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In silico and *in situ* characterization of the zebrafish (*Danio rerio*) *gnrh3* (sGnRH) gene

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Abstract

Background: Gonadotropin releasing hormone (GnRH) is responsible for stimulation of gonadotropic hormone (GtH) in the hypothalamus-pituitary-gonadal axis (HPG). The regulatory mechanisms responsible for brain specificity make the promoter attractive for *in silico* analysis and reporter gene studies in zebrafish (*Danio rerio*).

Results: We have characterized a zebrafish [Trp[7], Leu[8]] or salmon (s) GnRH variant, *gnrh*3. The gene includes a 1.6 Kb upstream regulatory region and displays the conserved structure of 4 exons and 3 introns, as seen in other species. An *in silico* defined enhancer at -976 in the zebrafish promoter, containing adjacent binding sites for Oct-1, CREB and Sp1, was predicted in 2 mammalian and 5 teleost GnRH promoters. Reporter gene studies confirmed the importance of this enhancer for cell specific expression in zebrafish. Interestingly the promoter of human GnRH-I, known as mammalian GnRH (mGnRH), was shown capable of driving cell specific reporter gene expression in transgenic zebrafish.

Conclusions: The characterized zebrafish Gnrh3 decapeptide exhibits complete homology to the Atlantic salmon (*Salmo salar*) GnRH-III variant. *In silico* analysis of mammalian and teleost GnRH promoters revealed a conserved enhancer possessing binding sites for Oct-1, CREB and Sp1. Transgenic and transient reporter gene expression in zebrafish larvae, confirmed the importance of the *in silico* defined zebrafish enhancer at -976. The capability of the human GnRH-I promoter of directing cell specific reporter gene expression in zebrafish supports orthology between GnRH-I and GnRH-III.

Background

Gonadotropin releasing hormone (GnRH) controls reproduction in vertebrates through the HPG axis [1–3]. In fish, the GnRH synthesizing neurons innervates the pituitary gland [4–6], and upon binding of GnRH to the receptor, GtH production is stimulated. This receptor mediated signal transduction pathway stimulates gonadal maturation, gametogenesis and production of sex steroids, which impose feedback on the HPG axis [7–12]. The evolutionary conserved GnRH decapeptide has been characterized

Zebrafish	sGnRH	(*Danio rerio*)	Gnrh3	QHWSYGWLPG
Atlantic salmon	sGnRH	(*Salmo salar*)	GnRH-III	QHWSYGWLPG
Sea lamprey	lGnRH-III	(*Petromyzon marinus*)	GnRH-?	QHWSHDWKPG
Chicken	cGnRH-II	(*Gallus gallus*)	GnRH-II	QHWSHGWYPG
Tunicate	tGnRH-II	(*Chelyosoma productum*)	GnRH-?	QHWSLCHAPG
Human	mGnRH	(*Homo sapiens*)	GnRH-I	QHWSYGLRPG
Gilt head seabream	sbGnRH	(*Sparus aurata*)	GnRH-I	QHWSYGLSPG
Chicken	cGnRH-I	(*Gallus gallus*)	GnRH-I	QHWSYGLQPG
Herring	hrGnRH	(*Clupea harengus pallasi*)	GnRH-?	QHWSHGLSPG
African catfish	cfGnRH	(*Clarias gariepinus*)	GnRH-I	QHWSHGLNPG
Spiny dogfish	dfGnRH	(*Squalus acanthias*)	GnRH-?	QHWSHGWLPG
Guinea pig	gpGnRH	(*Cavia porcellus*)	GnRH-I	QYWSYGVRPG
Tunicate	tGnRH-I	(*Chelyosoma productum*)	GnRH-?	QHWSDYFKPG
Sea lamprey	lGnRH-I	(*Petromyzon marinus*)	GnRH-?	QHYSLQWKPG
Pejerry	pjGnRH	(*Odontesthes bonariensis*)	GnRH-?	QHWSFGLSPG
Dybowski's Brown Frog	[Trp^8]GnRH	(*Rana dybowskii*)	GnRH-?	QHWSYGLWPG
Common octopus	GnRH-like	(*Octopus vulgaris*)	GnRH-like	QNYHFSNGWHPG

Figure 1
GnRH decapeptide conservation Sequence comparison of mature GnRH peptides [13–15], show complete similarity between Atlantic salmon GnRH-III and zebrafish Gnrh3. The common name, species of origin nomenclature, scientific name, GnRH-I, -II or -III nomenclature and corresponding mature GnRH peptides are shown.

directly or indirectly in a number of vertebrates and a few invertebrates and currently includes 15 different decapeptide variants [13,14], though if the common octopus (*Octopus vulgaris*) GnRH-like mature peptide (dodecapeptide) is included, a total of 16 variants have been characterized till day [15] (Fig. 1). The variants are commonly named according to species of origin, but may also be named GnRH-I, -II or -III, based on sequence alignments solely [16] (Fig. 1). The GnRH expression pattern in the brain of fish has revealed segregation into a posterior and anterior system [6], where the former exhibits the releasing variants and the latter GnRH-II, also known as chicken GnRH-II (cGnRH-II) [4,17–19]. Differential expression of three GnRH forms was initially shown in the brain of the gilthead sea bream (*Sparus aurata*) [20], and more recently in African cichlid (*Haplochromis burtoni*) and the ancient herring (*Clupea harengus pallasi*) [21–23]. In the European sea bass (*Dicentrarchus labrax*), GnRH-I and GnRH-III, also known as the sea bream (sbGnRH) and salmon (sGnRH) variants, have been found co-expressed in the olfactory bulbs, ventral telencephalon and preoptic region and ventral thalamus, with a bias towards stronger GnRH-I activity in the preoptic area, compared to GnRH-III [24]. Expression and *in situ* data from the African cichlid have demonstrated presence of GnRH-I (sbGnRH) in the preoptic area and GnRH-III (sGnRH) in the areas of the terminal nerve and pituitary, suggesting GnRH-I to be the releasing form [25–27]. The genomic structure of GnRH is highly conserved, and comprises 4 exons and 3 introns [28,29]. The 5' untranslated region (UTR) is encoded by exon I, whereas the signal peptide, GnRH decapeptide, proteolytic cleavage site (GKR) and the N-terminus of the GnRH associated peptide (GAP) are encoded by exon II. Exon III encodes the mid section of GAP and exon IV encodes the GAP C-terminus and the 3' UTR.

