Drosophila melanogaster SL2 cells contain a hypoxically inducible DNA binding complex which recognises mammalian HIF-1 binding sites

Masaya Nagao, Benjamin L. Ebert, Peter J. Ratcliffe, Christopher W. Pugh*

Room 420, Institute of Molecular Medicine, John Radcliffe Hospital, Headington, Oxford OX3 9DU, UK

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Abstract Nuclear extracts from *Drosophila* SL2 cells were found to contain a hypoxically inducible complex capable of binding to hypoxia response elements from mammalian genes. This complex (HIF-D) resembled mammalian hypoxia inducible factor (HIF-1) in DNA sequence specificity, abrogation of induction by cycloheximide, induction by desferrioxamine and redox sensitivity of DNA binding. However, HIF-D was not induced by cobalt and was less sensitive to phosphatase than HIF-1. Endogenous phosphoglycerate kinase mRNA in SL2 cells showed similar inducible characteristics to HIF-D. These findings are evidence that the mammalian HIF-1 dependent system of oxygen regulated gene expression has a functional homologue in *Drosophila*.

Key words: Hypoxia; Erythropoietin; O_2 sensing; Redox; Homology; Evolution

1. Introduction

Most living organisms make important changes in gene expression in response to the availability of oxygen. Analysis of mammalian gene expression has demonstrated that many responses to hypoxia can be linked to a common mechanism of regulation involving the activation of a sequence specific DNA binding complex termed hypoxia inducible factor-1 (HIF-1) [1–7]. The prototype for this system is erythropoietin (Epo), the hormone involved in the feedback control of red cell production in response to the adequacy of blood oxygen delivery (for reviews see [8,9]). HIF-1 was identified as an inducible nuclear factor binding a site which is critical for the operation of the erythropoietin 3′ enhancer [1]. The universal operation of this enhancer in non-erythropoietin producing mammalian cell lines and the widespread expression of HIF-1 suggested that this might be a generally important mechanism for hypoxic regulation of gene expression [2,3]. The definition of HIF-1 binding sites in *cis*-acting sequences of several other genes [4–7], and alterations in hypoxic gene regulation in mutant cells lacking functional HIF-1 activity [10], have confirmed the importance of this system in the response of mammalian cells to hypoxia.

* Corresponding author. Fax: (44) (1865) 222500.

Abbreviations: CIP, calf intestinal alkaline phosphatase; EMSA, electrophoretic mobility shift assay; Epo, erythropoietin; EPO-wt, wild type erythropoietin oligonucleotide; EPO-mut, mutant erythropoietin oligonucleotide; HIF-1, mammalian hypoxia inducible nuclear factor; HIF-D, *Drosophila* hypoxia inducible nuclear factor; PAS, Per-AHR-ARNT-Sim; PGK, phosphoglycerate kinase; PGK-wt, wild type phosphoglycerate kinase oligonucleotide; PGK-mut, mutant phosphoglycerate kinase oligonucleotide

In understanding cellular responses to hypoxia it is important to know whether this system of mammalian gene regulation is conserved in lower organisms. Here we report the identification in *Drosophila* of responses to hypoxia which show striking similarities to the mammalian HIF-1 system.

2. Materials and methods

2.1. Cell culture

SL2 cells (ECACC No. 90070554) were cultured to 70% confluence in Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal calf serum, glutamine (2 mM), penicillin (50 IU/ml), and streptomycin sulphate (50 μg/ml) at 27°C in air. Hypoxic stimulation (1% oxygen, 99% nitrogen) was provided by incubation at 27°C in a Napco 7001 incubator for 16 h or other times as specified in the text.

2.2. Nuclear extracts

Nuclear extracts were prepared by a modification of the protocol described by Semenza et al. [1]. In brief, after parallel normoxic and hypoxic incubations, cells were harvested and resuspended in four volumes of a hypotonic buffer (10 mM Tris-HCl pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl supplemented with 0.5 mM PMSF, 1 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 0.5 mM benzamidine, 2 mM levamisole, 10 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 0.5 mM DTT (all from Sigma)). Cells were then lysed using a Dounce homogenizer. The nuclear pellet was mixed with three volumes of extraction buffer (20 mM Tris-HCl (pH 7.8), 1.5 mM $MgCl_2$,, 420 mM KCl, 20% glycerol, supplemented as above) and incubated for 30 min. After centrifugation (15000×*g* for 30 min) the supernatant was twice dialysed against 20 mM Tris-HCl (pH7.8), 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1 mM sodium orthovanadate and 0.5 mM DTT. All the above procedures were performed at 4°C.

