Hypoxia-induced Synthesis of Hemoglobin in the Crustacean
*Daphnia magna Is Hypoxia-inducible Factor-dependent*[^S]

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Of the four known globin genes that exist in the freshwater crustacean *Daphnia magna*, several are individually induced by hypoxia, lending pale normoxic animals a visible red color when challenged by oxygen deprivation. The promoter regions of the *Daphnia* globin genes each contain numerous hypoxia response elements (HREs) as potential binding sites for hypoxia-inducible factors (HIFs). *Daphnia* HIF, bound to human HRE sequences, was detected in extracts from hypoxic (red), but not normoxic (pale), animals. Taking advantage of the phylogenetically conserved HIF/HRE recognition, we employed heterologous transfections of HIF-expressing human and *Drosophila* cells to model HIF signaling in *Daphnia*. These experiments revealed that three functional HREs within the promoter of the *D. magna* globin-2 gene cooperate for maximal hypoxic induction of a downstream luciferase reporter gene. Two of these three cis-elements, at promoter positions −258 and −107, are able to specifically bind human, *Drosophila*, or *Daphnia* HIF complexes in vitro. The same two sites are also necessary for maximal induction of reporter transcription under low oxygen tension in the presence of either endogenous human or overexpressed *Drosophila* HIF proteins. The third motif of the globin-2 gene promoter, a CACGTG palindrome at position −146, functions as a docking site for an unknown constitutive transcription factor. In human cells, this −146 complex interferes with HIF occupancy at the adjacent −107 HRE and thus controls the extent of HIF-mediated activation of the downstream target.

In 1758, the Dutch naturalist J. Swammerdam described the water flea *Daphnia* living in ponds or ditches and appearing with a red color “like that of beef” (1). Swammerdam did not realize that the development of coloration depends upon exogenous cues. Nearly 200 years elapsed before classical spectroscopy surveys in the 1940s and 1950s by Fox and co-workers established that the visible transition from pallor to a bright red color is due to the emergence of hemoglobin, particularly in response to low oxygen. This dramatic transformation was found to be a common feature among many lower crustaceans (i.e. “branchiopods”), including species of *Daphnia* (Cladocera), the brine shrimp *Artemia* (Anostraca), and *Triops* (Notostraca) (see Fig. 1a) (2, 3). Fox and Phear (4) realized the reversible nature of this hypoxic hemoglobin induction in daphnids, wherein resupply of oxygen results in hemoglobin loss and resumption of pallor at a rate similar to the gain in coloration. These early studies culminated in the realization that the amount of dissolved ambient oxygen is inversely related to the hemoglobin content in the hemolymph of many lower crustaceans (2, 5, 6). On the other hand, Fox and Phear (4) acknowledged that it was “unknown how a low oxygen pressure increases hemoglobin synthesis, either in Crustacea or in human mountain dwellers.” During the past 10 years, it has become clear that high altitude erythrocytosis, and thus enhanced hemoglobin synthesis, is due to regulation of erythropoietin expression by an oxygen-responsive transcription factor: HIF[^1] (see Refs. 7 and 8 for review). However, there is no information to date on the mechanism underlying the hypoxic induction of *Daphnia* hemoglobin.

When deprived of oxygen, *Daphnia magna* is able to raise its hemoglobin concentration by 15–20-fold. In the hemolymph of normoxic (pale) animals, the hemoglobin concentration is ~0.1 g/100 ml, whereas in hypoxic (red) animals, it increases to ~1.7 g/100 ml, approximately one-tenth the levels in human blood (9, 10). Remarkably, oxygen acquisition in the hemolymph of hypoxic *D. magna* improves because of increases not only in hemoglobin concentration, but also in oxygen affinity (11, 12). Measurements in purified hemoglobin solutions from pale and red animals revealed half-saturation oxygen pressures of 3.8 and 1.6 torr, respectively (13). These adaptations benefit hemoglobin-rich daphnids by extending the *pO2* range of their aerobic and regulated metabolism down to critical oxygen tensions (pO2) of ~1.3% oxygen, whereas pale animals begin to transition into anaerobiosis at pO2 values of ~4.8% oxygen (10, 12). Being able to raise both levels (see Refs. 14 and 15 for review) and oxygen affinity (11–13) of hemoglobin therefore imparts to red daphnids a higher tolerance toward environmental hypoxia and improvement in survival (9, 10) and reproduction rates (16), along with maintenance of numerous vital functions such as filtering activity and swimming behavior. As red animals approach anoxia, O2 consumption is enhanced, and acidosis is ameliorated compared with pale animals (see Refs. 12 and 14 for review).

