Hypoxic Regulation of Lactate Dehydrogenase A

INTERACTION BETWEEN HYPOXIA-INDUCIBLE FACTOR 1 AND CAMP RESPONSE ELEMENTS*

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The oxygen-regulated control system responsible for the induction of erythropoietin (Epo) by hypoxia is present in most (if not all) cells and operates on other genes, including those involved in energy metabolism. To understand the organization of cis-acting sequences that are responsible for oxygen-regulated gene expression, we have studied the 5′ flanking region of the mouse gene encoding the hypoxically inducible enzyme lactate dehydrogenase A (LDH). Deletional and mutational analysis of the function of mouse LDH-reporter fusion gene constructs in transient transfection assays defined three domains, between −41 and −84 base pairs upstream of the transcription initiation site, which were crucial for oxygen-regulated expression. The most important of these, although not capable of driving hypoxic induction in isolation, had the consensus of a hypoxia-inducible factor 1 (HIF-1) site, and cross-competed for the binding of HIF-1 with functionally active Epo and phosphoglycerate kinase-1 sequences. The second domain was positioned close to the HIF-1 site, in an analogous position to one of the critical regions in the Epo 3′ hypoxic enhancer. The third domain had the motif of a cAMP response element (CRE). Activation of cAMP by forskolin had no effect on the level of LDH mRNA in normoxia, but produced a magnified response to hypoxia that was dependent upon the integrity of the CRE, indicating an interaction between inducible factors binding the HIF-1 and CRE sites.

The regulation of gene expression by oxygen is an important feature of many biological processes. In energy metabolism for instance, mRNA levels for a number of glycolytic and gluconeogenic enzymes are subject to transcriptional regulation by oxygen in a coordinate and reciprocal manner (1–9). Other genes that are regulated by oxygen include vascular growth factors (10), transcription factors (11), glucose transporters (12), tyrosine hydroxylase (the rate-limiting enzyme for dopamine synthesis) (13), and erythropoietin (Epo),1 a hormone that regulates erythropoiesis in accordance with the oxygen carrying capacity of the blood (14).

Recent studies of Epo gene regulation have provided impor-

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1 The abbreviations used are: Epo, erythropoietin; HIF-1, hypoxia-inducible factor 1; LDH, lactate dehydrogenase; bp, base pair(s); CRE, cAMP response element; GH, growth hormone; H8, N-(2-[methylamino]ethyl)-5-isouquinolinesulfonamide; PGK, phosphoglycerate kinase 1.
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crucial for oxygen-regulated function. One of these is a HIF-1 binding site and is absolutely required for inducible activity. The other two domains lie on either side of this motif. One, which lies 5'- to the HIF-1 site, occupies a position similar to that of a critical cooperative site in the Epo enhancer. The other lies 3'- to the HIF-1 site and has the characteristic motif of a cAMP response element (5'-TGAAGTC-3') (28). Functional cooperativity is shown between these sites; concatamers that contain the HIF-1 site alone are not capable of driving oxygen-regulated expression of a reporter through a heterologous promoter, whereas concatamers containing the HIF-1 site in conjunction with either of the other sites are, and the combination of all three sites together confers the greatest level of hypoxic inducibility. Moreover, forskolin, which modulates activity of the cAMP system by activation of protein kinase A, is shown to increase the magnitude of hypoxic induction of the LDH-A gene by interacting with the CRE.

EXPERIMENTAL PROCEDURES

Cell Lines and Culture Conditions—All experiments were performed on HeLa (human cervical carcinoma) cells grown in minimum essential medium with Earle's salts supplemented with 10% fetal calf serum, glutamine (100 mM), penicillin (50 IU/ml), and streptomycin sulfate (50 μg/ml). In studies of endogenous gene expression plates were used when 70% confluent. Hypoxic stimulation (1% O2, 5% CO2, 75% N2) in most experiments; in some 0.5%, 3%, and 5% O2, each with 5% CO2 and N2 to balance) was provided by incubation for 14–16 h in a Napco 7110 incubator.

Transfection and RNA Analysis—In all experiments the test plasmid (20–40 μg), encoding human growth hormone as a reporter, was transfected by electroporation in combination with a control plasmid (20–40 μg), which expressed human α-globin. Transfected cells were split equally and incubated in parallel under normoxic (20% O2, 5% CO2, 75% N2) or hypoxic conditions as described above. RNA was extracted, analyzed by RNase protection, and quantitated as described previously (22). The presence of an element within a transiently transfected plasmid was confirmed by double hybridization with probes that protected 120 bp of GH mRNA and 132 bp of α-globin mRNA was performed on 3–5 μg of RNA. For assay of endogenous gene expression, 25 μg of RNA was hybridized with a cRNA probe that protected 92 bp of human LDH-A mRNA transcribed from exons 2 and 3. A small quantity of antisense RNA generated by in vitro transcription of a fragment of the rat endothelin 1 gene was added to each of these samples, together with a sense probe that protected 333 bp of that transcript. Estimation of the recovery of the endothelin hybridization product served to control for the effects of variation in sample processing and gel loading between specimens.

