



Cloning of *hif-1 α* and *hif-2 α* and mRNA expression pattern during development in zebrafish

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Abstract

Hypoxia-inducible factors (HIFs) regulate gene expression in response to hypoxia and in vertebrates they are known to participate in several developmental processes, including angiogenesis, vasculogenesis, heart and central nervous system development. Over the last decade, major progress in unraveling the molecular mechanisms that mediate regulation of HIF proteins by oxygen tension has been reported, but our knowledge on their developmental regulation during embryogenesis in model organisms is limited. Expression of *hif-1 α* and *hif-2 α* genes has been characterized during normal mouse development and they were found to be expressed from stages E7.5, later in E9.5 and E15.5 in several different tissues such as the brain, heart and blood vessels. However, there is no detailed temporal information on their expression at other embryonic stages, even though orthologous genes have been described in several different vertebrate species. In this study, we describe the cloning and detailed expression pattern of zebrafish *hif-1 α* and *hif-2 α* genes. Sequence analysis revealed that zebrafish Hif proteins are highly homologous to other vertebrate orthologues. Zebrafish *hif-1 α* and *hif-2 α* are both expressed throughout development in discrete territories in a dynamic pattern. Interestingly, in the notochord the expression of *hif-1 α* is switched off, while *hif-2 α* transcription is turned on, signifying that the two genes might have partially overlapping, although non-redundant functions in development. This is the first time that a detailed comparison of the expression of *hif-1 α* and *hif-2 α* is directly assessed in a vertebrate model system throughout development.

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1. Results and discussion

In eukaryotic organisms, oxygen homeostasis is critical for normal development and physiology and therefore, hypoxia has been proposed as a key regulator of various morphogenetic processes such as angiogenesis (Pugh and Ratcliffe, 2003), lung, brain and cartilage development (Compernelle et al., 2002; Ema et al., 1997; Lee et al., 2001; Madan et al., 2002; Royer et al., 2000; Schipani et al., 2001). Regulation of oxygen homeostasis primarily depends on

the hypoxia-inducible factors (HIFs), a family of heterodimeric transcriptional complexes composed of HIF- α and HIF- β subunits (Wang and Semenza, 1995). Whereas the expression of β -subunits is constitutive, HIF- α subunits are rapidly degraded by the ubiquitin-proteasome system in normoxia, whereas in hypoxic condition, they are stabilized and accumulate in the nucleus. Once in the nucleus, α -subunits dimerize with HIF- β and activate the transcription of a wide array of hypoxia-inducible genes. Target genes include the vascular endothelial growth factor (VEGF), which is involved in angiogenesis; erythropoietin (Semenza, 1999) that promotes red blood cell differentiation, and most glycolytic genes (Bruck, 2003; Semenza, 2001).

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A critical role for HIF genes in vertebrate development has been defined after the generation of mouse knock-out lines for *Hif-1* and *2 α* as well as for *Hif-1 β* . In these mutants, embryonic development is arrested between embryonic days 8 and 11. Loss-of-function embryos exhibit dramatic cardiovascular defects, impairment of angiogenesis, and failure of neural tube closure derived from mesenchymal cell death (Compernelle et al., 2002; Iyer et al., 1998a; Kotch et al., 1999; Maltepe et al., 1997; Peng et al., 2000; Ryan et al., 1998; Tian et al., 1998). In cell culture, regulation of HIF- α is controlled by oxygen-dependent post-translational regulatory mechanisms (i.e. protein half-life modulation, recruitment of transcriptional co-activators and control of sub-cellular localization). Nevertheless, in vertebrate model systems *in vivo*, up-regulation of HIF-1 α mRNA levels has been reported to occur in response to hypoxia (Catron et al., 2001; Ton et al., 2003; Royer et al., 2000).

Furthermore, *in situ* hybridization studies have revealed spatial regulation of HIF-1 α mRNA in normoxic mouse embryos, with high levels of the transcript detected in the brain, heart and kidney (Ema et al., 1997; Luo et al., 1997; Madan et al., 2002). In addition, evidence for temporal regulation of HIF-1 α has also been reported in mammals, with mRNA expression being relatively high during early development, and decreasing as gestation progresses (Madan et al., 2002). Likewise, in *Xenopus laevis* a function of XHIF-1 α during normal embryogenesis has been suggested, since the expression of a XHIF-1 α promoter-GFP construct shows dynamic spatial expression pattern and regulation (Sipe et al., 2004), exhibiting low levels throughout development, and a sharp increase at the gastrula stage. Later in development, XHIF-1 α promoter-GFP is expressed in specific regions of the central nervous system and also in axial tissues (Sipe et al., 2004). Direct studies addressing the expression of HIF- α mRNAs in *Xenopus* or other vertebrate species have not been conducted so far.

