Zebrafish BarH-like genes define discrete neural domains in the early embryo

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Abstract

BarH (Barhl) genes encode for highly conserved homeodomain-containing transcription factors involved in critical functions during development, including cell fate specification, migration and survival. Here, we report the dynamic and restricted expression of three zebrafish *barhl* within the developing central nervous system. *barhl2* becomes expressed in the late gastrula as a transverse diencephalic domain located immediately caudal to the prospective eyes. At early somitogenesis, *barhl1.1* and *barhl1.2* are expressed in the diencephalon in domains that partially overlap with the ventral and dorsal aspects of *barhl2* expression, respectively. At later stages, expression of all zebrafish *barhl* shows large extent of overlap in the pretectum, tectum and dorsal hindbrain. The presence of a unique territory of *barhl2* expression in the dorsal telencephalon and the high levels of expression in the retina are both consistent with expression reports of other Barhl2 orthologues, and support the subdivision of vertebrate Barhl into two paralogue groups based on the phylogenetic analysis of nucleotide and amino acid sequences.

Keywords: BarH; Barhl; Homeodomain proteins; FIL domains; Zebrafish; Central nervous system; Eye

1. Results and discussion

BarH1 and *BarH2* encode for homeodomain-containing proteins that play essential roles during embryogenesis in *Drosophila* including morphogenesis and fate determination of the eye and external sensory organs (Hayashi et al., 1998; Higashijima et al., 1992a,b; Kojima et al., 1991, 1993; Lim and Choi, 2003, 2004), regional pre-patterning of specific structures of the nervous system such as the notum (Sato et al., 1999), and formation and specification of the distal leg segments (Tsuji et al., 2000). Vertebrate homologues have been identified in medaka (Poggi et al., 2002), *Xenopus* (Patterson et al., 2000), rat (Saito et al., 1998), mouse (Bulfone et al., 2000; Saba et al., 2003; Mo et al., 2004) and human (Bulfone et al., 2000) (Fig. 1), and appear to share some functional properties with the fly counterparts, such as the ability to regulate bHLH proteins (Saito et al., 1998; Sato et al., 1999). Different reports have named these genes as either *BarH* (*bh*) (e.g. Saito et al., 1998; Patterson et al., 2000) or *BarH*-like (*Barhl*) (e.g. Bulfone et al., 2000). Confusingly, assignation of paralogue numbers in the two nomenclatures is inverted. For example, mouse BarH1 (*mbh1*) is the same as mouse Barhl2 (*mBarhl2*) and viceversa, and the same apply for *Xenopus* BarH genes. In this paper, we have adopted the Barhl nomenclature as it has a HGNC-approved symbol (HGNC:954). Alternative/original names for each of these genes are indicated in the Experimental Procedures section of this article.

Vertebrate *Barhl* are expressed primarily in the developing nervous system where they appear to control subtype cell identity, migration and survival. Members of the *Barhl2* group confer commissural neuron identity on dorsal cells in the spinal cord (Saba et al., 2003, 2005), are involved in the specification of retina ganglion (Poggi et al., 2004) and glycineric amacrine (Mo et al., 2004) cells, and have pro-apoptotic activity in cells of the gastrula that pattern the neural plate (Offner et al., 2005). Vertebrate *Barhl1* members, on the other hand, regulate the migration of cerebellar granule cells (Li et al., 2004), and are involved in the survival of specific cell types within the cerebellum (Li et al., 2004) and cochlea (Li et al., 2002).

