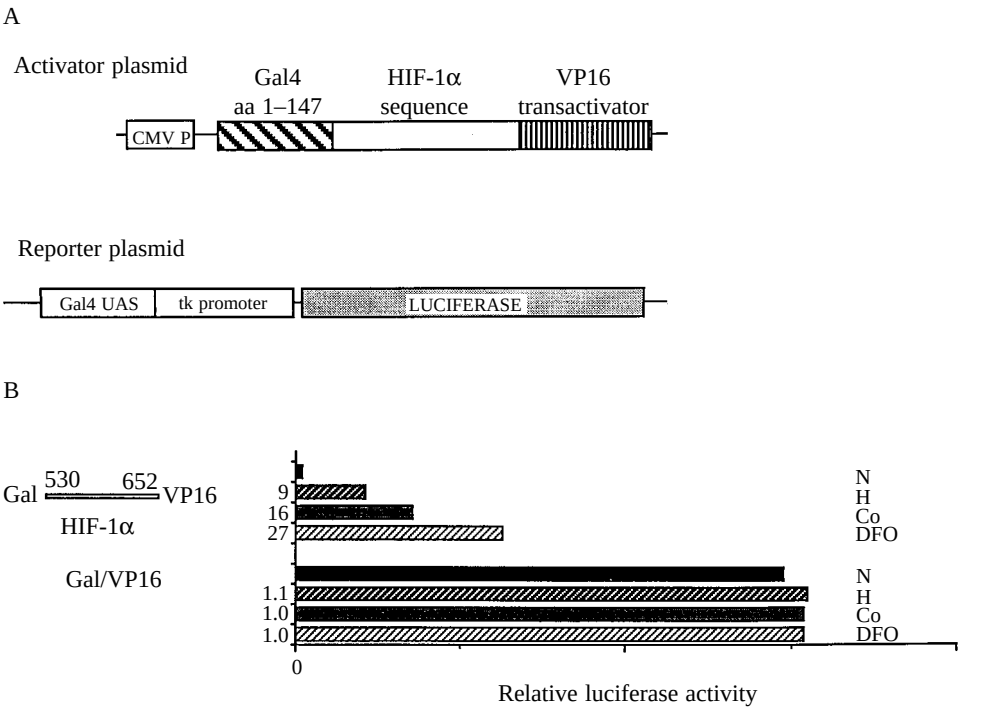

Fig. 2. Definition of a regulatory domain in HIF-1α. (A) Schematic diagram of the activator and reporter plasmid construction. In the activator plasmid, the HIF-1α sequence is incorporated between the DNA-binding domain of Gal4 (amino acids 1–147) and the transactivator from the viral protein VP16 (amino acids 410–490). CMV P, cytomegalovirus promoter; UAS, upstream activating site; tk, thymidine kinase. (B) Activity after transfection in Hep3B cells cultured in normoxia (N), in hypoxia (1% oxygen) (H) or exposed to 100 μmol l^{-1} cobaltous ions (Co) or 100 μmol l^{-1} desferrioxamine (DFO). The Gal/VP16 fusion shows a high level of constitutive activity. Inclusion of the HIF-1α sequence (amino acids 530–652) results in much reduced activity in normoxic cells that is increased by stimulation. The numbers to the left-hand side of each bar represent the ratio of stimulated to normoxic activity. Data from Pugh *et al.* (1997).

potentially confounded by dependence on a series of interrelated events (e.g. dimerization, DNA binding, interaction with transcriptional activators) for an assayable response. For this reason, we have sought to define regions of HIF-1 genes that can independently confer oxygen-regulated activity on heterologous transcription factors. In this work, we have used two types of chimeric gene: those in which the heterologous transcription factor encoded a DNA-binding activity but lacked *trans*-activation; and others in which an activation domain was either included with the heterologous DNA binding domain or added from a second heterologous gene. This allowed for the analysis of regulatory domains from HIF-1 genes which did not necessarily contain intrinsic activation potential. In these experiments, we found that sequences from HIF-1α but not from HIF-1β could convey the hypoxia-inducible property, indicating a regulatory role for the α subunit (Pugh *et al.* 1997).