*D. magna* hemoglobin differs from mammalian homologs in

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[^1]: The abbreviations used are: HIF, hypoxia-inducible factor; HRE, hypoxia response element; ARNT, aryl hydrocarbon receptor nuclear translocator; EMSA, electrophoretic mobility shift assay; igDNA, intergenic DNA; EpoE, erythropoietin 3’ enhancer.
several essential structural features that are pertinent to its regulation. Most important, *Daphnia* hemoglobin and indeed all other crustacean hemoglobins are extracellular respiratory pigments (see Fig. 1a) (2, 3, 17). This means that any oxygen-sensitive control of hemoglobin abundance should operate on the *Daphnia* globin genes per se rather than through elevation of red blood cell mass as in the “mountain dwellers” of Fox and co-workers (7, 8). Moreover, as inferred from sedimentation equilibrium studies, the native complex was found to be a 494-kDa 16-subunit polymer, with each of the ~31-kDa subunits containing not one, but two hemes (18). The subsequent cloning of a *Daphnia* globin cDNA fully confirmed the presence of a signal peptide, necessary for secretion, and of the two-heme domain 330-amino acid subunit sequence (19). Thus, each fully oxygenated *D. magna* hemoglobin is able to carry up to eight times as many O₂ molecules compared with human hemoglobin. Later reports identified similar two-domain globin cDNAs in the cladocerans *Daphnia pulex* and *Moina macrocopa* (20, 21).

The genome of *D. magna* contains at least four globin genes, all sharing two uniform heme domains, as a result of Paleozoic duplication/fusion events (22), and the placement of highly compact introns (61–140 bp in length) in homologous positions. Separated by short intergenic spacer DNAs, the *D. magna* globin genes exist in the following arrangement: 5'-hb4-hb3-hb1-hb2-3' (see Fig. 1b) (23). At the protein level, these globin genes respond differentially to hypoxia with rates of induction ranging from ~5-fold (hb1) to ~14-fold (hb2) and even ~20-fold (hb3) (23–26), suggesting that the preferential expression of the hb2 and hb3 genes at low *P*O₂ could contribute to the increased O₂ affinity seen in hypoxic hemolymph (see above) (24, 26). Subsequently, Zeis et al. (24, 26) observed that oxygen deficiency hardly impacts concentrations of hb1 transcripts, whereas it rapidly (<1 h) and markedly (up to 5-fold) elevates hb2 and particularly hb3 mRNA levels. Thus, hypoxia regulates globin genes in *D. magna* at the mRNA level (19, 25, 26).

Because of its central role in the oxygen-sensing signaling pathways in mammals (7, 8, 27–30), the primary candidate for mediating this hypoxic gene control would be the *Daphnia* homolog of HIF. HIF proteins are members of the family of *invertebrates* (36, 37), and *Drosophila* (38–41). This heterodimer is present only at low *P*O₂ when it activates expression of target genes through specific binding to short cis-regulatory E-box motifs called hypoxia response elements (HREs) in the promoter and/or enhancer regions of these genes. Functional HREs share the following consensus sequence: 5'-B(A/G)CGT-GVBBB-3' (where B is all bases except A, V is all bases except T, and the 4-base core required for HIF binding is underlined) (42). Oxygen-dependent activation of HIF, from mammals to invertebrates (e.g., *Drosophila*), is mediated through specific oxidative modifications of the α-subunit (43–46), which, as a consequence, is degraded under normoxic *P*O₂. At low oxygen tension, the α-subunit is stable and can assemble with constitutively expressed β-subunits (ARNT) to form the functional HIF transcription factor. In this study, we show that the hypoxic induction of *Daphnia* globin gene expression is HIF-dependent.

**MATERIALS AND METHODS**

**Animal Culture and Exposure to Hypoxia**—Daphnids (CE-14-2330, Carolina Biological Supply Co.) were determined by various species keys to be *D. magna* (47, 48). Animals were kept in active charcoal-percolated tap water, adjusted to pH 7.8, under 16/8-h light/dark photoperiod regimes and fed daily an enriched blend of four different species of green algae (CE-15-1950, Carolina Biological Supply Co.). Hypoxic exposure of 500–1000 daphnids in algal water were carried out in a three-arm flask flow-through system for the time periods indicated relative to normoxic (air/algal water) controls. The hypoxic in-flow gas was composed of computer-generated mixtures of *O*₂ and *N*₂ (Environics Systems 200). The *P*O₂ of the animal milieu was controlled through an oxygen/temperature sensor (CellOx 325, WTW Measurement Systems). Following an “adaptation period” of gradually intensifying hypoxia (usually overnight), the final hypoxic exposure was maintained between 2 and 3% oxygen.

**Cell Culture**—Hep3B human hepatoma cells (HB-8064, American Type Culture Collection) were cultured at 37 °C in air and 5% CO₂ atmospheres (balance *N*₂) in a minimal essential medium (Invitrogen) supplemented with 10% fetal bovine serum (Gemini Bioproducts) and 1% antibiotic/antimycotic preservative (100 IU/ml penicillin, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B; Invitrogen). Standard hypoxic challenge of Hep3B cells involved a 16-h exposure to 1% oxygen and 5% *CO*₂ (balance *N*₂) at 37 °C using an Espec BNP-210 incubator (Tabai Spec Corp.). *Drosophila* SL2 cells were purchased from American Type Culture Collection (CRL-1863) or provided by Drs. A. Michelson and N. Perrimon. The cells from *American Type Culture Collection* and Dr. Perrimon were cultured in Schneider's insect medium (Invitrogen); the cells from Dr. Michelson were cultured in Shields and Sang M3 insect medium (Sigma). Both media were enriched with 8.5% fetal bovine serum (Invitrogen), 0.5% yeastolate solution (Invitrogen), 0.25% Bacto-peptone (BD Biosciences), and 1% antibiotic/antimycotic preservative. SL2 cells were grown at 22 °C in 1% antibiotic/antimycotic preservative (100 IU/ml penicillin, 100 μg/ml streptomycin sulfate, 0.25 μg/ml amphotericin B; Invitrogen).

