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## Structure and Sequence Conservation of a Putative Hypoxia Response Element in the Lactate Dehydrogenase-B Gene of *Fundulus*

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*Many aquatic habitats are characterized by periodic or sustained episodes of low oxygen concentration, or hypoxia, and organisms that survive in these habitats do so by utilizing a suite of behavioral, physiological and biochemical adjustments to low oxygen (1–3). In the killifish *Fundulus heteroclitus*, one response to prolonged exposure to hypoxia is an increase in the activity of lactate dehydrogenase-B (LDH-B), the terminal enzyme of anaerobic glycolysis, in liver tissue (4). An increase in glycolytic enzyme activity also occurs in mammalian cells during hypoxia, a process due, in part, to increased rates of gene transcription mediated by the hypoxia-inducible transcription factor, HIF-1 (5). Given that a homolog of HIF-1 has been identified in fish (6), we hypothesized that HIF might be involved in the observed up-regulation of LDH-B in *F. heteroclitus*. Herein, we describe the presence of DNA elements in intron 2 of the Ldh-B gene from *F. heteroclitus* that resemble hypoxia response elements (HRE) described for mammalian genes (7–10). Specifically, over a region of approximately 50 base pairs we identified two consensus HIF-1 binding sites, as well as DNA elements that may bind other transcription factors (e.g., cyclic AMP response elements; CRE). We found that these sites were perfectly conserved among geographically diverse populations of *F. heteroclitus*, as well as being highly conserved among multiple species in the genus *Fundulus*. The spacing, orientation, and sequence conservation of these putative regulatory*

*elements suggest that they may be functionally involved in the hypoxic regulation of Ldh-B in these fish.*

Hypoxia is common in a variety of biological settings, and one area of great current interest is the role of hypoxia in regulating gene expression. In mammals, the hypoxia-inducible factor 1 (HIF-1) has been shown to play a key role in many cases of hypoxia-dependent gene expression (5). HIF-1 is a heterodimeric transcription factor, composed of subunits HIF-1 $\alpha$  and HIF-1 $\beta$ . Hypoxia leads to the accumulation of HIF-1 $\alpha$ , dimerization with HIF-1 $\beta$ , and binding of the dimer to specific nucleotide sequences (hypoxia response elements, HRE) of target genes. Upon binding DNA, HIF-1 interacts with other transcription factors and accessory proteins to stimulate rates of gene transcription (10–12). Originally described as a mediator of increased rates of transcription of the glycoprotein hormone erythropoietin (*Epo*) during hypoxia (7), HIF-1 has subsequently been implicated in the hypoxia-dependent transcription of a variety of mammalian genes, including vascular endothelial growth factor, glucose transporters, and several glycolytic enzymes (5).

Whether HIF-1 (or the related proteins, HIF-2 or HIF-3; ref. 5) is involved in gene expression in organisms that face fluctuations in oxygen availability in their natural environment has received less attention. Because aquatic habitats frequently show spatial and temporal variation in oxygen (13, 14), fish and other aquatic organisms represent ideal systems in which to study gene regulation during ecologically relevant hypoxia. Evidence is emerging that fish have functional analogs or homologs of HIF that may be involved in hypoxic regulation of gene expression. For example, when nuclear proteins were extracted from liver tissue of gulf killifish, *Fundulus grandis*, that had been subjected to hypoxia, one or more nuclear proteins that bound to mam-

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Abbreviations: CRE, cyclic AMP response element; *Epo*, erythropoietin; HIF-1, hypoxia-inducible factor 1; HRE, hypoxia response element; LDH, lactate dehydrogenase.

malian HIF-1 binding sequences were found (B.B. Rees, unpub. obs.). Furthermore, a putative HIF-1 $\alpha$  homolog has recently been cloned and sequenced from rainbow trout (6). However, the genes that may be subject to regulation by this transcription factor in fish remain largely uncharacterized (although see ref. 15).