An example of such an experiment is shown in Fig. 2. The fusion of the powerful activation domain (amino acids 410–490) from the herpes simplex virus protein VP16 to a DNA-binding domain derived from amino acids 1–147 of the yeast transcription factor Gal4 creates a powerful transcription factor which activates expression from promoters containing a Gal4 binding site. When co-transfected into Hep3B cells with a gene containing such a promoter linked to a luciferase reporter, this Gal4/VP16 fusion is highly active. When particular sequences derived from the HIF-1α gene are encoded between the Gal4 and the VP16 sequences, two striking effects are observed. First, in normoxic cells, the overall level of activity is much reduced. Second, when cells are exposed to hypoxia, cobaltous ions or desferrioxamine, the apparent repression is greatly relieved, producing a regulated activity which reflects that of HIF-1. The experiment illustrated in Fig. 2B demonstrates a regulatory domain of HIF-1α between amino acids 530 and 652. In other work, we have defined sequences lying further towards the amino terminus of HIF-1α that also have this property (J. F. O'Rourke *et al.*, unpublished work).

As a first analysis of the mechanisms by which such regulatory domains operate, we assayed levels of protein product in cells transfected with similar fusion genes. Studies so far have demonstrated that, at least with the regulatory sequences between amino acids 530 and 652, a major effect is observed on the level of expressed fusion protein. In normoxic cells, the fusion protein levels are much reduced by the inclusion of this sequence, with this reduction being relieved not only by exposure to hypoxia, cobaltous ions and desferrioxamine, but also by a variety of inhibitors of proteosomally mediated proteolysis. This suggests that one mechanism of regulation might be mediated by proteolysis of HIF-1α, with activation involving an inducible blockade of a constitutively high rate of degradation targetted through these sequences.

However, it is unlikely that this is the only mechanism of regulation. In our chimeric transcription factor assays, regulated activity was also observed which appeared to be independent of the level of expressed protein. This was particularly the case with the most C-terminal HIF-1α sequences. When fused to the Gal4 DNA-binding domain, C-terminal sequences from HIF-1α demonstrated oxygen-regulated *trans*-activation. Detailed analysis of these sequences showed that, while HIF-1α amino acids 786–826 showed constitutive *trans*-activation, amino acids lying immediately N-terminal to position 786 suppressed total

activity and conferred inducibility (J. F. O'Rourke *et al*., unpublished work). Thus, a Gal4 fusion bearing amino acids 775–826 showed reduced activity in normoxic cells which was relieved by hypoxia. In this case, the fusion protein appeared to be expressed at a similar level in normoxic and stimulated cells.

Overall, these findings suggest a model involving at least two mechanisms of HIF-1 activation, with induction or derepression of activation domains being amplified by regulation of transcription factor abundance, occurring through changes in protein stability (Pugh *et al.* 1997). Such a model would be consistent with assays of endogenous HIF-1 immunoactivity. These demonstrate rapid nuclear accumulation of HIF-1α during hypoxia from very low levels in unstimulated cells (Wang *et al.* 1995; Huang *et al.* 1996). Upon reoxygenation, levels decline within minutes, indicating a very short half-life in normoxic cells. Similar studies of HIF-1β/ARNT in whole-cell extracts have shown modest or even absent induction by hypoxia, with considerable levels being present in unstimulated cells (Huang *et al.* 1996). Consistent with a constitutive excess of the β subunit, forced overexpression of the α subunit in normoxic cells is itself sufficient to drive HIF-1-dependent reporter gene expression. However, in keeping with a dual mechanism of activation, hypoxic stimulation of these cells further enhances activity (Jiang *et al.* 1996).