The gene product of zebrafish *gnrh*3 has previously been detected by use of HPLC and RIA [30], and here we describe the cDNA and gene, including upstream sequence. Since the sGnRH variant is classified as GnRH-III [16], we propose to name the characterized zebrafish gene *gnrh*3 and the product Gnrh3, according to the zebrafish nomenclature convention [http://zfin.org/zf_info/nomen.html]. Zebrafish genes use lowercase and *italics* and the gene products are given a first capital letter and the remaining in lower case non-*italics*. For non-zebrafish genes and proteins we use GnRH-I, -II or -III nomenclature [16]. The upstream region of zebrafish *gnrh*3 has been analyzed with a set of bioinformatic tools and we show experimentally that *in silico* predicted transcription factor (TF) elements are involved in tissue specific expression in the forebrain. We also report tissue specific control of tran-

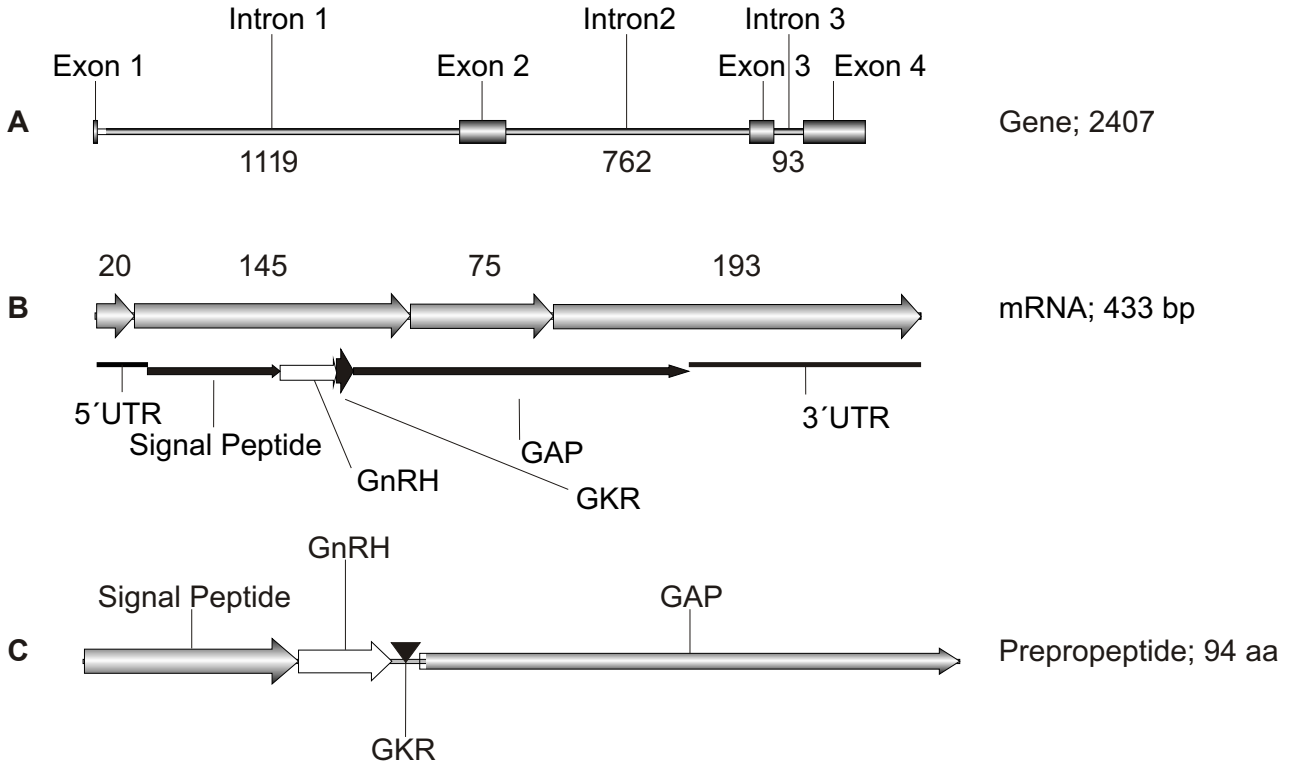


Figure 2
A) Schematic intron-exon diagram of the zebrafish *gnrh*3 gene The exons and introns are marked as boxes and lines, respectively. The intron lengths are shown underneath the lines. B) Exon lengths are shown above the mRNA sequence. The functional domains of the prepro polypeptide and untranslated regions (UTR) are marked. C) Post-translational cleavage of the signal peptide and GAP at the proteolytic cleavage site (GKR), generate the mature Gnrh3 decapeptide from the prepro polypeptide.

scription, conserved between zebrafish and man, using the human GnRH-I promoter for driving cell specific expression in transgenic zebrafish.

Results

Zebrafish gnrh3 gene

Zebrafish cDNA was cloned from poly A selected RNA using PCR primers complementary to the conserved GnRH-III decapeptide and GAP mRNA regions. Contig assembly of 5' and 3' RACE (rapid amplification of cDNA ends) clones resulted in a 433 bp long cDNA, featuring a 282 bp open reading frame (ORF) with 5' and 3' UTR of 27 and 124 bp. BLAST analysis of the prepro polypeptide and multiple sequence alignment of the decapeptide resulted in a high score of 10^{-34} to the Roach (*Rutilus rutilus*) prepro GnRH-III [31] and complete homology to the Atlantic salmon GnRH-III [29], respectively (Fig. 1). The 94 aa prepro polypeptide consists of a 23 aa signal peptide, GnRH decapeptide, proteolytic cleavage site (GKR) and 58 aa GAP (Fig. 2). The proteolytic cleavage site is conserved in all GnRH genes analyzed to date, whereas the signal peptide and GAP domains exhibit conservation of low scoring patterns (data not shown).

Zebrafish gnrh3 promoter sequence

The PCR promoter capture strategy (Fig. 3) resulted in 1656 bp upstream of the transcription start site (TSS) determined by 5' RACE. Analysis of the zebrafish exon I and 5' flanking sequence using Neural Network Promoter Prediction, revealed 4 putative TSS, hence Pol II promoters at -7, -114, -1156 and -1406, with corresponding scores of 0.85, 0.95, 0.98 and 0.92. Verification of TSS with Promoter Scan II failed, as no Pol II promoters were recognized with this software.

