REGULATION OF HYPOCRETIN (OREXIN) EXPRESSION IN EMBRYONIC ZEBRAFISH
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Running Title: Hypocretin in Larval Zebrafish
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Hypocretins/orexins (HCRT) are neuropeptides involved in the regulation of sleep and energy balance in mammals. Conservation of gene sequence, hypothalamic localization of cell bodies, and projection patterns in adult zebrafish suggest that the architecture and function of the hypocretin system are conserved in fish. We report on the complete genomic structure of the zebrafish and Tetraodon hypocretin genes and the complete predicted hypocretin protein sequences from 5 teleosts. Using whole-mount in situ hybridization, we have traced the development of hypocretin cells in zebrafish from onset of expression at 22hpf through the first week of development. Promoter elements of similar size from zebrafish and Tetraodon were capable of driving efficient and specific expression of EGFP in developing zebrafish embryos, thus defining a minimal promoter region able to accurately mimic the native hypocretin pattern. This EGFP expression also revealed a complex pattern of projections within the hypothalamus, to the midbrain, and to the spinal cord. To further analyze the promoter, a series of deletion and substitution constructs were injected into embryos, and resulting promoter activity was monitored in the first week of development. A critical region of 250 base pairs was identified containing a core 13 base pair element essential for hypocretin expression.

The hypocretin/orexin peptides (HCRT-1 and HCRT-2) are neurotransmitters involved in the regulation of sleep and energy balance (1). HCRT-1 and HCRT-2 have significant homology and result from the cleavage of a common precursor encoded by the hcrt locus. In mammals, the hypocretin expressing cell clusters are located in the lateral hypothalamus and send widespread projections that innervate multiple brain areas and the spinal cord. The population is relatively small, and involves approximately 70,000 neurons in the human brain (2). Projections are especially dense on several aminergic and cholinergic nuclei, most notably the adrenergic locus coeruleus and histaminergic tuberomammillary neurons (3).

Loss of HCRT transmission causes the sleep disorder narcolepsy in humans, canines and rodents (4-6). The symptoms of narcolepsy in humans typically present in the second decade of life, usually in association with undetectable levels of HCRT-1 in the cerebrospinal fluid (7,8). Postmortem studies indicate a 90-95% loss of hypocretin-producing cells in human narcolepsy (2,6,9,10). The process leading to cell death is unknown, but because of a strong association with Human Leukocyte Antigen (HLA) DQB1*0602 (11,12), it is generally presumed that hypocretin cells are the target of an autoimmune process (13). This cell destruction in human narcolepsy is likely triggered by environmental factors interacting with a specific genetic background.

An understanding of the processes underlying hcrt cell specification, differentiation, and maintenance, as well as why they are susceptible to loss are key questions of both basic and clinical relevance. The zebrafish is well suited for such studies by virtue of nearly transparent and rapid external development. Another important feature is the ease and efficiency of genetic manipulation of zebrafish embryos to create transgenic marker lines or to achieve rapid...
overexpression or phenotypic knockdown of selected genes (14, 15). The zebrafish is a simple and powerful model for studying promoter function, and recently genetic manipulation has been extended to functional characterization of putative regulatory sequences (16-20). Transgenesis is far simpler than in the mouse, with the additional advantage of being able to assess transient promoter activity in the context of the whole animal for accurate study of spatial and temporal gene regulation. Hundreds of independent transgenic animals can be easily monitored, thus position-dependant integration effects do not bias the results.

The hypocretin gene is conserved in teleost genomes and is similarly expressed in lateral hypothalamic cell clusters in the adult zebrafish (21). It may thus be involved in the regulation of states of "wakefulness" and energy homeostasis as in mammals. Larval zebrafish display a diurnal rhythm of locomotor activity and have periods of nocturnal immobility that are associated with a stereotypic posture and an increased arousal threshold, key behavioral indicators of a sleep-like state (22-24); Sleep homeostatic mechanisms are also present: following rest deprivation, larvae display a compensatory rest rebound with an increased arousal threshold (22). Hypnotic compounds of the benzodiazepine and barbiturate families induce a sleep-like behavior in zebrafish, thus the role of GABA in behavioral sleep is likely to be functionally conserved (22). This may also be the case with other sleep-modulatory neurotransmitters that have been identified in the zebrafish (25, 26) including acetylcholine, histamine, 5-HT, and dopamine (27-31).

In our efforts to understand the origins and unique character of hypocretin cells, we have applied both embryologic and transgenic studies in zebrafish. We have extended the genetic characterization of the zebrafish hypocretin gene to include the first coding exon, and demonstrate a selective pattern of lateral hypothalamic expression. Hcrt transcripts were localized in a cluster of approximately 9 cells per hemibrain by the end of the first week of development. A similar genomic characterization was performed in Tetraodon nigroviridis. Transgenesis revealed a compact, phylogenetically conserved promoter driving enhanced green fluorescent protein (EGFP) expression exclusively in native HCRT producing cells. We have used this promoter to create transient and stably transgenic EGFP marker strains to characterize the process of formation of hypocretin projections. Finally, a 13 base pair functional element essential to promoter function was also identified.

