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# Hypoxia-mediated regulation of gene expression in mammalian cells

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**Summary.** The molecular mechanism underlying oxygen sensing in mammalian cells has been extensively investigated in the areas of glucose transport, glycolysis, erythropoiesis, angiogenesis and catecholamine metabolism. Expression of functionally operative representative proteins in these specific areas, such as the glucose transporter 1, glycolytic enzymes, erythropoietin, vascular endothelial growth factor and tyrosine hydroxylase are all induced by hypoxia. Recent studies demonstrated that both transcriptional activation and post-transcriptional mechanisms are important to the hypoxia-mediated regulation of gene expression. In this article, the cis-acting elements and trans-acting factors involved in the transcriptional activation of gene expression will be reviewed. In addition, the mechanisms of post-transcriptional mRNA stabilization will also be addressed. We will discuss whether these two processes of regulation of hypoxia-responsive genes are mechanistically linked and co-operative in nature.

*Keywords*: hypoxia, regulation, gene, expression

## Background

Hypoxia induces the expression of a number of genes that enable cells to adapt to this stress factor (Helfman & Falanga 1993). These genes can be roughly divided into three classes. The first class consists of intracellular factors involved in the metabolic adaptation of the cell to hypoxia: for example, the ubiquitously expressed glycolytic enzymes which provide ATP through anaerobic glycolysis (Webster 1987), the glucose transporters (GLUT) which increase the intracellular glucose supply (Bashan *et al.* 1992) and transcription factors like hypoxia inducible factor 1 (HIF-1) (Wang *et al.* 1995a), activator protein 1 (AP-1) (Webster *et al.* 1993) and NF-κB (Koong *et al.* 1994a) which can activate the transcription of hypoxia specific genes. The second class comprises factors which act locally to ensure the survival of tissues exposed to hypoxia due to high oxygen consumption, reduced blood supply or injury: for example, vascular endothelial growth factor (VEGF), also known as vascular permeability factor, which activates tissue permeability and increases neovascularization of tissues and tumours (Shweiki *et al.* 1992). The third class includes molecules that are favourable for the adaptation of the whole organism to reduced oxygen tension, such as the erythropoietin (Epo), which stimulates the proliferation and differentiation of erythroid progenitor cells (Jelkmann 1992), and tyrosine hydroxylase (TH) which is the rate limiting enzyme in catecholamine synthesis (Czyzyk-Krzeska *et al.* 1994).

Both transcriptional activation and post-transcriptional

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mechanisms contribute to the hypoxia-mediated regulation of gene expression. In the first part of this review, we will introduce the metabolic state of cells in hypoxia. In the second part, we will discuss transcription factors involved in the regulation of hypoxia-responsive gene expression, and the third part will cover the current understanding in the regulation of mRNA stability through 3′untranslated regions (3′UTR) and their respective binding proteins. The fourth part will describe the known mechanisms in induction of hypoxia-responsive genes and discuss common themes demonstrated by these mechanisms.

## Metabolic state of cells in hypoxia

When oxygen is unavailable as the final electron acceptor in the respiratory chain, the cell must abandon oxidative phosphorylation, minimize nonessential cell functions and rely solely on glycolysis for energy production (Bunn & Poyton 1996). Since mitochondria can no longer operate oxidative phosphorylation pathways, the electron carriers that are normally oxidized will become reduced. These reduced electron carriers may react directly with any available oxygen and be reduced to superoxide or peroxide, thereby generating excess reactive oxygen intermediates (ROI) (Bunn & Poyton 1996). The redox state and the generation of free radicals in hypoxia can activate transcription factors like HIF-1 (Huang *et al.* 1996), AP-1 (Abate *et al.* 1990), and NF-κB (Koong *et al.* 1994b), which in turn, induce the expression of a group of hypoxia-regulated genes. Production of mitochondrial superoxide is an unlikely candidate for hypoxia-mediated signalling since cyanide and azide, which inhibit mitochondrial cytochromes and electron transfer, has no effect on the induction of hypoxia-responsive genes (Minchenko *et al.* 1994). Divalent cations such as $Co^{+2}$ and $Ni^{+2}$, which are known to substitute for ferrous iron in the haem moiety, stimulate Epo, VEGF, TH, GLUT-1 and glycolytic enzyme production (Goldberg *et al.* 1988; Kietzmann *et al.* 1993; Minchenko *et al.* 1994; Goldberg & Schneider 1994). When cells are treated with desferrioxamine, an iron chelator, or dioxohepatnoic acid, an inhibitor of heme synthesis, the hypoxic induction of gene expression can be reduced (Wang & Semenza 1993b). Carbon monoxide, which is known to bind noncovalently to ferrous heme groups in haemoglobin, myoglobin, certain cytochromes, and other heme proteins, also blocked the expression of these hypoxia-responsive genes (Goldberg *et al.* 1988; Kietzmann *et al.* 1993; Goldberg & Schneider 1994). Thus, under hypoxia, the oxygen sensor has been hypothesized to be a protein or proteins which are heme-containing and bind oxygen and can transduce hypoxia-dependent signals. However, apart from the assumption that the oxygen sensor might be a haemprotein, neither the nature of this molecule nor the mechanisms leading to enhanced gene expression have been characterized to date.