In the present study, we report the identification of two members of the HIF family in zebrafish, *hif-1 α* and *hif-2 α* , and describe their expression pattern during embryonic and larval development. We show that *hif-1 α* shows a very dynamic pattern in the embryo, in several tissues such as the epithelia of brain ventricles, notochord, blood vessels, somites, neuromasts, retina and the optic tectum. *hif-2 α* is expressed early in the embryonic brain and in developing blood vessels, even though it was also detected late in embryogenesis in the somites, notochord, intestine, retina and optic tectum. Comparison of the expression of *hif-1 α* and *hif-2 α* revealed that, although the two genes overlap in some territories, in other territories their patterns are mutually exclusive and in some tissues the expression of *hif-1 α* is replaced by that of *hif-2 α* .

1.1. Cloning and sequence analysis of *hif-1 α* and *hif-2 α*

Sequence database analysis revealed the occurrence of several zebrafish ESTs highly homologous to human HIF-

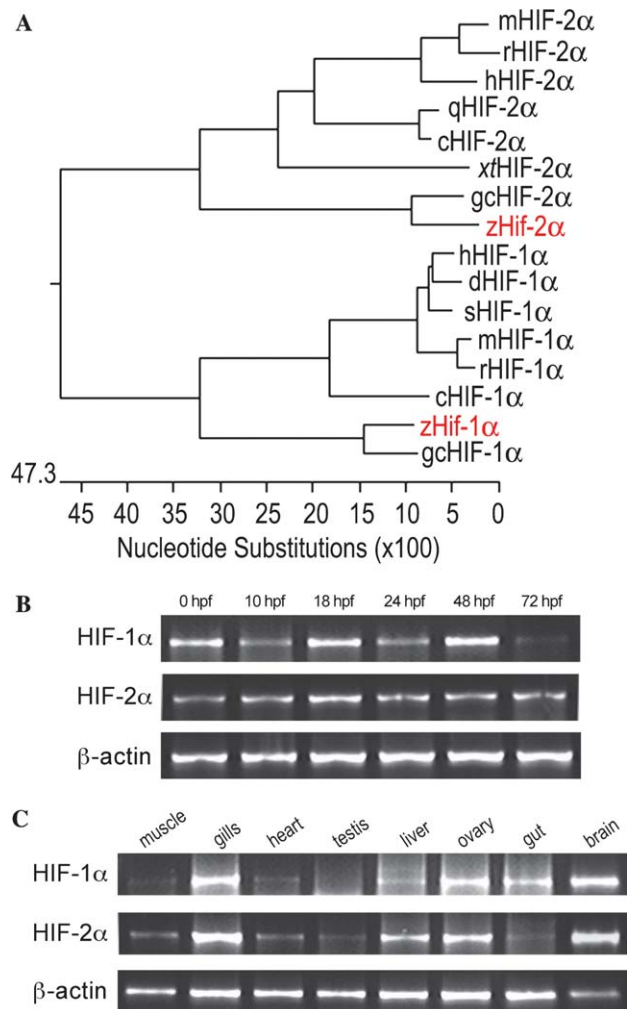


Fig. 1. Phylogenetic tree and expression of *hif-1 α* and *hif-2 α* during embryonic development. (A) Phylogenetic tree resulting from CLUSTAL analysis of HIF protein sequences. Horizontal lines indicate the degree of relatedness. Sequences are from human (h), mouse (m), rat (r), quail (q), chicken (c), *Xenopus tropicalis* (xt), grass carp (gc), dog (d), squirrel (s) and zebrafish (z). (B) Expression of *hif-1 α* and *hif-2 α* at different stages of development as assayed by RT-PCR from total RNA prepared from the indicated stages. *hif-1 α* and *hif-2 α* are expressed in all the stages analyzed. (C) Expression of *hif-1 α* and *hif-2 α* in adult tissues. Total RNA was prepared from the indicated tissues from adult fishes (one year old); both genes are expressed in all adult tissues analyzed, although at variable levels. As an internal control, expression of β -actin was assessed in the same samples (lower panel in B and C).