The definition of regulatory and activation domains for HIF-1α now points the way to defining the next steps in regulation. Quite possibly, this will involve regulated cofactors. For instance, the proteosome is constitutively active, and any regulated activity on HIF-1α could be modulated by a cofactor or by a modification of the target. Similarly, the known activation domains possess no clear homology with classical activator sequences, and it may be that they operate to recruit a cofactor. Interestingly, one known transcriptional coactivator, p300, is known to affect the activity of the system (Arany *et al.* 1996).

Evolutionary origins of HIF-1

One important question regarding a system which appears to play such a central role in mammalian gene regulation by oxygen is what its role might be in non-mammalian cells – in particular, in simpler organisms which might facilitate genetic analysis of the sensing mechanism. To address this, we have studied the binding of nuclear extracts from non-mammalian cells to an oligonucleotide from the mouse erythropoietin enhancer (Nagao *et al.* 1996). So far, our clearest results have been from *Drosophila melanogaster* SL2 cells and are shown in Fig. 3. These cells contain a sequence-specific hypoxia-inducible DNA-binding activity which has a similar electrophoretic mobility to HIF-1, making it highly likely that a homologous system is operative in insects. In ongoing experiments, we are exploring the nature of this activity, but it is clearly of interest that the first basic-helix–loop–helix PAS proteins were defined in *Drosophila* and that more recent

