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Cholecystokinin mRNA in Atlantic herring, *Clupea harengus*—molecular cloning, characterization, and distribution in the digestive tract during the early life stages

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Abstract

The mRNA of the peptide hormone cholecystokinin (CCK) was isolated from juvenile Atlantic herring, *Clupea harengus*, by RT-PCR. The open reading frame encodes a 137 amino acid-long precursor protein. The peptide sequence of herring CCK-8, DYMGWMDF, is identical to that of higher vertebrates and elasmobranchs, and contains methionine in the sixth position from the C-terminus, which has not been reported previously in teleosts. Expression analysis by in situ hybridization shows that positive endocrine-like cells were mainly located in the pyloric caeca and to a less extent in the rectum of the juvenile. A few positive cells were also found in the pyloric portion of the stomach and the intestine. CCK cells were present in all the larvae examined from the day of hatching onwards. Although the CCK cells were scattered throughout the whole midgut, no signals were detected in either the foregut or the hindgut. Since herring larvae have a straight gut, the distribution pattern of CCK cells seems to be reflected in the anatomy of the gut.

Keywords: Cholecystokinin; Cloning; Distribution; Digestive tract; Herring; In situ hybridization; Juvenile; Larvae; Ontogeny

1. Introduction

The distribution of CCK-producing cells in the digestive tract of teleosts has recently been studied by immunohistochemistry, and the data collected so far suggest that there is a relationship between the distribution pattern of CCK-immunoreactive (CCK-IR) cells and the macroscopic anatomy of the digestive tract, i.e. CCK-IR cells are concentrated in the anterior midgut in larvae with a rotated gut (Japanese flounder, *Paralichthys olivaceus* [13]; Atlantic halibut, *Hippoglossus hippoglossus* [11]; bluefin tuna, *Thunnus thynnus* [10]), while they are scattered throughout the whole midgut in larvae with a straight gut (ayu, *Plecoglossus altivelis* [9]). The first three species represent evolutionarily more advanced teleosts (Acanthopterigii) and have a rotated gut in their early life stages. However, larvae of more primitive teleosts (e.g., clupeoids, salmonids, and cyprinoids) are known to have a straight gut in their early life stages, and to extend these studies to such species, the herring, *Clupea harengus*, was selected as the subject of this study.

The herring belongs to the order Clupeiformes and is a commercially important fish in the North Atlantic. For this reason, large numbers of studies of its ecology, behavior, growth, digestive physiology, etc. have been performed [3,6,19,20,22]. Some preliminary studies of CCK in relation to protein digestion have been carried out in Atlantic herring [12,25], using a radio immunoassay developed for

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human CCK-8. We have also tested an immunohistochemical method using antiserum against Japanese flounder CCK, which has proven successful on other species. However, in Atlantic herring this resulted in presumably false signals on mucus-producing cells (Kamisaka et al., unpublished). The primary aim of this study was therefore to isolate the herring CCK gene in order to detect the CCK-producing cells by in situ hybridization.

CCK sequences have recently been reported in several teleosts, and the amino acid sequence of CCK-8 has been demonstrated to be DYLGWMDF in goldfish, *Carassius auratus* [23], and Japanese flounder, *Paralichthys olivaceus* [14,27], DY(L/N/T)GWMDF in rainbow trout, *Oncorhynchus mykiss* [7], and DY(L/V)GWMDF in spotted river puffer, *Tetraodon nigroviridis* [14]. In higher vertebrates, there is no variation such as in the teleosts, and the conserved CCK-8 sequence (DYMGWMDF) with methionine in the sixth position from the C-terminus seems to be the rule. As a step towards gaining further insight into the evolution of CCK in the highly diversified group of teleosts, the Atlantic herring is interesting as a representative of one of the more primitive fish.

The main purpose of this study was thus to establish an in situ hybridization method to study the localization of CCK-producing cells in this species, and further to study the appearance and distribution pattern of CCK-producing cells in the digestive tract during the ontogeny of Atlantic herring. The amino acid sequence of herring CCK was also compared with those of other teleosts, and the phylogeny of the CCK family was discussed.

