

In vivo studies of liver-type fatty acid binding protein (L-FABP) gene expression in liver of transgenic zebrafish (*Danio rerio*)

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Received 30 October 2002; revised 27 January 2003; accepted 27 January 2003

First published online 21 February 2003

Edited by Ned Mantei

Abstract Mammalian liver fatty acid binding protein (L-FABP) is a small cytosolic protein in various tissues including liver, small intestine and kidney and is thought to play a crucial role in intracellular fatty acid trafficking and metabolism. To better understand its tissue-specific regulation during zebrafish hepatogenesis, we isolated 5'-flanking sequences of the zebrafish L-FABP gene and used a green fluorescent protein (GFP) transgenic strategy to generate liver-specific transgenic zebrafish. The 2.8-kb 5'-flanking sequence of zebrafish L-FABP gene was sufficient to direct GFP expression in liver primordia, first observed in 2 dpf embryos and then continuously to the adult stage. This pattern of transgenic expression is consistent with the expression pattern of the endogenous gene. F2 inheritance rates of 42–51% in all the seven transgenic lines were consistent with the ratio of Mendelian segregation. Further, *hhex* and *zXbp-1* morphants displayed a visible liver defect, which suggests that it is possible to establish an in vivo system for screening genes required for liver development.

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Key words: Hepatogenesis; Liver primordia; Morpholino; Morphant; Green fluorescent protein

1. Introduction

The liver fatty acid binding protein (L-FABP) is a 14-kDa cytoplasmic protein that binds long-chain fatty acids (LCFAs) with high affinity [1]. The putative functions assigned to L-FABP include the desorption of LCFAs from the plasma membrane to the cytoplasm, the promotion of intracellular fatty acid (FA) diffusion [2], the targeting of FAs to different metabolic pathways, and protection against the cytotoxic effects of free FA [3–6]. Three FABP types have been found in zebrafish organs/tissues: intestinal-type FABP (I-FABP), brain-type FABP (B-FABP), and liver-type FABP (L-FABP). The zebrafish FABPs were originally named according to their site of initial isolation. The zebrafish I-FABP is uniformly expressed throughout the intestine. The zebrafish B-FABP mRNA is expressed in the periventricular gray zone of the optic tectum of the adult zebrafish brain. The L-FABP is expressed exclusively in the liver of the adult zebrafish [7].

To understand the regulatory networks responsible for L-FABP gene expression in liver, one solution would be to create a transgenic zebrafish line in which green fluorescent protein (GFP) is expressed in liver. Following this strategy, we isolated a liver-specific promoter region from zebrafish L-FABP gene and generated germline transgenic zebrafish expressing the GFP reporter gene under the control of this promoter region. Here we present a detailed characterization of transgene expression in developing zebrafish liver. This analysis showed that the 5'-flanking sequences of the L-FABP gene harbor all the necessary elements for specifically directing GFP gene expression to the developing liver.

The study of mouse and rat L-FABP genes has previously been used as an excellent model for understanding the mechanisms that determine distinct regional expression along the gut tube, as well as within the liver [8]. Mammalian liver development is a cumulative effect of dynamic events [9–14]. In contrast to mammals, zebrafish organogenesis takes only a few days to produce functional organs [15–17]. Zebrafish embryos are external and optically clear, which allows visual analysis of the development of internal structures and cells in living animals [15]. Thus, our zebrafish transgenic lines offer several advantages for the study of liver development.

In addition, to demonstrate an in vivo system allowing rapid screening for genes interrupting liver development, we used morpholinos (*hhex*-MO and *zXbp-1*-MO) targeting zebrafish *Hex* (*hhex*) and *Xbp-1* (*zXbp-1*) mRNA to produce zebrafish morphants with liver phenotypes. The adult transgenic fish may provide a convenient source of GFP-labeled liver cells for in vitro functional analysis, and the large number of transgenic progeny (embryos) may also be used as an expression-based mutagenesis screen where disruption of GFP expression patterns can be observed in live embryos. Our characterization of zebrafish L-FABP genes provides an entry point for investigating these aspects.

2. Materials and methods

2.1. Fish maintenance

Adult zebrafish were obtained from the local aquarium store and maintained in our own fish facility with a controlled light cycle of 14 h light/10 h dark at 28°C. They spawned soon after the onset of the light period, and the fertilized eggs were collected at the one-cell stage.

2.2. Inverse polymerase chain reaction (IPCR)

For IPCR amplification, 10 µg of zebrafish genomic DNA was digested with *NcoI* for 16 h. The digested DNA was phenol/chloroform-extracted, ethanol-precipitated, and then resuspended in 100 µl ligation buffer (50 mM Tris-HCl pH 7.4, 10 mM MgCl₂, 10 mM

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Abbreviations: GFP, green fluorescent protein; L-FABP, liver fatty acid binding protein

dithiothreitol, 1 mM adenosine triphosphate (ATP)) to reach a final concentration of 50–100 ng/μl. The reaction was initiated by addition of T4 DNA ligase (Promega) to 0.1 units/μl and allowed to proceed for 24 h at 16°C. The circularized DNA was then ethanol-precipitated, dried, and resuspended in 50 μl distilled water. The IPCR reactions were made up with 1 μl of recircularized genomic DNA in a final volume of 50 μl containing 1× *Adv* PCR buffer (Clontech), 0.2 mM of each dNTP, 0.25 μM of each primer and 0.5 units of *Adv* DNA polymerase (Clontech). The IPCR primers (LF-1, 5'-CAA AGA TGT GAA GCC AGT GAC AGA-3'; LF-2, 5'-TTT AAT GAC CTC TTC TGG CAG AGA-3') complementary to a 450-bp zebrafish expressed sequence tag (EST) (GenBank accession number AI545956) were designed in such a way so that their extension results in the synthesized strands polymerized in opposite directions to each other in the initial cycle. As a first step, the samples were denatured at 95°C for 2 min. This was followed by 35 cycles of 0.5 min denaturation at 95°C, 0.5 min primer annealing at 60°C and 3 min extension at 68°C, with a final extension at 68°C for 4 min. The 2.8-kb IPCR product generated from zebrafish genomic DNA was ligated into the pEGFP-C1 vector (Clontech). The resulting plasmid DNA was named pLF2.8-EGFP. The proximal promoter regions were then sequenced for verification based on the 5'-sequences from the L-FABP cDNA sequences.