**Whole Cell Extracts, Nuclear Extracts, and Electrophoretic Mobility Shift Assay (EMSA)**—Subsequent to 16–120-h normoxic and hypoxic exposures, respectively, daphnids were harvested (i.e., sifted, washed with ice-cold 1× phosphate-buffered saline, and quick-frozen in liquid nitrogen), homogenized, and resuspended in four volumes of a hypotonic buffer A (10 mM Tris-HCl (pH 7.5), 1.5 mM MgCl₂, and 10 mM *KCl*). Homogenates were either converted into whole cell extracts or subfractionated into cytosolic and nuclear extracts (49). In brief, cells (Hep3B and SL2) and tissue pellets (*Daphnia*) were suspended in buffer A, homogenized with a Dounce homogenizer, and centrifuged at 1000 *g* for 5 min. The hypotonic supernatant comprised the cytosolic extract. The organellar (nuclear) pellet was mixed for 30 min in 3 volumes of hypertonic extraction buffer C (20 mM Tris-HCl (pH 7.5), 1.5 mM MgCl₂, 420 mM KCl, and 20% glycerol), during which time proteins dissociated from DNA. Following centrifugation at 16,000 × *g* for 30 min, the high salt nuclear extract was dialyzed against one change of buffer D containing 20 mM Tris-HCl (pH 7.5), 100 mM *KCl*, 0.2 mM EDTA, 20% glycerol, 1 mM sodium vanadate, and 0.5 mM dithiothreitol. To minimize proteolytic loss of target proteins, both buffers A and C were supplemented with the following mixture of proteolytic inhibitors and reducing agents: 1 mM sodium vanadate, 0.5 mM benzamidine, 10 mM β-glycerophosphate, 2 mM levamisole, 0.5 mM phenylmethylsulfonfyl fluoride, 1 μM aprotinin, 1 μM leupeptin, 1 μM pepstatin, and 0.5 mM dithiothreitol (49). All steps were carried out either on ice or at 4 °C. For EMSAs, either 5 μg of nuclear or 50 μg of whole cell protein were incubated in binding buffer (15 mM Tris-HCl (pH 7.5), 75 mM KCl, 1 mM MgCl₂, 1.05 mM EDTA, 2.5 mM dithiothreitol, 0.5 mM sodium vanadate, 10% glycerol, and 0.05% Igepal CA-630) with [γ-32P]ATP-labeled HRE-containing oligonucleotides (~1 ng, “probe”) as summarized in Supplemental Table 1. Poly(dI-dC) and a large excess of the respective mutant oligonucleotides (0.75–1.5 μg) (Supplemental Table 1) were used in these binding reactions to minimize nonspecific binding to the HRE probe. Native 5% polyacrylamide gels were employed to resolve DNA-protein complexes from free probe.

**Luciferase Constructs of the Globin-2 Promoter**—Starting with *D. magna* genomic DNA as template, intergenic regions between the globin-4 and globin-3 genes (4.35-gDNA, 3332 bp), globin-3 and globin-1 genes (3.1-gDNA, 2848 bp), and globin-1 and globin-2 genes (1.2-gDNA, 1319 bp) (23) were amplified via nested PCRs (see Supplemental Table 2). TA-cloning to the pCRII plasmid and, following SacI/KpnI (4.3-gDNA and 1.2-gDNA) or Nhel (3.1-gDNA) digestion, inserted into the equivalent sites of the pGL3-Basic luciferase vector (Promega; denoted by prefix “b” in constructs). Thus, intergenic construct bases 4–33/4–33-gDNA cloned upstream of the pGL3-Basic vector luciferase gene, 5′-Ib and 1′-Ib were generated. As a 5′-Ib control, a 52-bp region of the 5′-Ib of human erythropoietin gene (EpoE) (49, 50), including the EpoE HRE used as an EMSA probe as described above (Supplemental Table 1), was cloned into the KpnI/BgII sites of the pGL3-Basic luciferase plasmid, creating EpoE/Ib.
**Fig. 1. Crustacean respiratory proteins and genes.**

*a*, Crustacea system, adopted from Ref. 54, showing higher (Malacostraca) and lower (Entomostraca) crustacean subclasses and the superimposed distribution of hemocyanin (Hc; blue) versus hemoglobin (Hb; red) hemolymph proteins. **Black background protein labels**, hemocyanin or hemoglobin proteins either constitutively present or with unknown O₂ responsiveness; **gray background protein labels**, reported cases of increased hemoglobin (see text) or hemocyanin (M. Brouwer, personal communication) concentration in response to declining oxygen tension. The **orange arrow** indicates the position of Daphnia. The **lower part** exemplifies branchiopods with hypoxia-stimulated hemoglobin production. 