EXPERIMENTAL PROCEDURES

Zebrafish Husbandry—Wild-type zebrafish were maintained at 28°C on a 14:10 Light dark cycle, under optimal maintenance conditions (32) in accordance with the animal protocol approved by Stanford University. Embryos were generated by natural spawning and were raised in egg water containing methylene blue (0.3ppm). For in situ hybridization and EGFP observation, embryos were incubated in 0.003% 1-phenyl-2-thiourea after 24 hpf to prevent pigment formation.

BAC clones—Zebrafish Hcrt clones were isolated from two BAC libraries through hybridization, and analyzed by hybridization, PCR, sequence analysis, and subcloning. Genome systems (Incyte Genomics) clones 87J11, 91K13 and 147H8 were a kind gift of Will Talbot; Chori 211 clones 3K3, 37N13, 59P20, 134F5, 135D16 were obtained through CHORI (Oakland, CA). Tetraodon nigroviridis BAC clones 50L6, 38121, 49B24, 10G8, 20D23 were identified through blast searches of Genbank the GSS database and obtained from Genoscope, Paris.

RT-PCR and RACE—The full transcripts of the zebrafish and Tetraodon nigroviridis hcr genes were determined through RT-PCR and 5’ and 3’ RACE. Total RNA was extracted from excised, pooled juvenile zebrafish brains and adult Tetraodon brain, then treated by on-column DNase I digestion (RNeasy, Qiagen). cDNA was prepared with AMV reverse transcriptase. 5’ and 3’ RACE were performed according to the GeneRacer protocol (Invitrogen) with primers 3’zhcrt-RACE-GSP2:

\[5'-GGAAGAGCGCTGGAGGAGCCGGCTA-3', \]

and 5’zhcrt-RACE-GSP2:

\[5'-TAGCAGGCGCCATGAAGACCGACGCAC-3'. \]

PCR products were cloned into pCRII TOPO®.
(Invitrogen), and sequenced. The expression and splicing of Tetraodon and zebrafish hcrt were confirmed through RT-PCR (Superscript II, Invitrogen). Primers were tet-F1: 5'-ACTTGTTCAAAAAGGAAGATCAG-3', tet-R1: 5'-CAACACGTAGAGTCGACAGGAGCG-3'.

**Bioinformatics:** Fish hcrt sequences were identified through Blast searches of databases including: Zv6 v38 May 2006 (zebrafish), TETRAODON 7 April 2003, FUGU 4.0 June 2005, and Gasterosteus aculeatus BROAD S1 (stickleback), all available at [http://www.ensembl.org/index.html](http://www.ensembl.org/index.html); Oryzias latipes (Medaka) databases golw_scaffold_last_seq, golw_scaffold Hd-rR (200506, 200406), golw_contig Hd-rR (200506, 200406) are available at [http://dolphin.lab.nig.ac.jp/medaka/src_db/search](http://dolphin.lab.nig.ac.jp/medaka/src_db/search) and the public version of Match 1.0 ([http://www.gene-regulation.com/pub/programs.html#match](http://www.gene-regulation.com/pub/programs.html#match)) using the best selection profile and minimize sum of both error rate options. Fenges, Fex and Spl (Softberry.com) were used to identify potential first exons and/or splice donor sites. Signal peptide cleavage sites were predicted with SignalP 3.0 ([http://www.cbs.dtu.dk/services/SignalP/](http://www.cbs.dtu.dk/services/SignalP/)). Putative first exons were next compared for conserved encoded amino acids, predicted intron length, and relative conservation of promoter elements (TATAA box) using ClustalW ([http://www.ebi.ac.uk/clustalw/](http://www.ebi.ac.uk/clustalw/)). Footprinter ([http://bio.cs.washington.edu/software.html](http://bio.cs.washington.edu/software.html)) (34) and MEME ([http://meme.sdsc.edu/meme/intro.html](http://meme.sdsc.edu/meme/intro.html)) were used to identify conserved motifs.

**Constructs-** Zebrafish hcrt promoter constructs 2kb-zhcrt-EGFP (-941 to +62) and 1kb-zhcrt-EGFP (-941 to +62, Genbank DQ831347) fragments were amplified by PCR of genomic DNA and ligated into the Sall-BamH1 sites of pEGFP-1. A 1043 bp Tetraodon nigroviridis hcrt promoter fragment (1-1040 of Genbank DQ831348) was amplified from BAC 10G8 DNA by PCR and ligated into the BglII site of pEGFP-1 (Clontech). Deletion and substitution mutations were created using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) as instructed by the manufacturer. Resulting clones were tested through restriction analysis and confirmed through sequencing.