High sensitivity Repeat Masker analysis of the zebrafish *gnrh*3 promoter demonstrated 75% similarity to a 340 bp DANA-SINE element [32] and a 21 bp low complexity region at -1208 and -831, respectively. An incomplete 81 bp TOL2 transposable element motif [33] was registered at -

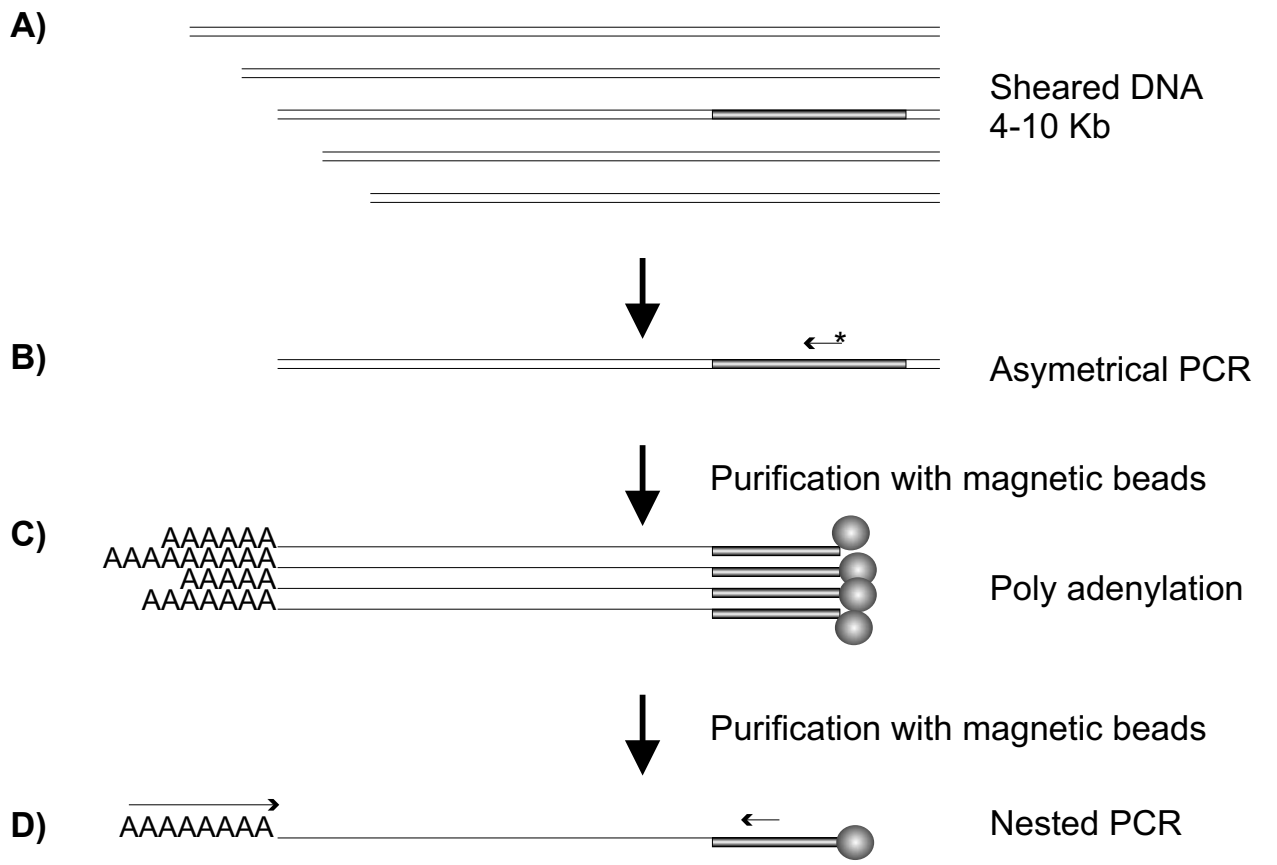
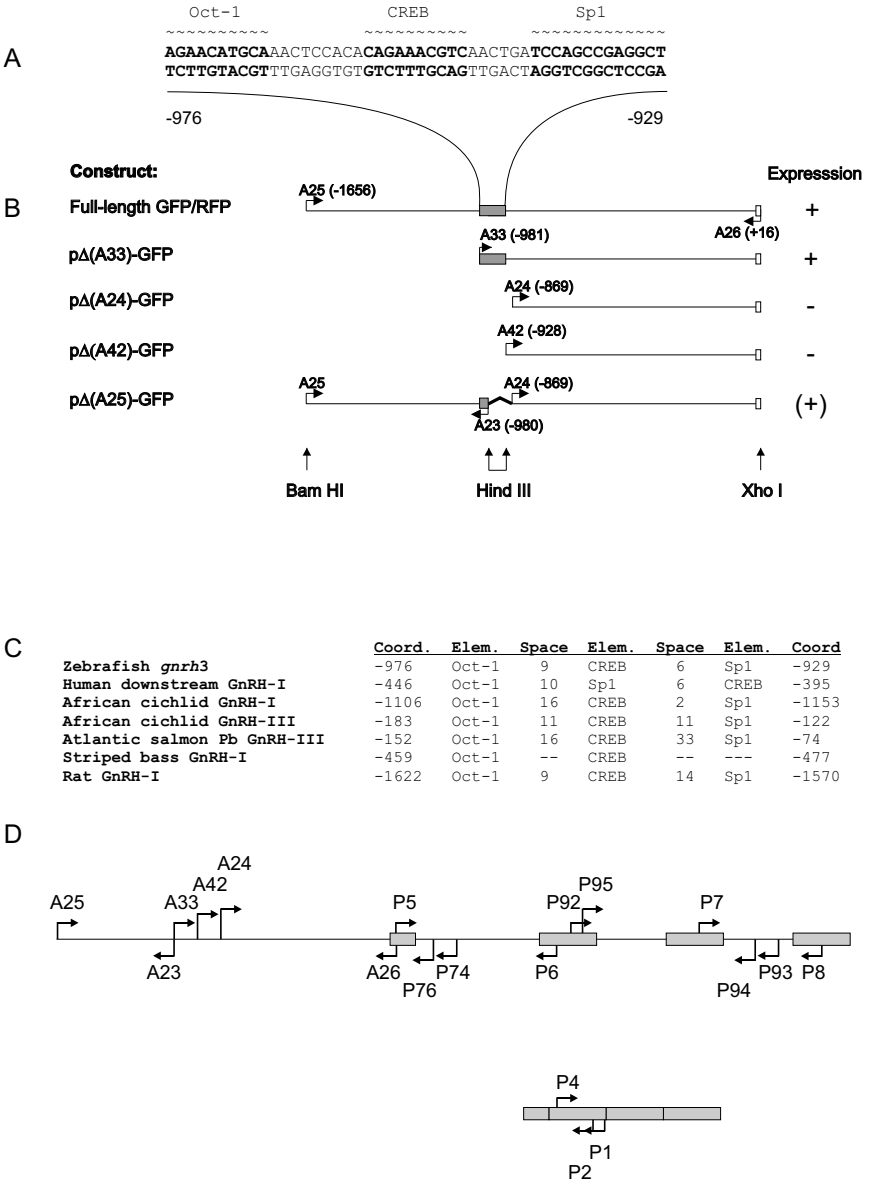


Figure 3
Overview of the PCR promoter capture method A) Sheared genomic DNA of 4–10 Kb harboring the gene of interest (thick line) is used as template in B) asymmetrical PCR reactions. The reactions are conducted with a biotinylated high Tm anti-sense primer and C) the resulting single stranded fragments are purified with streptavidin coated magnetic particles. D) A secondary PCR reaction is conducted with an oligo dT_{20} and a nested anti-sense primer.