*b*, the four *D. magna* globin genes, *hb4-hb3-hb1-hb2*, indicated in 5' → 3' order, with stereotypical two-heme domain structures. **Red exons** 1–4 encode domain 1 (Dm.1), and **orange exons** 5–7 encode domain 2 (Dm.2). **HRE** denotes a candidate HIF-binding site. Note that almost all HREs are located within igDNAs and tend to form dense HRE clusters immediately upstream of transcription start sites (TATA). Individual -fold hypoxic inductions of three hemoglobin proteins (hypoxic/normoxic (Hyp/Nor)) as determined previously (23) are shown in **blue**. **utr**, untranslated region.
To successively delete all HREs contained within 1.2-kbDNA, a series of five 5'-constructs of this region was amplified from complete intergenic templates (1319 bp) by employing five different KpnI-anchored forward PCR primers and the same MluI-anchored reverse PCR primer (Supplemental Table 2). In addition to the cloning of a slightly smaller 1319 bp reiterations series of 5'-deletion series of 1319 bp (i.e. complete igDNA) to 746/2 luciferase constructs. Full-length insertions and correct 5' → 3' orientations of all above-mentioned pGL3-Basic luciferase vector sites, thus yielding the 5'-deletion series of 1319 bp (i.e. complete igDNA) to 746/2 luciferase constructs. Full-length insertions and correct 5' → 3' orientations of all above-mentioned pGL3-Basic luciferase vector constructs were confirmed through DNA sequencing.

Site-directed Mutagenesis of Globin-2 Gene Promoter (phb2) HREs—Substitution of the essential 5'-CCGTG-3' HRE core bases with a 4-base 5'-ATGT-3' mutation is known to stereotypically abort binding of any HIF, be it of mammalian (42, 49) or insect origin (51). For this reason, the CGTG cores of phb2 HREs at positions −258, −146, and −107 (relative to the globin-2 transcription start site) were replaced with ATGT bases individually as well as with all possible double-site and triple-site mutations. Starting with the wild-type HRE −462/b construct as template, a QuickChange site-directed mutagenesis kit (Stratagene) was employed along with high melting point 48- and 46-mer sense and antisense mutagenesis primers (Supplemental Table 2) to introduce the ATGT mutation into the −146 and −107 sites, respectively. However, a rather AT-rich DNA surrounding the −258 HRE did not allow us to carry out PCR-based mutagenesis. Instead, we made use of a 60-mer mutagenesis primer that spanned the 5'-PvuII site located at positions −299 to −294 and beyond the −258 HRE (Supplemental Table 2) together with an MluI site-containing reverse primer located at the 3'-end of −462/b (Supplemental Table 2) to amplify and eventually exchange the mutated PvuII/MluI subfragment with its wild-type counterpart within −462/b. We next used plasmid DNAs of m = −258 (−258 HRE mutated), m = −146, and m = −107 clones as templates in mutagenesis PCRs to yield first all three double-site mutations (m = −258/m = −146, m = −258/m = −107, and m = −146/m = −107) and finally the triple-site mutation (m = −258/m = −146/m = −107). All mutations were confirmed via double-stranded sequencing of the respective −462/b construct.

Transient Transfections—Heterologous (co)transfections of Hep3B cells (10 ml at 30% confluency) or SL2 cells (10 ml at 0.5 × 10^6 cells/ml) were conducted using a calcium phosphate kit (Invitrogen) as recommended by the manufacturer (52, 53). Hep3B cells were transfected for 16 h with 15 μg of various Daphnia HRE-luciferase reporter constructs (see above) and 10 μg of lacZ plasmid to normalize luciferase activities to varying transfection efficiencies. On the other hand, SL2 cells completely lacked the endogenous capability to transactivate any of the above Daphnia HRE-reporter constructs under hypoxia. To obtain robust hypoxic inductions of HRE-reporter constructs, SL2 cells needed to be cotransfected not only with the HRE-luciferase constructs (1 nuc2/b plasmid) but also with 0.25 μg of pAc5.1/Sim a/Tango heterodimer). In each transfection, the pUC18 plasmid was added to achieve a total of 20 μg of DNA. Following 16-h transfections, post-transfection processing of Hep3B and SL2 cells was identical and is described in detail in the accompanying article (69).