Deletional and Mutational Analysis—The plasmid LDHGH formed the basis for deletional and mutational analysis of the mouse LDH-A promoter (22). This contained 233 bp of mouse LDH-A promoter and adjacent 5' sequence, extending from position –186 to 47 (where 1 is the transcription initiation site), cloned into the BamHI site in the 5' untranslated region of the growth hormone (GH) reporter. Progressive 5' truncations of the LDH element in this construct were made by PCR using appropriate oligonucleotides (Fig. 1). The region that was defined as critical for oxygen-regulated control of the reporter was then examined using a series of 14 consecutive 4-bp mutations of the LDHGH plasmid in which purines were substituted for their non-complementary pyrimidines, and vice versa (Fig. 2). These were made using the pALTER-based Altered Sites in vitro mutagenesis system as directed by the manufacturer (22), promega, Southampton, United Kingdom). The nucleotide sequence of the LDH element in all plasmids was confirmed by dideoxy sequencing.

Functional Testing of Concatamerized Elements—Since mutational analysis defined three domains that were functionally important in driving oxygen-regulated expression of the GH reporter, constructs were made to determine whether these domains, concatamers singly or in combination with one another, were capable of conferring the ability to respond to hypoxia on a minimal heterologous promoter (Fig. 3). The three largest elements (Fig. 3, panel B: A–C) were made from LDHGH by PCR using appropriate oligonucleotides with 5' ends that could be digested with XbaI (5'-GCTCTAGA-LDH sequence)-3'. The three smallest elements (Fig. 3, panel B: D–F) were synthesized as oligonucleotides, which, when phosphorylated and annealed, produced elements with 5' XbaI- and 3' Spel-compatible ends (top strand: 5'-CTAGA-LDH sequence)-A-3'; bottom strand: 5'-CTAGT-LDH sequence)-T-3'). The elements were each then cloned into an XbaI site located 10 bp 5' to the herpes simplex thymidine kinase TATA box of the reporter plasmid, which had low basal activity and was not itself inducible by hypoxia (22). The nucleotide sequence of dimers or trimers of each element was confirmed by dideoxy sequencing.

Nuclear Extract and Electrophoretic Mobility Shift Assay—A modification of the protocol of Dignam et al. (30) was used to prepare nuclear extract. Oligonucleotides used as probes or competitors (Table I) were purified by polyacrylamide gel electrophoresis. Labeling of the 5' nucleotide in the probes was performed with [γ-32P]ATP (3000 Ci/mmol) using phage T4 polynucleotide kinase. Labeler oligonucleotides were annealed with a 4-fold molar excess of the complementary strand. Unlabelled oligonucleotides were annealed in molar equivalent quantities. Binding reaction mixtures (20 μl) contained 200 mCi KCl, 1 mM MgCl2, 0.5 mM EDTA, 5 mM dithiothreitol, 5% (v/v) glycerol, and 0.15–0.30 μg of sonicated poly(dI–dC). Nuclear extract (3 μg) was incubated with this mixture for 5 min at room temperature, before probe (approximately 0.1 ng) and specific competitors were added. Incubation was continued for another 10 min. Reactions were electrophoresed (12.5 V/cm) at 4°C using 5% polyacrylamide in 0.3 × TBE (30 mM Tris, 30 mM borac acid, 0.06 mM EDTA, pH 7.3, at 20°C).

Modulation of Cyclic AMP—The mutational analysis and the functional testing of concatamerized elements both suggested that one of the functional domains important for oxygen-regulated expression had the characteristic sequence motif of a CRE (5'-TGAAGTC-3') (28). The effect of agents that modulate activity of the cAMP system on oxygen-regulated expression of the endogenous LDH gene and of transiently transfected constructs was therefore examined. Two agents were used: H8 (2'-deoxy-5'-O-(ethylamino)ethyl)-5-isoyquinolinesulfonamide), an inhibitor of protein kinase A; and forskolin, an activator of the cAMP protein kinase A system. For assay of endogenous LDH expression, H8 (15 μM) or forskolin (50 μM) were applied to cells incubated in normoxia, and 8 h later the plates were transferred into the hypoxic incubator, where they remained for 16 h. Controls were exposed to the agents for 24 h of normoxic incubation. In transient transfection experiments, cells were incubated with the same concentrations of H8 or forskolin for 8 h before they were harvested for electrophoresis, and the chemicals were applied again immediately after the cells had been plated out following transfection. Control transfections were performed in parallel using cells that were not exposed to either agent.