2.3. Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed as described previously [4]. Binding buffer consisted of 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 or 15 mM DTT, 0.03% NP40 and 5% glycerol. 50 ng polydIdC were mixed with 5 μg of SL2 nuclear extract in a 20 μl reaction prior to addition of approximately 0.1 ng of labeled probe. Oligonucleotides EPO-wt (GCCCTACGTGCTGCCTCGCATGGC) or PGK-wt (CGCGTCGTGCAGGACGTGACAAAT) were used as probe or wild type competitors and EPO-mut (GCCCTAATGTCTGCCTCGCATGGC) or PGK-mut (CGCGTCGTGCAGGAATGTACAAAT) as mutant competitors. Oligonucleotides were purified by polyacrylamide gel electrophoresis and labeled with [γ-^{32}P]ATP (3000 Ci/mmol) using T4 polynucleotide kinase. Labeled oligonucleotides were annealed with a 4-fold molar excess of the complementary strand. Unlabeled oligonucleotides were annealed in molar equivalent quantities.

2.4. Treatment of nuclear extract with phosphatase, diamide or DTT

Extracts prepared as described above were dialysed without sodium orthovanadate and incubated with calf intestinal phosphatase (Boehringer Mannheim) (0.1–1 unit/5 μg extract for 15 min at room temperature) prior to EMSA analysis as described above.

Nuclear extracts prepared without DTT were added to binding buffer without DTT and incubated for 10 min at room temperature with diamide (1 mM) with, or without, subsequent incubation with

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DTT (10 mM) prior to EMSA analysis. Alternatively, standard nuclear extracts in binding buffer were incubated with diamide at a final concentration of 25 mM for 10 min and then incubated with or without 30 mM DTT for 5 min prior to EMSA analysis as described above.

2.5. RNase protection assays

RNA preparation and RNase protection assays were performed as described previously [11]. For analysis of *Drosophila* PGK expression a riboprobe against parts of exon 2 and intron 2 (nt 1901–2018 from *Drosophila* PGK genomic DNA (ACC No. Z14029)) was generated.

3. Results

To test whether *Drosophila* cells contained a species with characteristics similar to HIF-1, the binding of nuclear extracts prepared from normoxic and hypoxic SL2 cells to oligonucleotides containing HIF-1 binding sites was analysed in EMSA. Oligonucleotides were derived from the HIF-1 binding site in the mouse erythropoietin 3′ enhancer [11], or mouse phosphoglycerate kinase 5′ enhancer [4]. Results are shown in Fig. 1. For comparison an EMSA using normoxic and hypoxic nuclear extracts from HeLa cells is shown to demonstrate the inducible species (HIF-1) and constitutive species from mammalian cells which bind this site. The DNA binding activities observed using *Drosophila* nuclear extracts were similar, but not identical, to those using mammalian extracts. The most striking similarity was the presence of a strongly inducible species in *Drosophila* nuclear extracts (HIF-D) which had a similar, though somewhat lower, mobility than mammalian HIF-1. This inducible species was competed by an excess of unlabeled wild type oligonucleotide but not by an excess of unlabeled oligonucleotides containing a functionally disabling four base pair mutation at the HIF-1 binding site, indicating specific binding to the HIF-1 site. In keeping with this, these extracts produced no inducible binding when the mutant oligonucleotides were used as probes (data not