570 with 72% similarity. Optimization of the transcription factor binding tools revealed that both TESS and Matinspector, using default parameters, correctly predicted the previously described -1501, but not the -1569 estrogen receptor (ERE) motif in the Atlantic salmon GnRH-III upstream promoter (Pa) [34]. Less stringent conditions resulted in an increased number of false positive ERE binding sites. Analysis of the Atlantic salmon Pa promoter using AliBaba 2.1, showed correct prediction of both ERE motifs with the default set up and was used for analysis of the Atlantic salmon GnRH-III Pa and downstream (Pb) promoter [29], human GnRH-I upstream [35] and downstream [36], striped bass (*Morone saxatilis*) GnRH-I [37], rat (*Rattus norwegicus*) GnRH-I [38], and African cichlid GnRH-I and GnRH-III promoters [22], using optimized and decreased stringency when necessary. Transcription factors experimentally shown to be involved in the regulation of GnRH expression, or to possess binding affinity to the promoter, include the POU factors Oct-1 and Oct-6 [39,40], ERE [7,8,34,41–43], glucocorticoid receptor (GR) [44], progesterone receptor (PR) [8,45,46], GATA-1 [47,48], cyclic AMP responsive element binding protein (CREB) [49], SF-1 [50] and Otx2 [51]. *In silico* predicted TF binding sites include Ap-1, ERE, GHF-2, GH-CSE2 and Pit-1 in Pacific salmon (*Oncorhynchus nerka*) [52], Ap-1, GR, PR, and Sp1 in African cichlid [22], Ap-1, CREB, ERE, GATA, GR, and Oct-1 in masu salmon (*Oncorhynchus masou*) [53], and members of the POU family, Ap-1, Brn-2, CREB, ERE, GR, Pit-1, and SF-1 in striped bass [37]. Of the above listed TF's, no putative binding sites could be predicted for SF-1, Brn-2, Oct-6 and Otx2 in either of the promoters. No obvious frequencies or patterns could be detected with respect to the binding sites of ERE, PR, GR, GATA-1, AP-1, Sp1, Pit-1 and Oct-1, as they were found abundantly in all promoters, with the exception of the salmon Pa and human downstream promoter, which



	Coord.	Elem.	Space	Elem.	Space	Elem.	Coord
Zebrafish *gnrh*3	-976	Oct-1	9	CREB	6	Sp1	-929
Human downstream GnRH-I	-446	Oct-1	10	Sp1	6	CREB	-395
African cichlid GnRH-I	-1106	Oct-1	16	CREB	2	Sp1	-1153
African cichlid GnRH-III	-183	Oct-1	11	CREB	11	Sp1	-122
Atlantic salmon Pb GnRH-III	-152	Oct-1	16	CREB	33	Sp1	-74
Striped bass GnRH-I	-459	Oct-1	--	CREB	--	---	-477
Rat GnRH-I	-1622	Oct-1	9	CREB	14	Sp1	-1570

Figure 4
GnRH tissue specific enhancer and promoter constructs A) The *in silico* predicted zebrafish *gnrh*3 enhancer region located between -976 and -929, featuring binding sites for Oct-1, CREB and Sp1. Coordinates are negatively numbered with the experimental TSS as +1. B) Schematic overview of all zebrafish constructs tested, with nomenclature by sense primer used. The human RFP construct is not shown. The 91 bp deletion (-960 to -869) in the pΔ(A25)-GFP is denoted by an angle in the thin line. The gray and the white boxes correspond to the enhancer and exon 1, respectively. Arrows describe PCR primers, showing name and distance from the upstream end relative to TSS. Restriction sites at the bottom correspond to the tail of the PCR primers used for amplification of alternative promoter fragments. Transient expression data are indicated with +, - or (+), the latter denoting broader cell specificity. PCR primer sequences: A23 (5'aagcttggagtttgcatgttctccct3'), A24 (5'aagcttcctttcttaaaatattgaattatgat3'), A25 (5'ggatcccttcagggatgccaggtctt3'), A26 (5'ctcgaggctgtgtttgttcaagatgagttct3'), A33 (5'aagcttcagggagaacatgcaaact3') and A42 (5'aagcttggaatcagagaccttcttgct3'). C) The putative enhancer featuring binding sites for Oct-1, CREB and Sp1 is 100 % conserved with respect to presence of Oct-1 and CREB for the promoters tested [22,29,36–38]. The distance to TSS (+1), the transcription factor binding site and number of nucleotides between binding sites (space), are shown for each promoter. The location of the predicted enhancer in rat GnRH-I is according to data published [54]. D) Schematic overview of primer binding sites and the orientation of the respective primers. The upper line denotes genomic DNA and the lower mRNA, where both are displayed in 5'-3' direction. Exons are shown as gray boxes and primers as arrows.

lacked Sp1 and Pit-1 motifs, respectively. Binding sites for CREB were predicted in all brain specific promoters tested, whereas the Atlantic salmon Pa and the gonad specific human upstream promoter lacked the motif. An additional CREB motif was also detected at -878 in the zebrafish promoter. Interestingly, single Oct-1 and Sp1 binding sites were found adjacent to the CREB motif at -957 in the zebrafish promoter (Fig. 4a). Based on this information, one reporter gene vector exhibiting the predicted enhancer [pΔ(A33)-GFP], another lacking the predicted enhancer [pΔ(A42)-GFP], and a third lacking the -878 CREB motif and upstream sequence [pΔ(A24)-GFP] were constructed and tested for capability of driving reporter gene expression. As a result of the observed expression or lack thereof (see below), the remaining promoter sequences were analyzed for presence of these three binding sites in cluster, with more weighting of Oct-1 and CREB. The African cichlid GnRH-I and Atlantic salmon Pb GnRH-III promoters described the three motifs at 75% min. matrix conservation, whereas striped bass GnRH-I lacked the Sp1 binding site. Stringency had to be decreased to 70% for detection in the rat and human downstream GnRH-I, and African cichlid GnRH-III promoters (Fig. 4c). In the GnRH-I promoter of rat, an additional Oct-1 / CREB dimeric binding site with 13 nt spacing was predicted between -1159 and -1127, using 70% min. matrix conservation. The location of the predicted motifs and the brain specific rat enhancer are relative to TSS [54].