RESULTS

We and others have previously reported that LDH-A mRNA is inducible by exposure to 1% O2 (5, 9, 22). In this study we found that it was inducible by hypoxia over a range of oxygen concentration from 5% to 0.5%. In comparison with paired normoxic control plates we found induction as follows: 5% O2, 9.5% (n = 5); 3% O2, 2.03 ± 0.41 (n = 4); 1% O2, 1.67 ± 0.26 (n = 4); 0.5% O2, 2.00 ± 0.10 (n = 5). For comparison with previously published work, all experiments were performed with the 1% O2 stimulus (5, 9, 16, 17, 22, 29). The effect of a series of truncations of the 5' end of the LDH sequence on the ability to drive expression of the reporter in normoxia and hypoxia are shown in Fig. 1. Deletion of 16 bp between L3 and L3.2 caused a reduction of only 16% (n = 4) in the level of normoxic expression, but a fall in the ratio of hypoxic/normoxic expression from 7.9 ± 1.5 to 1.5 ± 0.3, thus
defining the 5’ extent of the oxygen-regulated control element.

The effect of a series of 14 consecutive 4 bp mutations, the most of which begins 8 bp to the first truncation shown to ablate oxygen-regulated function (L3.2), is shown in Fig. 2. Three domains are important, defined by mutations (M) 1, M4-M5, and M10-M11, of which the one affected by M4-M5 seems to be the most significant, since these mutations completely abrogated the ability to respond to hypoxia.

The capacity of the three domains to drive hypoxic expression through a heterologous minimal promoter, when concatamered either singly or in combination with one another, is shown in Fig. 3. Neither a dimer nor a trimer containing solely the domain defined by M4-M5 could confer oxygen-regulated expression. By contrast, dimers containing both the M4-M5 domain and either of the other domains were capable of oxygen-regulated action, although one construct containing both the M4-M5 and M10-M11 domains was not, for reasons that are unknown. A dimer containing all three domains conferred the greatest level of hypoxic inducibility.

The domain defined by M4-M5 has the motif of a HIF-1 site (5’-(C/G/T)ACGT(G/C)(G/T)-3’). For analysis of the ability of this element to bind hypoxically inducible nuclear factors, a double-stranded oligonucleotide based on the domain was incubated with nuclear extract from normoxic and hypoxic HeLa cells. Figs. 4 and 5 show a pattern of binding to this oligonucleotide that closely resembles that seen using oligonucleotides derived from HIF-1 binding sites in the Epo 3’ enhancer and the PGK 5’ flanking sequence. They also demonstrate that there is cross-competition for binding of hypoxia-inducible factor(s) between these sequences, and that this is lost when the HIF-1 binding site of either the Epo or PGK oligonucleotide is mutated.

The domain defined by M10-M11 has the motif of a CRE. To test for functional involvement of cAMP in basal normoxic and hypoxically induced expression of LDH-A, the effects of the protein kinase inhibitor H8 and the protein kinase A activator forskolin were examined. The results are shown in Fig. 6. Neither agent altered the level of normoxic expression of the
endogenous gene. Both M4 and M10 produced a lower level of normoxic expression than did the wild type LDHGH construct. The level of normoxic expression of all three constructs was reduced in the presence of forskolin, whereas H8 was without effect. Forskolin induced a substantial increase in the ratio of hypoxic/normoxic expression of both the endogenous gene (control 1.40 ± 0.09, forskolin 3.80 ± 0.89, n = 6, p < 0.05) and the reporter after transfection with wild type LDHGH (control 6.7 ± 1.4, forskolin 17.1 ± 1.5, n = 3, p < 0.05). As expected, the M4 construct was not capable of driving oxygen-regulated expression and did not become so in the presence of either H8 or forskolin. Construct M10 did exhibit hypoxic inducibility, but in contrast with wild type LDHGH, the magnitude of this inducibility was not altered by forskolin (ratio of hypoxic/normoxic expression: control 5.2 ± 1.5, forskolin 6.4 ± 0.6, n = 3, p not significant), providing functional evidence that the ability of cAMP to magnify the hypoxic induction of LDH is modulated by the CRE motif placed between –48 and –41 bp upstream of the transcription initiation site.