**Substitution Primers:** forward primers are listed, overlapping reverse primers are reverse-complement

| Region 1: Sall | 5' - |
|---------------|------|
| GTTAGAAATACCAACAGGTCGACTGTGT | |
| GACGAGGCCCTTGGATT-3' | |
| Region 2: Sall | 5' - |
| CACTCATTTCTTGGATTTGTGGTCTGACG | |
| CTCGTTCCTCCTGTCATAAT-3' | |
| Region 3: Not I | 5' - |
| AGCTCGTCTCCTGTCATAAGCGGCGCCT | |
| TAAGAACATTGCCCGGATA-3' | |
| Region 4: Not I | 5' - |
| AAGAACATTTGCCGGGATACAGGGCGGC | |
| GTTACACGCTAATGACAA-3' | |

**Deletion primers:**

1000 to 750-Right: 5'-pATATCTCGTTTAGAGTGGTCTGATT-3'  
1000 to 750-Left: 5'-pTTTTCTGCTCTGAGCACAGTGTAG-3'  
750 to 500-Right: 5'-pTTAAATCTGACTGCTCTGACCT-3';  
750 to -500-Right Left: 5'-pGCTTGTGTTTCCAGGTTAAGTTG-3';  
500 to 250-Right: 5'-pGACAAAGATGCTAACAACCCCGAA-3';  
500 to 250-Left: 5'-pTGATGAGCCGCTGACACATTTA-3';  
251 to 99 (aka -250 to TATAAA)-Right: 5'-pGTGCGACATCTGTATAAAAACGAG;  
251 to 99-Left: 5'-pATTAGCGTGTAACCAGGATTACCT

**Region 2-Right:**

5'-pCGTACTCCCGTGCTCATAATAGTTGTTTAA-3';  
Region 2-SnabI-Left: 5'-pTAATACCAACAGAATGAGTGTGACG-3';
Transient and stable expression assay—Upon fertilization, 100-400 eggs were harvested and placed in agarose grooves for injection. Supercoiled plasmid DNA (transient experiments) or gel-purified, linearized DNA (stable transgenic lines) was diluted to 50ng/µl, and approximately 2nl were injected into the cytoplasm of one-cell stage wild-type embryos using a micromanipulator and a PLI 90 injector (Harvard Apparatus). Embryos were transferred to 10 cm dishes (<60 per dish) and maintained as above. For promoter analysis EGFP positive embryos were counted at two time points (30 hpf and 54 hpf) using a Zeiss M2Bio fluorescence microscope fitted with a 10X objective lens and an EGFP filter. Hypothalamic fluorescence of one or more cells (unilateral or bilateral) in the hypothecin cells region was scored as specific. For HCRT cell projection analysis, embryos were injected with the 1kbHCRT-EGFP construct and monitored during the first week of development.

Wholemount in situ hybridization—Whole mount RNA in situ hybridization was carried out as described previously (35,36). Digoxigenin or fluorescein labeled full-length antisense riboprobes for egfp and zebrafish hcrt were transcribed in vitro using standard reagents (Promega).

RESULTS

Genomic structure of zhcrt and hcrt genes—We performed 5′ RACE to identify the transcriptional start site of zebrafish hypocretin (zhcrt) and identified a small first exon encoding 6 amino acids and containing 62 bp of 5′ untranslated sequences (GenBank™/EBI Accession Number DQ831346). This exon was not identified in prior published work (21). The two-exon structure is similar to the structure of mammalian hcrt genes. Through sequence and Southern analysis of BAC clones from two libraries (Genome systems, Chori 211), we identified the presence of a single zhcrt locus with a polymorphic intron sized 3.6 or 6 kb associated with a 2408 bp insertion located 137bp upstream of exon 2, with homology to the helentron Hel_Dr6 repetitive element, as well as numerous SNPS and indel polymorphisms in BAC clones from the AB strain (GenBank™/EBI Accession Number DQ831349) (Figure 1A). This polymorphism was confirmed through Southern analysis of genomic zebrafish strains from various strains. The Tetraodon hcrt (thcrt) gene was identified through Blast searches, and re-sequenced on BAC DNA revealing also a 2 exon hypocretin gene. Expression and splicing of the predicted Tetraodon gene were confirmed by rTPCR and sequencing. Further studies of the zebrafish and Tetraodon BAC clones indicated conservation of synteny in this region, with the Stat3, Stat5, hert and Bec2/KCNH4 genes located in the BAC contigs as reported in mouse (37). In the most recent zebrafish and Tetraodon whole genome assemblies (Sanger Institute, Ensembl), hert corresponds to FGENESH00000056346 on chromosome 3 in zebrafish, and GSCT000132001 on chromosome 3 in Tetraodon.

We also identified two-exon hert genes in other teleosts through searches of genome databases and subsequent analysis of the regions surrounding the hits. These other teleost hert genes were identified in Fugu (NEWSINFRUG00000161995), Stickleback (GENSCAN00000033466, with 5′ sequence and exon 1 reported here located on contig 4260: 5838783-5839782), and Medaka (5′sequence and exon 1 on scaffold5097:24560-23509, and exon 2 on GOLWno5793_117.g1: 494-132). The medaka, stickleback, and both pufferfish genes all have multiple in-frame ATG codons in the predicted first exons, but these correspond to an out offrame ATG in zebrafish in alignments. As there is no data on the transcriptional or translational start sites in these animals, predicted translational start sites were selected as the last ATG based on conservation (7 encoded amino acids in mammals, 6 in zebrafish) (Figure 1B). Flanking 5′ non-coding sequences showed substantial conservation between various fish species, supporting our exon predictions (data not shown). Predicted signal peptide cleavage sites were in a localized region of the precursor protein similar to that known in mammals (Fig. 1B). In the two pufferfish, two potential sites were predicted (22/23 and 24/25) and the first site (22/23) was reported. In all fish studied, an additional stretch of amino acids is present within the mature HCRT-I peptide when compared to mammals (Fig. 1B).
Alignment of human and predicted protein sequences of HCRT in 5 teleosts revealed a strikingly high homology at the predicted C-terminal end of both hypocretin peptides and at dibasic cleavage sites. As these amino acids are indispensable for proper peptide processing and ligand binding to the receptor (38), the data add credence to the functional similarity of the hypocretin system across fish and mammals.