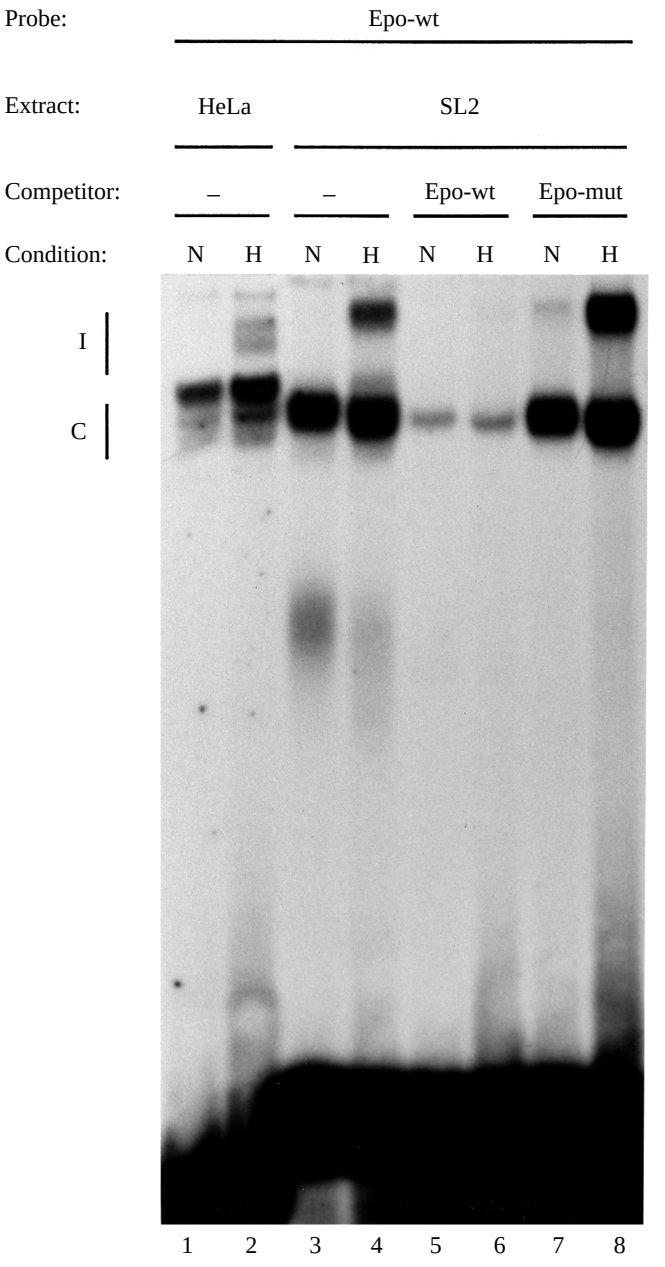

Fig. 3. DNA-binding assay demonstrating HIF-1-like activity in *Drosophila* SL2 cells. 5 μg of nuclear extract from normoxic (N) or hypoxic (H) HeLa cells (lanes 1,2) or SL2 cells (lanes 3–8) were incubated with ^{32}P-labelled wild-type Epo (EPO-wt) oligonucleotide from the mouse erythropoietin enhancer. An inducible complex is observed in both cells types and is removed by competition with excess unlabelled Epo-wt oligonucleotide, but not by a similar sequence which contains a 4 bp mutation at the HIF-1 binding site (EPO-mut). Data from Nagao *et al.* (1996). I, inducible complex; C, constitutive complex.

studies have isolated new genes, *sima* (Nambu *et al.* 1996) and *trachealess* (Wilk *et al.* 1996), which show even better sequence similarity and (at least in the case of *trachealess*) have a clear role in the organization of oxygen-delivery systems in the fruit fly. Thus, it seems likely that the

uncovering of this widespread system of oxygen-regulated gene expression in mammalian cells will soon also lead to the recognition of a connection with oxygen-regulated gene expression in non-mammalian systems.

The work conducted in the authors' laboratory was supported by the Wellcome Trust and the Medical Research Council, UK.

References

ADAIR, T. H., GAY, W. J. AND MONTANI, J.-P. (1990). Growth regulation of the vascular system: evidence for a metabolic hypothesis. *Am. J. Physiol.* **259**, 393–404.

ARANY, Z., HUANG, L. E., ECKNER, R., BHATTACHARYA, S., JIANG, C., GOLDBERG, M. A., BUNN, H. F. AND LIVINGSTON, D. M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. *Proc. natn. Acad. Sci. U.S.A.* **93**, 12969–12973.

BECK, I., RAMIREZ, S., WEINMANN, R. AND CARO, J. (1991). Enhancer element at the 3′-flanking region controls transcriptional response to hypoxia in the human erythropoietin gene. *J. biol. Chem.* **266**, 15563–15566.

BLANCHARD, K. L., ACQUAVIVA, A. M., GALSON, D. L. AND BUNN, H. F. (1992). Hypoxic induction of the human erythropoietin gene: cooperation between the promoter and enhancer, each of which contains steroid receptor response elements. *Molec. cell. Biol.* **12**, 5373–5385.

BODI, I., BISHOPRIC, N. H., DISCHER, D. J., WU, X. AND WEBSTER, K. A. (1995). Cell-specificity and signaling pathway of endothelin-1 gene regulation by hypoxia. *Cardiovasc. Res.* **30**, 975–984.

BUNN, H. F. AND POYTON, R. O. (1996). Oxygen sensing and molecular adaptation to hypoxia. *Physiol. Rev.* **76**, 839–885.

COTES, P. M., DORÉ, C. J., LIU YIN, J. A., LEWIS, S. M., MESSINEZY, M., PEARSON, T. C. AND REID, C. (1986). Determination of serum immunoreactive erythropoietin in the investigation of erythrocytosis. *New Engl. J. Med.* **315**, 283–287.

CZYZYK-KRZESKA, M. F., BAYLISS, D. A., LAWSON, E. E. AND MILLHORN, D. E. (1992). Regulation of tyrosine hydroxylase gene expression in the rat carotid body by hypoxia. *J. Neurochem.* **58**, 1538–1546.

EBERT, B. L., GLEADLE, J. M., O'ROURKE, J. F., BARTLETT, S. M., POULTON, J. AND RATCLIFFE, P. J. (1995). Isoenzyme specific regulation of genes involved in energy metabolism by hypoxia, cobalt and desferrioxamine: similarities with the regulation of erythropoietin. *Biochem. J.* **313**, 809–814.