2.3. Reverse transcriptase (RT)-PCR

For RT-PCR, one-step RT-PCR (Life Technologies) was performed, using total RNA from various developmental stages. β-Actin was used as a control and was amplified in a same PCR reaction tube for detecting L-FABP or GFP transcripts. The primers used were: L-FABP: 5'-GCTCTA GAA TGA AGA GAT ACC AGT GTC TGT TC-3' (forward), 5'-CCG CTC GAG TTT GTC GTG ACC CCG GAT GTG GCT-3' (reverse); β-actin: 5'-GTC CCT GTA CGC CTC TGG TCG-3' (forward), 5'-GCC GGA CTC ATC GTA CTC CTG-3' (reverse). The RT-PCR program was one cycle of 50°C for 30 min and 94°C for 2 min, followed by PCR amplification with 35 cycles of 94°C for 0.5 min, 57°C for 0.5 min, 72°C for 1 min and a final extension of one cycle at 72°C for 7 min. The RT-PCR products were subjected to 3% agarose gel electrophoresis. All PCRs were carried out using a Perkin-Elmer/Cetus Thermocycler 9600.

2.4. Microinjection of zebrafish embryos and production of transgenic zebrafish lines

To construct a permanent transgenic line, the vector backbone of pLF2.8-EGFP was removed by digesting with *Sfi*I and *Not*I. Digested DNA was adjusted to 500 ng/μl in 5 mM Tris, 0.5 mM ethylenediamine tetraacetic acid (EDTA), 100 mM KCl and 0.1% phenol red. For transient expression, an intact circular form of plasmid DNA constructs was adjusted to 100 ng/μl. Approximately 200 pl of DNA solution was injected into the blastomere of early one-cell stage embryos with a glass micropipette. At 36 h postinjection, fish were examined using fluorescence microscopy, and GFP-expressing fish were saved. Germline integrated transgenic zebrafish were selected from these GFP-positive fish by raising them to sexual maturity and breeding them with wild-type fish. Progeny from these fish (at least 100 progeny) were screened for GFP expression and GFP-positive fish were saved for further analysis and breeding.

2.5. Morpholino injections

Morpholino antisense oligonucleotides targeted to hhex (zebrafish Hex) (GenBank accession number AF131070) and zXbp-1 (zebrafish Xbp-1) (GenBank accession number AF420255) gene were obtained from Gene Tools (Corvallis, OR, USA). hhex MO sequence: 5'-GCG CGT GCG GGT GCT GGA ATT GCA T-3'; zXbp-1 MO sequence:

5'-CGG TCC CTG CTG TAA CTA CGA CCA T-3'. Control morpholinos of hhex and zXbp-1 were designed to include four base mutations compared to the original MO sequences.

2.6. Whole-mount *in situ* hybridization

The antisense digoxigenin-labeled RNA probe for the 5'-untranslated region (UTR) of zebrafish L-FABP was produced using a DIG-RNA labeling kit (Roche), following the manufacturer's instructions. *In situ* hybridizations were carried out on whole-mount embryos as previously described [18,19].

2.7. Tissue sections

LF2.8-EGFP transgenic fish were perfused with 4% paraformaldehyde, washed with phosphate-buffered saline (PBS), cryoprotected in 30% sucrose, frozen in ornithine carbamoyltransferase (OCT) (Miles Inc.) and sectioned at 15 μm on a cryostat.

2.8. Optics

Whole-mount *in situ* hybridization patterns were observed with a Zeiss Axioscope microscope. For analyzing GFP fluorescent patterns, embryos and larvae were anesthetized with 0.05% 2-phenoxyethanol (Sigma) and GFP expression was examined under a fluorescein isothiocyanate (FITC) filter on the ECLIPSE E600 microscope (Nikon) equipped with the DXM 1200 CCD camera (Nikon). For fluorescence imaging by confocal laser scanning microscopy (CLSM), we used a Leica TCSNT system fitted to a Leica microscope with a 20× objective (Nikon). Optical sections were scanned at regular increments of 0.5–1 μm. Three-dimensional reconstructions and rotations were computed using TCSNT version 1.6.587 software (Leica).

3. Results

3.1. IPCR cloning and transient transgenic analysis of the L-FABP gene promoter

In order to isolate a zebrafish liver-specific promoter region, we used the IPCR technique described in Section 2 and isolated a 2.8-kb 5'-flanking region of the L-FABP gene [7]. The pLF2.8-EGFP expression vector produced by coupling 2783 bp of 5'-flanking region of the L-FABP gene and a partial 5'-proximal coding region to an eGFP reporter gene, was examined for its promoter activity after removal of bacterial vector sequences. In a transient transgenic analysis, although the number of fluorescent cells and intensity of fluorescence varied a little among the transient transgenic fish, GFP expression was highly specific and seen almost exclusively in the liver primordia of the embryos injected with the transgene sequence (Table 1). In three independent experiments, 50–60% of embryos were observed to have green fluorescent cells in the liver primordia of 3 dpf larvae.