1 α and HIF-2 α . Two of these ESTs were fully sequenced and selected for our studies. EST BM182886 (3.5 Kb) showed high identity with human *hif-1 α* (50% identity) and the deduced sequence corresponds to a 777 amino acid protein. The second EST (BI845166) was highly similar to *hif-2 α* from human and grass carp (55% and 87% of identity, respectively) and encodes a predicted 832 amino acid protein. Phylogenetic analysis revealed that *Hif-1 α* and *Hif-2 α* zebrafish proteins cluster respectively with HIF-1 α and HIF-2 α of other vertebrate species (Fig. 1A). Their sequences display a remarkable degree of conservation in the overall protein organization (Lee et al., 2004), since the

bHLH, PAS-A, PAS-B, ODD, TAD-N and TAD-C domains were all present in the predicted zebrafish proteins (data not shown). When compared individually, all these domains showed more than 90% identity with the carp and human HIF- α proteins (Table 1). Both zebrafish HIF- α proteins displayed prolyl residues, presumably hydroxylated by the oxygen sensor and required for ubiquitin-mediated degradation (P₄₀₂, and P₅₆₄, in human HIF-1 α), as well as the Asparagine residue in the TAD-C domain (N₈₀₃, in human HIF-1 α), that is hydroxylated to regulate transcriptional co-activator recruitment.

The genomic structure of the two loci shows that the *hif-1 α* gene includes 15 exons (data not shown), which was coincident with the number of exons described in the human and mouse genes (Iyer et al., 1998b); *hif-2 α* includes 16 exons (data not shown). The two-zebrafish genes are located on separate linkage groups (LG), *hif-1 α* in LG20 and *hif-2 α* in LG13.

1.2. Temporal and spatial expression patterns of HIF genes

Temporal and spatial expression patterns of *hif-1 α* and *hif-2 α* were analyzed during embryogenesis by RT-PCR and mRNA *in situ* hybridization. Expression of both genes begins just after fertilization and continues during embryonic and larval development. Expression of *hif-1 α* is more dynamic as compared with that of *hif-2 α* (Fig. 1B). In the adult, *hif-1 α* and *hif-2 α* mRNAs are expressed in muscles, gills, heart, testis, liver, ovary, gut and brain, although expression levels differ across different organs (Fig. 1C).

1.2.1. Expression of *hif-1 α*

mRNA *in situ* hybridization analysis revealed a dynamic expression pattern for *hif-1 α* (Fig. 2). The transcript is expressed maternally (Fig. 2A) and thereafter remains evenly distributed up to 10 h post-fertilization (hpf) (Fig. 2B). At 18 hpf, expression becomes restricted to the ventral part of the embryo (Fig. 2C) and by 24 hpf, it was detected in the brain, ventricle epithelium and caudal vein

(Fig. 2D and insets). At 28 hpf, the expression of *hif-1 α* continues to be dynamic, being detected in brain structures, notochord, and the caudal vein territory (Fig. 2E and inset therein). Later on, at 32 hpf, expression could also be detected in the brain, notochord, somites, dorsal aorta, and intersegmental blood vessels (Fig. 2F and insets). At 48 hpf the transcript was found to be expressed in the brain, notochord, somites, and intestine (Fig. 2G) and at 60 hpf, *hif-1 α* expression occurs in the branchial region, notochord, somites and intersegmental blood vessels (Fig. 2H). Later in development, at 72 hpf, the transcript was detected in tail and brain blood vessels, the neural tube, notochord, retina and intestine (Fig. 3I and insets). To detect possible sites of low-level expression of *hif-1 α* mRNA, we over-stained the hybridized embryos at this stage. Interestingly, this treatment revealed weak expression of *hif-1 α* in lateral line neuromasts (Fig. 3J and inset therein). In order to confirm that expression of *hif-1 α* indeed occurs in blood vessels, we compared its mRNA *in situ* hybridization pattern with the expression of an intrinsic blood vessel marker. To this end, we carried out experiments in a transgenic fish line (Tg *flil:EGFP^{v1}*) that expresses GFP in vascular endothelial cells under control of the *flil* promoter (Lawson and Weinstein, 2002). These experiments confirmed that *hif-1 α* is expressed in blood vessels (Fig. 2I), although its expression was difficult to detect by mRNA *in situ* hybridization due to the small size of the blood vessels. Finally, *hif-1 α* expression analysis at 144 hpf revealed clear expression in brain blood vessels, the gut, retina, and optic tectum (Fig. 2K and L).