FANDREY, J., FREDE, S. AND JELKMANN, W. (1994). Role of hydrogen peroxide in hypoxia-induced erythropoietin production. *Biochem. J.* **303**, 507–510.

FIRTH, J. D., EBERT, B. L., PUGH, C. W. AND RATCLIFFE, P. J. (1994). Oxygen-regulated control elements in the phosphoglycerate kinase 1 and lactate dehydrogenase A genes: similarities with the erythropoeitin 3′ enhancer. *Proc. natn. Acad. Sci. U.S.A.* **91**, 6496–6500.

FIRTH, J. D., EBERT, B. L. AND RATCLIFFE, P. J. (1995). Hypoxic regulation of lactate dehydrogenase A: interaction between hypoxia inducible factor 1 and cyclic AMP response elements. *J. biol. Chem.* **270**, 21021–21027.

GALSON, D. L., TSUCHIYA, T., TENDLER, D. S., HUANG, E., REN, Y., OGURA, T. AND BUNN, H. F. (1995). The orphan receptor hepatic nuclear factor 4 functions as a transcriptional activator for tissue-specific and hypoxia-specific erythropoietin gene expression and is antagonized by EAR3/COUP-TF1. *Molec. cell. Biol.* **15**, 2135–2144.

GLEADLE, J. M., EBERT, B. L., FIRTH, J. D. AND RATCLIFFE, P. J. (1995*a*). Regulation of angiogenic growth factor expression by hypoxia, transition metals and chelating agents. *Am. J. Physiol.* **268**, C1362–C1368.

GLEADLE, J. M., EBERT, B. L. AND RATCLIFFE, P. J. (1995*b*). Diphenylene iodonium inhibits the induction of erythropoietin and other mammalian genes by hypoxia: implications for the mechanism of oxygen sensing. *Eur. J. Biochem.* **234**, 92–99.

GOLDBERG, M. A., DUNNING, S. P. AND BUNN, H. F. (1988). Regulation of the erythropoietin gene: evidence that the oxygen sensor is a heme protein. *Science* **242**, 1412–1415.

GOLDBERG, M. A., GAUT, C. C. AND BUNN, H. F. (1991). Erythropoietin mRNA levels are governed by both the rate of gene transcription and posttranscriptional events. *Blood* **77**, 271–277.

GOLDBERG, M. A., GLASS, G. A., CUNNINGHAM, J. M. AND BUNN, H. F. (1987). The regulated expression of erythropoietin by two human hepatoma cell lines. *Proc. natn. Acad. Sci. U.S.A.* **84**, 7972–7976.

GOLDBERG, M. A. AND SCHNEIDER, T. J. (1994). Similarities between the oxygen-sensing mechanisms regulating the expression of vascular endothelial growth factor and erythropoietin. *J. biol. Chem.* **269**, 4355–4359.

GONZALEZ, C., ALMARAZ, L., OBESO, A. AND RIGUAL, R. (1994). Carotid body chemoreceptors: from natural stimuli to sensory discharges. *Physiol. Rev.* **74**, 829–877.

HANKINSON, O. (1979). Single-step selection of clones of a mouse hepatoma line deficient in aryl hydrocarbon hydroxylase. *Proc. natn. Acad. Sci. U.S.A.* **76**, 373–376.

HELLKAMP, J., CHRIST, B., BASTIAN, H. AND JUNGERMANN, K. (1991). Modulation by oxygen of the glucagon-dependent activation of the phosphoenolpyruvate carboxykinase gene in rat hepatocyte cultures. *Eur. J. Biochem.* **198**, 635–639.

HOFFMAN, E. C., REYES, H., CHU, F.-F., SANDER, F., CONLEY, L. H., BROOKS, B. A. AND HANKINSON, O. (1991). Cloning of a factor required for activity of the Ah (Dioxin) receptor. *Science* **252**, 954–958.