We are interested in describing targets of hypoxia-dependent gene regulation in the killifish, *F. heteroclitus*. This species occurs in estuaries along the eastern seaboard of North America, habitats in which oxygen levels vary with depth, salinity, temperature, and degree of eutrophication (13, 16). We focused on the *Ldh-B* gene in *F. heteroclitus* because previous research had shown that the activity of LDH-B increased about 2-fold during exposure of this fish to hypoxia (4). Furthermore, much of the *F. heteroclitus Ldh-B* gene sequence is known from the studies by Powers and his colleagues on the thermal evolution of LDH-B isozymes in this species (17–20). In addition to the complete coding sequence, about 5 kb of promoter and 5' flanking sequence (18–20) and up to 300 bp on each end of all seven introns (P.M. Schulte, unpub. obs.) have been sequenced. Finally, there is evidence of HIF homologs in this and closely related species (B.B. Rees, unpub. obs.; W. Powell and M. Hahn, Woods Hole Oceanographic Institution, pers. comm.; D. Crawford, University of Missouri, pers. comm.).

In the present study, we asked whether the *F. heteroclitus Ldh-B* gene contains conserved DNA regions that could serve as putative HIF-1 binding sites. We searched the available gene sequence for a hexanucleotide element (5'-ACGTGC-3') representing the consensus HIF-1 binding site (21). In about 7 kb of DNA sequence, we found a single match to this sequence, located in the latter half of intron 2. We subsequently sequenced this intron in individuals from populations distributed across the geographic range of *F. heteroclitus* and found an intriguing pattern of sequence conservation in this intron (Fig. 1A). The 5' half of the intron displayed a degree of variation consistent with the level of geographic variation noted in noncoding sequence from the 5' flanking region and promoter of the same gene (19, 20). The 3' half of the intron, however, was highly conserved among these individuals, and the putative HIF-binding site was perfectly conserved. To determine the degree of sequence conservation at greater phylogenetic distances, we sequenced *Ldh-B* intron 2 from 12 additional species within the genus *Fundulus* (Fig. 1B). Again, the 5' half was found to be quite variable, whereas the 3' half of the intron was highly conserved. The putative HIF-binding site, while not perfectly conserved among species, was present in 10 of the 13 species examined.

Closer examination of the 3' half of *Fundulus Ldh-B* intron 2 revealed that this putative HIF-binding site was followed by DNA elements that may represent additional transcription factor binding sites. Immediately downstream of the HIF-binding site was the sequence 5'-AACAG-3'.

Similar or identical sequences are present in the HREs of certain mammalian genes, and these sequences are required for hypoxic induction of those genes (7, 8, 22). The next downstream DNA element in the *Fundulus Ldh-B* intron 2 was 5'-CGTG-3', which corresponds to the core DNA sequence of a second potential HIF-binding site (5). Finally, intron 2 ended with a DNA element that differed by only one nucleotide from the consensus CRE, 5'-(T/G)(T/A)CGTCA-3' (23). Each of these sites was perfectly conserved among geographically diverse populations of *F. heteroclitus* (Fig. 1A), as well being remarkably well conserved among several species of *Fundulus* (Fig. 1B).

The HREs that have been characterized in mammalian genes typically have a di- or tripartite structure, in which HIF-binding sites are located close to other DNA elements necessary for hypoxic regulation of transcription. Figure 2 shows the arrangement of these elements in the mammalian *Epo* (7, 8) and *Ldh-A* (9, 10) genes and compares this arrangement with the consensus sequence we determined for the 3'-half of intron 2 from *F. heteroclitus Ldh-B*. This comparison shows that the occurrence and spacing of multiple putative binding sites in the *F. heteroclitus Ldh-B* intron 2 conform to the general structure of HREs defined for other genes. Indeed, there is a striking similarity among DNA elements identified to be important in hypoxic induction of mammalian *Ldh-A* (two HIF-binding sites and a CRE) and the putative binding sites we identified in the *F. heteroclitus Ldh-B* intron 2. The high degree of sequence conservation of these DNA elements within and among species of *Fundulus* (Fig. 1), combined with the similarity in spacing and orientation of these elements with known HREs (Fig. 2), suggests that the 3'-half of intron 2 from *F. heteroclitus Ldh-B* may act as an HRE and contribute to the regulation of this gene during hypoxia.

Previous research on the promoter and 5' flanking region of *F. heteroclitus* indicated that sequence variation in non-coding regions of the *Ldh-B* gene can be quite high among populations, approaching 15 nucleotide changes per 100 bp across the geographic range of this species (19, 20). In light of this level of variation, the very low variability (one change in 50 bp, Fig. 1A) in the 3' half of intron 2 of the *F. heteroclitus Ldh-B* gene was surprising. The observed degree of sequence conservation in this intron could be explained if the putative binding sites we described are functionally important and are maintained by selection. Although the location of this putative HRE within an intron is somewhat unusual, intronic locations for enhancers are not unprecedented. Binding sites for HIF-1 have been found within the introns of the mammalian glycolytic genes phosphofructokinase and aldolase (21), although in the case of aldolase the intronic site may not contribute to hypoxic regulation of this gene (22). Among fish, functional enhancers occur in introns of the immunoglobulin gene of the channel catfish (24) and the sonic hedgehog gene in ze-

A.