Reporter gene expression

The full-length zebrafish *gnrh*3 promoter construct expressed GFP in 10% and 30% of microinjected embryos at 3 and 4 days post fertilization, respectively (Fig. 5). In all cases the reporter gene activity was located to cells in the areas of olfactory bulb and telencephalon, featuring short protruding axons. A number of reporter gene constructs with alternative promoter deletions were created and named according to the sense PCR primer used (Fig. 4). The pΔ(A33)-GFP construct possessing the complete *in silico* defined enhancer, was found to be capable of driving forebrain specific expression, with a similar pattern of expression as for the full-length construct. Both pΔ(A24)-GFP and pΔ(A42)-GFP had the enhancer region deleted and revealed however no reporter gene activity, whereas pΔ(A25)-GFP possessed forebrain activity in 6% of the larvae at 4 days post fertilization (p.f.). The latter construct possessed deleted CREB and Sp1 motifs. A discrepancy in cell specificity, between the full-length and pΔ(A25)-GFP construct was observed, the latter showing broader cell specificity and revealed green fluorescent protein (GFP) activity in other neural cells in the mid and hindbrain area (Fig. 5). To examine the discrepancy further, a co injection with full-length RFP and pΔ(A25)-GFP was conducted. The resulting expression pattern consisted of co-localized GFP and red fluorescent protein (RFP) activity in the forebrain of some, but not all individuals (fig. 5). Transient expression data from the human GnRH construct showed RFP activity in 2% of the larvae at day 7 p.f. and transgenic offspring (F1 generation) harboring the full-length zebrafish or human promoters, possessed GFP and RFP activity in the exact same cell population in the area of olfactory bulb and telencephalon at 5 days p.f. (Fig. 5). Laser confocal microscopy of full-length GFP transgenic F1 larvae at 5 days post fertilization, revealed similar location and morphology of the GFP expressing cells, as observed transiently (data not shown).

Discussion

The nearly finalized genome project and importance as a model in vertebrate developmental biology, renders increased value for zebrafish as a model in functional genomics. We have chosen the zebrafish *gnrh*3 promoter to compare three tools for prediction of transcription factor binding sites and investigate factors involved in tissue specificity of zebrafish *gnrh*3. The genomic organization with four exons and three introns, confirms the overall conserved structure of GnRH genes previously characterized [28,55]. In the analysis for alternative Pol II promoters and TSS in the sequence upstream of exon I, only Neural Network Promoter Prediction recognized promoter elements in the 1.6 kb sequence upstream of the gene and thus confirmed the TSS from our experimental 5' RACE data, although with a discrepancy of 7 nt. Three additional TSS were predicted, of which the high scoring putative Pol II promoter at -1156 would resemble the putative Pa promoter in Atlantic salmon located upstream of exon Ia at -1247, relative to the TSS of the proximal promoter Pb [29]. Since our data are based on mRNA extracted from brain, we have no experimental data on alternative promoter activity in the gonads, as observed with human GnRH-I [35].

Regarding prediction of regulatory elements involved in tissue specific control of the zebrafish *gnrh*3 promoter, a number of bioinformatic tools are available [56]. Briefly they recognize putative binding sites by use of IUPAC consensus (TESS filtered) (Schug J, Overton CG [http://www.cbil.upenn.edu/tess/index.html]), context (AliBaba 2.1) [57], or transcription factor binding site matrix information (Matinspector) [58]. Alternatively, Hidden Markov Model (HMM) and other algorithms for pattern recognition may be used. Currently the context-based method seems to be the most specific alternative for TF binding site analysis [57], as also confirmed by our comparative *in silico* analysis of the ERE motifs in the salmon GnRH-III Pa promoter [34]. Of importance when predicting TF binding sites is sensitivity and specificity, since too low specificity will bury correct motifs in a wealth of false positives, hindering correct interpretation of the data. Transcriptional response to cellular signals is frequently