## Hypoxia-mediated regulation of transcription factor expression

### *HIF-1 transcription factor and HIF-1 responsive cis-element*

HIF-1 is an $\alpha/\beta$ (120 kD/94 kD) heterodimer with each subunit containing a basic helix-loop-helix motif and a Per and Sim (PAS) protein–protein interaction domain (Wang *et al.* 1995a). HIF-1 interacts with a specific DNA sequence, 5′-TACGTGCT-3′, and the kinetics of HIF–1 association with, and dissociation from, its DNA binding site are extremely rapid, with a half–life of association and dissociation being less than 1 min (Wang & Semenza 1993a). This DNA binding activity can be inhibited when hypoxic cells are treated with 2-aminopurine, a protein kinase inhibitor (Wang & Semenza 1993a). In addition, phosphatase treatment of HIF-1 abolished the DNA-binding activity, suggesting that activation of protein kinase cascade is involved in the hypoxia-induced HIF-1 signalling pathway. HIF-1 expression is tightly regulated by cellular oxygen concentration and the expression level appears to be determined primarily by the abundance of the HIF-1$\alpha$ subunit. Studies have shown that HIF-1$\beta$ (identical to the aryl hydrocarbon receptor nuclear translocator, ARNT) is constitutively expressed in all cells while HIF-1$\alpha$ is continuously synthesized and degraded under normoxic conditions but rapidly accumulates following exposure to low oxygen tension (Huang *et al.* 1996). The rapid degradation of HIF-1$\alpha$ under normoxic condition is mediated by the ubiquitin-proteasome system whereas its stabilization by hypoxia depends on redox-induced changes (Huang *et al.* 1996; Salceda & Caro 1997).

HIF-1 was first identified as an activity which binds to Epo enhancer at the 3′ end of the gene and induces Epo expression under hypoxia (Semenza & Wang 1992). Subsequent studies showed that HIF-1 was widely expressed in many different cells and tissues (Wiener *et al.* 1996), and is responsible for the transcriptional induction of a number of hypoxia-responsive genes including the glycolytic enzymes (Semenza *et al.* 1994), glucose transporters (Ebert *et al.* 1995), tyrosine hydroxylase (Norris & Millhorn 1995), and VEGF (Levy *et al.* 1995). Several lines of experimental evidence

suggest that HIF-1 is a global regulator of hypoxia-responsive gene expression. A compendium of investigations have shown that:

- reporter genes containing HIF-1 binding sites were induced by hypoxia in non-Epo-producing cells (Wang & Semenza 1993c; Wiener *et al.* 1996);
- hypoxia, cobalt chloride and desferrioxamine induced the HIF-1 DNA-binding activity and hypoxia-responsive gene transcription with similar kinetics (Wang & Semenza 1993b);
- mutations in the HIF-1-binding site eliminated hypoxia-inducible enhancer activity of hypoxia-responsive genes reporter constructs (Semenza & Wang 1992; Wang & Semenza 1993c; Liu *et al.* 1995);
- cycloheximide, actinomycin D, and 2-amino-purine inhibited both HIF-1 induction and hypoxia-responsive gene transcription (Wang & Semenza 1993a).

However, recent studies of HIF-1$\beta$-deficient cells showed that HIF-1 was required for complete induction of hypoxia-responsive genes such as lactate dehydrogenase A (LDH-A) and phosphoglycerate kinase 1 (PGK-1), but the expression of VEGF and GLUT-1 showed only a minor reduction (Wood *et al.* 1996). In a similar study, cells deficient in HIF-1-$\alpha$ completely abrogated GLUT-1 expression whereas the expression of heme oxygenase-1 was only partly affected (Wood *et al.* 1998). Thus both HIF-1 dependent and independent mechanisms are operating in the regulation of hypoxia-responsive gene transcription under hypoxic conditions. HIF-1 may therefore be a major mediator, but not the only activator in controlling homeostatic responses to hypoxia.

### *Activator protein 1 (AP-1) transcription factor complex and AP-1 responsive element*

The AP-1 responsive cis-element is present in numerous cellular and viral genes and plays an important role in the regulation of cell growth and differentiation (Chiu *et al.* 1988). The AP-1 transcription factor complex is comprised of dimers of the c-fos and c-jun proto-oncogene products and modulates gene transcription by binding to the specific consensus AP-1 recognition DNA sequence TGA(C/G)TCA (Chiu *et al.* 1988). Dimerization of Fos and Jun is mediated through the interaction of leucine zipper domains from both proteins which is required for DNA binding (Abate *et al.* 1990). In the DNA-binding domain of the Fos-Jun heterodimer, reduction of a single conserved cysteine residue is essential for the binding. Reduction of this cysteine residue could potentially be modulated by the redox state of the cell which hypoxia can readily alter.