Here, we report the expression of three zebrafish *barhl*, which we have named *barhl1.1*, *barhl1.2* and *barhl2*, based on the comparative analysis of both nucleotide and amino acid sequences (Fig. 1). Protein sequence alignment of Barhl from zebrafish (Dr BARHL1.1, Dr BARHL1.2, Dr BARHL2), medaka (Ol BAR), *Xenopus* (Xl BARHL2, Xl BARHL1), rat

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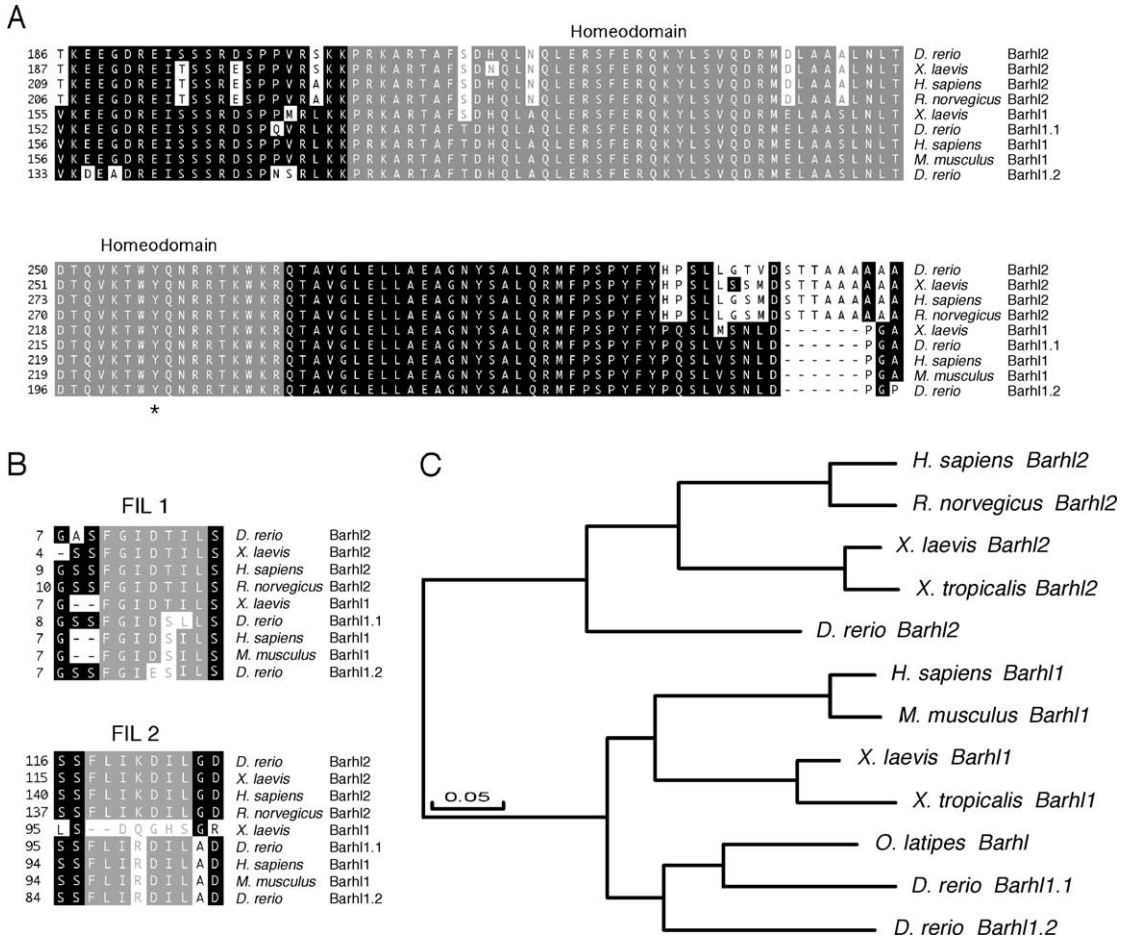