HOGENESCH, J. B., CHAN, W. K., JACKIW, V. H., BROWN, R. C., GU, Y.-Z., PRAY-GRANT, M., PERDEW, G. H. AND BRADFIELD, C. A. (1997). Characterization of a subset of the basic-helix–loop–helix-PAS superfamily that interacts with components of the dioxin signaling pathway. *J. biol. Chem.* **272**, 8581–8593.

HUANG, L. E., ARANY, Z., LIVINGSTON, D. M. AND BUNN, H. F. (1996). Activation of hypoxia-inducible transcription factor depends primarily on redox-sensitive stabilization of its α subunit. *J. biol. Chem.* **271**, 32253–32259.

HUANG, Z. J., EDERY, I. AND ROSBASH, M. (1993). PAS is a dimerization domain common to *Drosophila* period and several transcription factors. *Nature* **364**, 259–262.

JIANG, B.-H., RUE, E., WANG, G. L., ROE, R. AND SEMENZA, G. L. (1996). Dimerization, DNA binding and transactivation properties of hypoxia-inducible factor 1. *J. biol. Chem.* **271**, 17771–17778.

JUNGERMANN, K. (1995). Zonation of metabolism and gene expression in liver. *Histochemistry* **103**, 81–91.

KIETZMANN, T., SCHMIDT, H., UNTHAN, F. K., PROBST, I. AND JUNGERMANN, K. (1993). A ferro-heme protein senses oxygen levels, which modulate the glucagon-dependent activation of the phosphoenolpyruvate carboxykinase gene in rat hepatocyte cultures. *Biochem. biophys. Res. Commun.* **195**, 792–798.

KOUREMBANAS, S., HANNAN, R. L. AND FALLER, D. V. (1990). Oxygen

tension regulates the expression of the platelet-derived growth factor – β chain gene in human endothelial cells. *J. clin. Invest.* **86**, 670–674.

LEE, P. J., JIANG, B.-H., CHIN, B. Y., IYER, N. V., ALAM, J., SEMENZA, G. L. AND CHOI, A. M. K. (1997). Hypoxia-inducible factor-1 mediates transcriptional activation of the heme oxygenase-1 gene in response to hypoxia. *J. biol. Chem.* **272**, 5375–5381.

LORENTZ, A., JENDRISSEK, A., ECKARDT, K.-U., SCHIPPLICK, M., OSSWALD, P. M. AND KURTZ, A. (1991). Serial immunoreactive erythropoietin levels in autologous blood donors. *Transfusion* **31**, 650–654.

MARTI, H. H., JUNG, H. H., PFEILSCHIFTER, J. AND BAUER, C. (1994). Hypoxia and cobalt stimulate lactate dehydrogenase (LDH) activity in vascular smooth muscle cells. *Pflügers Arch.* **429**, 216–222.

MAXWELL, P. H., DACHS, G. U., GLEADLE, J. M., NICHOLLS, L. G., HARRIS, A. L., STRATFORD, I. J., HANKINSON, O., PUGH, C. W. AND RATCLIFFE, P. J. (1997). Hypoxia inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. *Proc. natn. Acad. Sci. U.S.A.* **94**, 8104–8109.

MAXWELL, P. H., PUGH, C. W. AND RATCLIFFE, P. J. (1993). Inducible operation of the erythropoietin 3′ enhancer in multiple cell lines: evidence for a widespread oxygen sensing mechanism. *Proc. natn. Acad. Sci. U.S.A.* **90**, 2423–2427.

MAZURE, N. M., CHEN, E. Y., YEH, P., LADEROUTE, K. R. AND GIACCIA, A. J. (1996). Oncogenic transformation and hypoxia synergistically act to modulate vascular endothelial growth factor expression. *Cancer Res.* **56**, 3436–3440.

