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Short Contribution

Intestinal fatty acid binding protein gene expression reveals the cephalocaudal patterning during zebrafish gut morphogenesis

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ABSTRACT Intracellular fatty acid-binding proteins (FABPs) are small and highly conserved cytoplasmic proteins that bind long-chain fatty acids and other hydrophobic ligands. We have examined, as a model for studying intestinal epithelial cell differentiation, the cell-specific and spatio-temporal expression of intestinal fatty acid-binding protein *(i-fabp)* gene during zebrafish larval development. After molecular cloning of zebrafish I-FABP cDNA, whole-*mount in situ* hybridization analysis revealed that *i-fabp* is expressed in the intestinal tube around day 3 postfertilization. By day 4, highest level of *i-fabp* transcript is encountered in the proximal columnar epithelium. From day 5 onwards, *i-fabp* is strongly expressed in the anterior intestine and its rostral expansion, slightly expressed in the esophagus mucosa and rectum, while no mRNA could be detected in the posterior intestine. Therefore, the regional differentiation of the intestine precedes first feeding and complete yolk resorption. *I-fabp* expression in the anterior intestine of the fed larvae is correlated with an intracellular storage of lipid droplets in the enterocytes and the massive synthesis of very low-density lipoproteins particles. In conclusion, the cephalocaudal expression pattern of *i-fabp* demarcates early during zebrafish gut morphogenesis the anterior fat absorbing to posterior cells of the intestine. This gene could be used as a marker for screening for mutations that affect the events of intestinal epithelial differentiation, cephalocaudal patterning, and asymmetric gut looping morphogenesis.

KEY WORDS: *fatty acid binding protein, intestine, zebrafish, lipid absorption, lipoprotein, gut morphogenesis*

The fatty acid-binding proteins (FABPs) are a family of proteins that are principally located in the cytosol and are characterized by the ability to bind to hydrophobic ligands, such as fatty acids (Banaszak *et al.*, 1994; Veerkamp and Maatman, 1995; Glatz and van der Vusse, 1996). Together with two other families of ligand-binding proteins, the lipocalins and the avidins, the FABPs form part of an overall structural superfamily: the calycins (Flower, 1996). Intestinal FABP (I-FABP) is thought to play a role in the intracellular transport of long-chain fatty acids and is presumably part of the gut absorptive process (Glatz and van der Vusse, 1996). The human holo protein is composed of two α-helices and eleven β-strands, organized in two β-sheets, thereby forming a clam shell-like structure with a binding pocket for hydrophobic ligands (Zhang *et al.*, 1997). In mammals and *Xenopus*, *i-fabp* is specifically and abundantly expressed in the enterocytes (Cohn *et al.*, 1992; Green *et al.*, 1992; Shi and Hayes, 1994). Here we report the isolation of I-FABP cDNA from zebrafish and examine the expression pattern of this gene during zebrafish gut morphogenesis.

Sequence information of zebrafish I-FABP cDNA (Genbank/EMBL accession number AJ132590) was obtained from nucleotides (nt) -12 to +591, excluding the poly(A) tail, and an open reading frame of 396 nt was shown coding for 132 amino acids (from nucleotides +1 to +396). A polyadenylation consensus signal (AATAAA) was found 16 nt upstream from the poly(A) tail. An alignment of the deduced amino acid zebrafish I-FABP se-

Abbreviations used in this paper:

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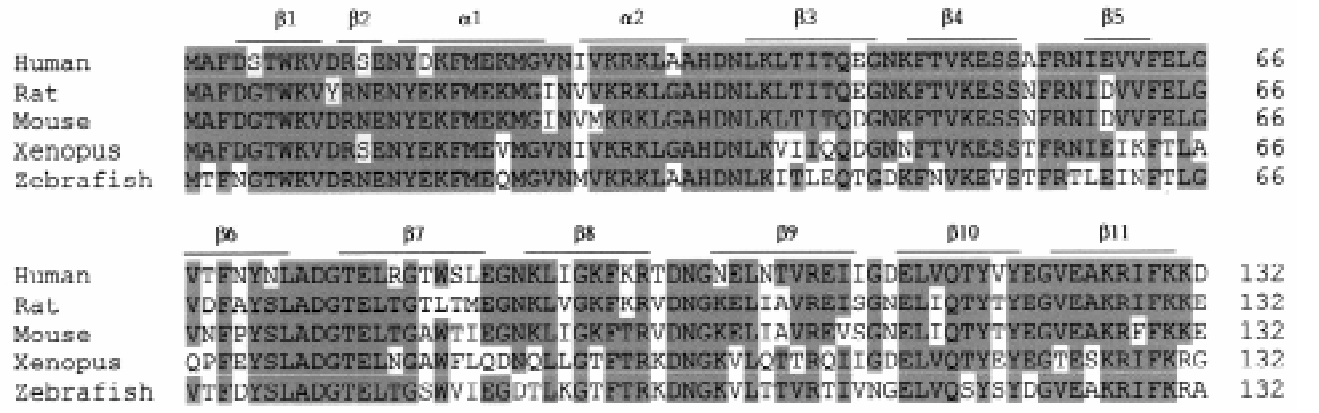

Fig. 1. Comparison of the deduced amino-acid sequence of zebrafish I-FABP with those of *Xenopus* *(Shi and Hayes, 1994)***, mouse** *(Green et al., 1992)***, rat** *(Alpers et al., 1984)***, and human** *(Sweetser et al., 1987)***.** *Conserved residues are shaded in gray. The positions of two alpha helices designed* α_1 *and* α_2*, and of 11* β*-strands, designed* β_1 *to* β_{11} *(Zhang et al., 1997) of the holo form of human I-FABP (Protein Data Bank entry: 3IFB), are underlined above the human sequence.*

quence with the corresponding mammals and *Xenopus* sequences is shown in Figure 1. Zebrafish I-FABP is identical in size with the homologs found in other vertebrates and exhibits 66.6% and 67.4% identities to human and *Xenopus* sequences, respectively. Phylogenetic analysis demonstrated that zebrafish, *Xenopus* and mammals I-FABPs, fell into one group and were highly significantly separated from the other FABP type sequences currently available in animals (data not shown).

Expression of *i-fabp* was detected by whole-mount *in situ* hybridization during zebrafish gut morphogenesis (Fig. 2). At the end of the hatching period, i.e. 3 days postfertilization (dpf), *i-fabp* is expressed in the intestinal tube, that has begun to differentiate as a columnar epithelium ventrally to the first pronephric tubules and dorsally to the yolk sac (Fig. 2A,B). By day 4, *i-fabp* is strongly expressed in the midgut while a low level of transcript is observed in the hindgut (Fig. 2C). Transverse sections demonstrate that the difference in intensity of staining reflect the level of *i-fabp* transcripts in epithelial cells and not the number of cells along the antero-posterior axis of the digestive tract (Fig. 2B, data not shown). By days 3 to 5, the anterior intestine undergoes a transition from a straight to a coiled tube. Gut looping on the left side of the larva is clearly detectable by staining with *i-fabp* transcript (Fig. 2D,E). This transcript is abundantly and homogeneously distributed in the intestinal mucosal epithelium of the anterior intestine (Fig. 2G). From day 5 onwards, *i-fabp* is strongly