*Embryonic and larval expression of hypocretin*- We examined the development of hypocretin-producing cells through whole mount *in situ* hybridization ISH. Starting at ~22 hours postfertilization (hpf) *hcr* expression was observed either unilaterally or bilaterally in loose clusters in the lateral ventral diencephalon corresponding to the developing hypothalamus (Fig. 2 A-C). There was variability in both the number and positioning of cells within the bilateral clusters through all time points. Signal was never detected outside this unique hypothalamic cluster at any larval time point, indicating that in the zebrafish, *hcr* transcripts are restricted to the lateral hypothalamus. The positions of the cell clusters changed with the ongoing development of the brain, first appearing rostral with respect to the lens axis, then moving directly between the lenses (2 dpf) finally reaching a position slightly dorsal and caudal to the lens axis (6 dpf) (Fig. 2 A-O). Cell counts were determined by observation of 20 animals aged from 1-7 dpf. At day 1, up to 5 cells per cluster were observed, with an average total number of 4.6±0.5 (SEM). Average total cell numbers were 14.4±0.9 at 2dpf, 17.1±0.7 at 3dpf, 20.5±0.9 at 4 dpf, 15.7±0.7 at 5 dpf, 15.9±0.5 at 6dpf, and 18.9±0.7 at 7dpf. Overall, an average total of 17.63±0.36 cells were seen at 2-7 dpf.

A one kilobase promoter fragment is sufficient to drive cell-specific hypocretin expression- The promoter activity of putative zebrafish *5'* genomic fragments was explored through expression of EGFP. Purified DNA was injected in zebrafish embryos at one-cell stage, and EGFP fluorescence observed after 24hpf. We found that reporter constructs containing 1 and 2 kb of sequence upstream of the translational start site (1kb-*hcr* GenBank™/EBI Accession Number DQ831348, and 2kb-*hcr*, containing bases -1941 or -941 through +62, see Fig. 1) were efficient at driving EGFP expression in a pattern consistent with time of onset and position of native hcr expression (Fig. 3). In addition, a 1040 bp Tetraodon fragment (1kb-*hcr*, spanning 1 -1040 of GenBank™/EBI Accession Number DQ831348) was able to drive expression of EGFP in zebrafish embryos in a similar manner (Fig. 3). Strikingly, ectopic EGFP expression was never observed. Co-expression of *egfp* and endogenous hcr mRNAs was demonstrated by double *in situ* hybridization (Fig. 3). A stable strain of zebrafish with fluorescent hcr cells was derived using the 1kb-*hcr*-EGFP Tetraodon construct (two germline founders among 100 animals raised). We also tested the 3.2 human hypocretin promoter fragment effective in mouse2 (39) and never observed any fluorescence.

*Elaboration of hcr projections*- Development of HCRT fibers was characterized by observation of EGFP fluorescence under the control of the 1kb-*hcr* and 1kb-*hcr* promoters. Mosaic transient expression resulted in the intense expression of EGFP in a variable number of cells (up to a number similar to native expression; compare Fig. 3F and G), allowing observation of projections. Cell projections were initiated by 1dpf, during the proliferative phase, and first extended caudo-dorsally at an oblique angle (Fig 4 A,B). After 2dpf, the projections tend to attain a sharp 90-degree turn located in the center of the midbrain (Fig 4 C), with subsequent trajectory running strictly posteriorly in the mesoventral part of the midbrain, hindbrain and spinal cord (Fig 4 C,D,L,J). Viewed dorsally, the fibers maintain a strictly parallel orientation, transitioning from a deep midbrain-hindbrain position to a peripheral location within the spinal cord (Fig 4 L,J). The length of these posterior projections was found to increase with time, being restricted to the midbrain at day one (Fig 4 A,B), crossing the hindbrain at day 2 (Fig 4 C) and extending through the spinal cord at day 3 (see Fig 4 D). These caudal projections branch in the vicinity of the rhombomere 1-2 boundary (Fig 4 C,D,J). Between 3dpf and 5dpf, projections from single cells become complex. In addition to the posterior projection described above, more localized and arborized projections were observed most notably
laterally within the hypothalamus and midbrain (Fig 4 E,F,H).