MCQUILLAN, L. P., LEUNG, G. K., MARSDEN, P. A., KOSTYK, S. K. AND KOUREMBANAS, S. (1994). Hypoxia inhibits expression of eNOS *via* transcriptional and posttranscriptional mechanisms. *Am. J. Physiol.* **267**, H1921–H1927.

MELILLO, G., MUSSO, T., SICA, A., TAYLOR, L. S., COX, G. W. AND VARESIO, L. (1995). A hypoxia-responsive element mediates a novel pathway of activation of the inducible nitric oxide synthase promoter. *J. exp. Med.* **182**, 1683–1693.

NAGAO, M., EBERT, B. L., RATCLIFFE, P. J. AND PUGH, C. W. (1996). *Drosophila melanogaster* SL2 cells contain a hypoxically inducible DNA binding complex which recognises mammalian HIF-1 binding sites. *FEBS Lett.* **387**, 161–166.

NAMBU, J. R., CHEN, W., HU, S. AND CREWS, S. T. (1996). The *Drosophila melanogaster similar bHLH-PAS* gene encodes a protein related to human hypoxia-inducible factor 1α and *Drosophila* single-minded. *Gene* (in press).

NECAS, E. AND NEUWIRT, J. (1972). The effect of inhibitors of energy metabolism on erythropoietin production. *J. Lab. clin. Med.* **79**, 388–396.

O'DONNELL, V. B., TEW, D. G., JONES, O. T. G. AND ENGLAND, P. J. (1993). Studies on the inhibitory mechanism of iodonium compounds with special reference to neutrophil NADPH oxidase. *Biochem. J.* **290**, 41–49.

O'ROURKE, J. F., PUGH, C. W., BARTLETT, S. M. AND RATCLIFFE, P. J. (1996). Identification of hypoxically inducible mRNAs in Hela cells using differential display PCR. *Eur. J. Biochem.* **241**, 403–410.

PLATE, K. H., BREIER, G., WEICH, H. A. AND RISAU, W. (1992). Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas *in vivo*. *Nature* **359**, 845–848.

PUGH, C. W., EBERT, B. L., EBRAHIM, O. AND RATCLIFFE, P. J. (1994). Characterisation of functional domains within the mouse erythropoietin 3′ enhancer conveying oxygen-regulated responses in different cell lines. *Biochim. biophys. Acta* **1217**, 297–306.

PUGH, C. W., O'ROURKE, J. F., NAGAO, M., GLEADLE, J. M. AND RATCLIFFE, P. J. (1997). Activation of hypoxia inducible factor-1; Definition of regulatory domains within the α subunit. *J. biol. Chem.* **272**, 11205–11214.

PUGH, C. W., TAN, C. C., JONES, R. W. AND RATCLIFFE, P. J. (1991). Functional analysis of an oxygen-related transcriptional enhancer lying 3′ to the mouse erythropoietin gene. *Proc. natn. Acad. Sci. U.S.A.* **88**, 10553–10557.

RATCLIFFE, P. J. (1993). Molecular biology of erythropoietin. *Kidney Int.* **44**, 887–904.

REYES, H., REISZ-PORSZASZ, S. AND HANKINSON, O. (1992). Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. *Science* **256**, 1193–1195.

ROLFS, A., KVIETIKOVA, I., GASSMANN, M. AND WENGER, R. H. (1997). Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. *J. biol. Chem.* (in press).

SANDNER, P., GESS, B., WOLF, K. AND KURTZ, A. (1996). Divergent regulation of vascular endothelial growth factor and of erythropoietin gene expression *in vivo*. *Pflügers Arch.* **431**, 905–912.

SEMENZA, G. L., JIAN, B.-H., LEUNG, S. W., PASSANTINO, R., CONCORDET, J.-P., MAIRE, P. AND GIALLONGO, A. (1996). Hypoxia response elements in the aldolase A, enolase 1 and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J. biol. Chem.* **271**, 32529–32537.