2. Materials and methods

2.1. Molecular cloning

The whole brain and intestine of Atlantic herring (TL 13 cm, 8 months; reared at Bergen High-Technology Center, Norway) was dissected, and total RNAs were isolated using the Trizol reagent (Life Technologies, Rockville, MD). Poly $(A)^+$ mRNA was purified with Oligotex resin (Qiagen, Hilden, Germany). Double-stranded RACE-ready cDNA and adaptor-ligated RACE-ready cDNA were made using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer's instructions.

The CCK mRNA was amplified by polymerase chain reaction (PCR) using Taq polymerase and degenerate primers designed from the conserved regions of known vertebrate CCK genes [14]; forward primer-1 (FP-1; GGN ATC TGY GTR TGY GT) and reverse primer-1 (RP-1; CT SCG KCG NCC RAA RTC CAT CCA). The PCR parameters were 35 cycles of 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 2 min. These PCR products were diluted with tricine–EDTA buffer (1:50) and used as templates for nested PCR performed with FP-2 (TGY GTR TGY GTN CTN YTG GCW GC) and RP-1. PCR conditions were the same as the primary PCR. The PCR amplification yielded a major band of expected size about 350 bp. This band was gel-purified using the QiaEX II Agarose Gel Extraction kit (Qiagen), ligated into the pGEM-T Easy vector (Promega, Madison, WI), and multiple clones were screened by sequencing (ABI PRISM 377 system; Perkin-Elmer, Cupertino, CA).

Full-length sequence of the cloned CCK product was obtained by 3′- and 5′-RACE with the gene-specific primers CCK-F1 (GCC CTC CTC CCG TTC ACT AAA GC) and CCK-R1 (TCT TGC TCC TCC TCG TCC TCC AC). The first round of PCR was carried out using FP-1 and adaptor primer (AP)-1, RP-1 and AP-1, respectively, using adaptor-linked cDNA as template, and nested PCR with CCK-F1 and AP-2, CCK-R1 and AP-2, respectively. In both RACE reactions, the annealing temperature was 62 °C and a total of 35 cycles were performed. Several strong bands in each PCR reaction were ligated into Topo TA Cloning vector (Invitrogen, Carlsbad, CA) and multiple clones were screened as described previously. Finally, the gene-specific primers, CCK-F2 (TGC GCT TCT TCT CCC TCC TCT TCT) and CCK-R2 (CAA ATT GAC ATC GAA TGT ATC GCA), were used to obtain full-length cDNA sequences using the PCR condition described above.

2.2. Sequence and phylogenetic analysis

Nucleotide sequences were compared with the GenBank database using the BLAST algorithm [1]. ClustalX (1.64b) was used to align amino acid sequences, and the cleavage site was estimated by SignalP-HMM (http://www.cbs.dtu.dk/services/SignalP-2.0/; [16,17]). Phylogenetic analysis was based on these alignments and was performed with the Puzzle 4.0.2 program (http://www.tree-puzzle.de/; [26]) using the maximum-likelihood method with the substitution model of Jones et al. [8]. Phylogenetic trees were constructed with bootstrap confidence values based on 1000 replicates.

2.3. In situ hybridization

Digestive tracts were sampled from two herring juvenile individuals (TL 13.5 ± 0.5 cm). They were cut into the following eight segments as shown in Fig. 1; stomach (cardiac portion, blind sac, pyloric portion), pyloric caeca, intestine (anterior, middle, posterior), and rectum. Samples were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2) for 48 h at 4 °C, and kept in 25% sucrose PBS solution with 25% OCT (Tissue Tek Sakura Finetek Europe, Zoeterwoude, The Netherlands) overnight at 4 °C. The tissues were embedded in 100% OCT by placing it in a plastic mould on an iron block precooled in liquid nitrogen. Embedded tissues were wrapped in Parafilm and aluminum foil, and stored at −80 °C. Sections were cut at 10 μm in a cryostat (Leitz Cryostat 1720, Wetzlar, Germany), air dried, and stored at −20 °C until use.