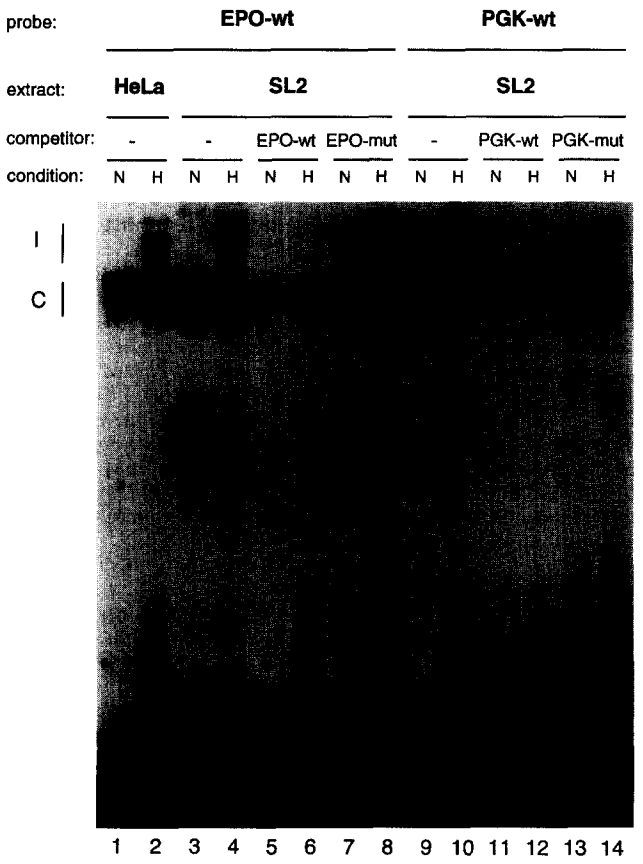

Fig. 1. Specific binding of HIF-D to mouse EPO or PGK probes which contain HIF-1 sites. 5 μg of nuclear extract from normoxic (N) or hypoxic (H) HeLa (lanes 1–2) or SL2 cells (lanes 3–14) were incubated with ^{32}P-labelled Epo-wt (lanes 1–8) or Pgk-wt (lanes 9–14) oligonucleotides. Competition with a 200-fold molar excess of unlabelled oligonucleotide was as follows: Epo-wt in lanes 5–6; Epo-mut in lanes 7–8; Pgk-wt in lanes 11–12; Pgk-mut in lanes 13–14. Extracts were incubated in binding buffer for 5 min. Competitors, followed immediately by probe, were added and after a further 10 min incubation samples were separated on a 5% polyacrylamide EMSA gel. Positions of inducible (I) and constitutively (C) retarded species are marked. An additional high mobility band was seen inconsistently with the *Drosophila* extracts.