Statistics—The statistical analysis for this study was generated using SAS software (version 8 of the SAS System for Windows). Percentages of normalized luciferase expression levels of the wild-type HRE −462/b plasmids were compared with each of the seven mutant HRE constructs for identical oxygen tensions as obtained in five Hep3B and four SL2 (co)transfections, respectively. Because of small samples sizes (n < 30) and because the distribution of some of the expression sets deviated from normality, the Wilcoxon rank sum test, a non-parametric two-sided test, was used to evaluate the difference in rank sums of normoxic or hypoxic expression of wild-type HRE-reporter versus mutant HRE-reporter constructs. Relative to t test-based comparisons of mean wild-type versus mutant expression data, the rank sum calculations used for Fig. 4 (b and c) resulted in more conservative, hence
FIG. 3. Hep3B transfections with *D. magna* globin constructs. *a*, globin igDNAs. Reporter assays are shown for the following experimental luciferase constructs (see inset): 4-3/b (*i.e.* 4-3igDNA cloned upstream of the luciferase gene in the pGL3-Basic luciferase vector (b)), 3-1/b, 1-2/b, and 3-1flip/b (*i.e.* 3-1igDNA cloned into the luciferase plasmid in the 5' → 3' direction opposite to the 3-1/b construct). The negative control
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preferred, significance estimates whenever luciferase expression levels of mutant HRE constructs were altered. p < 0.05 and p ≤ 0.01 were considered significant.

RESULTS AND DISCUSSION

The molecular mechanism responsible for the up-regulation of D. magna globin genes under low PO2 has not yet been unraveled, despite ample descriptions of the relative changes in steady-state levels of the individual globin mRNAs and protein subunits (23–26). To elucidate whether a HIF-dependent signaling pathway underlies the hypoxic induction of daphnid hemoglobins, we carried out a computational search for HIF-specific cis-elements called HREs within the globin locus of D. magna. We used as query the HRE consensus sequence 5′-BRCGGTGVB-3′, derived from numerous functional binding sites of mammalian HIF complexes (where B is all bases except A, R is purine bases A and G, V is all bases except T, and the HRE core essential for HIF binding is underlined) (42). A diagram of the compact globin locus in D. magna (Fig. 1b) depicts the conserved two-heme domain architecture of the four known globin genes (5′-hb4-hb3-hb1-hb2-3′), the oxygen-dependent regulation of these genes, and the multiple putative HIF-binding sites (HREs). Protein products of the hb3 and hb2 genes are strongly induced by hypoxia, whereas the up-regulation of the hb1 gene is only moderate (Ref. 25; see also Refs. 24 and 26). Intergenic HREs occur at a frequency two to four times that expected by chance and form high density clusters immediately upstream of the transcription start sites (TATA boxes) of the hb1 and hb2 genes. This non-random distribution of HREs suggests that some of these sites might be functional HIF elements and thus are under selection pressure.

The abundance of HREs within the globin locus prompted an assessment of Daphnia HIF activity by EMSA in comparison with the homologous human and Drosophila DNA-binding complexes. The radiolabeled W18 (wild-type 18mer) HRE-containing oligonucleotide (Supplemental Table 1) used in the EMSA in Fig. 2 is derived from the 3′-enhancer of the human erythropoietin gene, whose HIF-controlled hypoxic induction critically depends on this cis-element (49, 50, 55–57). We used nuclear extracts from known HIF-expressing Hep3B hepatoma cells (left panel) (58) and SL2 cells (middle panel) (51) as sources for human and Drosophila HIFs, respectively (see accompanying paper for more details on Drosophila HIF (69)). Because of the absence of any established Daphnia cell line, Daphnia HIF had to be obtained as nuclear extract from animals directly. 16-h exposures at 1% PO2 (H lanes) were sufficient to markedly stimulate HRE-binding activities relative to normoxic controls (N lanes) in Hep3B cells (lane 2 versus lane 1) and SL2 cells (lane 5 versus lane 4), whereas daphnids required low PO2 incubations (−2–3%) of several days to respond with detectable gel shifts (lane 9 (3 days of hypoxia) versus lane 7 (4 days of normoxia)). Addition of 100-fold excess unlabeled W18 competitor oligonucleotide (+ competitor) to the binding reactions either markedly reduced (i.e. Daphnia HIF, lane 11) or completely abolished (i.e. Hep3B HIF, lane 3, and SL2 HIF, lane 6) the intensities of all three W18-shifted complexes, thus identifying each interaction as specific. Although extracts from hypoxic daphnids reproducibly contained higher W18-binding activities relative to normoxic controls, the kinetics of the hypoxic induction of Daphnia HIF along with its yields were rather variable. The inset in Fig. 2 shows that Daphnia HIF also recognized homologous HREs, as exemplified by its binding to the hb2 −258 HRE in lane 2 (see below and Supplemental Table 1). This time, however, activity was visible in extracts from daphnids exposed to 24 h (inset, lane 2), but not to 3 days (inset, lane 3), of hypoxia (compare with lanes 8 and 9 in Fig. 2). Known confounding factors include different propensities to induce hemoglobin based on age and size of the animals when comparing different broods (15, 25) and may also result in the highly variable HIF activities seen here. The recent cloning of Daphnia HIF subunits by one of us (H. Y.) will certainly aid in future structure/function analyses, including improved induction kinetics.