3.2. Generation of LF2.8-EGFP transgenic zebrafish

To confirm the tissue specificity of the L-FABP promoter and to generate stable GFP expression in the zebrafish liver, the LF2.8-EGFP construct was used to produce germline-transmitting transgenic zebrafish lines. Transgenic fish were produced by microinjection of the LF2.8-EGFP construct (after removal of bacterial vector sequences) into one-cell

Table 1
Efficiency of transient transgenic GFP expression in the LF2.8EGFP-injected transgenic zebrafish larvae at 3 dpf

Experiment	number of embryos injected (one cell stage)	number of surviving embryos (20–30 h)	Green fluorescence patterns in liver (%)	Green fluorescence patterns in other regions (%)
1	225	212	135 (60%)	4 (1.9%)
2	217	198	111 (51%)	1 (0.5%)
3	209	202	119 (57%)	0 (0%)

DNA concentration of each experiment is 100 ug/ml. 200 nl of the DNA solution was injected for experiment 1, 100 nl for experiment 2, and 50 nl for experiment 3.

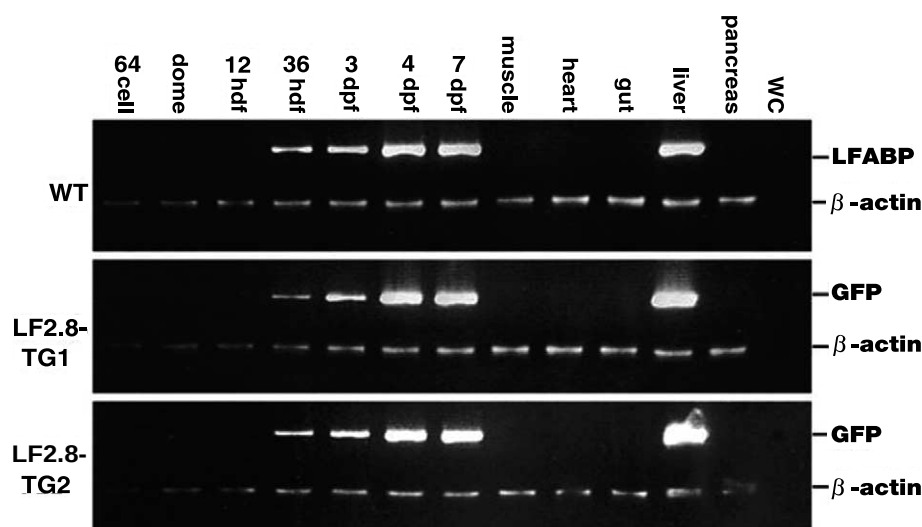


Fig. 1. Comparison of the developmental and tissue-specific expression of the endogenous L-FABP gene in wild-type zebrafish and the eGFP transgene in two transgenic lines (LF2.8-TG1 and LF2.8-TG2). RT-PCR was performed to detect message of endogenous L-FABP gene and GFP transgene. PCR products (400 bp for EGFP and L-FABP) from transcripts of EGFP and L-FABP zebrafish gene were detected by RT-PCR at the indicated developmental stages, and in the indicated tissues at 49 dpf. β -Actin (200 bp) was used as a control and was amplified in a same PCR reaction. WC indicates the negative water control. The PCR products were confirmed by sequencing.

stage zebrafish embryos. The injected embryos were examined at 3–5 dpf by fluorescence microscopy, grouped according to the intensity of fluorescence, raised to sexual maturity, and screened for potential founders. The founder fish were mated to wild-type fish and the fluorescence of their 3–5 day-old progeny was examined using fluorescence microscopy. We raised the embryos injected with the LF2.8-EGFP construct and isolated seven transgenic founders (three male and four female) from 268 adult fish. Founder fish had highly mosaic germ lines, with F1 inheritance rates ranging from 7 to 32%. The 42–51% F2 inheritance rates seen in all the seven lines were consistent with those expected for Mendelian segregation and with rates described in previous reports [21–23]. The frequency of germline transmission is summarized in Table 2.

3.3. The LF2.8-EGFP transgenic expression can mimic endogenous L-FABP expression

Liver-specific expression of L-FABP had been shown in adult zebrafish [7]. However, no temporal and spatial expression of L-FABP had been further analyzed in zebrafish embryonic stages. To provide additional evidence that L-FABP-positive cells are expressed in early liver primordia formation,

we compared the expression of L-FABP with that of ceruloplasmin (Cp), which has recently been shown to be expressed in zebrafish liver primordia [20]. At 3 dpf, the expression of L-FABP (LF) in embryonic liver primordia was very similar to that of ceruloplasmin (Cp) (data not shown). In order to assess whether the transgene conferred developmental and tissue-specific expression, expression of L-FABP and the eGFP transgene were compared at various developmental stages. Total RNA was individually purified from the various stages of embryos and from tissues of transgenic and wild-type fish. RT-PCR was performed to detect expression of the endogenous L-FABP gene and the GFP transgene. β -Actin message was also amplified as a control for the quality of the RNA. In the developmental process of zebrafish, maternally supplied L-FABP mRNA is not detected from the stages of one cell to early embryonic stage (12 hpf stage) and the L-FABP mRNA is first expressed in the 36 hpf embryos. Zebrafish L-FABP mRNA was abundantly expressed in the liver and was not detected in other organs/tissues including gut, heart, pancreas and muscle (Fig. 1). Thus, the expression pattern of the LF2.8-EGFP transgene was very similar to that of the endogenous L-FABP gene.