We conclude that the expression pattern of *hif-1 α* mRNA is highly dynamic throughout development, paralleling the developmental regulation of *Xenopus laevis* HIF-1 α (Sipe et al., 2004) using the promoter region of this gene driving GFP expression, as well as HIF-1 α expression in mouse embryos (Jain et al., 1998), where its mRNA was also restricted to specific developmental stages (presently analysed at E9.5, 13.5 and 15.5).

1.2.2. Expression of *hif-2 α*

Expression of *hif-2 α* (Fig. 3) was first detected early at the 8 cell-stage, indicating that it is also expressed maternally. Expression is ubiquitous until 24 hpf, when it concentrates in the brain, brain ventricle epithelia and somites (Fig. 3D). At 28 hpf *hif-2 α* was detected in brain blood vessels, somites and intersegmental blood vessels (Fig. 3E), but in contrast to *hif-1 α* , it was neither detected in the notochord nor in the caudal vein, (compare Fig. 3E and Fig. 2E). Later in development, at 32 hpf, the expression pattern does not change (Fig. 3F); however, at 48 hpf, the transcript was detected in brain blood vessels, somites, notochord, and intersegmental blood vessels (Fig. 3G and insets therein). Note that *hif-1 α* is not expressed in the notochord at this stage (compare Figs. 2G and 3G). At 60 hpf the expression of *hif-2 α* becomes evident in the notochord, neural tube (lower levels of expression), somites, dorsal aorta, intersegmental blood vessels and in the intestine

Table 1

Identity of HIF domains of zebrafish Hif-1 α and Hif-2 α with grass carp and human orthologues

HIF Domain	Zebrafish HIF-1 α		Zebrafish HIF-2 α	
	Carp	Human	Carp	Human
bHLH	100	93.3	95.6	93.3
PAS A	98.2	76.4	98.2	91.1
PAS B	98.1	87.8	92.4	86.7
TAD-N	91.3	89.1	91.8	67.3
TAD-C	100	100	93.3	86.7
NLS1	100	100	80	80
NLS2	100	90	90	70
NLS3	90	50	80	20
NLS4	100	81.8	100	100
NLS5	100	100	–	–

Multiple domains of zebrafish Hif-1 α and Hif-2 α where matched using CLUSTAL-W software, with grass carp and human versions, the identity percentages are shown in the table.