Hypoxia transiently induces both c-fos and c-jun mRNA expression in a wide range of cells and tissues and the induction can be seen within minutes of exposure to the stress with a typical decline after 4 h (Webster *et al.* 1993; Ausserer *et al.* 1994). The increased mRNA levels result from both transcriptional activation and message stabilization (Ausserer *et al.* 1994). The promoter region of c-jun also contains a positively acting AP-1 site, so that c-jun transcription can be directly stimulated by its own gene product (Angel *et al.* 1988; Ausserer *et al.* 1994). Superinduction of c-jun message was observed during simultaneous oxygen and glucose deprivation, and the induction was blocked by preincubating cells with reducing agent, protein kinase inhibitors, or PMA, suggesting that transcriptional activation of c-jun during both hypoxic and hypo-glycemic stress involves redox control and PKC-mediated mechanisms (Webster *et al.* 1993; Ausserer *et al.* 1994). The AP-1 transcription factor complex has been found to be involved in the up-regulation of genes for enzymes functioning in the detoxification of end products derived from oxidative metabolism (Vogt & Bos 1990). Thus genes transcribed by the AP-1 complex may protect cells from damaging metabolites under hypoxia, especially the accumulation of reactive oxygen species which leads to extensive cell damage and death.

### *NF-κB transcription factor and κB motif*

The heterodimeric NF-κB complex is composed of two DNA-binding subunits, p50 and p65, which share structural homology with the c-rel proto-oncogene product (May & Ghosh 1998). The major form of NF-κB is found in an inactive state complexed with the inhibitory subunit, IκBα, in the cytoplasm. Upon activation, IκBα is phosphorylated and released from the complex, and the p50-p65 heterodimer complex is translocated from the cytoplasm to the nucleus where it binds with high affinity to the κB motif on DNA (May & Ghosh 1998).

Studies have shown that NF-κB may play an important role in gene transcription under hypoxia. NF-κB DNA-binding activity was modulated with variation to the redox state of the protein (Toledano & Leonard 1991). Moreover, prolonged exposure of cells to hypoxia will activate an IκBα degradation process which in turn will facilitate NF-κB activation and transactivate genes which contain the κB motif (Koong *et al.* 1994a). Src activation by hypoxia may be one of the earliest events that precede Ras activation in the signalling cascade which ultimately leads to the phosphorylation and dissociation of the IκBα (Koong *et al.* 1994b). Recent studies have shown that Fos and Jun are capable of physically interacting with

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NF-$\kappa$B p65 through the Rel homology domain. This complex of NF-$\kappa$B and Fos/Jun exhibited enhanced DNA binding and biological function via both $\kappa$B and AP-1 reponse elements (Stein *et al.* 1995). Thus, a co-operative mechanism of gene regulation involving two different classes of transcription factors which form novel protein complexes, may potentiate their respective biological activities, especially under hypoxia.

## RNA stabilization, binding proteins and their recognition sequences

Messenger RNA stability influences gene expression in virtually all organisms, from bacteria to mammals. In mammalian cells, the half-lives of many mRNAs vary in response to diverse environmental stimuli such as hypoxia, starvation, and viral infection. These fluctuations affect the expression of specific genes thereby providing the cell with the flexibility to adapt to a changing environment.

The decay rates of mRNA can range from several minutes, in the case of some proto-oncogenes and cytokine mRNAs, to days, as is the case for globulin mRNA (Ross 1995). The turnover rate of a given mRNA is determined by both mRNA sequence and the binding proteins which recognize them. Mature eukaryotic mRNAs are normally protected against 5′ exonucleases by the 5′m7G cap structure along with cap-binding proteins. Protection against 3′ exonucleases at the 3′poly(A) tail is mediated through poly(A)-binding protein (Ross 1995). The observation that a poly(A) tail which is shortened to approximately 12 nucleotides induces 5′ decapping, suggests that an interaction between the 5′ and 3′ termini is important in determining the rate of mRNA decay (Ross 1995). However, when either of these terminal structures are removed, or when endonucleolytic attack exposes unprotected 3′ and 5′ ends, rapid mRNA degradation occurs. Other than 5′m7G cap and the poly A tail, specific cis elements in conjunction with mRNA binding proteins also contribute to mRNA steady-state level by promoting degradation or stabilization. Although these cis elements could occur anywhere on the mRNA, the majority of them are localized to the 3′UTR (Chen & Shyu 1995; Decker & Parker 1995). In several examples, mRNA stability appears to be controlled by a protective factor that binds within the 3′UTR at or near the endonucleolytic cleavage site and competes with endonucleases. Conversely, binding of degradative factors will promote the endonucleolytic attack. There are three unique cis-acting elements identified in 3′UTRs which have been implicated in the regulation of mRNA stability in eukaryotic cells.