**DISCUSSION**

Functional similarities between the oxygen-dependent regulation of genes encoding glycolytic enzymes and the regulation of the Epo gene have led to the analysis of glycolytic gene control sequences for sites that bind HIF-1, a nuclear factor that is critical for inducible operation of the Epo 3′ enhancer (22, 23). Such a site was demonstrated by deletional analysis to be crucial for the activity of the element driving oxygen-regulated expression in the phosphoglycerate kinase 5′ flanking sequence (22), providing firm evidence linking the hypoxic induction of glycolytic enzymes and Epo to a common cellular oxygen sensing mechanism. Other potential HIF-1 sites have been defined by sequence analysis of glycolytic gene loci. For several of these, HIF-1 binding activity has been shown, and for some the functional activity of concatamerized sequences has been demonstrated on heterologous reporters (23). However, no detailed analysis of the organization of an hypoxia-inducible nuclear factor, and that it cross-competes for the binding of such factors with oligonucleotides from the PGK and Epo hypoxic enhancers (for oligonucleotide sequences, see Table I). 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).

![Electrophoretic mobility shift assay showing that an oligonucleotide from the LDH-A 5′ flanking region (LDH) binds to an hypoxia-inducible nuclear factor, and that it cross-competes for the binding of such factors with oligonucleotides from the PGK and Epo hypoxic enhancers (for oligonucleotide sequences, see Table I). 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).](image_url)
promoter and that in the Epo enhancer relates to its basal activity. Whereas the latter has little activity in normoxic cells (17), the former has strong constitutive action, and mutation at the HIF-1 site has a substantial effect on this. Either HIF-1 or the constitutive complexes that bind to the HIF-1 site in the LDH-A promoter (see Figs. 4 and 5) could be responsible. Mutation M1 had no action on constitutive promoter function but severely reduced inducible promoter function. It is interesting that there is a striking similarity between the positions of this domain with respect to the HIF-1 site in LDH, and the arrangement of functional domains in the Epo 3′ enhancer (17, 20, 24–26). Table II shows an alignment of the LDH-A promoter (in reverse orientation) with the sequences of the mouse and human Epo enhancers. It can be seen that in each case mutational analysis has defined a functionally critical domain in the same position, between 7 and 11 bp to the HIF-1 site. There is sequence homology between this region in mouse LDH-A and mouse Epo, with 3 of 4 bp identical, but not between the mouse sequences and that of human Epo. The nature of the factor that binds to this site is unknown. In LDH-A, but not Epo, the sequence that is mutated in M1 is part of a Myo-D left binding site (5′-CACGTG-3′) (31). However, the consensus extends into the mutants M0 and M2, neither of which affected function. Nevertheless, it is possible that in muscle cells Myo-D could affect the inducible operation of the LDH promoter by binding at this site.

The third domain (M10-M11) involved in hypoxically inducible function of the LDH-A promoter has the characteristic motif of a cyclic AMP response element (5′-TGACGTCA-3′) (28). Mutation of this site reduced, rather than abolished, the magnitude of induction by hypoxia, and in keeping with previous analyses it was also found to control constitutive promoter function (1, 2, 32).

The minimal requirements for hypoxically inducible function were determined by assay of concatamers of sequenced subunits placed adjacent to a minimal promoter. None of the constructs that omitted the HIF-1 site showed any inducible activity. However, the HIF-1 element alone was unable to support inducible expression, even as a multimer. By contrast, constructs that contained the HIF-1 site, accompanied by either the upstream domain or the CRE, could convey inducible function.

![Figure 5](image1.png)

**Fig. 5.** Electrophoretic mobility shift assay showing cross-competition for the binding of hypoxia-inducible nuclear factor to the LDH oligonucleotide by unlabeled wild type PGK and Epo oligonucleotides, but not by PGK and Epo oligonucleotides in which the HIF-1 site has been mutated (PGK-M and Epo-M). Details are as described in Fig. 4.