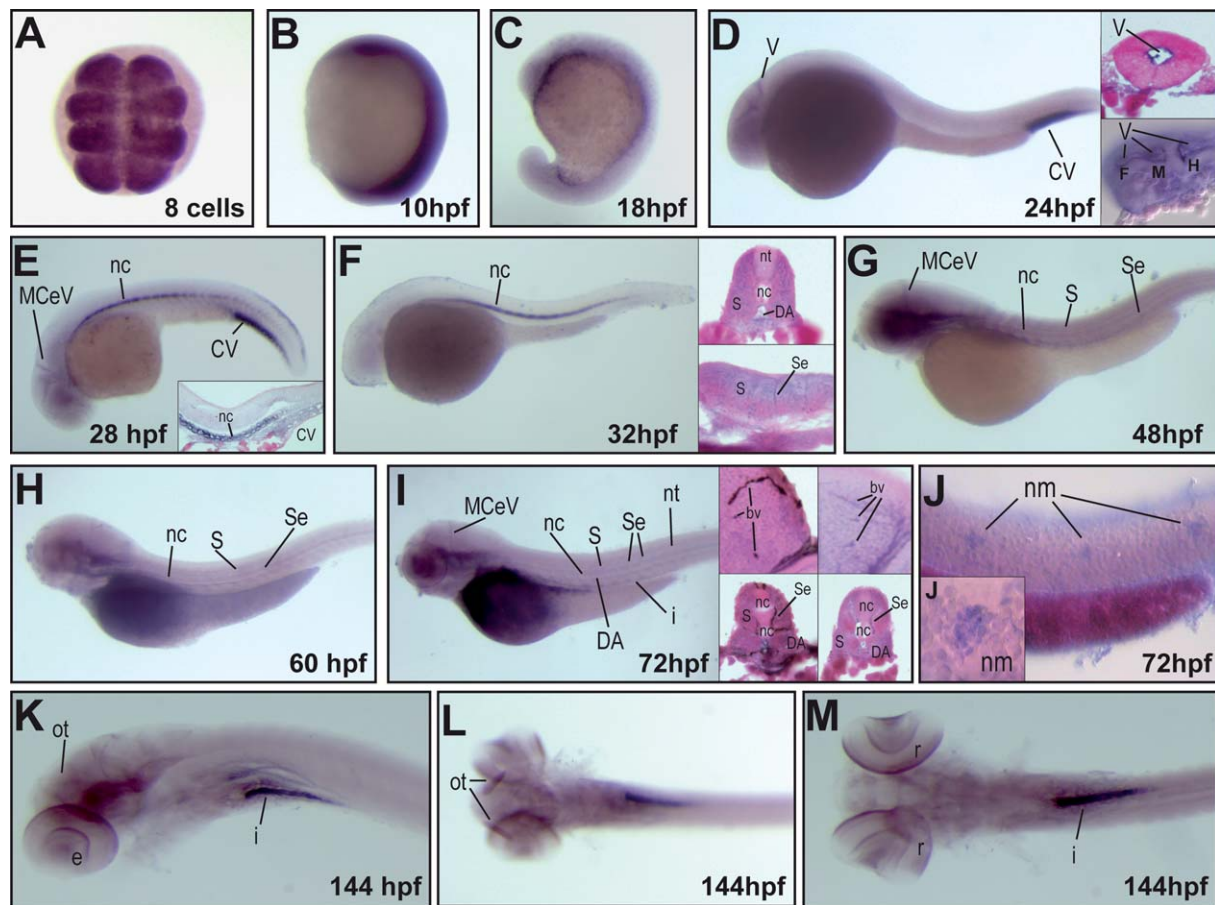


Fig. 2. Whole-mount *in situ* hybridization for *hif-1α*. Expression of *hif-1α* mRNA was analyzed at the indicated developmental stages. *hif-1α* mRNA is detected at early stages in an ubiquitous expression pattern (A and B), becoming ventrally restricted by late embryogenesis (C); A, dorsal view; B and C, lateral view with anterior on top. At 24 hpf *hif-1α* is expressed in ventricle epithelia (D, paraffin transverse cross-section in top inset), as confirmed by Nomarski microscopy (D, bottom inset). At 28 hpf, expression is present in the notochord, caudal vein and brain blood vessels (E), as confirmed by histological sagittal cross-section (inset in E). At 32 hpf, *hif-1α* mRNA is detected in brain and notochord (F), paraffin cross-section of embryos shows that expression occurs in the somites, notochord and dorsal aorta (transverse cross-section, top inset in F) as well as in intersegmental blood vessels (sagittal cross-section, bottom inset in F). At 48 and 60 hpf (G and H, respectively) the expression of *hif-1α* is detected in the brain, notochord, somites and intersegmental blood vessels. At 72 hpf the mRNA is detected in the brain, branchial region, intersegmental blood vessels, dorsal aorta and intestine (I), left insets in I show a Tg (*fli1*:EGFP)^{y1} embryo subjected to GFP immunohistochemistry and *hif-1α* mRNA *in situ* hybridization; right insets in I show the mRNA *in situ* hybridization pattern of *hif-1α* in a wild type fish (all insets are transverse cross-sections). Overstaining of 72 hpf embryos revealed expression in caudal neuromasts (J), as confirmed by DIC optics (inset in J). At 144 hpf expression was observed in the optic tectum, retina and intestine (K). A dorsal view of embryos at 144 hpf shows expression in the optic tectum (L) and retina (M). In (D) to (M) lateral view, with anterior to the left. Insets showing transverse cross-sections with dorsal side up; sagittal cross-sections show the anterior to the left. Abbreviations: V, ventricle epithelia; F, forebrain; M, midbrain; H, hindbrain; MCEV, middle cerebral vein; CV, caudal vein; Se, intersegmental vessel; DA, dorsal aorta; nc, notochord; S, somites; nt, neural tube; I, intestine; bv, blood vessel; nm, neuromasts; ot, optic tectum; e, eye; r, retina.