*AU-rich element (ARE).* Many unstable mammalian mRNAs contain AREs at the 3′UTR. The AREs range in size from 50 to 150 nucleotides and generally consist of either scattered AUUUA pentanucleotides, contiguous AUUUA repeats, the nonamer motif UUAUUUAUU, or, in some cases a stretch of AU residues or U-rich region lacking either motif (Chen & Shyu 1995). AUUUA motifs facilitate degradation of the mRNA body, while the U-rich domain promotes deadenylation and enhances the destabilising function of the AUUUA motifs. These destabilising elements were first described in the proto-oncogenes and cytokines such as c-fos (Shyu *et al.* 1991), c-myc (Brewer & Ross 1988), and GM-CSF (Shaw & Kamen 1986). The rapid transcriptional induction of these genes by other growth factors and cytokines is followed by rapid degradation of their transcripts due to interaction of AREs with specific RNA-binding proteins. Removal of the AREs from either the GM-CSF or c-fos transcripts results in significant transcript stabilization. Insertion of the c-fos ARE into the 3′UTR of the $\beta$-globulin transcript reduced its stability from 8 h to 30 min (Shaw & Kamen 1986; Shyu *et al.* 1989). Several proteins have been described to interact AREs with high affinity. These include hnRNP A1 and hnRNP C (Hamilton *et al.* 1993), Hel-N1 (Levine *et al.* 1993), and AUF1 (Zhang *et al.* 1993). However, it is not clear how these proteins destabilize mRNAs. Studies have shown that ARE-directed mRNA degradation is influenced by many factors, including phorbol esters, calcium ionophores, redox shift and transcription inhibitors (Chen & Shyu 1995). These observations suggest that modification of the RNA-binding proteins, such as phosphorylation or a redox shift, may play a critical role in the regulation of mRNA stabilization and processing.

*Stem-loop elements.* The 3′UTR of transferrin receptor mRNA contains five distinct stem-loop structures (also called iron-response elements, IRE). The structure is capable of interacting with iron response proteins (IRP-1 and IRP-2) when intracellular iron stores are low, thus protecting the mRNA from endonucleolytic attack (Mullner & Kuhn 1988). Interaction of histone mRNA 3′terminal stem-loop with a 50-kD stem-loop-binding protein also dramatically increases histone mRNA stability (Pandey *et al.* 1991).

*Pyrimidine-rich element.* The globulin mRNAs are among the most stable eukaryotic mRNAs, with estimated half-lives of as long as 60 h. The stability of $\alpha$-globulin mRNA is regulated by a pyrimidine-rich cis element in the 3′UTR and a specific ribonucleoprotein (RNP) complex ($\alpha$-complex) of RNA binding proteins (Wang *et al.* 1995b;

Kiledjian *et al.* 1995). The RNP complex is composed of two poly(C)-binding proteins, $\alpha$-complex protein 1 ($\alpha$CP1), $\alpha$-complex protein 2 ($\alpha$CP2), and one nonpoly(C) binding protein. These three proteins do not individually bind 3′UTR but can do so within the context of the entire complex. The $\alpha$-complex also interacts with two other stable mRNAs, $\alpha$(I)-collagen, and 15-lipoxygenase suggesting that the $\alpha$-complex might be a general mRNA stabilization factor for genes required to perform the housekeeping functions of the cell.

## Induction of hypoxia-responsive genes

### *Glucose transporter*

Most animal cells take up glucose by a process that depends on the gradient of glucose concentration through glucose transporters. The ATP yield from the glycolytic pathway is 18-fold lower than that from the Krebs-oxidative pathway, therefore the glucose transporter expression and the rate of glucose consumption must increase substantially during hypoxic stress (Bashan *et al.* 1992). Five isoforms of glucose transporters, which are encoded by different genes, are differentially expressed in various tissues: GLUT-1 is widely distributed and is expressed at low levels in most cells and tissues and responsible for constitutive glucose transport whereas GLUT-4 is responsible for insulin-activated uptake of glucose by muscle and fat cells (Simpson & Cushman 1986).