To provide additional evidence for the similarity of the expression patterns, we performed a series of in situ hybridizations in one of the LF2.8-EGFP transgenic lines at different developmental stages (Fig. 2). From 12 to 30 hpf, no signals can be detected by either in situ analysis of L-FABP expression in wild-type embryos or by eGFP fluorescence in transgenic embryos (data not shown). Only a few hundred cells expressing endogenous L-FABP in ventral endoderm near the heart chamber were faintly detected by in situ hybridization in 36 hpf embryos (data not shown), while transgene expression was easily seen in a group of cells near the same region at the same stage (data not shown). This difference may be due to the high stability of GFP. The transcripts were detectable around 2 dpf first in the liver primordia (Fig. 2A) and small green fluorescent liver primordia were also seen in the 2 dpf transgenic embryo (Fig. 2B). L-FABP

Table 2
Inheritance of LF2.8-EGFP in transgenic zebrafish lines

Founders	Sex	Inheritance of GFP expression			
		F1	%	F2	%
LF2.8-TG1	M	42/201	21%	166/345	48%
LF2.8-TG2	M	15/225	7%	126/279	45%
LF2.8-TG3	M	41/259	16%	111/253	44%
LF2.8-TG4	F	29/266	11%	155/303	51%
LF2.8-TG5	F	61/307	20%	146/298	49%
LF2.8-TG6	F	43/151	28%	132/312	42%
LF2.8-TG7	F	55/171	32%	125/255	49%

The F1 transgenic progeny from each line were derived from single pairs of fish by crossing founder males or females to wild-type females or males. The F2 transgenic progeny from each line were derived from single pairs of fish by crossing F1 transgenic males or females to wild-type females or males.

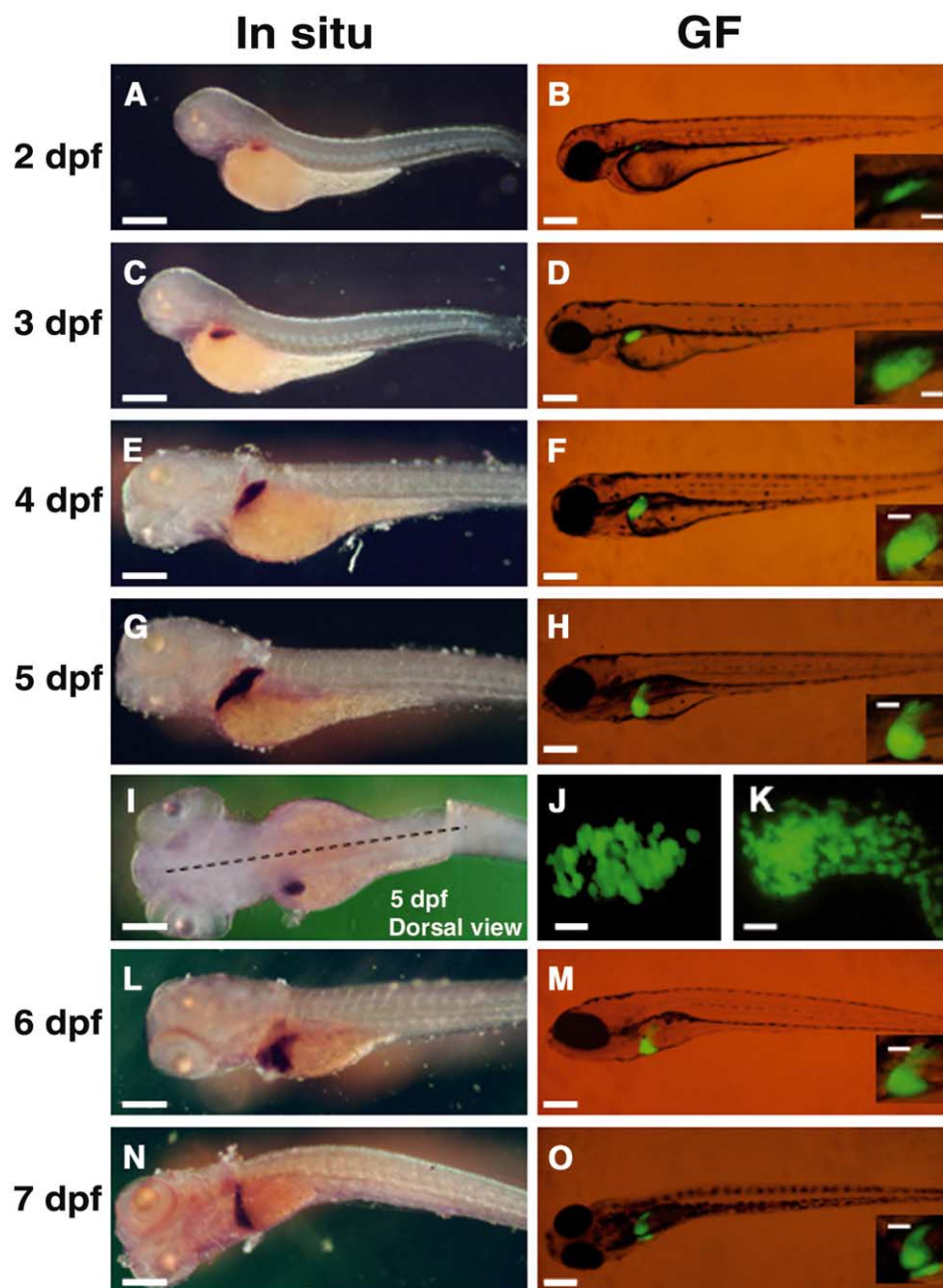


Fig. 2. Comparison of the endogenous L-FABP expression in embryonic progeny of wild-type zebrafish with expression of eGFP in progeny of the transgenic line LF2.8-TG1. Panels A, C, E, G, I, L and N show in situ hybridization to detect endogenous L-FABP. Green fluorescence (GF) photomicrographs were obtained at various stages of transgenic zebrafish development (B, D, F, H, M and O). Insets in B, D, F, H, M and O show higher magnification images of the GFP-positive liver primordia. Confocal images are shown in J and K. Scale bars: 50 μ m (A–I and L–O); 125 μ m (J, K); 50 μ m (inserts).