(Fig. 3H), a pattern maintained in 72 hpf larvae (Fig. 3I). In order to confirm that the expression of *hif-2α* also occurs in blood vessels, we again used the Tg *fli1*:EGFP^{y1} fish line. As shown in the insets of Fig. 3I, 72 hpf larvae, displayed a *hif-2α* expression pattern that coincides with the anti-GFP signal in the intersegmental blood vessels and dorsal aorta, thereby confirming that expression of *hif-2α* indeed occurs in blood vessels. In addition, we have been able to detect the transcript in the notochord and somites (Fig. 3I top insets), revealing that in these structures expression occurs in non-vascular tissues. Finally, at 120 hpf, expression of *hif-2α* could be also detected in blood vessels, as well as in the notochord, somites, gut, brain, optic tectum and retina (Fig. 3I and inset therein).

The expression pattern of *hif-2α* described in this report is similar to the one partially described in mouse (Flamme et al., 1997; Jain et al., 1998) and quail embryos (Elvert et al., 1999). Murine *Hif-2α* mRNA was detected from stage E7.5 where it showed an ubiquitous expression pattern, that was later restricted to the brain capillary dorsal aorta and cardinal veins at E11.5. At stage E15.5 the expression was finally detected in the endothelial cells of the brain as well as in endothelial cells of other structures. The analysis of the expression pattern of these two genes shows for the first time that some embryonic tissues express *hif-1α* but not *hif-2α* at a given developmental stage, but the later in development the expression of *hif-1α* is replaced in these tissues by the expression of *hif-2α*.