Interestingly, hypoxia induces the expression of glucose transporters in an isoform-specific manner; GLUT-1 and GLUT-3 are up-regulated, whereas GLUT-4 is unaffected and GLUT-2 is down-regulated (Bashan *et al.* 1992; Ebert *et al.* 1996). The hypoxic induction of GLUT-1 can be mediated by both transcriptional activation through HIF-1 as well as post-transcriptional mRNA stabilization mechanisms (Stein *et al.* 1995). When L6 muscle cells were incubated in 3% oxygen for 48 h, the level of GLUT-1 protein increased 14-fold which led to a 6.5-fold increase in glucose uptake (Bashan *et al.* 1992). This up-regulation can be mimicked by cobalt and the iron chelators but was blocked by cycloheximide, indicating a haemprotein is acting as an oxygen sensor and the need for *de novo* protein synthesis. Other than the HIF-1 recognition sequence, the GLUT-1 promoter also contains a serum response element (SRE), two phobol ester response elements (TRE), and a cAMP response element (CRE) (Murakami *et al.* 1992; Ebert *et al.* 1995). Therefore, GLUT-1 expression can arize from a variety of transcriptional activation pathways including activation of SRE through mitochondria inhibitors.

Hypoxia also regulates GLUT-1 expression through increased mRNA stability. GLUT-1 mRNA half-life increased from 0.52 h to 8 h with hypoxia (Stein *et al.* 1995). The GLUT-1 3′UTR contains a destabilising AUUUA motif as well as AU-rich sequences. The 3′UTRs of VEGF and GLUT-1 show only a 42% overall homology, but both contain AREs that are highly homologous (Levy *et al.* 1996a). Interestingly, GLUT-1 3′UTR (nucleotides 1967–2359) forms a 65-kD hypoxia-inducible RNA-protein complex with similar sequence and protein binding characteristics to that of VEGF 3′UTR (Levy *et al.* 1996a). These initial observations suggest there may be a common role for the ARE and its cognate binding proteins in mediating the hypoxic stabilization of these two mRNAs.

### *Glycolytic enzymes*

The HIF-1 binding consensus sequence has been found in the 5′ flanking promoter regions of many glycolytic enzyme genes. For example the human PGK-1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), LDH-A, aldolase A, pyruvate kinase and enolase 1 genes as well as in intervening sequences of mouse phosphofructokinase L all contain HIF-1 binding sites (Semenza *et al.* 1994; Firth *et al.* 1994). Thus, these glycolytic enzymes can be up-regulated by hypoxia through HIF-1 activation, although the level of induction varies among cell types. Synthesis of GAPDH was induced 2-to 5-fold after incubation of endothelial cells for 18 h under 3% oxygen and the induction was mediated primarily through transcriptional activation (Graven *et al.* 1994). Similar hypoxia-induced increases in glycolytic enzyme expression was also observed in primary human skeletal muscle cells during differentiation from myoblasts to myotubes (Webster *et al.* 1990). For all of these glycolytic genes, the hypoxic induction can be mimicked by both cobalt and iron chelators but not cyanide, as well as being blocked by cycloheximide (Firth *et al.* 1994; Ebert *et al.* 1996). Thus, the signal transduction mechanisms leading to hypoxia activation of genes encoding these glycolytic enzymes are likely to be regulated in a similar manner to the GLUT-1 transporter.

### *Vascular endothelial growth factor (VEGF)*

VEGF, also known as vascular permeability factor (VPF), is a potent angiogenic cytokine which is an endothelial cell-specific mitogen. VEGF is expressed as four isoforms (121, 165, 189, and 206 amino acids) through alternative splicing (Tischer *et al.* 1991). The predominantly expressed VEGF165 isoform forms a 45-kD

glycosylated homodimer which binds to heparin and is 18–24% homologous with PDGF A and B chains (Senger *et al.* 1993). VEGF has been demonstrated to be expressed at high levels by many tumours *in situ* and tumour cell lines *in vitro*.

Hypoxia is a strong inducer of VEGF expression *in vivo*and *in vitro* (Minchenko *et al.* 1994) and both transcriptional activation and increased mRNA stability account for the overall increase in steady-state VEGF mRNA levels under hypoxia (Stein *et al.* 1995; Ikeda *et al.* 1995; Claffey *et al.* 1998). Studies have shown that the transcription rates of VEGF plateau following hypoxic induction, while mRNA stabilization remains elevated level throughout the duration of the exposure (Ikeda *et al.* 1995). Hence, hypoxia-induced VEGF expression is activated in a biphasic manner: initial activation is through transcriptional induction and then secondary stimuli such as metabolic changes give rise to the increased stability of VEGF mRNA. Analysis of the human VEGF promoter revealed that in addition to HIF-1 binding site, it also contains one AP-1 site, one AP-2 site and a cluster of SP1 sites (Tischer *et al.* 1991). Studies have indicated that AP-1 (Abate *et al.* 1990) is a redox-sensitive transcription factor, thus the redox environment of hypoxia favours AP-1 activation and binding to DNA. Consistent with the presence of AP-1-and AP-2-enhancer elements, VEGF expression is inducible in most cell types by phorbol esters, calcium ionophore, growth factors and cAMP-analogues (Tischer *et al.* 1991; Claffey *et al.* 1992; White *et al.* 1995). Altogether, transcriptional activation of VEGF is likely mediated not only by HIF-1-induced transcription but also by other factors like AP-1.