is predominately expressed in functional liver due to its biological functions for lipid metabolism [3–6]. Zebrafish liver may start its function after the stage of 2 dpf (hatchout). Thus, weak or no GFP fluorescence can be seen in the early stage of transgenic embryos. The liver primordia continued to be restricted to this similar region at 3 dpf (Fig. 2C, D). The size of the liver primordia was increased in the 4 dpf larvae (Fig. 2E, F), and further increased in the 5 dpf larvae (Fig. 2G, H). The 5 dpf larvae showed a similar oval shape structure, but this was much larger than seen in the 3 and 4 dpf liver primordia. At 5 dpf the liver became an asymmet-

rical organ, and was seen at the left-hand side of the trunk (Fig. 2I).

To estimate the size of embryonic liver, three-dimensional images of liver structure of the 4 dpf and 5.5 dpf larvae were obtained by confocal laser scanning microscopy (CLSM). GFP-expressing cells were organized into an oval-shaped cluster in the 4 dpf larvae (Fig. 2J) and a conical structure in the 5.5 dpf larvae (Fig. 2K). The size of the 4 dpf larval liver as measured by CLSM was about 105 μ m in width, 200 μ m in length and 20–75 μ m in thickness; at 5.5 dpf these values increased to about 91 μ m in width, 320 μ m in length, and

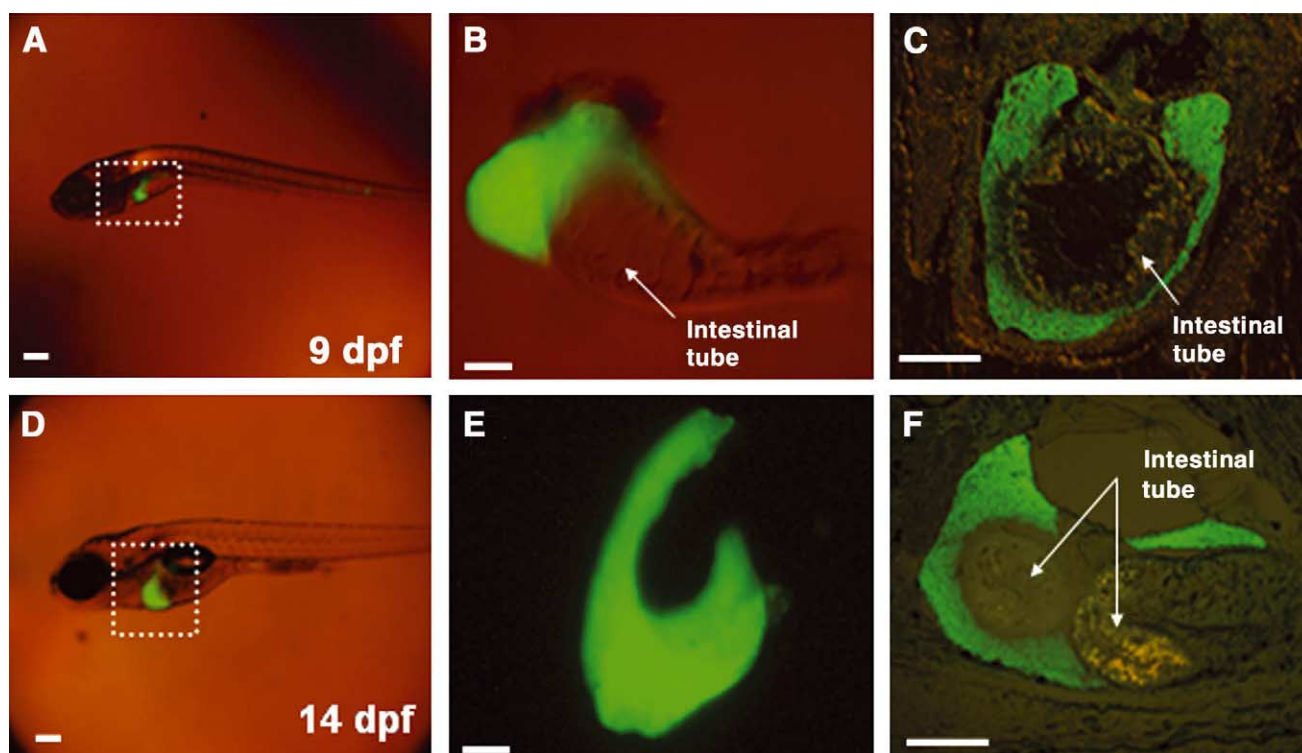


Fig. 3. Analysis of GFP expression in the liver of larval transgenic fish. A: The liver exhibited a conical shape at 9 dpf. B, C: The anterior end of the intestinal tube is surrounded by the liver in 9 dpf transgenic larvae. B: The dissected liver and intestine in the boxed region (A) is depicted at higher power. C: Cross-section of 9 dpf liver. D: The liver becomes a crescent-shaped structure at 14 dpf. E: The dissected liver in the boxed region (D) is depicted at higher power. F: Sagittal section of 14 dpf liver. The liver significantly increases its length in 14 dpf fish. Scale bars: 100 μ m (A, D); 50 μ m (B, E); 100 μ m (C, F).

45–102 μ m in thickness. A significant increase in cell number was seen in the 6 dpf liver, and GFP-expressing cells were reorganized into a larger conical structure (Fig. 2L, M). The liver becomes a crescent-shaped structure at 7 dpf (Fig. 2N, O). These results suggest that the pattern of transgenic expression is consistent with the expression pattern of the endogenous genes.