Functional characterization of zebrafish hcrt promoter- We began the analysis of the hcrt promoter by studying the 1kb functional regions from Tetraodon and zebrafish to identify conserved areas using alignments and motif-search software. To help distinguish functionally conserved bases in alignments from those identical through random neutral evolution, we included the corresponding regions from Fugu, Medaka and Stickleback to allow identification of conserved bases and motifs. Conservation was notable in the 500 bp upstream of the identified TATA boxes (917 in 1kb-zhcrt, 1030 in 1kb-thcrt). We next performed a two-tiered functional characterization, first creating nested deletions then disrupting regions of conservation (Fig. 5). Constructs were injected (50 ng/µl) into single-cell embryos, and promoter activity assessed by quantifying the proportion of animals expressing fluorescence in one or more hcrt cell at 2dpf. The proportion of fluorescent embryos injected with the 1kb-zhcrt-EGFP and 2kb-zhcrt-EGFP constructs were equivalent, indicating that deletion of the 5’ 1 kb of sequence had no effect on the efficiency of EGFP expression, and was unlikely to contain regulatory elements of importance. The 1kb-zhcrt-EGFP construct was selected as the wild-type baseline promoter, and subsequent constructs normalized against this 1000 bp benchmark for comparison of promoter efficiency. A further series of deletions were characterized, each including 250 bp of the baseline promoter. The final deletion was 153 bp to maintain the activity of the TATA box and 12 highly conserved adjacent bases. Deletions within the baseline 1kb promoter decreased the efficiency of expression by 75% or more, but always produced a normal pattern of expression. Ectopic expression was never observed. Strikingly, deletion of the region from -500 to -250 resulted in a complete loss of EGFP expression in all animals.

This critical 250 bp region was analyzed to determine the extent of conservation, conserved motifs and potential transcription factor binding sites between zebrafish and Tetraodon. Four regions containing clusters of identical residues were selected for further study by site-directed mutagenesis (Fig.5). Each construct was designed to substitute 3-7 conserved bases within the conserved region. All four of the resulting mutations had significant effects on the percentage of embryos displaying EGFP fluorescence. Three of these mutations (regions 1, 3 and 4) had moderate effects, reducing the efficiency of the promoter by approximately one half compared to the native 1kb fragment. These regions had homology to core binding sites of various transcription factors in the TRANSFAC database including a zeste core binding site in region 1, a core site for nix-2.5 in region 3, and an Ap-1 consensu site in region 4. In addition to these potential sites within selected regions, potential interferon response elements were detected at position 337 in zebrafish and at positions 154 and 488 of the Tetraodon promoter. The zebrafish IRF is in a position that contains mismatches compared to Tetraodon, while the Tetraodon elements are in regions that do not appear to be conserved with zebrafish.

In contrast to the moderate effects associated with mutations in regions 1,3 and 4, altering three bases in region 2 dramatically reduced the activity of the 1 kb promoter fragment to 4% of the wild-type. We also deleted all 13 bases comprising region 2 at positions 670 through 682 of 1kb-zhcrt (TGCTAACGAAGCT). Of these 13 bases, 9 are conserved between zebrafish and Tetraodon, and 7 are conserved among zebrafish, Tetraodon and Fugu (and also 7 between zebrafish Tetraodon and medaka). The result of this deletion was a complete loss of hcrt promoter activity. Bioinformatics analysis suggests that region 2 contains a potential core binding site for the Epstein-Barr virus (EBV) Zta factor and an overlapping core site for c-MYB, but the regions of homology do not extend through the full recognition motifs. We did not note similarity between the region 2 motif and the OE1/OE2 elements of the human promoter (40).

DISCUSSION

We report the complete 2-exon structure of the hypocretin gene in several teleosts, correcting a previous report suggesting a single exon (21). The position of the intron is conserved among these species and in mammals. The
conservation of coding and noncoding sequences in the vicinity of our reported first exons adds support to our predicted gene structure in animals we studied strictly in silico (Fugu, stickleback, medaka). We also noted conserved synteny of local genes including Stat3 Stat5α, hcrt, Bec2/KCNH4 in zebrafish and Tetraodon compared to human and mouse. When compared to mammals, the predicted fish preprohypocretin protein is conserved in areas of known functional importance. Conservation of amino acids is most prominent at the C-terminus of HCRT-1 and HCRT-2 (a region homologous between both peptides and of critical importance for HCRT receptor binding), as well as at the sites required for peptide cleavage. The pattern of conservation in teleost HCRT-2 (see Fig. 1B) is in excellent agreement with previously reported functional studies (38). Substitutions of the 8 C-terminal amino acids of human HCRT-2 (A22-M28) caused significant drops in the affinity of the processed peptide for its receptor, whereas substitution in other areas, particularly in the N-terminal area, have minimal effects. The terminal 8 HCRT-2 residues have been retained in teleosts, with one conservative substitution. Interestingly, the zebrafish HCRT precursor protein is also somewhat different from other teleosts. A stretch of additional amino acids in fish compared to mammalian HCRT-1 is larger in zebrafish than in other fish (Fig. 1B). This region is not well conserved even among fishes and is therefore not predicted to have an important functional role.

We also establish a conserved position of hcrt cells in zebrafish compared with mammals, with Hcrt expression detected in a selected area of the hypothalamus in both zebrafish larvae (Fig. 2) and adults, although the location was more anterior than in mammals, in the rostral-medial hypothalamus. The onset of expression was remarkably early, at 22 hpf, at a time before differentiation and proliferation is complete, and suggests that the ligand is present before projections have been fully formed. Cell counts indicated that the hcrt cell groups form early in development and quickly reach 7-10 cells per hemisphere by day 7. During the first week of development, the cluster changes from a rostro-ventral to a more caudo-dorsal position. This apparent early single peak of hcrt cell birth is consistent with that seen in the rat (41). Hcrt cells were generated in a single peak on day E12, based on BrdU labeling. Interestingly, this peak of hcrt cell birth was between two peaks of MCH cell genesis at E11 and E13 with an overall peak of cell birth at E12. We note that in zebrafish mch has an onset of expression later than hcrt, becoming detectable at 36 hpf (data not shown). Adult zebrafish hypothalami contain approximately 40 hcrt cells indicating additional proliferation after day 10. The zebrafish thus offers an exciting and simplified vertebrate model for the study of these cells, in comparison to the roughly 70,000 hcrt cells in the adult human brain.