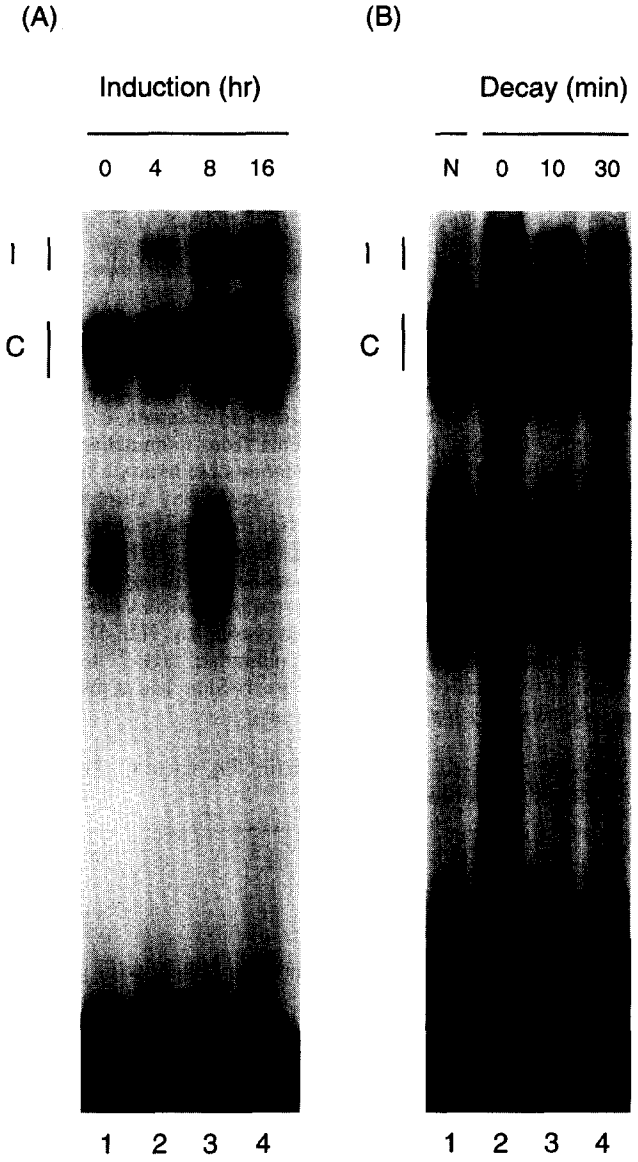

Fig. 2. Time course of induction and decay of HIF-D activity. (A) EMSA using the Epo-wt probe and nuclear extracts of SL2 cells prepared after incubation in normoxia (lane 1) or hypoxia (1% O_2) for 4, 8 or 16 h (lanes 2, 3 and 4 respectively). (B) EMSA using the Epo-wt probe and nuclear extracts from SL2 cells incubated in normoxia (lane 1), hypoxia for 16 h and then returned to normoxia for 0, 10 or 30 min (lanes 2, 3 and 4 respectively). Positions of inducible (I) and constitutively (C) retarded species are marked.

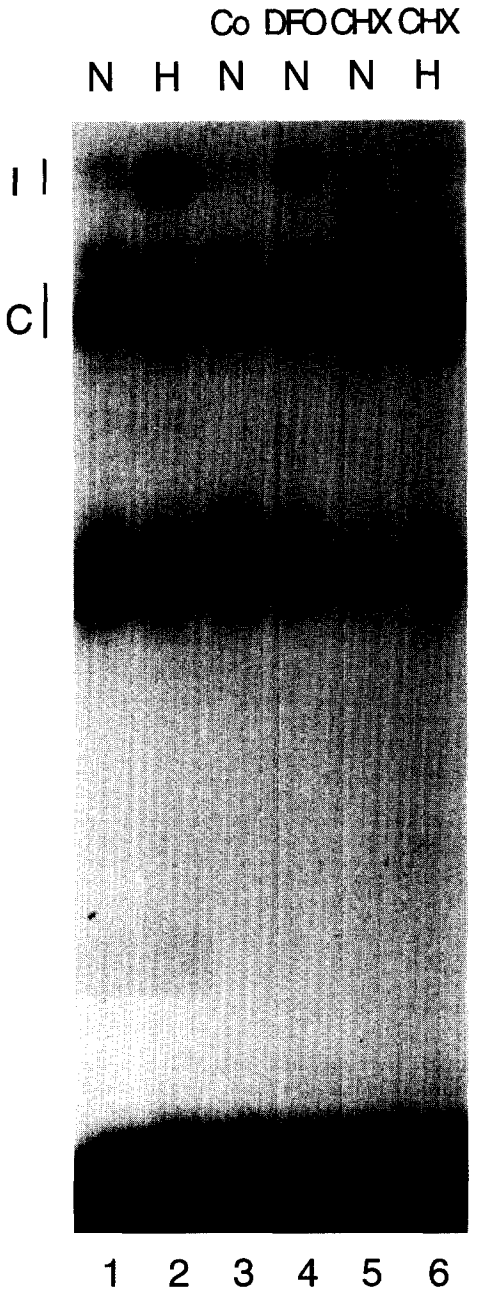

Fig. 3. Induction of HIF-D in SL2 cells cultured in the presence of cobaltous chloride, desferrioxamine or cycloheximide. Nuclear extracts were prepared from SL2 cells cultured in normoxia (N) with the addition of 100 μM cobaltous chloride (lane 3), 100 μM desferrioxamine (lane 4) or 100 μM cycloheximide (lane 5) or cultured in hypoxia (H) (lanes 2 and 6), with the addition of cycloheximide (lane 6). EMSA was performed using the Epo-wt probe. Positions of inducible (I) and constitutively (C) retarded species are marked.

shown). Both the Epo and PGK oligonucleotides also bound species of higher mobility which were constitutively present in normoxic *Drosophila* cells and of similar mobility to the constitutive species in HeLa cell extracts that bind this site.

The time course of induction of HIF-D in SL2 cells exposed to hypoxia for different lengths of time and the decay of HIF-D in hypoxic SL2 cells transferred to a normoxic environment are shown in Fig. 2. Induction of HIF-D was observed in cells exposed to hypoxia for 4 h but was more prominent at 8 and 16 h. Decay following transfer of cells to normoxia was rapid, although a small amount of HIF-D was still detectable 30 min after transfer to normoxic conditions.