Fig. 1. Sequence analysis of vertebrate Barhl. (A) Amino acid alignment of the extended homeodomain region. Similarities between the different vertebrate proteins extend further down- and up-stream of the homeodomain to cover a region of around 100 amino acids. Identical amino acids within the homeodomain and in the extended regions are highlighted in grey and black, respectively. Vertebrate Barhl homeodomains contain a characteristic tyrosine residue at position 49 (asterisk) instead of the more common phenylalanine. (B) Alignment of regions containing conserved N-termini FIL domains, defined by the characteristic composition of phenylalanine (F), isoleucine (I) and leucine (L) amino acids (Saito et al., 1998). Vertebrate Barhl contain two such FIL domains with the exception of *Xenopus laevis* Barhl1 that possesses only one. Amino acid identities within FIL domains and in the surrounding regions are highlighted in grey and black, respectively. (C) A distance tree of *Barhl* genes isolated from different vertebrate species. The distance tree was drawn with the Maximum-likelihood program from the Phylip package using nucleotide sequences encoding for the extended homeodomain region depicted in A. As the tree indicates, zebrafish has two *barhl1* genes (named *barhl1.1* and *barhl1.2*) and only one *barhl2* gene.

(Rn BARHL2), mouse (Mm BARHL1) and human (Hs BARH1, Hs BARH2) revealed high degree of amino acid identity within three regions: one containing around 100 amino acids encompassing the homeodomain (Fig. 1A) and two regions located on the N-termini containing FIL domains (FIL1 and FIL2) (Saito et al., 1998) (Fig. 1B). FIL domains have been found in homeoproteins of the Engrailed, Goosecoid, Nk-1, Nk-2 and Msh classes, where they have been associated with transcriptional repression activity (Jaynes and O'Farrell, 1991; Smith and Jaynes, 1996). Phylogenetic analysis based on either Maximum-Likelihood (Fig. 1C) or Neighbour (data not shown) methods, and considering nucleotide sequences encoding for the extended homeodomain region (Fig. 1A), consistently subdivide vertebrate *Barhl* into two paralogue groups (Fig. 1C). This analysis also reveals that zebrafish has two *barhl1* and only one *barhl2*. This division finds further support in the fact that zebrafish Barhl1.1 and Barhl1.2 share 100% amino acid identity in the homeodomain, whereas have only 93.1% identity in this region with Barhl2.

Expression of zebrafish *barhl* is highly dynamic and restricted to discrete domains within the developing central nervous system. Transcripts of *barhl2* are first detected at 80–90% epiboly as bilateral stripes extending across the medio-lateral axis of the neural plate (Fig. 2A). At this stage, expression of *barhl1.1* and *barhl1.2* is still undetectable. Double labelling of *barhl2* in combination with early markers of the prospective midbrain (*pax2*, Fig. 2A), telencephalon (*emx1*, Fig. 2B), eye field (*rxb*, Fig. 2C), and dorsal epithalamus (*flh*, Fig. 2D) indicate that *barhl2* demarcates a transverse diencephalic domain within the anterior neural plate, located immediately caudal to the prospective eyes (Fig. 2E). After closure of the neural tube, the bilateral stripes of expression fuse at the midline and *barhl2* defines a diencephalic domain perpendicular to the axis that comprises