The exclusively hypothalamic localization reported here is in contrast to other reports in zebrafish, goldfish and amphibians. In addition to a ventral hypothalamic hcrt cluster in zebrafish, Kaslin et al. (21), reported a much larger preoptic cluster of neurons that was observed only by immunostaining with antibodies to mammalian HCRT. The nature of this cluster was acknowledged as “putatively” hypocretinergic by these authors (21). In adult brain slices, we also observed strong immunostaining in the pretectal area clustered close to the ventricle in addition to weakly stained cells in the hypothalamus (data not shown). Consistent with previously reported results, however, only the small hypothalamic cluster of cells was seen with ISH and these strongly stained cells were clearly more caudal and located in a region covered by the tectum. Cell counts at day 10 and in adults for this cluster were consistent with our ISH data (21). Other recent studies in three frogs and in goldfish also identified putatively hypocretinergic anteriorly placed cells in the preoptic/suprachiasmatic area (42-45) using various antibodies directed against mammalian HCRTs. Overall, we believe that much of the previous anatomical work published to date has been confounded by antibody cross reactivity. These results emphasize the importance of using species-specific reagents to minimize detection of cross-reacting epitopes in highly diverged proteins such as HCRT. Although there could be very low levels of expression in the preoptic area that result in detectable amounts of antigen, the relative intensity of ISH and immunostaining argue against this hypothesis. Rather, it appears that hcrt expression is highly
localized in a single nucleus in zebrafish, leading us to examine the underlying regulation.

Our work also identified remarkably selective and compact promoters for the hypocretin gene in two teleosts, Tetraodon nigroviridis and Danio rerio. Efficiency and specificity of the zebrafish promoter were entirely retained when reducing the size from 2 kb to 1kb, but beyond this, every change we introduced had variable but substantial effects on efficiency, without any loss of specificity. Most strikingly, even in the case of specific mutations, no ectopic expression was ever found. The region approximately 500 bases upstream of the reporter had the strongest effect. In this segment, we identified a 13 bp motif that, if disrupted, was able to entirely abolish promoter activity. Thus a hypocretin-specific transcriptional activator may exist that binds this motif. In a parsimonious model, such an activator may be required for hcr expression, and act in the context of multiple additional transcriptional activators distributed along the 1kb span to effect maximal transcriptional efficiency. The results of the serial deletions do not support the existence of essential repressor elements. This model is in contrast to regulation in mammals. Two regulatory elements, OE1 and OE2 were identified through human-mouse sequence comparisons and functionally tested by transgenesis (40). Deletion of OE1 induced ectopic expression in medial regions of the hypothalamus, including the arcuate nucleus, and restoration of proper localization was only obtained in the presence of OE2.

The Tetraodon promoter was also able to accurately drive expression, although at significantly lower levels than the wild-type zebrafish construct. The two promoters are highly diverged, reflecting the split of these lineages approximately 280 million years ago (MY) (46), a distance similar to that between humans and birds (300 MY). Short, conserved regulatory elements were not easily recognized in alignments or motif searches using only zebrafish and Tetraodon, and including Fugu, medaka or stickleback in alignments allowed functionally conserved signal to emerge through the diverged nonfunctional background. A combination of alignments and motif discovery tools proved to be the most useful approach to identify potential functional areas and motifs, complementing our in vivo experimental evaluations using serial promoter sequence deletions. This approach was effective at identifying the 13 base region 2 as a putative functional element within this promoter, to be addressed by functional analysis.

The human hcr promoter construct did not promote egfp expression in zebrafish. Promoter conservation spanning such phylogenetic distances has been reported, however in the majority of cases, these are from genes critical for early development. Such promoter sequences are sufficiently constrained to allow identification of conserved noncoding sequences across vertebrates (19,47,48), and are found to promote EGFP expression in patterns consistent with the associated genes when assayed in zebrafish. Functional conservation across phylogenetic groups in the absence of apparent sequence conservation has also been recently reported at the RET receptor tyrosine kinase locus (49). Similar to results reported here, pufferfish sequences that were conserved with zebrafish drove EGFP expression consistent with the endogenous RET pattern. Surprisingly, a majority of sequences conserved among non-primate mammals and without similarity to teleosts also drove expression consistent with zebrafish ret when assayed in zebrafish embryos. The interpretation is that the functional 4-20 bp elements were present but undetectable by conventional means. We were unable to find any striking homology between our most active promoter element (13 bp core of region 2) and the reported core functional 55 bp OE1 sequence. It is possible that the transcription factors binding this core 13 bp sequence could also be important for mammalian hypocretin gene regulation, however the overall results of Moriguchi et al suggest this is not the case. Rather, determination of hcr expression by various transcription factors is likely to be somewhat different between fish and mammals. Indeed the basal promoters of mammals and zebrafish are different, with a CAAT box identified in human as opposed to a TATA box in fish. Several reports suggest an active IRF-1 site in the human hcr promoter acts to downregulate transcription (50,51). There are also potential core interferon response elements in zebrafish and
Tetraodon. No effects could be attributed to the deletion of these sites in our assay, however it is possible that interferon could have modulatory effects on hcrt transcription in zebrafish.