Since both the iron chelator, desferrioxamine, and cobaltous ions may induce HIF-1 [3,12], we examined the effect of these compounds on *Drosophila* cells. No induction of HIF-D was observed with cobaltous ions (100 μM). Only slight induction was observed after exposure to desferrioxamine. HIF-1 induction in mammalian cells is sensitive to the protein synthesis inhibitor cycloheximide [1]. Similarly, exposure of *Drosophila* cells to 100 μM cycloheximide completely abolished induction of HIF-D (Fig. 3).

Since HIF-1 DNA binding activity is sensitive to phosphatases [13] we compared the sensitivity of HIF-1 and HIF-D DNA binding activities to calf intestinal phosphatase (CIP). Nuclear extracts were incubated at room temperature for 15 min with CIP prior to setting up oligonucleotide binding reactions. Results are shown in Fig. 4. As has been reported the DNA binding activity of HIF-1 was sensitive to CIP. In con-

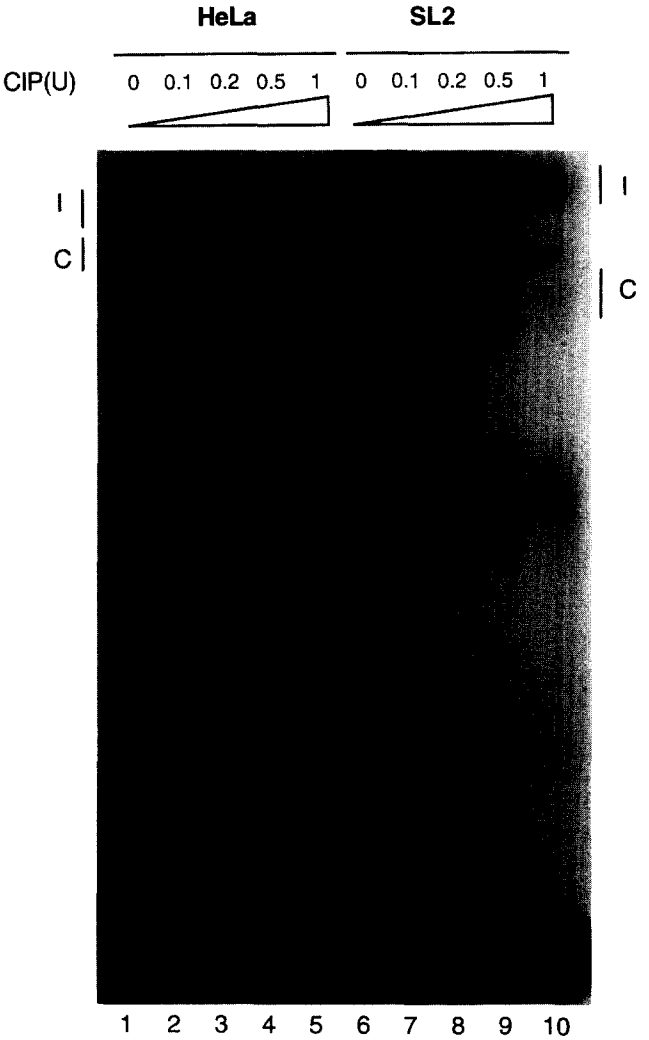

Fig. 4. Comparison between human HIF-1 and HIF-D in phosphatase sensitivity of DNA binding. Hypoxic nuclear extracts (5 μg) from HeLa (lanes 1–5) or SL2 cells (lanes 6–10) were incubated with the indicated amounts of calf intestinal alkaline phosphatase at room temperature for 15 min and analyzed by EMSA using the Epo-wt probe. Positions of inducible (I) and constitutively (C) retarded species are marked.