![Figure 6](image2.png)

**Fig. 6.** Effect of agents that modulate cAMP activity on basal normoxic expression and the response to hypoxia of the endogenous LDH-A gene (panels A and B), and of transiently transfected LDGH constructs (panels C and D, wild type; panels E and F, mutant involving the HIF-1 site (M4); panels G and H, mutant involving the CRE site [M10]). Values shown are the means ± S.E. of six (endogenous gene) or three (transient transfection) independent experiments. Basal normoxic expression is given relative to that in untreated control cells (endogenous gene) or to that in cells transfected with wild type LDGH (both defined as 1). *p < 0.05 versus control (Student's t test, with Bonferroni correction for multiple comparisons). For experimental details, see "Experimental Procedures."
indicating that cooperative interactions necessary for hypoxic function could take place with factors binding either of these sites. A requirement for cooperation between HIF-1 and other binding sites is consistent with analysis of the Epo enhancer (17, 20, 24–26). However, in our analysis of the mouse phosphoglycerate kinase-1 enhancer, we found that a multimerized 18-bp sequence containing the HIF-1 site was sufficient to confer hypoxic inducibility on a heterologous promoter (22). Semenza and colleagues (23) have recently demonstrated this property using a dimer of 24 bp of sequence from the human phosphoglycerate kinase gene, and with a trimer of a 26-bp oligonucleotide derived from mouse phosphofructokinase L sequence. It is not clear whether the HIF-1 complexes at these sites are in some way different from those in Epo and LDH-A in being able to drive oxygen-regulated expression on their own, or whether the elements studied contain other cooperative domains.

The finding that the CRE was one of the functional domains in the LDH-A promoter required for the response to hypoxia was clearly of interest in relation to the ability of cAMP to stimulate LDH-A expression in some cells (1, 2, 32). In HeLa cells we found that neither the protein kinase A inhibitor H8 nor the cAMP agonist forskolin produced significant changes in LDH-A mRNA in normoxia. By contrast, hypoxic induction of the endogenous gene was much magnified in the presence of forskolin. The transfected wild type LDHGH construct also had this property, but the M10 promoter mutated construct did not, demonstrating that the interaction occurred within the LDH-A promoter, and was dependent on an intact CRE. The M4 mutated promoter was completely unresponsive to hypoxia in the presence of forskolin, indicating that the HIF-1 site remained absolutely necessary for hypoxic function.

Although we did not measure protein kinase A activity, the observation that forskolin did not augment the response to hypoxia in the CRE-mutated construct strongly suggests that this action was modulated by the adenyl cyclase/cyclic nucleotide kinase A/CRE-binding protein system, rather than through one of forskolin’s other effects (33). We do not know which of the many CRE binding species are involved in this interaction; however, the finding that while forskolin augmented the hypoxic response, H8 did not reduce it, may indicate that both constitutive and inducible CRE species are involved. The small reduction in expression of all LDHGH plasmids in normoxic cells treated with forskolin, an effect not seen on expression of the endogenous gene, was not dependent on the CRE and is unexplained. One possibility is that sequence(s) excluded from the promoter construct may act on the endogenous gene to negatively regulate the effect that forskolin had on normoxic expression of that construct.

Overall, these results indicate the potential for complex interaction between the HIF-1 site and the CRE in the LDH-A promoter, the most marked being that stimulation of the CRE leads to a magnified hypoxic response. The precise nature of this interaction may vary between cell types, since in a study of vascular smooth muscle cells Marti and colleagues (9) found that forskolin induced LDH in normoxic cells, but did not produce enhancement of the response to hypoxia, and H8 reduced the induction of LDH by hypoxia. Agonists and antagonists of the cAMP system have been reported to affect a variety of cellular responses to hypoxia, including expression of the Epo gene (34). Since cAMP levels can be increased by hypoxia, it has been proposed that this second messenger is directly involved in the cellular signaling system that responds to this stimulus (9, 34). The current experiments demonstrate a different type of involvement of the cAMP system in hypoxic gene regulation, where factors binding to two distinct cis-acting sequences, the HIF-1 site and the CRE, interact.

In summary, we have performed a detailed analysis of the mouse LDH-A promoter, demonstrating that oxygen-regulated function depends upon cooperation between three domains: a HIF-1 binding site, which is absolutely necessary, but not by itself sufficient, for hypoxically inducible function; a domain adjacent to this, located in the same position relative to the HIF-1 site as a site critical for function of the Epo enhancer; and a CRE. These findings indicate both that HIF-1 is critical to the mechanism of hypoxic induction of LDH-A and demonstrate that the mechanism requires cooperation of HIF-1 with neighboring sites. The interactions of stimuli acting at the HIF-1 site and the CRE illustrate how different characteristics of an inducible response can be generated from interactions between specific binding sites.

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| Table II |
| Comparison of LDH and Epo sequences |
| Sequence |
| mLDH | CACACGTGCTGCTGTCTCACAGCC |
| mEpo | CACACGTGCTGCTGTCTCACAGCC |
| hEpo | CACACGTGCTGCTGTCTCACAGCC |

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