The role of HIF in the oxygen-dependent control of D. magna globin genes was assessed by transfections that were heterologous because of the lack of either adequate amounts of primary Daphnia cells or a continuous Daphnia cell line. Despite considerable effort, we were unable to immortalize viable primary cells from parthenogenetic D. magna embryos. Transfection of various human oncomones consistently resulted in arrested growth of the few surviving progeny. However, the conserved HRE recognition by vertebrate and invertebrate HIF complexes demonstrated in Fig. 2 (and Fig. 5) allowed the use of human Hep3B and Drosophila SL2 cells as substitute sources for high level hypoxic induction of HIF.

Because of the multiplicity of D. magna globin genes, we first needed to select the most informative model for these reporter assays. We therefore inserted the three HRE-rich igDNA regions that link genes hb4-hb3, hb3-hb1, and hb1-hb2 (Fig. 1b) upstream of a luciferase reporter gene in the pGL3-Basic vector, thus generating the HRE-luciferase constructs 4-3b, 3-1b, and 1-2b, respectively (Fig. 3a, inset). Hep3B cells were transfected with these experimental constructs in comparison with negative (plasmid-free transfection (Null) and insert-free vector transfection (bVec)) and positive (52-bp 3′-enhancer HRE region of the erythropoietin gene upstream of the pGL3-Basic vector luciferase gene (EpoE)) controls. Following transfections, cells were split into equal size aliquots for parallel 16-h exposures to 21% versus 1% oxygen. Fig. 3a shows percentages of mean (±S.E.) oxygen-dependent luciferase expression levels (normoxic (gray bars) and hypoxic (black bars) expression) together with -fold inductions by hypoxia for all constructs assayed (hypoxic (black bars)/normoxic (gray bars) luciferase activity ratio). Although the negative controls (plasmid-free transfection and insert-free vector transfection) showed, as expected, PO2-insensitive base-line expression of the reporter, the EpoE conferred an −8-fold hypoxic induction of the luciferase gene. In agreement with the differential hypoxic regulation of individual D. magna globin genes (Fig. 1b) (23–26), each igDNA (HRE)-luciferase construct responded uniquely. Reporter expression driven by 4-igDNA was negligible under high PO2, and was induced −4-fold by hypoxia, consistent with the marked hypoxia-specific activation of the downstream en
FIG. 4. Mutagenesis of D. magna hb2 promoter HREs. a, sequences and CGTG core substitutions with the ATGT mutation in HREs at positions −258, −146, and −107. b, Hep3B transfections. Reporter assays are shown for control (plasmid-free (Null), insert-free pGL3-Basic luciferase vector (bVec), and EpoE/b; see Fig. 3a) and experimental transfections of wild-type or mutant HRE sequences of the −462 deletion of the hb2 promoter (phb2) cloned upstream of the pGL3-Basic vector luciferase gene as indicated under LucRep. Site-specific mutations are presented.
dogenous \textit{hb3} gene (23–26). In contrast, the weak oxyregulation of \textit{hb1} expression (25, 26) was supported by the 3-1/b construct (i.e. the \textit{hb1} promoter) with its strong and nearly oxygen-insensitive luciferase activation. In addition to the 3-1/b construct, we also generated a “flip” plasmid that had the identical 3-1igDNA inserted into the reporter vector in the opposite 5′ → 3′ orientation upstream of the luciferase gene (3-1flip/b) (Fig. 3a, inset). The lack of transactivation by 3-1lip/b indicates that the response conferred by 3-1/b and, by inference, the 4-3/b and 1-2/b constructs is directional and mediated by promoter elements residing within the idgDNA.

Finally, 1-2igDNA (i.e. the \textit{hb2} promoter) was able to induce luciferase weakly in normoxia and strongly under hypoxia (−6-fold), arguing for a somewhat broader \(p_{O_2}\) expression profile for \textit{hb2} in comparison with \textit{hb3}, in agreement with the hypoxic induction of the endogenous \textit{hb2} gene (25, 26). The robust response mediated by 1-2igDNA (the \textit{hb2} promoter) prompted us to choose this region for further dissection of the involvement of HIF in the hypoxic regulation of \textit{Daphnia} globin.

We next generated and cloned into the pGL3-Basic luciferase vector a series of five 5′-deletions of 1-2igDNA ranging from the full-length intergenic insert, which extended to position −1148 relative to the transcription start site of \textit{hb2} (21) and contained four HREs, to the −74 truncation, which contained recognition motifs for the basal transcription machinery (TATA box), but no HREs (Fig. 3b, inset). The deletions sequentially removed each of the four HREs. Thus, the −462, −209, −131, and −74 constructs contained three, two, one, and zero HREs, respectively. The successive removal of HREs via the 1-2igDNA deletions resulted in progressively declining reporter transactivations in hypoxic Hep3B cells from a maximal 11-fold induction (−1148/b) to a complete lack of \(O_2\)-sensitive regulation (−74/b). A single HRE (−131/b) was inadequate to confer robust inductions under hypoxia. In contrast, the −462 deletion retained most of the hypoxia-driven gene control (−7-fold induction) and therefore may be regarded as the minimal hypoxia-responsive promoter of \textit{hb2} (phb2). In common with certain mammalian genes encoding glycolytic enzymes (59, 60), cooperation between bound HIF complexes is required for hypoxic regulation.