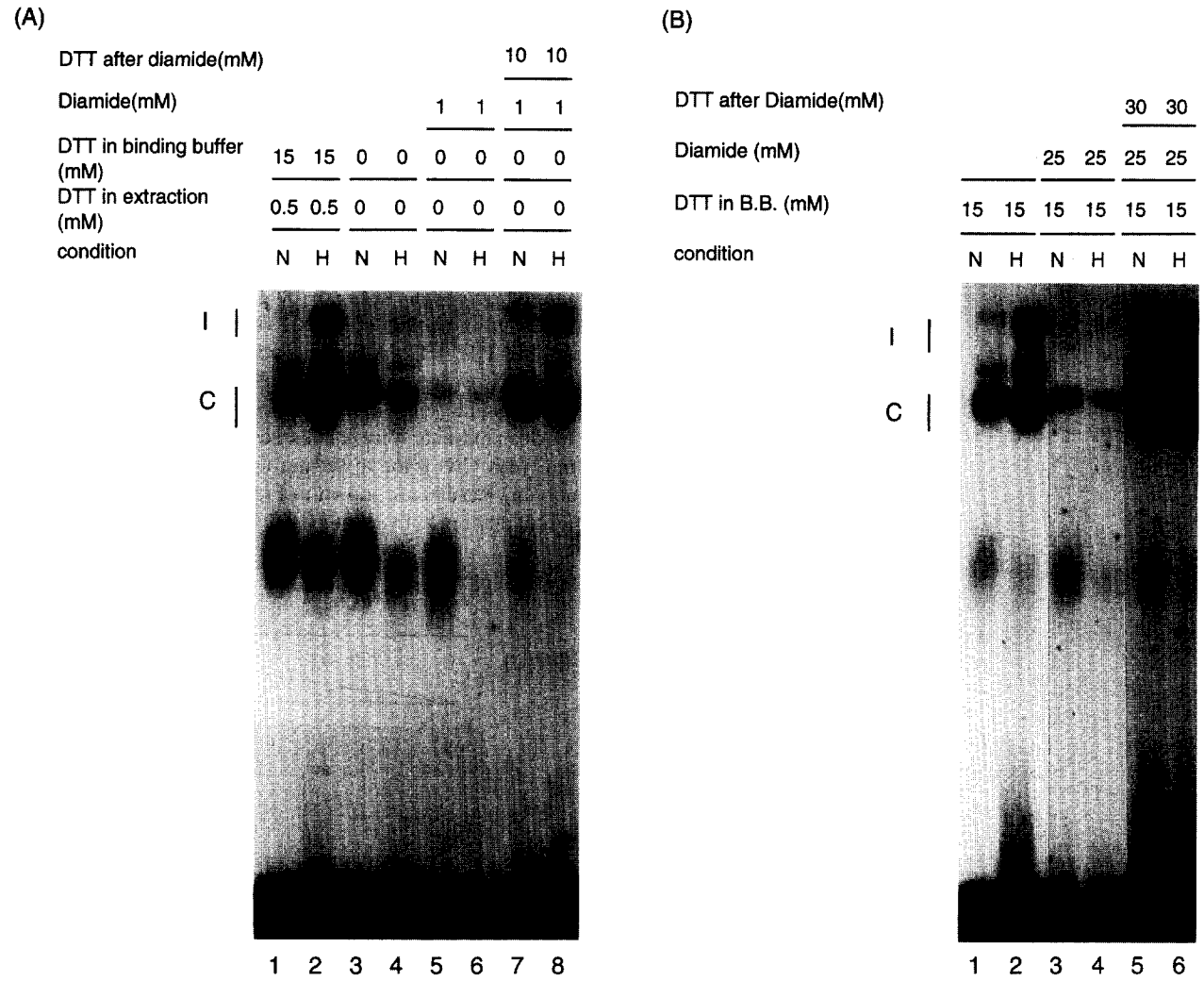

Fig. 5. Influence of diamide and DTT on DNA binding by HIF-D. (A) Nuclear extracts were prepared from SL2 cells cultured in normoxia (N) or hypoxia (H) as indicated. EMSA analysis was performed using the Epo-wt probe. Standard conditions were used in lanes 1 and 2. Extracts prepared in the absence of DTT were used in lanes 3–8. Extracts were incubated with diamide (1 mM) added to the binding buffer (lanes 5–8). The effects of subsequent incubation with DTT (10 mM) prior to probe addition are shown (lanes 7–8). (B) Nuclear extracts from SL2 cells cultured in normoxia (N) or in hypoxia (H) were prepared in the presence of 0.5 mM DTT and used for EMSA. EMSA was done in the presence of 15 mM DTT (lanes 1–6) with 25 mM diamide (lanes 3–6) and after addition of 30 mM DTT following diamide treatment (lanes 5–6).

trast, HIF-D DNA binding activity was relatively resistant to CIP, whereas the 'constitutive' DNA binding complexes in both mammalian and *Drosophila* extract showed an intermediate sensitivity to phosphatase treatment.