The human VEGF165 mRNA is a 3.7kb transcript which contains 2.2kb of coding region and 1.5kb of 3′UTR (Tischer *et al.* 1991). Under hypoxia, the half-life of the VEGF mRNA is usually increased 2-to 3-fold (Levy *et al.* 1996b; Claffey *et al.* 1998). Deletion of AREs from VEGF 3′UTR results in a significant stabilization of VEGF mRNA in an *in vitro* degradation assay (Levy *et al.* 1996b). Conversely, insertion of VEGF 3′UTR into a relatively stable mRNA can markedly destabilize it (Shima *et al.* 1996). Analysis of VEGF 3′UTR revealed multiple AUUUA and polypyrimidine sequence motifs (Levy *et al.* 1995; Claffey *et al.* 1998), suggesting that the regulation of VEGF mRNA half-life appears to be mediated by both destabilising and stabilising AREs. Studies of human VEGF showed a 126-base element in 3′UTR (nucleotides 231–357 3′ to the translation stop codon) involved in hypoxia-mediated human VEGF mRNA stability (Claffey *et al.* 1998). Rat VEGF possesses a 600-base region in 3′UTR (nucleotides 1251–1877 from translation stop codon) required for rat VEGF mRNA stability (Levy *et al.* 1996b). However, little homology exists between the two VEGF 3′UTRs. Recent studies identified the 34 kD rat VEGF ARE-binding protein as HuR, which interacts with a 45-base element within the 600-base ARE and stabilized the mRNA under hypoxia (Levy *et al.* 1998). Inhibition of HuR expression abrogated the hypoxia-mediated increase in VEGF mRNA stability whereas overexpression of HuR increased the stability, indicating that HuR plays a critical role in mediating the hypoxic stabilization of VEGF mRNA.

Signal transduction pathways leading to VEGF expression are still largely unknown. VEGF expression can be mimicked by cobalt and iron chelators (Goldberg & Schneider 1994) but blocked by carbon monoxide and nitric oxide (Liu *et al.* 1998). VEGF mRNA levels were significantly higher in cells overexpressing PKC$\alpha$ and this induction was lost after down-regulation of PKC (Finkenzeller *et al.* 1992). Both Ras and Raf (Grugerl *et al.* 1995) as well as dominant negative mutant p53 (Kieser *et al.* 1994) have also been shown to stimulate VEGF expression through PKC activated pathways. Interestingly the von Hippel-Lindau (VHL) tumour suppresser gene represses the constitutive expression of VEGF by blocking PKC isoforms $\zeta$ and $\delta$ (Pal *et al.* 1997). Furthermore, genistein, a general tyrosine kinase inhibitor, blocked the hypoxic induction of VEGF mRNA through its action on Src, and reduced the hypoxia-induced stabilization of VEGF 3′UTR transcripts by inhibiting binding of proteins to the VEGF 3′UTR (Mukhopadhyay *et al.* 1995; Levy *et al.* 1996b). Moreover, reactive oxygen intermediates (ROI) can induce the rapid expression of VEGF in many cell types, mainly

| | Transcriptional Activation Sites | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| mRNA | HIF-1 | AP-1 | AP-2 | SP-1 | SRE | TRE | CRE | Reference |
| VEGF | + | + | + | + | | | | Tischer *et al.* 1991 |
| Epo | + | | | | | | | Blanchard *et al.* 1992 |
| TH | + | + | + | | | | + | Millhorn *et al.* 1997 |
| GLUT-1 | + | | | | + | + | + | Murakami *et al.* 1992 |

**Table 1.** Transcriptional activation sites on different hypoxia-responsive genes. To ensure adequate mRNA transcripts during sustained hypoxia, a synergistic co-operation of HIF-1 with distinct transcription factors may be required

**Table 2.** Stabilization of hypoxia-responsive gene mRNA. Representative hypoxia-regulated RNA-binding proteins, their apparent molecular weights, and their binding sites on several hypoxia-responsive genes

| | mRNA Stabilization | | | |
|---|---|---|---|---|
| mRNA | Binding | Protein | Binding Site on 3′UTR (location from stop codon) | (Reference) |
| VEGF | HuR | 34 kD | AAUUCUACAUACUAAAUCUCUCUCCUUUUUUAAUUUUAAUAAUAUUUG (nucleotide 1634-1678 of rat VEGF 3′UTR) | (Levy *et al*., 1998) |
| Epo | ERBP | 70/135 kD | binding site is unknown (nucleotide 0-114 of human Epo 3′UTR) | (Scandurro *et al*., 1997) |
| TH | HIP | 50 kD | UCCCCU (nucleotide 1584-1590 of rat TH 3′UTR) | (Czyzyk-Krzeska *et al*., 1997) |
| GLUT-1 | | 65 kD | binding site is unknown (nucleotides 1967-2359 of rat GLUT-1 3′UTR) | (Levy *et al*., 1996a) |

operates through increased mRNA stability (Kuroki *et al*. 1996). Taken together, these studies suggest that PKC, PKA, protein tyrosine kinase c-Src, and GTP-binding protein Ras signalling pathways can all contribute and transduce signals which increase VEGF expression.