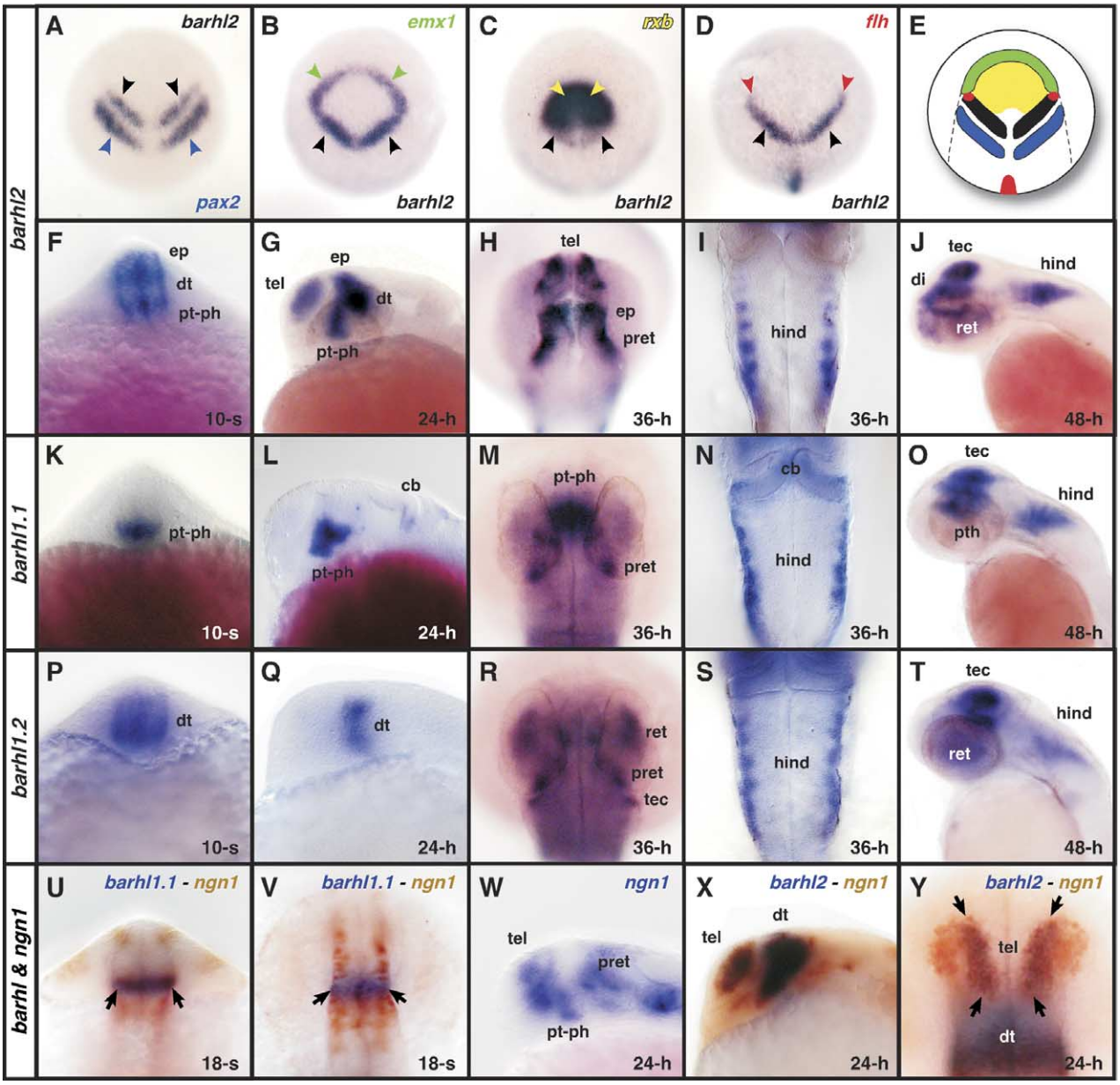

Fig. 2. Expression of zebrafish *barhl* in the developing central nervous system. (A–E) Dorsal views of 90%-epiboly embryos showing *barhl2* expression in the anterior neural plate (black arrowheads), in relation to the expression domains of *pax2* (blue arrowheads), *emx1* (green arrowheads), *rxb* (yellow arrowheads) and *flh* (red arrowheads). Expression of these genes is summarised in E. Anterior to the top. Frontal (F,K,P,U; dorsal to the top), lateral (G,J,L,O,Q,T,W,X; anterior to the left) and dorsal (H,I,M,N,R,S,V,Y; anterior to the top) views of embryos showing expression of *barhl2* (F–J), *barhl1.1* (K–O), *barhl1.2* (P–T), *ngn1* (W), and a combination of immunohistochemistry against GFP in the *tg*(*ngn-1-GFP*) line together with whole mount in situ hybridisation for *barhl1.1* (U,V) and *barhl2* (X,Y). Stages are 10-somite (F,K,P), 18-somite (U,V), 24 hpf (G,L,Q,W,X,Y), 36 hpf (H,I,M,N,R,S), and 48 hpf (J,O,T). *Abbreviations:* cb (cerebellum), dt (dorsal thalamus), ep (epithalamus), hind (hindbrain), ph (posterior hypothalamus), pret (pretectum), pt (posterior tuberculum), ret (retina), tec (tectum), tel (telencephalon).