The compact promoter is able to completely mimic the native hcrt pattern, and allowed us to characterize hypocretin cell development and projections during early development. We found all projections to be ipsilateral at these stages. Two major types of projections were noted: (1) a long posterior projection reaching the spinal cord, with accessory branching on the way (Fig. 4 A-D, H, J) and (2) more highly arborized local set of projections extending laterally in an umbrella-shaped network of terminals (Fig. 4 F and I). This pattern of projections is roughly consistent with mammalian neuroanatomy (3,52,53). In rats, the highest density of anterior projections is directed to the basal forebrain area (3,53), immediately anterior to the hypothalamus. Dense hypothalamic projections are also noted in mammals, although in contrast to zebrafish, many are also located medial to the hypocretin cell group. Posteriorly, mammalian hypocretin cells project heavily to the brain stem areas and beyond. In rats, HCRT strongly innervates the spinal cord from cervical to sacral segments (54), and has therefore been proposed to have some role in modulating sensory input, particularly nociception, and autonomic tone. Interestingly, the long posterior projection observed in zebrafish was associated with significant branching in the rostral hindbrain region, in a region equivalent to major innervation sites of the brainstem in mammals (such as the locus coeruleus) (3,53). Whether or not these additional projections will contact aminegic and cholinergic cell groups located nearby in zebrafish as reported in mammals and suggested by Kaslin (21) will require additional work. The regulation of aminegic (especially histaminergic) and cholinergic nuclei by hypocretin has been suggested to be of functional importance in the regulation of sleep (55-58); conservation in zebrafish would bolster the use of this model for the study of sleep and wake.

In conclusion, we have characterized the early stages of hcrt development in the zebrafish and have characterized a minimal promoter. Such a promoter will be an invaluable resource for the study of this system at the neurobiological and developmental level, and can be used to generate animal models for functional studies. At the molecular level, these tools may allow the identification of hypocretin-specific transcriptional activators, as well as providing a resource for identifying novel downstream factors modulated by hypocretin activity. There are clear links demonstrating a similarity between these cells in zebrafish and mammals, and an understanding of the development, differentiation and establishment of network will be valuable in translational studies for human narcolepsy. The simplified organization of the zebrafish hypocretin systems offers a unique opportunity: the study of the formation of a network of neurons of importance for the regulation of sleep and metabolism.

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FOOTNOTES

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* These authors contributed equally to this work

1 The abbreviations used are: Hypocretin Hcrt; Enhanced green fluorescent protein; ISH in situ hybridization; hpf hours postfertilization; dpf days postfertilization; million years MY

2 Human hert-EGFP promoter construct kindly provided by T. Sakurai, Japan

3 ISH on adult brains was performed by K. Eriksson, Stanford University. Data not shown.

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FIGURE LEGENDS

Fig. 1. A: Genomic structure of zhcrt. Exons 1 and 2 are separated by a polymorphic intron of 3.6 or 6 kb in zebrafish. B: Alignment of human and complete predicted protein sequences of HCRT in 5 teleosts derived from cloned DNA sequences (zebrafish, Tetraodon) and from sequences available through genome databases (Fugu, stickleback, medaka). Amino acids predicted to be encoded by exon 1 are within the boxed region. Shaded regions depict mature human HCRT-1 and HCRT-2 peptides. Predicted N-termini of mature HCRT-1 in fish are displayed in bold. All fish listed contain additional amino acids within the HCRT-1 peptide compared to human (see dashed region).

Fig. 2. Whole mount in situ hybridization of zhcrt in the first week of development using antisense zhcrt riboprobes. Hcrt is exclusively detected in bilateral clusters of the developing hypothalamus and onset of approximately 22 hpf marked by arrowheads in A,B, and C. Rotation of cells along a dorsal-caudal path with respect to the lens axis is notable through the first week. Cells proliferate early and reach approximately 8-9 cells per hemibrain cluster during the first week of development. Frontal (C,F,I,L,O) and dorsal (A,D,G,J,M) views show the localization of the cluster in the lateral hypothalamus. Vitellus was removed in order to picture stained cells in A and D. Lateral views are depicted in (B,E,H,K, and N) and frontal views in (C,F,I,L,O) illustrate gradual movement of the clusters during development along the dorso-ventral and antero-posterior axes with lens position as a reference.