### *Erythropoietin (EPO)*

Epo is a 30-kD glycoprotein which plays a central role in erythropoiesis by supporting the proliferation and differentiation of erythroid progenitor cells in the bone marrow (Jelkmann 1992). Epo is primarily produced by the adult kidney and foetal liver and hypoxia is the principle physiological stimulus for Epo production (Jelkmann 1992). Extensive screening of cell lines has revealed only two hepatoma lines, Hep3B and HepG2, which produce Epo in an oxygen-regulated manner (Goldberg *et al*. 1987). Transcriptional response of the human Epo gene to hypoxia is mediated in part by promoter sequences located – 118 to – 65 upstream of the transcription start site and to a significant degree by a HIF-1 enhancer element located in the 3′ flanking region (Blanchard *et al*. 1992). Using an *in vitro* reporter assay system, the Epo promoter alone confers a 6-fold induction in response to hypoxia whereas the 3′enhancer alone confers a 14-fold induction. When the 3′enhancer and the Epo promoter are combined, they co-operate to produce a 50-fold hypoxia-dependent activation. Other than promoter and enhancer elements, the hypoxic induction of Epo can also be augmented by an orphan receptor, hepatic nuclear factor (HNF-1), which is located at the 3′ end of HIF-1 enhancer (Galson *et al*. 1995). The hypoxia-induced Epo production can be blocked by cytokines like IL-1, TNF and TGF$\beta$ (Faquin *et al*. 1992) as well as PKC activators such as PMA and calcium ionophore (Faquin *et al*. 1993). However, it is not clear how these factors down-regulate the hypoxia-dependent Epo expression.

Hypoxia-mediated Epo expression also involves mRNA stabilization and studies have shown that hypoxia increases Epo mRNA half-life from 2 h to 8 h (McGary *et al*. 1997). The 3′UTR of the Epo transcript lacks any known sequence homology with typical AU-rich motifs of cytokine mRNAs, however, some similarities exist with the 3′UTR of the tyrosine hydroxylase (TH) gene (Czyzyk-Krzeska & Beresh 1996). ERBP (Epo mRNA-binding protein), a cytosolic protein from Hep3B cells, binds specifically to a highly conserved pyrimidine-rich 120-bp region in the 3′UTR of Epo mRNA (Rondon *et al*. 1991). The region is located immediately downstream of the translation stop codon and contains a 28-base pyrimidine rich stretch that is homologous to a similar region in the TH mRNA (Czyzyk-Krzeska & Beresh 1996). UV cross-linked ERBP-mRNA complexes migrated as two bands of 70 kD and 135 kD and the complex formation requires association with heat shock protein 70 (Scandurro *et al*. 1997). A conserved 46 base sequence in the Epo 3′UTR (nucleotides 0–114 3′ to the translation stop codon), located 67 bases downstream of the ERBP recognition site, contains a destabilising cis-element (Ho *et al*. 1995). Deletion of this region prolonged Epo mRNA half-life from 2 h to 15 h. Part of this region formed a stem with the neighbouring ERBP binding site, thus raising the possibility that the ERBP complex may play a role in protecting Epo mRNA from endonucleolytic cleavage. The binding activity of ERBP to Epo mRNA was markedly increased by hypoxia and reducing agents, but abolished by an oxidizing agent (Rondon *et al*. 1991), suggesting that hypoxia-induced redox state favours prolonged Epo mRNA half-life.

### *Tyrosine hydroxylase (TH)*

Tyrosine hydroxylase (TH) is the rate limiting enzyme in catecholamine synthesis in type I cells of the carotid body which are crucial to central and peripheral neurohormonal

regulation of the cardiovascular and respiratory systems (Millhorn *et al.* 1997). Reduced arterial oxygen tension is a powerful physiological stimulus that induces type I cells to synthesize and release dopamine, resulting in a several-fold increase in TH mRNA levels. The increase in the rate of TH mRNA transcription is relatively fast with a peak that occurs 6 h following the onset of hypoxia (Czyzyk-Krzeska *et al.* 1994). In contrast, the increase in TH mRNA stability is much slower and is most evident during longer exposure times (> 12 h). Thus, hypoxia induction of TH mRNA is mediated by a dual mechanism involving an early increase in TH gene transcription and subsequently the sustained TH mRNA stability during the course of hypoxia.