all the dorso-ventral extent of the neural tube. At this stage (about 1–2 somites), expression of *barhl1.2* becomes detectable as a transverse diencephalic domain similar to *barhl2* but excluded from the most dorsal and ventral aspects of the neural tube. A few hours later *barhl1.1* expression appears restricted to the ventral diencephalon. By 10–14 somites, the three zebrafish *barhl* define complementary and partially overlapping domains within the diencephalon. *barhl2* is detected in the prospective epithalamic and thalamic regions, and in a ventral diencephalic domain that move towards anterior to finally cover the posterior hypothalamus/posterior tuberculum (Fig. 2F,G). *barhl1.1* and *barhl1.2*, on the other hand, are expressed in the prospective posterior hypothalamus/posterior tuberculum (Fig. 2K,L) and dorsal thalamus (Fig. 2P,Q), respectively. From around 18 h post-fertilisation (hpf), several novel domains of *barhl* expression appear in the brain. *barhl2* transcripts become detectable in the dorsal telencephalon (where expression is maintained until around 40 hpf) (Fig. 2G,H). Interestingly, telencephalic *barhl2*, and the most ventral part of the hypothalamic *barhl1.1* expression domain,

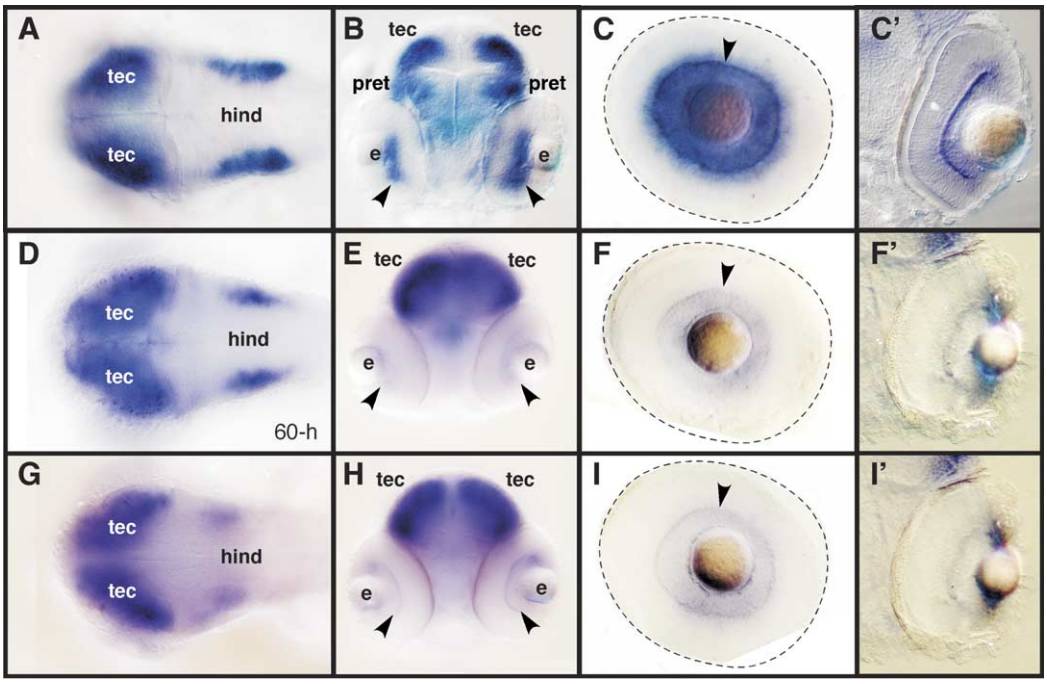

Fig. 3. Overlapping domains of zebrafish *barhl* expression during late embryogenesis. Expression domains of *barhl2* (A,B,C,C′), *barhl1.1* (D,E,F,F′), and *barhl1.2* (G,H,I,I′) at 60 h post-fertilisation, shown in dorsal (A,D,G; anterior to the left), frontal (B,C′,E,F′,H,I′; dorsal to the top) and lateral (C,F,I; anterior to the left) views. The border of the eye is depicted with a dashed line in C, F and I. Expression in the retina is shown in arrowheads. *Abbreviations:* e (eye), hind (hindbrain), pret (pretectum), ret (retina), tec (tectum).