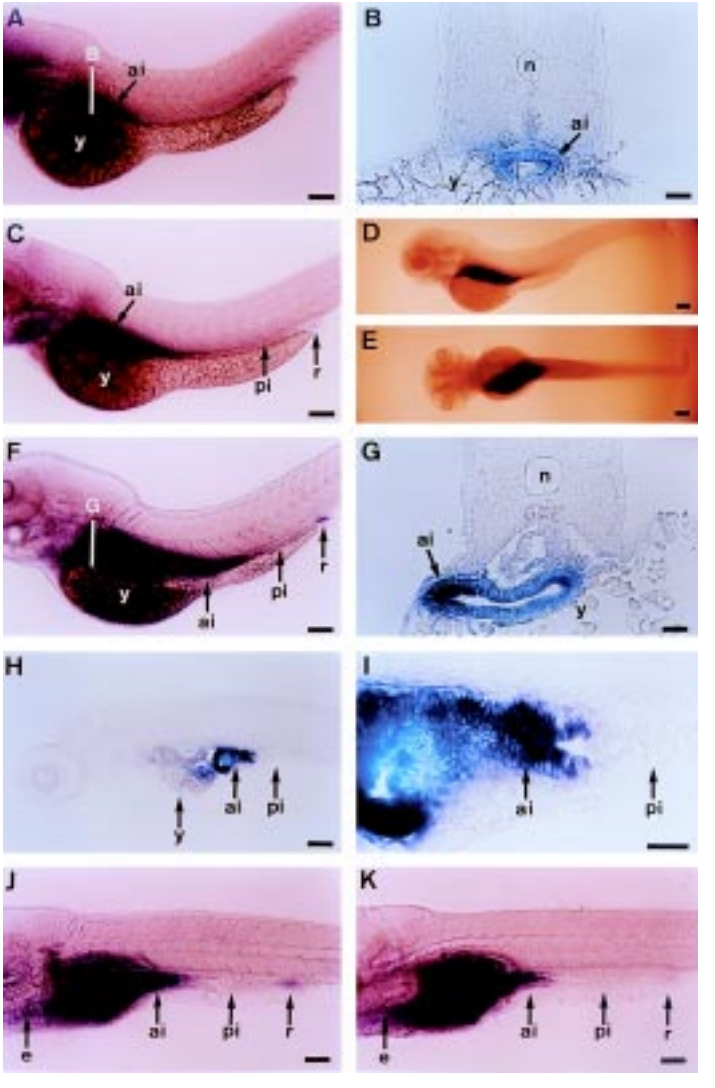

Fig. 2. Whole-mount *in situ* hybridization analysis of *i-fabp* expression during zebrafish larval development. (A) *3-day larva.* **(B)** *Transverse section of the anterior intestine (ai) region indicated in panel (A).* **(C,D)** *Four-day larva.* **(E)** *Dorsal view of the larva shown in (D).* **(F)** *Five-day larva.* **(G)** *Transverse section of the anterior intestine region indicated in panel (F). A strong i-fabp expression was observed in the anterior region of the intestinal tube, whereas only very low levels of transcripts were found in the posterior intestine (pi) region. The hybridization staining is colored dark brown to blue.* **(H)** *Parasagittal section of a 7-day larva.* **(I)** *Higher magnification view of the anterior to posterior intestine transition in (H). The expression of i-fabp to the intestinal epithelial layer demarcated the boundary between the anterior and posterior intestine.* **(J)** *Ten-day larva.* **(K)** *Fifteen-day larva. The larva is photographed with transmitted illumination in (A,C,F,J,K). With this method, a significant level of transcripts was visualized from 4 to 10 days in the rectum (r) region (C,F,J, and data not shown). The staining observed in the yolk (y) in (A,C,F) is a background color optic artifact. Transverse sections (B,G, and data not shown) and incident illumination of the early larva (D,E) demonstrate the absence of i-fabp transcripts in the yolk syncytial layer during larval development. e, esophagus; n, notochord. (A,C-F,H,J,K) Anterior is to the left and dorsal is up. Bar, 100* μ*m. (B,G,I), bar, 25* μ*m.*