The liver still showed a conical-shaped structure at 9 dpf (Fig. 3A), similar to what was seen at 7 dpf (Fig. 2N, O). However, the anterior end of the intestinal tube is surrounded by the liver in 9 dpf transgenic embryos (Fig. 3B, C). The liver becomes a crescent-shaped structure at 14 dpf (Fig. 3D, E), and is significantly longer at this time (Fig. 3E, F). For juvenile and adult transgenic fish, GFP expression is strong in juvenile fish at 20 dpf (Fig. 4A) and 51 dpf (Fig. 4B), and in adult fish at 96 dpf (Fig. 4C) and 120 dpf (Fig. 4D). Green fluorescence is only observed in the liver in sagittal sections from 51 dpf transgenic fish (Fig. 4E), and individual GFP-labeled liver cells (hepatocyte) can be clearly discerned at higher magnification (Fig. 4F). Surprisingly, GFP fluorescence in liver is still highly detectable and there is no visible defect in the liver after 13 months of development. In fact, seven independent transgenic lines show continual stable transmission and high level of GFP expression in liver and have been maintained for over six generations.

3.4. LF2.8-EGFP transgenic zebrafish lines enable the rapid or in vivo screen for genes or mutants (morphants) required for liver development studies

In mice, gene inactivation of Hex, Xbp-1, Sek1, c-Jun and

N-myc results in an interruption of liver development [24–29]. To demonstrate that LF2.8-EGFP transgenic lines could be used for high throughput analyses of liver mutants, and to compare the activity of these genes involving liver formation, morpholinos (hhex-MO and zXbp-1-MO) targeting zebrafish Hex and Xbp-1 were injected into one-cell stage LF2.8-EGFP transgenic embryos. Embryos injected with low concentration (100 ng/ μ l) of the hhex-MO solution start to show a reduced liver phenotype with no other defects at 4 dpf (Fig. 5A, B, C). The liver size of embryos injected with medium concentration (400 ng/ μ l) of the hhex-MO solution was significantly reduced, and the embryos start to show different trunk defects from 4 to 6 dpf (data not shown). The liver of embryos injected with high concentration (800 ng/ μ l) of the hhex-MO solution was barely visible at the stage of 4 dpf, and the embryos start to show severe edema (data not shown) at the stage of 5 dpf. There are no effects on the liver of embryos injected with the hhex control-MO (Fig. 5D, E, F). In previous studies, zebrafish Hex morphants showed phenotypes including reduced or absent liver, and lack of digestive organ chirality [30]. In mouse Hex mutant embryos, the liver diverticulum could be identified in both Hex^{+/−} and Hex^{−/−} embryos as a small region of cells at embryonic day 9.5 (E9.5). At E13.5, a normal liver was observed in Hex^{+/+} and Hex^{+/−} embryos but this organ was absent in Hex^{−/−} embryos, which also had brain defects [24]. Our data are in agreement with these studies, in that initial liver specification is seen to occur in both mouse Hex^{−/−} mutants and zebrafish hex morphants at early stages, but liver organogenesis fails later.