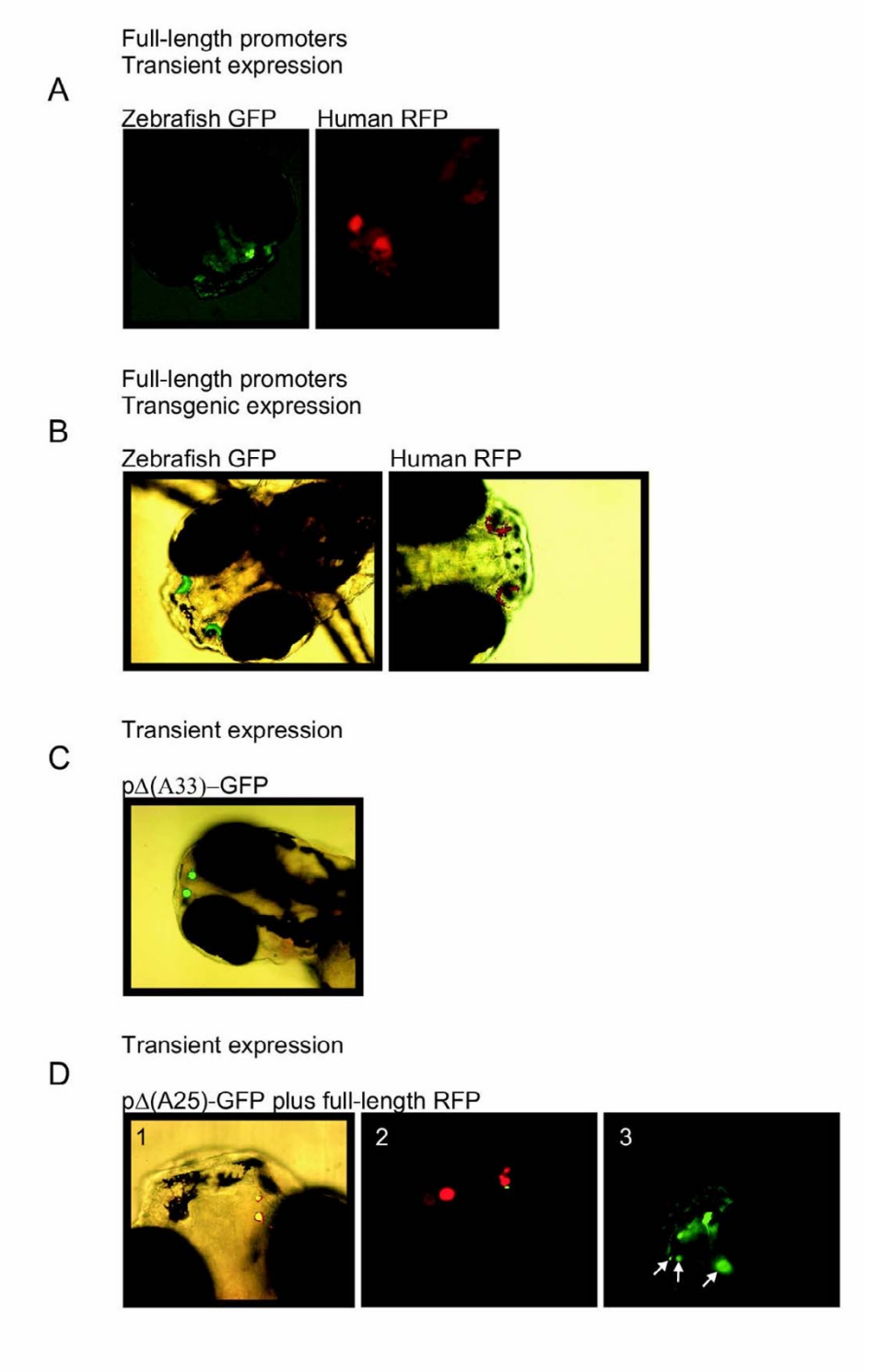


Figure 5
Reporter gene expression A) Transient GFP and RFP expression driven by the full-length zebrafish and human promoters. B) Zebrafish and human full-length -GFP and -RFP transgenics (F1) show reporter gene activity in the same cells. C) Retained cell specificity of pΔ(A33)-GFP. D) Expression patterns of pΔ(A25)-GFP. (1) Co-localized GFP and RFP activity (yellow) of pΔ(A25)-GFP and full length RFP. (2) Some of the co-injected larvae displayed broader cell specificity and lack of co-localization. (3) GFP activity in midbrain/hindbrain located neuronal cells (arrows).

mediated through co-operation between transcription factors binding to neighboring binding sites. The maximum or minimum distance between two co-operative sites/half sites are of importance, but as bending of DNA may bring two distant TF binding sites into vicinity, we have chosen to look for binding sites with up to 30 bp spacing. Two new bioinformatic tools have been developed to cope with factors interacting over longer distances, CISTER [59] and FastM [60], but as their sensitivity and specificity seems inferior to AliBaba, we chose not to use them. Certain TF's may contribute to the basal activity of a promoter being responsible for tissue specificity, whereas others modulate the level of expression. So far most studies investigating GnRH-I or GnRH-II promoter regulation have been conducted in mammalian systems [7,8,39–51]. It has been postulated that GnRH-III has replaced the GnRH-I variant as a result of mutations [23]. This also implies that the transcription factors involved should be conserved between the corresponding promoters. The reason why we could not predict binding sites for SF-1, Brn-2, Oct-6 and Otx-2, may be due to lack of SF-1 and Otx-2 matrix information in the TRANSFAC database (db) [61], used by AliBaba 2.1. A high stringency search for these factors using TESS revealed no consensus among the promoters, with respect to SF-1 and Otx-2. The matrix information for Brn-2 is sufficient in the TRANSFAC db, and the absence of this factor from the AliBaba predictions was judged reliable or false negative. Binding sites for the remaining TF's mentioned also revealed lack of consensus with the exception of CREB. This shows the complexity of GnRH regulation, as many transcription factors have been shown to be involved in tissue specificity and modulation of expression. The findings of CREB motifs in all GnRH promoters analyzed indicate that this factor may be responsible for the tissue specificity, even though we had to decrease the stringency for detection in the rat GnRH-I enhancer, human downstream GnRH-I and African cichlid GnRH-III promoters. Decreased search stringency may lead to false positives, but by including the proximate Oct-1 binding site and searching the upstream Atlantic salmon and human promoters at low stringency, without finding any adjacent CREB and Oct-1 motifs, we believe that the *in silico* predictions are reliable. Our findings are also supported by observation of altered expression when the human downstream promoter is truncated to -350 [36], as the putative enhancer is located between -445 and -397. Truncation of the rat enhancer from -1863/-1571 to -1863/-1636 has been shown to reduce expression dramatically [54] and also destroys the predicted enhancer. Modulation of GnRH expression mediated by Oct-1 has been shown in Gt1 cells, though the motif of importance is located upstream of the putative enhancer [39]. CREB has also been experimentally shown to induce human GnRH-II and to a lesser degree GnRH-I expression, in human neuronal cells [49]. Expression vectors were constructed using information from the TF binding site predictions. Expression from the vectors was confirmed by *in vivo* transient expression assays and by the formation of stable transgenic zebrafish lines harboring the full-length zebrafish or human GnRH promoters, fused to GFP and RFP respectively. The transgenic lines made it possible to study discrepancies between transgenic and transient expression data with respect to cell specificity between the zebrafish and human GnRH promoter driven reporter genes. The pΔ(A24)-GFP and pΔ(A42)-GFP were unable to express, presumably due to the lack of the Oct-1, CREB and Sp1 motifs between -976 and -929. The second CREB binding site at -878 present in pΔ(A42)-GFP is proposed to be non-functional. Expression from pΔ(A33)-GFP, was as strongly tissue specific as was observed with the full-length promoter, confirming our *in silico* predictions, since the difference between the pΔ(A33)-GFP and pΔ(A42)-GFP constructs corresponds to the predicted enhancer. From this we conclude that this region featuring the putative Oct-1, CREB and Sp1 sites, is necessary for cell specific expression of the *gnrh*3 gene in zebrafish, hence supporting our TF binding site predictions. The pΔ(A25)-GFP construct featuring deletion of the CREB and Sp1 binding sites, was tested for influence upon expression. Forebrain expression was observed, but with a broader cell specificity shown by lack of co-localization with the full-length zebrafish RFP construct, and GFP activity observed in midbrain and hindbrain neuronal cells. This denotes that the Oct-1, CREB and possibly Sp1 are necessary for full cell specificity, because the Oct-1 but not the CREB and Sp1 binding sites were retained in the deletion. Interaction between neighboring Oct-1 and CREB is to our knowledge not known in other promoters. Transgenic (F1) expression of the full-length GFP was observed in a number of cells located in the same area as that of transient expression, but the density of cells made visualizing GFP activity in the axons using fluorescence microscopy difficult. Confocal laser scanning microscopy confirmed the morphological similarities (data not shown), hence the same cell population express transiently and transgenically. Localization of the human driven RFP and zebrafish GFP to the same population of cells as also described for the Atlantic salmon GnRH-III promoter [62], show that the transcriptional machinery in zebrafish is capable of recognizing a human promoter and directing the tissue specific expression. These results confirm the hypothesis that teleost GnRH-III genes are orthologues of GnRH-I. Cells producing the releasing GnRH form originate from the olfactory placode and migrate to their final positions during brain development [63–68], though we made no attempts to investigate such events. Future experiments applying laser confocal microscopy of developing multi-transgenic zebrafish with alternative GnRH-I and GnRH-III, as well as GnRH receptor and GtH promoter constructs should be conducted to further investigate on-