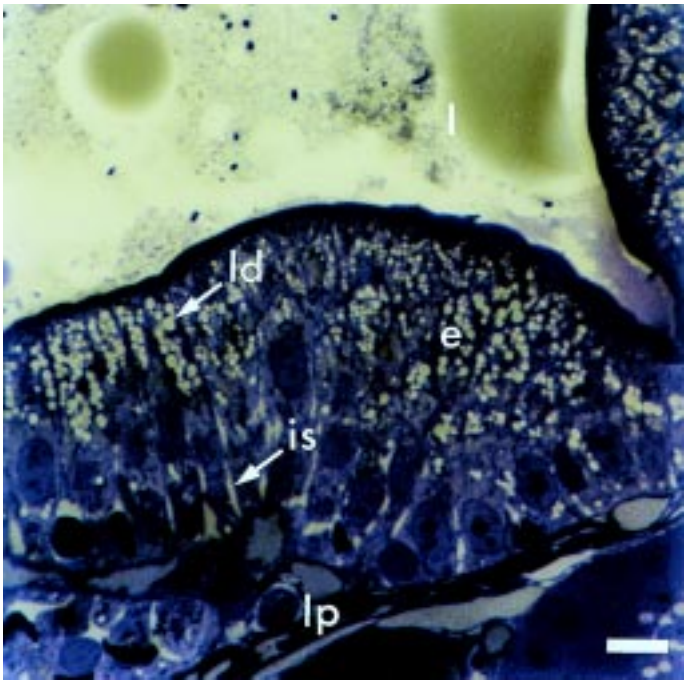

Fig. 3. Intestinal mucosa of the anterior intestine of a larva on day 15 showing enterocytes containing large lipid droplets. *Transverse semithin section stained with toluidine blue of the anterior intestine 3 h after feeding. Extremely elongated cells formed intestinal mucosal folds. The enterocytes (e), with a very high level of i-fabp transcripts (Fig. 2), were filled with numerous large supranuclear inclusions measuring up to 2 μm and described as lipid droplets (ld). is, interenterocyte space; l, lumen; lp, lamina propria. Bar, 8 μm.*

expressed in the anterior intestine and its rostral expansion, slightly expressed in the esophagus mucosa and rectum, while no mRNA could be detected in the posterior intestine (Fig. 2F,H,J,K). The digestive tract of zebrafish is then longitudinally differentiated into mouth and oral cavity, pharynx, esophagus, intestine, rectum, and anus. As in all *Cyprinidae*, the zebrafish is stomachless. The regional difference of *i-fabp* expression sharply demarcates the anterior to posterior parts of the intestine (Fig. 2I) despite the rapid growth of the intestinal epithelium during larval development (Fig. 2D,F,H,J,K). This is in contrast with mammals where *i-fabp* mRNA and protein contents gradually decrease along the antero-posterior axis of the gut epithelium (Sacchettini *et al.*, 1990; Cohn *et al.*, 1992; Green *et al.*, 1992; Simon *et al.*, 1995). Regional differences in *i-fabp* expression and protein immunoreactivity were also observed during development of the gut in *Xenopus (*Ishizuya-Oka *et al.*, 1994, 1997; Chalmers and Slack, 1998). High levels of the transcription factor *pdx-1* expression were detected in the pyloric caeca of zebrafish reflecting a differential expression of this gene along the antero-posterior axis of the adult digestive tract (Milewski *et al.*, 1998).

In fish (Sire *et al.*, 1981; Honkanen *et al.*, 1985) as in mammals (Borgstrom *et al.*, 1957; Jersil and Clayton, 1971) the proximal one-third of the intestine is the major site of fat absorption. In *Cyprinidae*, the columnar epithelial cells of the rostral part of the gut, including the intestinal bulb, synthesize and transfer lipoproteins during the lipid absorption process (Iwai, 1969; Gautier and Landis, 1972; Noaillac-Depeyre and Gas, 1974, 1976; Rombout *et al.*, 1984). The second intestinal segment, or posterior intestine, is responsible for the macromolecular absorption of proteins (Stroband *et al.*, 1979; Rombout *et al.*, 1985; Sire and Vernier, 1992) and the most caudal part of the gut, or rectum shows indications of a function in osmoregulation (Noaillac-Depeyre and Gas, 1973, 1976). The expression pattern of *i-fabp* reveals that the regional differentiation of the zebrafish intestine is established early during larval development, before first feeding (Fig. 2C-F) and complete yolk resorption (Fig. 2H,I). *I-fabp* expression in the anterior intestine of the fed larvae is correlated with an intracellular storage of lipid droplets in the enterocytes (Fig. 3) and the massive synthesis of very low-density lipoproteins particles (Fig. 4). From the onset of the exotrophic period of fish larvae, these ultrastructural features of lipid absorption characterize the epithelial cells of the anterior intestine (for a review see Diaz *et al.*, 1997). In conclusion, the cephalocaudal expression pattern of *i-fabp* demarcates early during zebrafish gut morphogenesis the anterior fat absorbing to posterior cells of the intestine. This gene could be used as a marker for screening for mutations (Pack *et al.*, 1996) that affect the early events of intestinal epithelial differentiation, cephalocaudal patterning, and asymmetric gut looping morphogenesis.