SEMENZA, G. L., NEJFELT, M. K., CHI, S. M. AND ANTONARAKIS, S. E. (1991). Hypoxia-inducible nuclear factors bind to an enhancer element located 3′ to the human erythropoietin gene. *Proc. natn. Acad. Sci. U.S.A.* **88**, 5680–5684.

SEMENZA, G. L., ROTH, P. H., FANG, H.-M. AND WANG, G. L. (1994). Transcriptional regulation of genes encoding glycolytic enzymes by hypoxia-inducible factor 1. *J. biol. Chem.* **269**, 23757–23763.

SEMENZA, G. L. AND WANG, G. L. (1992). A nuclear factor induced by hypoxia *via* de novo protein synthesis binds to the human erythropoietin gene enhancer at a site required for transcriptional activation. *Molec. cell. Biol.* **12**, 5447–5454.

SHWEIKI, D., ITIN, A., SOFFER, D. AND KESHET, E. (1992). Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. *Nature* **359**, 843–845.

TIAN, H., MCKNIGHT, S. L. AND RUSSELL, D. W. (1997). Endothelial PAS domain protein 1 (EPAS1), a transcription factor selectively expressed in endothelial cells. *Genes Dev.* **11**, 72–82.

WANG, G. L., JIANG, B.-H., RUE, E. A. AND SEMENZA, G. L. (1995). Hypoxia-inducible factor 1 is a basic-helix–loop–helix-PAS heterodimer regulated by cellular O_2 tension. *Proc. natn. Acad. Sci. U.S.A.* **92**, 5510–5514.

WANG, G. L. AND SEMENZA, G. L. (1993*a*). Desferrioxamine induces erythropoietin gene expression and hypoxia-inducible factor 1 DNA-binding activity: implications for models of hypoxia signal transduction. *Blood* **82**, 3610–3615.

WANG, G. L. AND SEMENZA, G. L. (1993*b*). General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc. natn. Acad. Sci. U.S.A.* **90**, 4304–4308.

WEIBEL, E. R., TAYLOR, C. R. AND HOPPELER, H. (1992). Variations in function and design: Testing symmorphosis in the respiratory system. *Respir. Physiol.* **87**, 325–348.

WEIR, E. K. AND ARCHER, S. L. (1995). The mechanisms of acute hypoxic pulmonary vasoconstriction: the tale of two channels. *FASEB J.* **9**, 183–189.

WENGER, R. H., ROLFS, A., MARTI, H. H., GUÉNET, J.-L. AND GASSMANN, M. (1996). Nucleotide sequence, chromosomal assignment and mRNA expression of mouse hypoxia-inducible factor-1α. *Biochem. biophys. Res. Commun.* **223**, 54–59.

WIENER, C. M., BOOTH, G. AND SEMENZA, G. L. (1996). *In vivo* expression of mRNAs encoding hypoxia-inducible factor 1. *Biochem. biophys. Res. Commun.* **225**, 485–488.

WILK, R., WEIZMAN, I. AND SHILO, B.-Z. (1996). *trachealess* encodes a bHLH-PAS protein that is an inducer of tracheal cell fates in *Drosophila*. *Genes Dev.* **10**, 93–102.

WOOD, S. M., GLEADLE, J. M., PUGH, C. W., HANKINSON, O. AND RATCLIFFE, P. J. (1996). The role of aryl hydrocarbon receptor nuclear translocator (ARNT) in hypoxic induction of gene expression: studies in ARNT deficient cells. *J. biol. Chem.* **271**, 15117–15123.

ZHOU, Y.-D., BARNARD, M., TIAN, H., LI, X., RING, H. Z., FRANCKE, U., SHELTON, J., RICHARDSON, J., RUSSELL, D. W. AND MCKNIGHT, S. L. (1997). Molecular characterization of two mammalian bHLH-PAS domain proteins selectively expressed in the central nervous system. *Proc. natn. Acad. Sci. U.S.A.* **94**, 713–718.