togeny, tissue specificity and regulatory mechanisms of GnRH and other genes involved in the HPG axis.

Methods

Cloning of zebrafish gnrh3 RACE products

Poly A selected RNA was isolated from frozen adult zebrafish brains, using an mRNA direct Kit (Dynal, Oslo, Norway). Primer sequences for 5' and 3' RACE reactions were selected from the conserved decapeptide and 5' GAP regions between goldfish (*Carassius auratus*) [69] and Atlantic salmon GnRH-III [29], and the resulting 5' RACE sequences, respectively. All primer-binding sites are shown schematically in fig. 4. Both 5' and 3' RACE reactions were conducted with a Boehringer Manheim 5'/ 3' RACE kit (Roche Diagnostics, Mannheim, Germany), using 1 μg poly A RNA. Primer P1 (5'gcctccasytcmccwacacttctctt3') was used for synthesis of 5' RACE cDNA, and P2 (5'ggwagccraccgtaygaccagtgct3') plus an Oligo $d(T)_{18}$ anchor primer for the 5' RACE PCR. A -21M13 tailed Oligo $d(T)_{18}$ primer was applied for 3' RACE cDNA synthesis and a -21M13 anchor primer plus P4 (5'gcatggagtggaaaggaaggtt3') in the 3' RACE PCR. All RACE PCR reactions were carried out with 0.1 μM primers, 1.5 mM $MgCl_2$ and 1 unit of Amplitaq polymerase (Applied Biosystems, Foster City, USA). The PCR cycling parameters were as follows: 1) 1 cycle of 10 min denaturation at 95°C, 2) 35 cycles with 20 sec 95°C denaturation, 30 sec 55°C primer annealing and 40 sec 72°C extension and 3) 7 min extension at 72°C.

PCR amplification

Genomic zebrafish DNA was isolated as described in the zebrafish book [70], with two additional chloroform extractions. PCR amplifications of intron 1 and 3 were performed with 0.1 μM primer P5 (5' tcttgaacaaacacagca3') plus P6 (5'cacaaactaacagcaacaa3') and P7 (5'ctgtctattcctgctgatt3') plus P8 (5'ctcatatcagcttcatcat3'), respectively. The reactions were carried out with Amplitaq Gold (Applied Biosystems), 2.0 mM $MgCl_2$ and 10 ng genomic DNA: 1) 10 min at 95°C, 2) 40 cycles of 60 sec at 94°C, 60 sec at 57,5°C and 90 sec at 72°C and 3) 7 min at 72°C. Intron 2 primary PCR was carried out with 20 ng genomic DNA, 1.5 to 3.5 mM $MgCl_2$, 1.0 unit Hot Star polymerase (Qiagen, Hilden, Germany), and 0.4 μM 5'-biotinylated P92 (5'agcatggagtggaaaggaaggttgctggtccagtt3') and P93 (5'gcaaaccttcagcatccacctcattcacctgtaa3'). PCR conditions: 1) 95°C for 15 min, 2) 40 cycles of 94°C for 20 sec and 90 sec at 72°C and 3) a 10 min 72°C step. The PCR reaction products were pooled and purified with a Dynapure™ Dye Terminator Removal Kit (Dynal). A secondary PCR was conducted with P94 (5'aacacacttacaaattaggctgccaatgtttt3') and P95 (5'gctgttagtttgtgtgttggaggtcagtcttt3'), using purified primary PCR products as template: 1) 95°C for 15 min, 2) 9 cycles of 94°C for 20 sec, annealing / extension touchdown from 72°C to 68°C for 90 sec, 3) 35 cycles of 94°C for 20 sec, and 72°C for 90 sec and 4) 10 min at 72°C. The human GnRH-I promoter was amplified from genomic DNA extracted from a 1 ml blood-sample, using a Wizard genomic purification kit (Promega, Wisconsin, USA). A 1762 bp long promoter fragment [36], including 97 bp of exon I [71], was amplified using 0.1 μM of the PCR primers P122 (5'actagtctacccagagataagtgattcacctga3') and P123 (5'accggtctgtgacttttctgttttcctatcttcct3'), 200 ng DNA, 1 × Q-solution and 1.0 unit of Hot Star polymerase: 1) 15 min at 95°C, 2) 35 cycles of 94°C for 15 sec, 55°C for 15 sec, 2 min. at 72°C and 3) 10 min at 72°C.