overlap with *neurogenin1* (Fig. 2U–Y), a finding that is consistent with the proposed ability of vertebrate Barhl to regulate bHLH proteins (Saito et al., 1998; Sato et al., 1999). At 18 hpf, *barhl1.1* is seen in a small subset of cells in the prospective cerebellum (Fig. 2L). At this stage, expression of *barhl1.1* is also detectable along the dorsal hindbrain (prospective clusters of interneurons), in a domain that is later shared with *barhl1.2* and *barhl2* (from around 36 hpf; see Fig. 2I,N,S). At 24 hpf, *barhl1.1* transcripts are also seen in a small subset of cells within the olfactory bulb (data not shown). At 36 hpf, novel domains of *barhl* expression become detectable in the pretectal area (*barhl1.1*, *barhl1.2* and *barhl2*), latero-ventral epithalamus (*barhl1.2*), tectum and retina (*barhl1.2*) (Fig. 2H,M,R). *barhl1.1* and *barhl2* also show expression in the tectum and retina from around 48 hpf (Fig. 2J,O). By 60 hpf, all zebrafish *barhl* share overlapping expression domains in the tectum, pretectum and hindbrain (Fig. 3), whereas *barhl2* maintains a unique expression domain in the dorsal diencephalon and is detected in the retina at considerably higher levels than *barhl1.1* and *barhl1.2* (compare Fig. 3C–C′ with 3F,F′,I,I′).

In summary, zebrafish *barhl* show a combination of unique and overlapping expression domains that support a subdivision into two paralogue groups based on both nucleotide and amino acid sequence analysis (Fig. 1). Comparison of *Barhl* expression domains between mouse and zebrafish is consistent with this subdivision and reveals that *Barhl2* members have unique territories of expression in the telencephalon, epithalamus, and (at least at high levels) in the retina (Table 1).

Table 1
Comparison of expression territories of mouse (Mm) and zebrafish (Dr) *barhl*

	Mm Barhl1	*Dr barhl1.1*	*Dr barhl1.2*	*Mm Barhl2*	*Dr barhl2*
Telencephalon				√	√
Retina		Low levels	Low levels	√	√
Epithalamus				√	√
Thalamus			√	√	√
Hypothalamus/posterior tuberculum	√	√			√
Tectum	√	√	√	√	√
Cerebellum	√	√		√	
Hindbrain	√	√	√		√
Spinal cord				√	
Reference	Bulfone et al. (2000)	This report	This report	Mo et al. (2004)	This report