The increase in TH gene transcription during hypoxia is mediated by proximal promoter sequences − 272 to + 27 relative to the transcription start site. This region contains several regulatory elements such as HIF-1, CRE, AP1, AP2, MyoD and SP1 (Norris & Millhorn 1995; Millhorn *et al.* 1997). AP-1 seems to be functionally important, since mutation at this site abolishes hypoxic induction and nuclear extracts from hypoxic PC-12 cells show increased binding of Fos/JunB heterodimers to the AP-1 DNA element (Norris & Millhorn 1995; Millhorn *et al.* 1997). Since cAMP levels increase in type I cells and PC12 cells during hypoxia, it is possible that the CRE (cAMP responsive element) might also be contributing to increased transcription of the TH during hypoxia, although it may not be the primary regulator (Czyzyk-Krzeska *et al.* 1994).

Hypoxia induces a 3-fold increase (from 10 h to 30 h) in TH mRNA stability in the PC12 cells *in vitro* (Czyzyk-Krzeska *et al.* 1997). The increased stability observed during hypoxia results from a rapid induction of binding of a cytoplasmic 50 kD hypoxia-inducible protein (HIP) to a pyrimidine-rich (U/C)(C/U)CCCU motif (UCCCCU is preferable; located between bases 1551 and 1578 3′ to the translation stop codon) (Czyzyk-Krzeska & Beresh 1996; Czyzyk-Krzeska *et al.* 1997). This novel cis-element is referred to as the HIP-binding site and is conserved in the TH mRNAs from different species. The HIP-binding site is also present in the 3′UTR of other hypoxia inducible genes such as Epo (Rondon *et al.* 1991), and VEGF (Levy *et al.* 1995). Therefore, interaction of HIP with the HIP-binding site may play an important role in hypoxia-mediated regulation of mRNA stability for several genes.

## Conclusion

Although still poorly understood, multiple mechanisms for the regulation of hypoxia-responsive gene expression have been demonstrated, and many of these mechanisms seem to share certain common features (Figure 1; Tables 1 and 2). For example, hypoxic induction can be favoured by cellular redox state, mimicked by transition metal ions such as cobalt and iron chelating agents but not by inhibitors of mitochondrial respiration. These have led to the proposal of specific haem protein(s) which act as an oxygen sensor for the cell. Signals from this proposed oxygen sensor would then activate HIF-1, which in turn provides a common regulatory mechanism for increased gene expression through transcriptional activation (Table 1). The promoter regions of many hypoxia-responsive genes also contain additional transcription factor binding sites, such as AP-1, AP-2, and NF-κB (Table 1). Thus, synergistic co-operation of HIF-1 with distinct transcription factors may be required to ensure adequate mRNA transcription during sustained hypoxia.

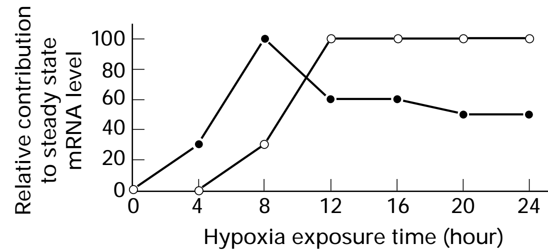


**Figure 1.** Schematic illustrations of transcriptional activation and mRNA stabilization of hypoxia-responsive gene expression. A biphasic induction of hypoxia-responsive gene during the course of hypoxic stress (i.e. VEGF, Epo, TH, and GLUT-1). The transcription rates (●) usually peak, plateau or decline over time, while steady-state mRNA levels (○) increases or remains elevated throughout the duration of hypoxic exposure.

Both transcriptional activation and mRNA stabilization mechanisms are involved in the maintenance of high levels of mRNA during the course of hypoxia. The transcription rates usually peak, plateau or decline over time, while steady-state mRNA levels increase or remain elevated throughout the duration of hypoxic exposure (Figure 1). Thus, it appears that cells first respond to decreased oxygen tension through activation of transcription and then secondary stimuli such as metabolic changes give rise to increased stability of hypoxia-regulated mRNAs. In many cases mRNA degradation requires ongoing protein synthesis because mRNA stabilization is potentiated in the presence of various protein synthesis inhibitors. The activation of PKC, protein tyrosine kinase c-Src, GTP-binding protein Ras, and MAP kinase signalling pathways have all been implicated in hypoxia signal transduction. However, our knowledge of hypoxia-mediated signal tranduction is limited and the molecular components to link hypoxic-activated transcription factors

and factors involved in mRNA stabilization are still unknown. Future studies hopefully will define the accessory proteins necessary for oxygen responsiveness, the pathways cells use to transduce hypoxia-specific signals and assemble transcription factors for gene activation, and define the proteins needed to maintain mRNA stability.

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