PCR promoter capture

The zebrafish *gnrh*3 promoter was amplified by a PCR promoter capture method (Fig. 3), comprising two PCR reactions. Genomic zebrafish DNA was sheared by sonication and size fractionated in a 10 to 40% stepwise (5% steps) sucrose gradient ultra centrifugation, for 18 hrs at 28.000 rpm. Fractions of DNA were collected from the bottom of the tube, through a pierced opening (23 G needle), and analyzed on 0.8% agarose gel-electrophoresis. Asymmetrical PCR reactions were performed in 50 μl, with 1 unit Hot Star polymerase, 200 μM dNTP, 400 ng 4–10 Kb DNA, 1.5 to 3.0 mM $MgCl_2$ and 20 nM of the biotinylated primer P74 (5'cttgctgacaaaacccacagcaat3'): 1) 15 min at 95°C, 2) 40 cycles of 15 sec at 94°C and 2 min of at 72°C and 3) 10 min at 72 °C. The PCR reaction products were pooled and centrifuged in an Amicon Centricon YM-100 Mw centrifugal filter (Millipore, Bedford, USA), to remove excess of biotinylated primer. The asymmetrical PCR products were coupled to streptavidin coated Dynabeads and purified with Dynapure™ Dye Terminator Removal Kit (Dynal). Equal volume of binding buffer was gently mixed with the pooled PCR products and incubated for 5 min at room temperature. The suspension was washed twice with 150 μl TE pH 8.0, once with 150 μl dH_2O and once with 50 μl 1 X Terminal Transferase buffer. The supernatant was discarded and the purified PCR products were poly adenylated in 20 μl 1 X terminal transferase buffer with 0.25 mM fresh dATP and 20 units of terminal transferase (Promega) for 1 hr at 37°C. The polyadenylated PCR products were washed as described above, using 1 X PCR buffer in the last step. Secondary nested PCR reactions were carried out with 1.5 to 3.0 mM $MgCl_2$ and Hot Star Taq polymerase, using 0.4 μM of the primers P75 (5'gggctcgagagt$_{20}$b3') and P76 (5'tgaacatttctatcacact3') in 25 μl reactions. The PCR cycling was performed with; 1) 15 min denaturation at 95°C, 2) 40 cycles of 20 sec at 94°C,10 sec at 55°C and 2 min at 72°C, and 3) a 10 min at 72 °C. The PCR fragments were analyzed on 2% agarose gel electrophoresis, excised and purified prior to cloning and sequencing. A 1656 bp promoter fragment was PCR amplified with the primers A25 (5'ggatcccttcagggatgccaggtctt3') and A26

(5'ctcgaggctgtgtttgttcaagatgagttct3'), specific for the sequence obtained from the PCR promoter capture reactions. The PCR reaction was conducted with Hot Star polymerase, 100 ng genomic DNA and 2.0 mM $MgCl_2$. Cycling conditions: 1) 15 min at 95°C, 2) 40 cycles of 15 sec at 94°C,10 sec at 60°C and 2 min at 72°C, and 3) 10 min at 72°C.

Sequencing

All PCR products were cloned using the pGEM T-easy vectors (Promega) and sequenced either with vector or sequence specific primers. All sequencing reactions were carried out in 10 µl reactions with Big Dye Terminator chemistry (Applied Biosystems) and analyzed on an ABI 377 automated sequencer (Applied Biosystems). The zebrafish *gnrh*3 cDNA and gene have Gene Bank accession number AJ304429 and AF490354.

Bioinformatics

Contig assembly of the zebrafish sequences was conducted with Contig Express (Informax, Bethesda, USA) and the ORF was translated and analyzed using BLAST [72], with BLOSUM 62. The zebrafish *gnrh*3 promoter sequence was analyzed for presence of repeat regions and transposable elements, using Repeat Masker (Smit AFA, Green P [http://repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker]). Confirmation of Pol II promoter and recognition of the transcriptional start site was conducted with Promoter Scan II [73] and Neural Network Promoter Prediction (Reese MG, Harris NL, Eeckam FH [http://www.fruitfly.org/seq_tools/promoter.html]). *In silico* analysis for transcription factor-binding sites was initiated with a search for TF's present in brain and neuronal cells, using TESS filtered search (Schug J, Overton CG [http://www.cbil.upenn.edu/tess/index.html]). Secondly, a binding site analysis was conducted to find binding sites common to and missing from brain and gonad specific promoters respectively. To increase the reliability of these predictions, TESS filtered search, AliBaba 2.1 [57] and Matinspector professional [58] were compared and stringency optimized for correct prediction of the ERE described in the salmon Pa GnRH-III promoter [34]. In addition to the zebrafish *gnrh*3 promoter, the Atlantic salmon GnRH-III Pa and Pb [29], human GnRH-I upstream (II) [35] and downstream (I) [36], striped bass GnRH-I [37], rat GnRH-I [38], and African cichlid GnRH-I and GnRH-III [22] promoters were analyzed for TF binding sites.

Reporter gene constructs and microinjection

The zebrafish (-1656 to +16) and human (-1762 to +97) GnRH promoter fragments were cloned into pGEM T-easy vectors prior to endonuclease digestion with Bam HI/ Xho I and Spe I / Age I, respectively. The promoter fragments were ligated into promoter-less hrGFP (Stratagene, La Jolla, USA) and non-humanized pDsRed (Clontech, Palo Alto, USA) vectors, respectively. A full-length RFP version of the zebrafish construct was also made. A total of 4 zebrafish GnRH promoter deletion constructs were created, using information from the TF binding site predictions. All PCR fragments for the promoter deletion constructs were fused to an hrGFP vector and named according to the sense primer used (Fig. 4b). Briefly, the PCR reactions were run with the primers listed in figure 4b legend, Hot Star polymerase and 2 mM $MgCl_2$. The cycling conditions were as follows; 1) 95°C for 15 min, 2) 3 cycles of 94°C for 15 sec, 54°C for 15 sec and 72°C for 30 sec, 3) 36 cycles of 94°C for 15 sec, 64°C for 15 sec and 72°C for 30 sec, and 4) 5 min at 72°C. All PCR products were ligated into pGEM T-easy vectors and digested with their corresponding restriction endonucleases (Fig. 4b), before fusion with the hrGFP vector backbone. The pΔ(A25)-GFP vector was created by fusing two PCR products, creating a 91 bp deletion within the full-length promoter (Fig. 4b). Transgenic zebrafish were created by microinjection of 10^6 copies of full-length zebrafish and human constructs into embryos at the one or two cells stage [74]. Transient and transgenic reporter gene expression was monitored with respect to onset and cell specificity of expression, from 24 hrs to 10 days post fertilization, using an Olympus BX 60 microscope (Olympus, Tokyo, Japan) equipped with NIBA and WG filter sets (Olympus). Fluorescence image capture and image processing was conducted with a Colourview-12 digital camera and Analysis Auto 3.1 software (Soft Imaging System, Münster, Germany).

Authors' contributions

Author 1, Torgersen, J., was involved in design of the study, cloning of cDNA, gene and promoter, vector construction, *in silico* analysis, fluorescence microscopy and preparation of the manuscript. Author 2, Nourizadeh-Lillabadi, R., participated in cloning of gene and promoter, and vector construction. Author 3, Husebye, H., participated in cloning of cDNA and gene, and preparation of the manuscript. Author 4, Aleström, P., was involved in experimental design, coordination and drafting of the manuscript.

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