In zebrafish Xbp-1 morphants, a reduced size of liver with a

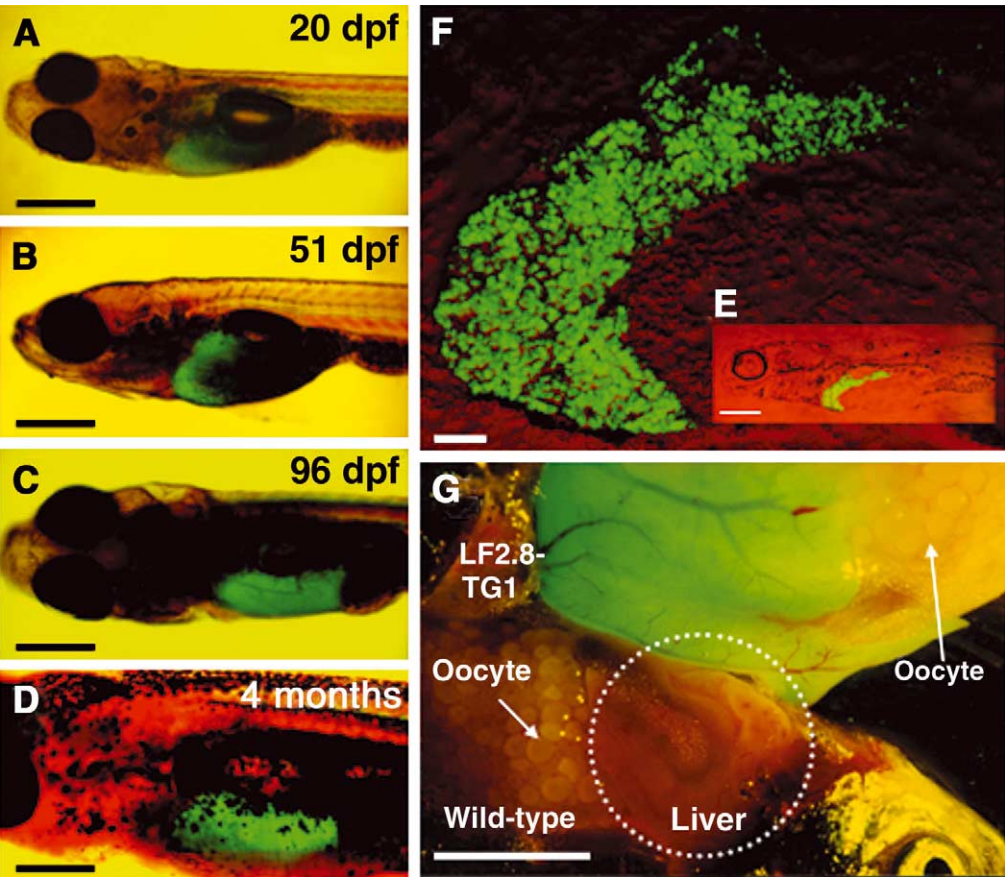


Fig. 4. Analysis of GFP expression in the liver of juvenile and adult transgenic fish. A–D: eGFP expression is strong in the juvenile (A: 20 dpf; B: 51 dpf) and adult zebrafish liver (C: 96 dpf; D: 4 months). E: Sagittal cryosection of the liver from the 51 dpf transgenic fish in B; green fluorescence is only observed in the liver. F: Individual GFP-labeled liver cells (hepatocytes) were clearly seen in the liver. G: GFP expression in liver is still quite detectable and there is no visible defect in the liver after 13 months of development. Lack of green fluorescence in a wild-type fish is shown for comparison. Scale bars: 0.2 cm (A, B, E); 0.25 cm (C); 100 μ m (F); 0.5 cm (D, G).

significant decrease in cell population was also seen in embryos injected with low concentration (200 ng/ μ l) of the zXbp-1-MO at 4 dpf (Fig. 5G, H, I). The liver size of embryos injected with medium concentration (800 ng/ μ l) of the zXBp-1-MO solution was similar to that of embryos injected with low concentration (200 ng/ μ l) of the zXbp-1-MO solution, but the embryos started to show low growth rate (smaller body length) at 5 dpf (data not shown). The liver of embryos injected with high concentration (1600 ng/ μ l) of the zXbp-1-MO solution was markedly reduced and the embryos showed severe edema at 4 dpf (data not shown). There were no effects

on the liver of embryos injected with the zXbp-1 control-MO solution (Fig. 5J, K, L). Mice lacking Xbp-1 displayed hypoplastic fetal livers and hepatocyte development itself was severely impaired by diminished growth rate [26]. Our data agree in that the delayed hepatocyte growth seen in mouse Xbp-1^{-/-} mutants is similar to the reduced liver cell population observed in zebrafish xbp-1 morphants at early stages, followed by impaired liver organogenesis at the later stages.

As shown in Table 3, injection of hhex-MO and zXbp-1-MO resulted in dose-dependent reduction of GFP expression in the 4 dpf LF2.8-EGFP transgenic embryos due to an in-

Table 3
Hepatogenesis in hhex and zXbp-1 morphants in 4 dpf LF2.8-EGFP zebrafish larvae

Phenotype	Control morpholino 400 ng/ μ l	hhex morpholino 800 ng/ μ l	hhex morpholino 400 ng/ μ l	hhex morpholino 100 ng/ μ l
Normal liver	96 (95%)	2 (1%)	5 (2.5%)	34 (17%)
Reduced liver	0 (0%)	151 (75.5%)	186 (93%)	160 (80%)
Other defect	2 (2%)	22 (11%)	3 (1.5%)	2 (1%)
Dead	3 (3%)	25 (12.5%)	6 (3%)	4 (2%)
Phenotype	Control morpholino 400 ng/ μ l	zXbp-1 morpholino 1600 ng/ μ l	zXbp-1 morpholino 800 ng/ μ l	zXbp-1 morpholino 200 ng/ μ l
Normal liver	97 (97%)	3 (1.5%)	8 (4%)	61 (30.5%)
Reduced liver	0 (0%)	163 (81.5%)	178 (89%)	126 (63%)
Other defect	1 (1%)	19 (9.5%)	5 (2.5%)	4 (2%)
Dead	2 (1%)	15 (7.5%)	9 (4.5%)	9 (4.5%)

Embryos were injected with approximately 2.0 nl of the morpholino (MO) solution per embryo. Control morpholinos for hhex and zXbp-1 were designed by four base mutations compared to their original MO sequence.