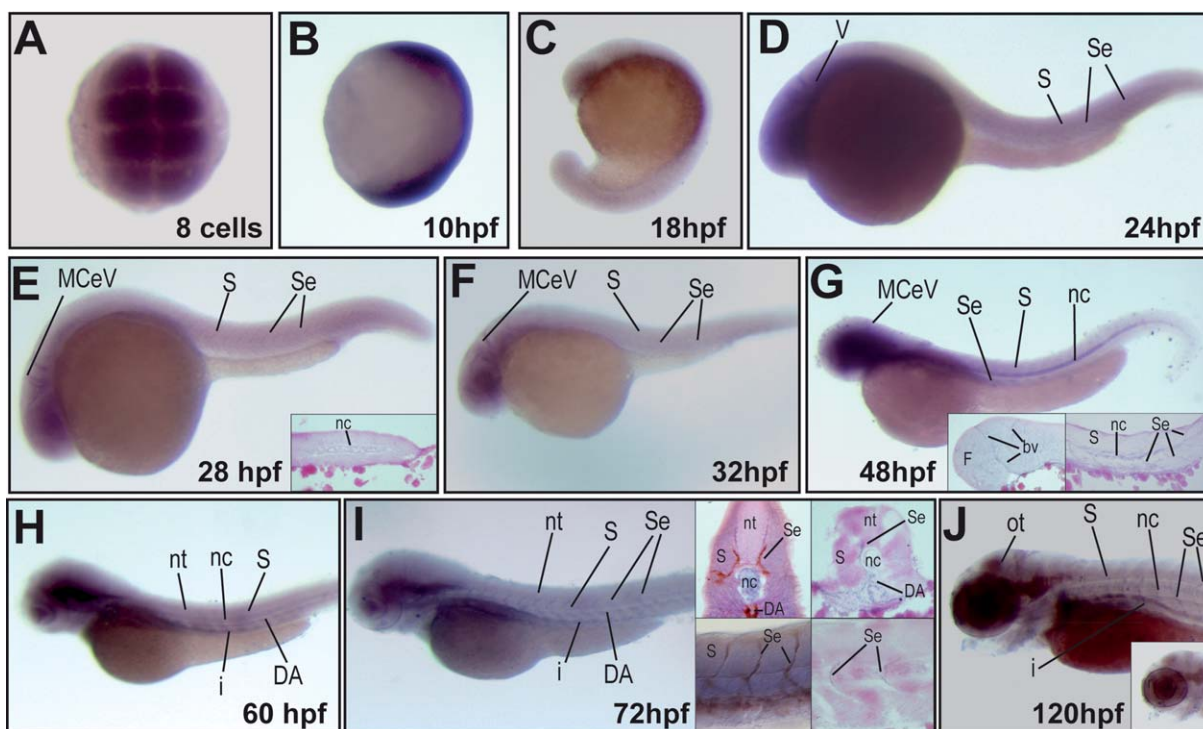


Fig. 3. Expression of *hif-2α* in zebrafish embryos and larvae as revealed by mRNA *in situ* hybridization. *hif-2α* mRNA is maternally expressed and ubiquitously distributed at early stages (A and B), becoming ventrally restricted later in embryogenesis (C); (A) dorsal view; (B) and (C), lateral views with the anterior side on top. At 24 (D), 28 (E) and 32 hpf (F) *hif-2α* was detected in the brain, somites, and blood vessels. At 48 hpf, *hif-2α* expression is present in brain blood vessels, notochord and intersegmental blood vessels (G), as it is shown in the brain (left inset in G) and tail paraffin cross-sections (right inset in G). At 60 hpf, *hif-2α* mRNA is expressed in the brain, somites, dorsal aorta, intestine and notochord (H). At 72 hpf, *hif-2α* expression was detected in the brain, brain blood vessels, intersegmental vessels, dorsal aorta and intestine (I). mRNA *in situ* hybridization for *hif-2α* and anti-GFP immunohistochemistry in Tg(*flil:EGFP*)^{y1} embryos show that expression occurs in intersegmental blood vessels (left-top inset in I, transverse cross-section; left-bottom inset, DIC lateral view). Paraffin cross-sections of wild type embryos at 72 hpf, show expression of *hif-2α* in somites, intersegmental blood vessels, notochord and aorta (right-top inset in I, transverse cross-section; right-bottom in I, sagittal cross-section). At 120 hpf, expression of *hif-2α* was detected in the optic tectum, intersegmental blood vessels, notochord, intestine and retina (J). (D)–(J) are lateral views with anterior to the left. Insets show transverse cross-sections with the dorsal part on the top, or sagittal cross-sections with anterior to the left. Abbreviations: V, ventricle epithelia; F, forebrain; M, midbrain; H, hindbrain; MCEV, middle cerebral vein; Se, intersegmental vessel; DA, dorsal aorta; nc, notochord; S, somites; nt, neural tube; I, intestine; bv, blood vessel; nm, neuromasts; ot, optic tectum; e, eye; r, retina.

1.2.3. Summary

We have cloned the zebrafish *hif-1α* and *hif-2α* orthologues that map at two different linkage groups. The two genes have maternal expression, and in early embryonic stages their transcripts display a widespread expression pattern in all tissues. Later in development, *hif-1α* mRNA expression pattern is very dynamic, especially in the central nervous system, notochord, somites and blood vessels. *hif-2α* mRNA localization has a distinct expression pattern, being restricted to the central nervous system, notochord, somites, retina and mainly to blood vessels. Thus, *hif-1α* and *hif-2α* expression patterns are coincident in some territories, as somites; whereas in other territories they are mutually exclusive, as the caudal vein for *hif-1α* and brain regions for *hif-2α*. Moreover, in tissues such as the notochord the expression of *hif-1α* is turned off to be immediately replaced by the expression of *hif-2α*. To our knowledge, this is the first study in which the expression of *hif-1α* and *hif-2α* genes is directly compared throughout the entire development of a vertebrate model animal.

2. Experimental procedures

2.1. Animals

Fish and embryos (AB strain) were maintained in our own facility according to standard procedures (Westerfield, 1995). The transgenic line Tg(*flil:EGFP*)^{y1} was obtained from the Zebrafish International Resource Center (ZIRC). All embryos were collected by natural spawning, staged according to Kimmel et al. (1995) and raised at 28 °C in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl₂, 0.33 mM MgSO₄, and 0.1% Methylene Blue) in petri dishes (Haffter et al., 1996). Larval stages are expressed in hours post-fertilization (hpf).