2. Experimental procedures

2.1. Sequences analyses

mRNA and protein sequences were obtained from: *H. sapiens* Barhl1 (acc. Nos NM020064 and NP_064448), *H. sapiens* Barhl2 (acc. Nos NM_020063 and NP_064447), *M. musculus* Barhl1 (acc. Nos NM_019446 and NP_062319, also found as mBH2), *R. norvegicus* Barhl2 (acc. Nos NM_022956 and NP_075245), *X. laevis* Barhl1 (acc. Nos AF283692 and AAG14451, found as XBH2), *X. laevis* Barhl2 (acc. Nos AF283691 and AAG14450, found as XBH1), *X. tropicalis* Barhl1 (acc. No. ENSXETG00000006257), *X. tropicalis* Barhl2 (acc. No. ENSXETG00000023986), *Danio rerio* Barhl1.1 (acc. Nos AAS92236 and AY596176, found as BarH2), *Danio rerio* Barhl1.2 (acc. Nos AAU00059 and AY596187, found as BarH3), *Danio rerio* Barhl2 (acc. Nos NP_991303 and NM_205740, found as B-H2), *O. latipes* Bar (acc. Nos AJ426046 and CAD19778). Alignments were made using MegAlign software (DNASTAR, Inc.). Distance trees were drawn using the Maximum-likelihood and the Neighbour-joining program from the Phylip package, using nucleotide sequences encoding for the extended homeodomain region of Barhl depicted in Fig. 1A.

2.2. Cloning of zebrafish barhl

ESTs containing part of the coding sequence of zebrafish *barhl2* (acc. Nos BI325578, BM023761, AI957963, BI840866) were identified through BLAST search using the nucleotide sequence encoding for the extended homeodomain of XBH1 (Patterson et al., 2000). Using the consensus sequence of these ESTs as a query, we obtained the full-length coding sequence of *barhl2* in the Sanger Database (acc. No. ENSDARG00000004760). An EST containing part of the coding sequence of zebrafish *barhl1.1* (acc. No. CA496570) was identified through BLAST search using nt. 700–1200 of Hs BARHL1 (acc. No. NM020064) encoding for amino acid sequences downstream of the homeobox, where the differences between Hs Barhl1 and Hs Barhl2 are more pronounced. Using the sequence of this EST, we identified two genomic sequences in the Sanger Database: ENSDARG00000019013 and ENSDARG00000006369 contained the entire coding sequences of *barhl1.1* (chromosome 21) and *barhl1.2* (chromosome 5), respectively. Full-length *barhl1.1* (containing also 5′ and 3′ UTR regions) was amplified by RT-PCR from mRNA isolated from 24 hpf-embryos by using specific primers (Forward: 5′-ATTAAATTTGC-CATCCGAGAGTAT-3′, Reverse: 5′-GTCCCGTTATTGCATTAGGTG-TAT-3′). The PCR product was cloned into pCRII vector (Invitrogen) and sequenced. Using the coding sequence of ENSDARG00000006369 we identified one EST through BLAST search containing the 5′ UTR and the first exon of *barhl1.2* (acc. No. AI957406). During the course of this work, the full coding sequences of zebrafish *barhl1.1* (BarH2), *barhl1.2* (BarH3) and *barhl2* (B-H2) were published at GenBank by others (acc. Nos AY596176, AY596187 and NM_205740).

2.3. Zebrafish strains

Danio rerio of wild type and transgenic lines were from the ICBM Zebrafish Facility. The *tg*(*ngn-1-GFP*) line was donated by P. Blader (Blader et al., 2004).

2.4. Whole mount in situ hybridisation and immunohistochemistry

Gene and protein expression was detected using standard procedures (Jowett, 1999). Anti-sense mRNA probes used for whole mount in situ hybridisation were generated from plasmids containing either part (5′UTR and first exon of *barhl1.2*; 5′UTR and first two exons of *barhl2*) or the entire (*barhl1.1*) cDNA coding sequence. For antibody labelling, rabbit anti-GFP (Chemokine) was used at 1:1000 dilution.

2.5. Vibrotome sections

Embryos treated by whole mount in situ hybridisation were embedded in 4% agarose in PBS, oriented in frontal views with both eyes placed symmetrically, and mounted on a motorised vibrotome (Vibroslice MA752, Lafayette Instrument, Co.). 50 μm sections were placed on glass slides for digital photography.

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