HIF-D DNA binding activity was sensitive to oxidation. Omission of DTT from the preparative protocol and binding reaction diminished HIF-D activity, which was abolished by the addition of the sulfhydryl oxidising agent diamide (1 mM). Addition of DTT (10 mM) to the binding reaction restored HIF-D activity to the diamide treated nuclear extract which had been prepared without DTT (Fig. 5A). This reversible sensitivity of HIF-D DNA binding could also be demonstrated using nuclear extract prepared using DTT although higher concentrations of diamide (25 mM) and DTT (30 mM) were required (Fig. 5B).

Among the mammalian genes known to be regulated by HIF-1 the most highly conserved are those encoding glycolytic enzymes. We therefore tested whether, as in mammalian cells, the *Drosophila* PGK gene was inducible by exposure of cells to an atmosphere of 1% oxygen. An increase of approximately 3-fold in *Drosophila* PGK mRNA was demonstrated after exposure of cells to hypoxia for 16 h. A much smaller level of induction was observed in response to desferrioxamine. In contrast with mammalian cells but in keeping with the inducible characteristics of HIF-D *Drosophila* PGK mRNA was not induced by exposure of cells to cobaltous ions (Fig. 6).

4. Discussion

The sites involved in a functional interaction between two or more molecules are often highly conserved in evolution [14]. The demonstration of a hypoxically inducible nuclear factor in SL2 cells, which shows sequence specific DNA binding activity to mammalian HIF-1 recognition sites, thus provides important evidence for the existence of a homologous system of gene regulation by oxygen in *Drosophila*.

The characteristics of this *Drosophila* nuclear factor, which we have termed HIF-D, resembles mammalian HIF-1 in a

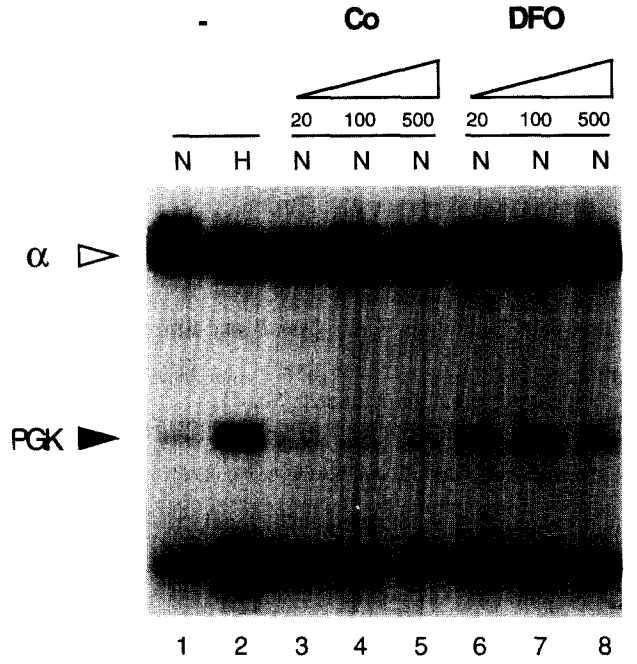

Fig. 6. Induction of the endogenous Pgk gene by hypoxia, cobaltous ion, or desferrioxamine analyzed by RNase protection assay. RNA was prepared from SL2 cells cultured in normoxia (N) or in hypoxia (H) in the presence of 20, 100 or 500 μM cobaltous chloride (lanes 3, 4 and 5 respectively), or in the presence of 20, 100 or 500 μM desferrioxamine (lanes 6, 7 and 8 respectively). Two protected species were consistently observed using the *Drosophila* Pgk riboprobe, and most probably represent alternatively spliced mRNAs; both were regulated in a similar manner. A fixed amount of RNA from K562 cells was added to each sample and analysed with a riboprobe protecting 133 bp human α-globin as a loading control (α).

number of other ways. The DNA binding complexes were of similar mobility, and in both cases induction was sensitive to cycloheximide, indicating a requirement for new protein synthesis at some stage in the activation mechanism. The requirement for dithiothreitol in the preparative buffers for HIF-D, and the reversible effects of diamide and dithiothreitol on DNA binding activity were again similar to findings for HIF-1 [15], and suggest that sensitive sulfhydryl groups must be in the reduced form for DNA binding activity. Though the potential involvement of redox reactions in the oxygen sensing process has led to interest in the possibility that changes in the redox status of protein sulfhydryl groups of HIF-1 might be involved in the mechanism of regulation, there is as yet no proof that such changes occur in the intracellular environment. It is nevertheless of interest that similar in vitro sensitivity to sulfhydryl reagents is observed for HIF-D.