2.2. Molecular methods and sequence analysis

EST clones were purchased from Open Biosystems (Springfield, USA). With the complete sequence of BM182886 we found previously reported sequence AY326951 corresponding to *hif-1α*. To amplify *hif-1α*, reverse transcription-polymerase chain reaction (RT-PCR) was performed using the following primers F₀-CTGAGTGGTTTCACCCAGGA-3' and R₀-5'-GCAGCAGAGCTCAGTTGACT-3' using cDNA prepared from 48 h post-fertilization (hpf) zebrafish embryos. To amplify the complete *hif-2α* ORF, RT-PCR was performed using the following primers F₁-5'-GGGAATTACGCGAGAACGG-3' and R₁-5'-CGGTCTAAGTCGCC

TGGTC-3', using cDNA prepared from 48 hpf zebrafish embryos. The amplified fragments (2364 bp for *hif-1α* and 2525 bp for *hif-2α*) were sub-cloned in the pGEM-T Easy vector system (Promega) and sequenced. The sequence of *hif-2α* fragment was used to search for a genomic clone (accession No. BX248102), to complete the 5' terminus of the mRNA of *hif-2α* (complete cDNA 3148 bp) (accession No. DQ375242). RT-PCR reactions for expression studies were performed, with the following primers: for *hif-1α* F₂-5'-CCAGTGGAAACCAGACATCAG-3' and R₂-5'-GAC-TTGGTCCAGAGCACGC-3', for *hif-2α* F₃-5'-CTTCCAACCCCTTGAGTTCAC-3' and R₁ (see above for the sequence). The amplified fragments were sub-cloned into the pGEM-T Easy plasmid, sequenced and used to generate anti-sense riboprobes for *in situ* hybridization (see below). The mRNA for expression studies was extracted from embryos or larvae at different stages of development (0, 10, 18, 24, 48 and 72 hpf) using the Trizol[®] Reagent according to the manufacturer's indications (Invitrogen, San Diego, CA, USA). Reverse transcription was performed with the RevertAid Kit (Fermentas, Lithuania) according to the manufacturers' instructions. As an internal control, we used β-actin primers: F₀-5'-TTCTGGTTCGGTACTACTGGTATTGTG-3' and R₀-5'-ATCTTCATCAGGTA-GTCTGTGTCAGGT-3' (amplified fragment 679 bp), as described previously (Chen et al., 2002; Mackenzie et al., 2004).

2.3. Phylogenetic tree construction

The phylogenetic tree was produced by a multiple alignment of zebrafish HIF-1α and HIF-2α deduced proteins with 10 other HIF family members using the MegAlign program (DNASTAR) and the CLUSTALW algorithm. The following proteins were chosen to represent all of the reported vertebrate HIFs present in Genbank: grass carp HIF-1α (AAR95697), human HIF-1α (AAP88778), mouse HIF-1α (Q61221), rat HIF-1α (CAA70701), squirrel HIF-1α (AAU14021), dog HIF-1α (AAR19225), chicken HIF-1α (JC7619), grass carp HIF-2α (AAT76668), human HIF-2α (NP_001421), mouse HIF-2α (P97481), rat HIF-2α (Q9JHS1), chicken HIF-2α (NP_990138), *Xenopus tropicalis* HIF-2α (AAH74648) and common quail HIF-2α (AAF21052).

2.4. Whole mount *in situ* hybridization, immunohistochemistry and histology

Embryos were raised at 28 °C and fixed for *in situ* hybridization in 4% paraformaldehyde and hybridization reactions were performed as previously described (Jowett and Lettice, 1994). Probes were designed to contain only the non-coding 3' region by using RT-PCR (see above). For *hif-1α* we used a 788 bases ribo-probe (nucleotides 1777–2565 from AY326951) and for *hif-2α* a 814 bases ribo-probe (nucleotides 1938–2752 from DQ375242). Embryos were mounted in glycerol, observed under a Leica MZ12.5 stereomicroscope or a Nikon Eclipse 80i microscope and photographs were taken with a Leica DC300F digital camera. For double staining, after *in situ* mRNA hybridization, embryos were fixed for 20 min in paraformaldehyde and immunohistochemistry was performed as described previously (Schulte-Merker, 2002), with anti-GFP antibody (FL) (Santa Cruz); then the embryos were post-fixed for 20 min in 1.25% glutaraldehyde/2% paraformaldehyde in BT buffer (0.15 mM CaCl₂, 4% sucrose, 0.1 M phosphate buffer; Westerfield, 1995; Bever and Fekete, 2002) and dehydrated in a graded ethanol series. Finally, the embryos were embedded in Paraplast plus[®] (UK), sectioned at 9 μm, mounted in 3-aminopropyltriethoxysilane (Polysciences, Inc., Washington, PA, USA) treated slides and stained with eosin.

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