To assess the role of each of the three −462 HRE elements in the \(p_{O_2}\)-controlled expression of \textit{hb2}, we substituted the 5′-CTGT-3′ core of these motifs with 5′-ATGT-3′ (Fig. 4a). Mutations within CGTG cores of HREs are known to abolish binding of both human (42, 49) and \textit{Drosophila} (51) HIFs. The sequence and orientation of the octanucleotide 5′-TACGTGAT-3′ (upper-case in Fig. 4a) are the same in the −258 and −107 HREs, whereas the −146 motif is, with 5′-CAGCTG-3′, a palindromic hexamer. In mammalian cells, CACGTG palindromes are notably underrepresented among functional HIF elements (42, 59, 60). Instead, these E-boxes often serve as high affinity sites for non-HIF complexes, e.g. normoxic Myc/Max heterodimers or constitutive ARNT/ARNT homodimers (61–64), and have been reported to confer hypoxic suppression, rather than induction, upon target genes (64, 65). As shown in Fig. 4b, the wild-type version of \textit{Daphnia} phb2 (−462/b) mediated an −5-fold induced luciferase activity under low \(p_{O_2}\). Mutating either the −258 HRE (−462/b m−258+) or the −107 HRE (−462/b m−107+) decreased this hypoxic response by 57 and 65%, respectively. Consequently, the −258/−107 HRE double mutant (−462/b m−258+/m−107+), and, of course, the −258+/−107 triple mutant had lost virtually all capacity for hypoxic gene activation (\(<0.01\) or 0.05). Therefore, Hep3B HIF, once bound to \textit{Daphnia} −258 and −107 phb2 HREs, is sufficient and necessary for the induction of target genes under low \(p_{O_2}\).

Unexpectedly, in Hep3B cells, the −146 mutation (Fig. 4b, −462/b m−146+) yielded a highly significant −3-fold enhancement of the hypoxic luciferase stimulation (−5 to −15-fold induction) rather than a reduction, suggesting potential interference of HIF-mediated phb2 regulation by −146-binding factors. Parallel HIF/HRE EMSAs using normoxic (\textit{N lanes}) versus hypoxic (\textit{H lanes}) Hep3B nuclear extracts along with \textit{Daphnia} −258, −146, and −107 single-site probes (Fig. 5a)

by a plus sign under the respective luciferase reporter (LucRep) construct (e.g. −462/b \textit{m}−258+ indicates the −462/b \textit{phb2} deletion with the −258 HRE mutated, and −462/b \textit{m}−258+/m−107+ indicates the −462/b \textit{phb2} deletion with both −258 and −107 HREs mutated). Percentages of mean (±S.E.) and \(\beta\)-galactosidase normalized normoxic (\textit{gray bars}) and hypoxic (\textit{black bars}) luciferase activities and −fold hypoxic inductions are indicated (\(n = five\) transfections). * and **, significant (\(p < 0.05\)) and highly significant (\(p < 0.01\)) changes, respectively, in reporter expression between the wild-type HRE −462/b construct and any of the mutant HREs −462/b constructs of the \textit{hb2} promoter, \(c\), SL2 (CD) transfections. Reporter assays are shown for control (plasmid-free (\textit{Null}) and insert-free pGL3-Basic luciferase vector (\textit{bVec})) and experimental transfections using the same set of wild-type and mutant HRE −462/b constructs of the \textit{hb2} promoter (\textit{phb2}) as described for \(b\). Percentages of \(\beta\)-galactosidase normalized mean normoxic (\textit{gray bars}) and hypoxic (\textit{black bars}) luciferase activities following transfections (\(n = 4\)) are indicated as described for Fig. 3a. Luciferase reporter cotransfections (\(n = 4\)) using expression plasmids for \textit{Drosophila} HIFs (i.e. \textit{Simia}) and HIF\(\beta\) (\textit{ARNT}, i.e. Tango) homologs resulted in the \(\beta\)-galactosidase normalized mean hypoxic (\textit{gray hatched bars}) and hypoxic (\textit{black hatched bars}) luciferase activities and −fold hypoxic inductions shown. *, significant (\(p < 0.05\)) changes in reporter expression between the wild-type HRE −462/b construct and any of the mutant HRE −462/b constructs of the \textit{hb2} promoter.
clearly demonstrated the in vitro doping of Hep3B HIF to the −258 and −107 TACGTTGATG motifs (lanes 2 and 6, black arrow). Although the −107 HRE bound Hep3B HIF with high specificity, the −258 motif interacted, in hypoxic extracts, with a combination of HIF and an oxygen-insensitive factor. In contrast, the −146 palindromic showed strong constitutive (oxy-
genome-insensitive) banding (lanes 3 and 4, white arrow). Unless accessory factors aid in its recruitment, human heterodimeric HIF by itself appeared to bind weakly to palindromic response elements (Fig. 5a), leaving these motifs accessible for competing (and possibly transcription-suppressing) E-boxcoactivator complexes (60, 64–67). Therefore, the increase in hypoxic transactivation potential (−5– to −15-fold induction) seen in the m−146 version of pE258 (Fig. 4b) is most likely due to a diminished interference from CACGTG-binding constitutive factors (ARNT-ARNT homodimers?), which leads to amplified HIF binding at the neighboring −107 and possibly −258 HREs. This implies that the Daphnia hypoxia-responsive hb2 promoter, far from representing a simple on (−HIF/−HIF switch, at least in Hep3B cells, might be regulated in a graded fashion, where the abundance and occupancy of constitutively active factors at the −146 element serve to fine-tune adjacent HIF/HRE interactions and thus pO2-dependent gene expression.