Fig. 3. EGFP fluorescence in zebrafish hert cells. EGFP fluorescence under the control of 1kb-thcrt- and 1kb-zhcrt promoter fragments were visualized in live animals on the second day of development. Observed fluorescence from the stable 1kb-thcrt-EGFP transgenic strain and transient expression from 1kb-zhcrt-EGFP construct are consistent with the timing and position of native hert expression (lateral and frontal views). Expression of egfp and hert are localized in the same cell-bodies, establishing that observed fluorescence accurately tracks hert cells. Single and double in situ hybridization experiments demonstrate identical expression of egfp (INT/BCIP, red color) and endogenous hert (BM purple, blue color) mRNA within the same cell population in transgenic animals (co-localized colors appear purple).
Fig. 4. Hcrt-cell projections as monitored by EGFP fluorescence. Mosaic transient expression (ranging from one cell to the complete pattern) allowed selected intensely fluorescent cells to be monitored. Animals from the stable 1kb-thcrt-EGFP line (A,B) had the same pattern of projections as those expressing from the 1kb-zhcrt-EGFP construct (C-J). Lateral views of developing projections are pictured at 24hpf (A), 30hpf (B), 2dpf (C) and 4dpf (D). Frontal view (E) depicts cells at 5 dpf in the lateral hypothalamus. The fluorescence, pictured at 5 dpf (F) is able to completely mimic native hertz expression compared to a 5 dpf ISH reference (G). At this stage, hertz cells are loosely clustered and apparently interconnected. Single cells at 5dpf are highly arborized (H, a cell from the left hemisphere at high magnification from dorsal view, top of figure is anterior axis). The most prominent early projections are directed dorso-caudally (1-3 dpf, A-D) in parallel tracts (I, 34 hpf; J), with prominent lateral branching in the ventral area of the rhombomere 1-2 boundary (J). By 5 dpf, projections extend to the spinal cord and are highly arborized, also projecting locally within the midbrain hypothalamic area (F,H,J).

Fig. 5. Functional analysis of the hertz promoter through transient expression of EGFP. Zebrafish DNA fragments containing 1 or 2 kb of sequences upstream from the initiation codon of hertz (2kb-zhcrt-EGFP, 1kb-zhcrt-EGFP, also called Δ2000-1000) are shown in grey. Vertical lines mark (250 bp) increments of the functional 1 kb promoter that were deleted. Black ovals mark conserved regions selected for mutagenesis. Region 1: Genbank DQ831347 position 653 to 662 CTCATTCTGT, region 2: position 670 to 682 TGCTAACGAAGCT, region 3: position 694 to 713 CCATAATAGTTGTTTTAAGA, region 4: position 725 to 744 TACAGGTAATCCTGTTACA. Arrow and hatched section denote the start of transcription and 5’ untranslated region, with GFP coding sequences beginning at +63 (substituting at the native translational start site) in zebrafish constructs. The Tetraodon 1kb-thcrt-EGFP construct was less efficient than the native zebrafish promoter, although entirely specific. Promoter activity was assessed as the proportion of embryos displaying 1 or more fluorescent hertz cells at 2 dpf, and normalized to the wild-type 1 kb results. The region 2-1 and 2-2 constructs contained 3 substituted bases, and 13 deleted bases, respectively. In addition to the teleost promoters, a characterized 3.2 kb human hertz construct was also tested and was not functional in zebrafish.
Figure 1

A

Exon 1
81bp
-25
Exon 2
372bp
+63+1-941
3'UTR
18bp

Zebrafish

B

KDJLFSKNSWAVLQLLLFALLSSGAAAPFLPCGRKTRCSELYLHELHGAC--

Human

MDTAK-----------KLQVLVMMVLLAH-------------HLARDAEQVASCACRAPPSCSKYMLCRGRNSSVSARHLVHLNND

Zebrafish

KSETR----------KVFVILYLLLS--------QLACADANSECNCRPSCRSCLYVVLCRSGKPLG----------RFLTDG

Tetraodon

KSETR----------KVFVILYLLLS--------QLACADANSECNCRPSCRSCLYVVLCRSGKPLG----------RFLTDG

Fugu

KSDR----------KVFVILYLLLLS--------QLACADANSECNCRPSCRSCLYVVLCRSGKPLG----------RFLTDG

Medaka

KETSAM----------KSLALVLMMLLS--------QADCDPSVAECRCPSRSCLYALFCEQKUNF-------GARAGD

Stickleback

KETSAM----------KFLALALMLLLS--------HVACAEHELSCGCRPARCSILVILCRSGKUNF-------GELFGD

A

B

14
Figure 2

22hpf 1dpf 2dpf 3dpf 6dpf
dorsal lateral frontal

A B C D E F H G I J K L M O N

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Figure 3

1kb-zhcrt-EGFP

1kb-thcrt-EGFP

hcout

egfp

hcout + egfp

dorsal

lateral
Figure 4
Figure 5

| Construct     | EGFP+ Total | Normalized |
|---------------|-------------|------------|
| 2kb-zhctf     | 111/247     | 99%        |
| Δ 1000-500    | 10/156      | 14%        |
| Δ 1000-750    | 35/328      | 23%        |
| Δ 750-500     | 13/129      | 22%        |
| Δ 500-250     | 0/113       | 0%         |
| Δ 250-98      | 17/142      | 26%        |
| Δ 2000-1000   | 69/152      | 100%       |
| Region 1      | 18/101      | 39%        |
| Region 2-1    | 2/111       | 4%         |
| Region 2-2    | 0/225       | 0%         |
| Region 3      | 45/183      | 54%        |
| Region 4      | 35/111      | 62%        |
| 1kb-thorl     | 15/97       | 34%        |
| 3.2kb-thorl   | 0/115       | 0%         |
Regulation of hypocretin (OREXIN) expression in embryonic zebrafish
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and Emmanuel Mignot

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