Experimental Procedures

Embryos and larvae were raised at 28.5°C. From the age of 5 dpf, larvae were fed with chicken dried egg-yolk powder. A cDNA clone encoding zebrafish I-FABP was isolated by RT-PCR from total RNA prepared with adult anterior intestine. PCR was performed using a set of sense (oligo ZFA1, 5'-CTG TCA TCA TCA TGA CCT TCA ACG G-3') and antisense (oligo ZFA3, 5'-CCG CAC ACT GGA AAT TAA CTTT AC-3') primers designed on a partial 5'-end I-FABP cDNA sequence available in dbEST database (accession AA566788, Gong *et al.*, 1997). The 203bp RT-PCR product obtained was subcloned in pGEM-T easy (clone ZF-FB) and sequenced. Full coding sequence of zebrafish I-FABP cDNA was

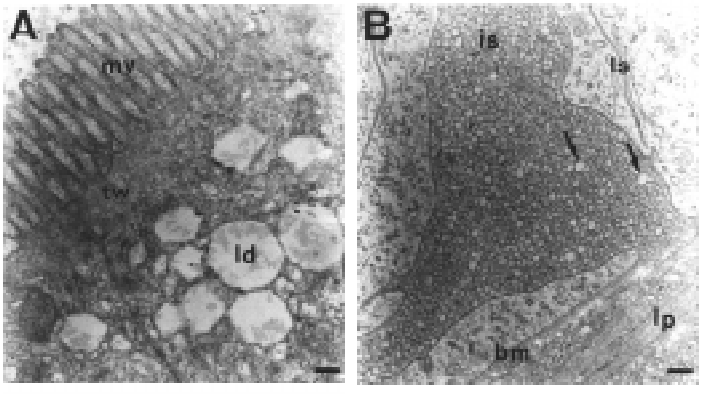

Fig. 4. Ultrastructure of enterocytes of the anterior intestine in 15 d-old zebrafish larva. (A) *Three hours after feeding, numerous large supranuclear lipid droplets (ld) were visible in the apical cytoplasmic region of enterocytes.* **(B)** *A very high amount of lipoproteins with diameters ranged from 40 to 90 nm were secreted in the interenterocyte space (is) of the infranuclear zone of the enterocytes. These very low density lipoproteins (VLDL)-sized particles (arrows) were located in cavities of the endoplasmic reticulum and the Golgi apparatus (data not shown), released into interenterocytes spaces, crossed the basal membrane (bm) and reached the sub-epithelial connective tissue and vessels of the lamina propria (lp). mv, microvilli; ls, lamellar structure; tw, terminal web. Bars, 250 nm.*

obtained using a universal primer for reverse transcription (oligo 3' PT-RACE, 5'-GGC CAC GCG TCG ACT AGT ACT TTT TTT TTT TTT TT-3'). The cDNA was amplified by PCR with sense oligo ZFA1 and antisense oligo 3' RACE (5'- GGC CAC GCG TCG ACT AGT AC-3').

Whole-mount *in situ* hybridization and histological sections were performed as previously described (Babin *et al.*, 1997; Monnot *et al.*, 1999). The fully detailed protocol is accessible on the web at the *zfin* server: http://www-igbmc.u-strasbg.fr/zf_info/zfbook/chapt9/9.82.html. Both antisense- and sense-digoxygenin-labeled RNA probes were generated using clone ZF-FB. As a control, sense probe was synthesized and did not give any staining. Larvae were raised in water containing 0.2 mM 1-phenyl-2-thio-urea (PTU) to prevent pigment formation. Duplicate experiments demonstrated no modification of *i-fabp* expression pattern after PTU treatment (data not shown). For light and transmission electron microscopy, the larvae were sampled each hour from 1 to 5 h after feeding, and were fixed at 4°C for 1 h in 2% osmium tetroxide buffered by sodium cacodylate (0.15 M, pH 7.3). Samples were then treated as previously described (Poupard *et al.*, 2000).

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