Approaching the Daphnia HIF response with a more closely related insect model, we also subjected HIF-positive Drosophila SL2 cells to transfections with the same series of wild-type and mutant phb2 HRE constructs (Fig. 4c). However, SL2 reporter assays deviated from Hep3B assays in two major respects. (a) Endogenous HIF levels, although detectable in EMSAs (Fig. 2), were insufficient to elevate hypoxic luciferase expressions with any of the phb2 constructs beyond base-line levels (Fig. 4c, normoxic [gray bars] versus hypoxic [black bars] luciferase activities). Thus, SL2 cotransfections were necessary to overexpress Drosophila HIFα (i.e. Sima) and ARNT (i.e. Tango) homologs (see accompanying article (69) for details). (b) In Sima/Tango cotransfections of SL2 cells, EpoE/b failed to yield any induced reporter activity. Lack of function of human erythropoietin element in Drosophila, seen here and in transgenic flies (46), underscores the required contribution of accessory transcription factors other than HIF (e.g. hepatic nuclear factor-4) (68) in Epo enhancer-confined hypoxic gene activation. Since the erythropoietin gene is not conserved in flies, homologs for some of these accessory factors might also be missing in Drosophila.

The Sima/Tango (i.e. fly HIF) cotransfections of the wild-type hb2 promoter (−462/b) in Fig. 4c (normoxic [gray hatched bars] versus hypoxic [black hatched bars] luciferase activities) demonstrated that the resulting −7-fold hypoxic induction was mediated solely by the binding of Drosophila HIF homologs to multiple daphnid HREs found within phb2. In contrast to Hep3B responses, mutating either the −258 or −107 HRE alone entirely aborted hypoxic transactivation (p < 0.05). As expected, all other m−258 or m−107 combinations also abolished induction (p < 0.05). Mutating the −146 motif resulted in a decline (−7.−3-fold induction) in hypoxia-driven reporter expression, in contrast to the enhanced induction observed in Hep3B cells (Fig. 4b). Fly HIF/daphnid HRE EMSAs (Fig. 5b) documented that SL2 HIF recognized both the −258 and −107 motifs (lanes 2 and 6, black arrow) either by itself (−107 motif) or in competition with constitutive DNA-binding proteins (−258 motif) and thus displays human HIF-like binding patterns (Fig. 5, a and b). The −146 E-box also bound the constitutive fly factor (lanes 3 and 4, white arrow), although much less strongly than the Hep3B equivalent. This relatively weak affinity of the fly proteins for the symmetrical −146 E-box might result in a less pronounced interference with neighboring HIF/HRE complexes and could therefore explain the diminished hypoxic response in m−146 SL2 cotransfections (Fig. 4c).

On the other hand, the strong interaction of constitutive protein(s) with the −146 site in Hep3B cells (Fig. 5a) seems to underlie an efficient inhibition of nearby HIF complexes, consistent with the marked enhancement of hypoxic induction in the luciferase reporter in which the −146 motif had been mutated (Fig. 4b, −462/b m−146−).

We also carried out additional EMSA surveys of the homologous Daphnia HIF/HRE interactions (data not shown), which, although hampered by the close co-migration of Daphnia HIF and the Daphnia −146-binding factor, supported nonetheless the role of the −107 site together with the −258 sequence (Fig. 2, inset) in functioning as HREs recognized by Daphnia HIF and by human (Hep3B) and Drosophila (SL2) HIF proteins (see above).

In conclusion, these studies provide a molecular explanation for a phenomenon that has intrigued biologists for the last 50 years: the dramatic induction of hemoglobin expression when daphnids are challenged by hypoxia. We have presented evidence here for the occurrence of HRE-binding HIF activity in hypoxic, but not normoxic, Daphnia. Utilizing heterologous transfusions of HIF-expressing human and insect cells, we have also shown that endogenous or overexpressed HIF proteins are sufficient and necessary to mediate the hypoxic induction of a luciferase reporter by binding cooperatively to two functional HREs at positions −258 and −107 in the promoter of the D. magna globin-2 gene (hb2). At least the hb2 gene is therefore hypoxically stimulated through a bipartite HRE promoter, which suggests that the synergy between tandem HIF sites, as mediated by coactivator proteins (67), is a crucial and conserved feature in the hypoxic response of vertebrate and invertebrate HIF target genes (59, 60). Binding of constitutive factors to the internal −146 palindromic E-box of the hb2 promoter affects adjacent HIF/HRE interactions and could potentially serve to fine-tune hb2 expression at low oxygen tensions.

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