The DNA binding activity of HIF-D was more resistant to treatment with calf intestinal phosphatase than HIF-1, although using high concentrations of the enzyme, binding was ultimately abolished in both cases. Based on this in vitro sensitivity to phosphatases, and the effects of protein kinase inhibitors on the induction of HIF-1 in hypoxic cells [13,16], it has been suggested that phosphorylation could be an important step in the activation of HIF-1. The difference between HIF-1 and HIF-D observed in these experiments might indicate a difference in the mechanism of activation of HIF-D; alternatively the critical phosphate group(s) may simply be less sensitive to this enzyme.

In addition to hypoxia, HIF-1 can also be activated in most mammalian cells by desferrioxamine and cobalt [3,12]. HIF-D was induced by desferrioxamine although the effect was very much less marked than has been observed for HIF-1. HIF-D was not induced by cobaltous ions. Interpretation of these differences will require a fuller understanding of the mechanisms underlying HIF-1 activation by desferrioxamine and cobalt. Although interactions with a putative hemoprotein sensor have been proposed [12,17], there is no direct evidence for such a mechanism, nor are the responses to hypoxia always mimicked by cobalt in mammalian cells [18].

The existence of a hypoxically inducible DNA binding complex in *Drosophila* SL2 cells raises the issue as to its function. In mammalian cells, HIF-1 has been implicated in the regulation of a number of hypoxically inducible genes. Examples include genes encoding erythropoietin, tyrosine hydroxylase, angiogenic growth factors, glucose transporters, and glycolytic enzymes [1,4—7]. For some of these genes, such as erythropoietin itself, the evolutionary origin is unknown. In other cases, such as the glycolytic enzymes, evolutionary conservation is clear. As has been found in mammalian cells, PGK mRNA was increased by hypoxia in SL2 cells. The induction of *Drosophila* PGK mRNA differed from that for mammalian PGK-1 in responding at a much reduced level to desferrioxamine and not discernably to cobalt. This pattern of response resembled that for HIF-D and would be compatible with the involvement of HIF-D in the regulation of *Drosophila* glycolytic enzymes. Further work will be required to establish this with certainty and to determine the function of HIF-D in this organism.

HIF-1 is a heterodimer complex consisting of two PAS domain containing basic-helix-loop-helix proteins, HIF-1α, a newly described member of this family, and HIF-1β, a transcription factor previously recognised as the aryl hydrocarbon receptor nuclear translocator (ARNT) [19,20]. It is probable that HIF-D consists of similar proteins. The *Drosophila* proteins Per and Sim are defining members of the PAS domain family. Since Per does not contain a basic-helix-loop-helix domain or any other known DNA binding domain, it is unlikely to be involved in the DNA binding of the HIF-D complex [21]. Analysis of Sim responsive genes has defined an asymmetric Sim response element (g/a t/a ACGTG) which is similar to the consensus for HIF-1 (g/t/c ACGTG) [22]. Based on comparative amino acid sequence analysis of the basic regions of basic-helix-loop-helix proteins it has been proposed that Sim might form a heterodimer with a *Drosophila* ARNT-like protein, with Sim binding the 5′ half-site and the ARNT homologue binding the 3′ half-site. Even though the basic region of Sim is closely similar to HIF-1α it is unlikely that Sim is a component of HIF-D, since expression of Sim is limited to embryonic midline neuroepithelium [23]. More likely, *Drosophila* possess other members of this gene family responsible for oxygen regulated gene expression.

In summary, recent evidence has implicated HIF-1 as an important regulator of mammalian gene expression in response to hypoxia. Evidence for a homologous complex in *Drosophila* suggests that this is a highly conserved system of transcriptional regulation by oxygen which probably has even more primitive origins.

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