Location	GTGAGAGGTCGCGCCACGCTCGGTTTCGCGCTCGCCCCCCCCCTCCGCTCCGGACGCTGCTCCAACAGGCCGTGTCTCTGTGCCTCTCCCGTCAG	GenBank
New Ham	.....T.....T.....T.....C..T.....	AF061090
New Bru	.....C.....-----T.....	AF061091
New Ham	.....C.....-----C.....	AF061092
Massach	.....C.C.....-----	AF322872
Georgia	.....C.C.....-----	AF322873
Georgia	.....-----	AF322874

B.

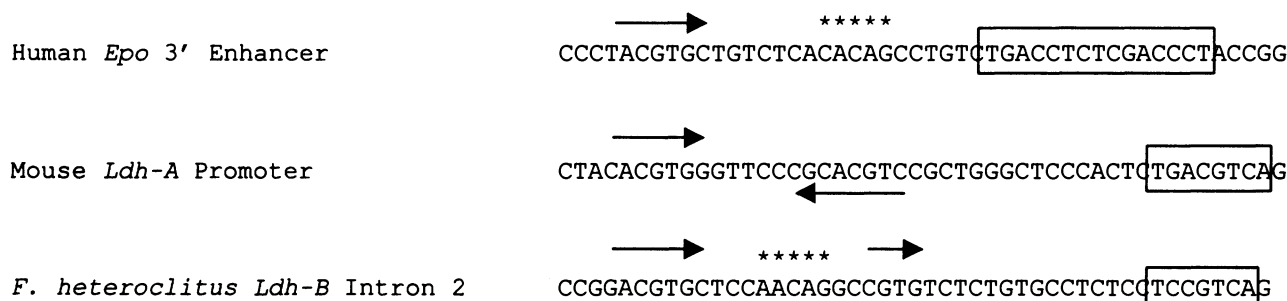
hete	GTGAGAGGTCGCGCCACGCTCGGTTTCGCGCTCGCCCCCCCCCTCC---GCTCCGGACGCTGCTCCAACAGGCCGTGTCTCTGTGCCTCTCCCGTCAG	GenBank
gran	.....CT.....T.....A.....C.....CCCC	AF322871
pulv	.....T.C.....-----A.....G.....	AF061100
diap	.....CT.....-G.....G.C.T.G.....	AF061099
simi	.....C.....-----G.T.G..CC-----T..A.....	AF061093
maja	.....C.....-G.....-----T..A.....	AF061103
cate	.....C.....-G.A.C.A.A-----A..A.....	AF061098
chry	.....C.....GCGC.CGC-AC...T.....A..A.....	AF061102
oliv	.....CA.T.....-G...C.A.AC...T.....A.....	AF061096
blai	.....C.....-TG..TCG..AC.AAT.....T..T.A.....	AF061097
line	.....C.....-G-----C.AC.AAT.....T..A.....	AF061095
nott	.....C.....-G...CG..AC.AAT.....T.C..A.....	AF061101
scia	.....-.....G...C...AC.T.T.....A.....	AF061094

**Figure 1.** (A) Sequence variation in intron 2 of *Ldh-B* from *Fundulus heteroclitus* representing populations from the geographic distribution of this species. Locations were New Brunswick, Canada (New Bru); Hampton, New Hampshire (New Ham); Woods Hole, Massachusetts (Massach); and Sapelo Island, Georgia (Georgia). (B) Sequence variation in intron 2 of *Ldh-B* from various species within the genus *Fundulus*. Species were *F. heteroclitus* (hete); *F. grandis* (gran); *F. pulvereus* (pulv); *F. diaphanus* (diap); *F. similis* (simi); *F. majalis* (maja); *F. catenatus* (cate); *F. chrysotus* (chry); *F. olivaceus* (oliv); *F. blairae* (blai); *F. lineolatus* (line); *F. notti* (nott); *F. sciadicus* (scia). In A and B, the consensus sequence for *F. heteroclitus* is given on the first line; dots indicate a nucleotide identical to that in the *F. heteroclitus* consensus, and dashes indicate a deletion. The boxed underlined letters indicate the consensus hexanucleotide HIF-1 binding site (21). Other boxed areas indicate other DNA regions of interest (see text). GenBank accession numbers are given at the end of each line. **Methods:** Genomic DNA was isolated by proteinase K digestion followed by high salt extraction (26). Spleen tissue was used as a source of genomic DNA for *F. heteroclitus* and *F. grandis*, and muscle tissue, kindly provided by Dr. Kate Shaw (University of Kansas), was used for *F. similis*, *F. lineolatus*, *F. sciadicus*, *F. notti*, *F. chrysotus*, *F. olivaceus*, *F. blairae*, *F. pulvereus*, and *F. catenatus*. Genomic DNA from *F. diaphanus* and *F. majalis* was kindly provided by Dr. Douglas L. Crawford (University of Missouri). PCR primers to amplify intron 2 of the *Fundulus Ldh-B* gene were designed based on an alignment of *Ldh-B* sequences from *Rattus norvegicus*, *Sus domesticus*, *Sceloporus undulatus*, *Xenopus laevis*, *F. heteroclitus*, and *F. parvipinnis* (27). One of two forward primers was used, either 139F (5'-GACGAGCTCGCTCTGGTGA-3') or 148F (5'-GCTCTGGTGGACGTGATGGA-3'), in combination with the reverse primer 368R (5'-GGGATGATGCACTTGAA-GAC-3'). The position of the primers is indicated relative to the *Ldh-B* coding sequence, starting with +1 for the A of the methionine start-codon. PCR conditions were as follows: 40 cycles (1 min at 94°C, 1 min at 57°C, and 1 min 15 s at 72°C) in 50- $\mu$ l reaction mixtures containing 10 mM Tris (pH 8.3), 1.5 mM MgCl<sub>2</sub>, 5 mM KCl, 0.01% Nonidet P-40, and 0.01% Triton X-100, 0.6  $\mu$ M of each primer, 200  $\mu$ M of each dNTP, and 1 U of *Taq* polymerase. Genomic DNA concentrations were optimized for each sample. PCR fragments were cloned with no further purification into the EcoRV site of either the Bluescript II KS (-) T-vector (Stratagene, La Jolla, CA) or the pGEM-5Zf (-) T-vector (Promega, Madison, WI). All clones were completely sequenced at least once in each direction, and all reported sequences represent the consensus from at least two independent clones.

brafish (25). Therefore, the location of the putative HRE we describe is not inconsistent with a role in transcriptional regulation.

To our knowledge, this is the first description of a putative HRE in a gene from a non-mammalian vertebrate. In combination with the previously observed increase in LDH-B activity during hypoxia in this species (4), this result is significant insofar as it suggests that HIF or its homologs, heretofore described as regulators of gene ex-

pression in mammalian development, physiology, and pathology (5), may play a role in mediating effects of environmental hypoxia in other animals. Future functional studies will be required to assess the role of this putative HRE in regulating *Ldh-B* gene expression in *F. heteroclitus*. Although the 3' half of this intron was highly conserved among the other members of the genus we examined, the observed nucleotide differences within putative binding sites (Fig. 1B) could impact the ability of this region to



**Figure 2.** Diagrammatic representation of the orientation and spacing of functional DNA elements in hypoxia response elements of the human erythropoietin 3' enhancer (7, 8) and mouse *Ldh-A* promoter (9, 10). Arrows represent HIF binding sites (forward or reverse orientation). For human *Epo*, the asterisks indicate a DNA element necessary for hypoxic induction, and the boxed nucleotides represent a direct repeat of a steroid response element. For mouse *Ldh-A*, the boxed nucleotides represent a CRE. The location of similar DNA elements in the 3' half of *Ldh-B* intron 2 from *F. heteroclitus* (consensus) are indicated using the same symbols (the boxed nucleotides form an imperfect CRE). The human *Epo* sequence is from (8) and begins at nucleotide +3455; the mouse *Ldh-A* sequence is from (10) and begins at -88 (nucleotide +1 is the transcription start site).

serve as an HRE in other species of *Fundulus*. Accordingly, this group of fish may provide a "natural experiment" on the role of these DNA elements in hypoxic regulation of gene expression.

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