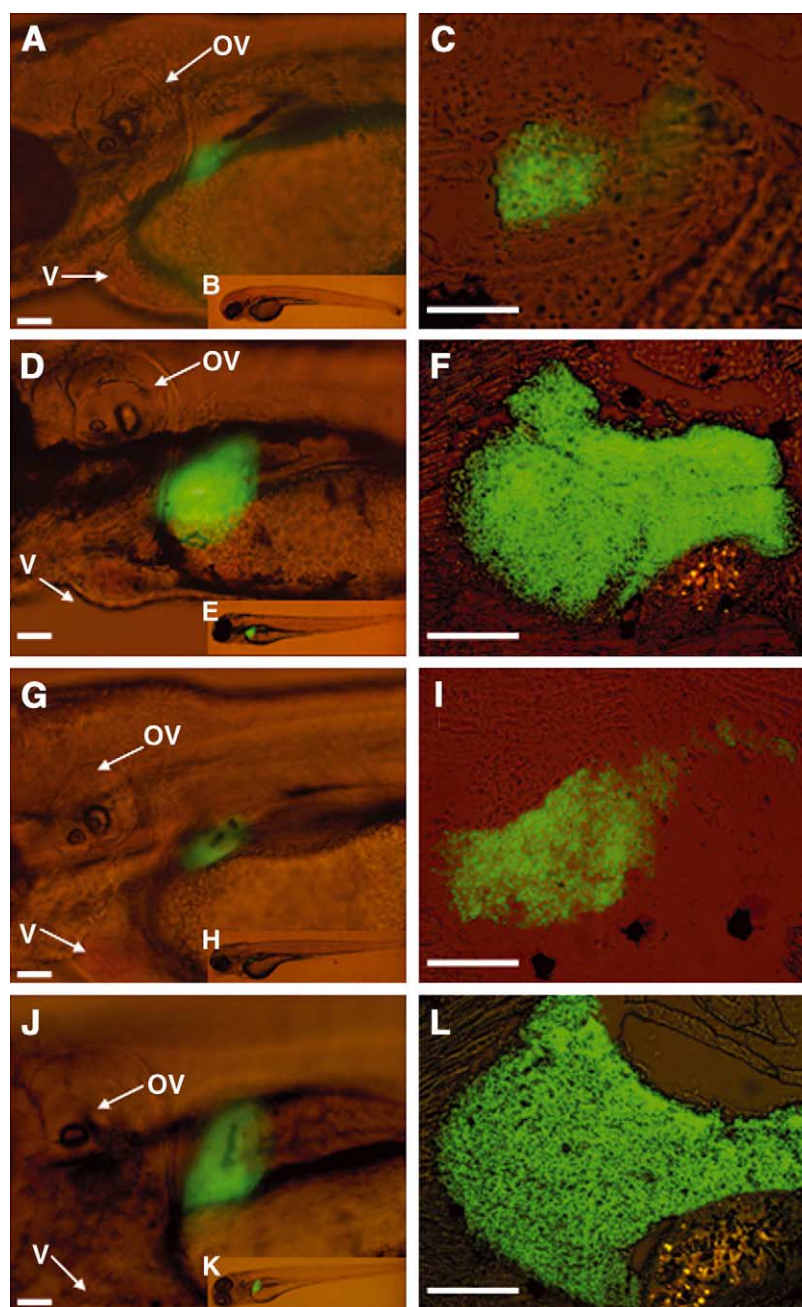


Fig. 5. Effect of zebrafish Hex and Xbp-1 morpholinos (hhcx-MO and zXbp-1-MO) on zebrafish hepatogenesis. Zebrafish embryos were injected at the one-cell stage with a low concentration (100 ng/ μ l for hhcx-MO; 200 ng/ μ l for zXbp-1-MO) of the morpholinos complementary to 5'-proximal regions of the cDNA as described in Section 2. A–C: The 4 dpf hhcx morphant. D–F: The 4 dpf hhcx control-injected morphant. A: The liver in the hhcx morphant is much smaller than in hhcx control-injected morphant (D). B: The hhcx morphant in A is depicted at low power. D: The liver of hhcx control morphant is normal. E: The hhcx control morphant in D is depicted at low power. C, F: Histological cross-sections of 4 dpf hhcx morphants and hhcx control-injected embryos. C: Interrupted liver development of hhcx morphants was easily seen in the histological section. F: Small amount of hepatic tissue in the hhcx morphant (C) compared with the hhcx control-injected morphant. G–I: The 4 dpf zXbp-1 morphant. J–L: The 4 dpf zXbp-1 control-injected morphant. G: The liver in the zXbp-1 morphant is little smaller than in zXbp-1 control-injected morphant (J). H: The zXbp-1 morphant in G is depicted at low power. J: The liver of zXbp-1 control morphant is normal. K: The zXbp-1 control morphant in J is depicted at low power. I, L: Histological cross-sections of the 4 dpf zXbp-1 morphant and zXbp-1 control-injected morphant. I: Interrupted liver development of zXbp-1 morphant was easily seen in the histological section. L: Low density of liver cells in zXbp-1 morphants (I) compared with zXbp-1 control-injected embryos. Scale bars: 50 μ m (A, D, G, J); 100 μ m (C, F, I, L). OV, otic vesicle; V, ventricle.

terruption of liver development. The embryos injected with high concentration of the hhcx-MO and zXbp-1-MO solution displayed other embryonic abnormalities, which may be due to a significant loss of liver function later. The small livers present in the embryos injected with low concentration of the

morpholinos do not arise from a non-specific developmental delay (Fig. 5D, E, J, K). Thus, the dramatic effects of size reduction and altered shape on the morphant livers appeared to be a result of a decrease in the cell population during liver formation. However, complete inhibition of liver development

was not obtained. These results illustrate how LF2.8-EGFP transgenic zebrafish can be used as a simple and efficient tool for isolating and analyzing genes involved in liver development or function in zebrafish.

4. Discussion

Many successful studies in transgenic zebrafish have been reported in the past several years [31]. In the majority of the previous studies, transgenic zebrafish were generated using a reporter gene driven by a tissue-specific promoter/enhancer of zebrafish origin [23,32–38] or from other species [21,39–44]. So far, none of the liver-specific gene promoters in zebrafish have yet been analyzed in detail, and stable transgenic lines carrying promoters of such genes have not been reported. Since zebrafish has become a popular model organism, the generation of transgenic zebrafish which express a reporter gene in specific tissues or organs will undoubtedly be useful in the study of vertebrate organogenesis. In this study, we have isolated and characterized the L-FABP promoter region in zebrafish, a gene whose expression has previously been shown in the liver of the adult zebrafish [7].

We showed that an approximately 2.8-kb 5'-upstream region contains sufficient regulatory elements to direct liver-specific expression of GFP in embryonic, larval, juvenile, and adult zebrafish. This pattern of transgene expression in the liver recapitulates that of the intact endogenous donor zebrafish L-FABP but not that of mammalian (human, rat and mouse) L-FABP gene, which is not only active in the liver but also in the gastrointestinal tract [8]. In virtually all seven lines established, no positional effect of the integration sites was found. These seven independent transgenic lines show continual stable transmission and high level of GFP expression in liver and have been maintained for over six generations. The transgenic embryos from each line displayed an identical fluorescent liver pattern, and no variegated GFP expression was seen in any other regions of the embryos. This presents a strategy for using the L-FABP promoter to drive GFP in liver without affecting either early embryonic liver development or adult liver function. Thus, the results indicate that the zebrafish L-FABP promoter can reliably drive reporter gene expression in an identical manner as the endogenous L-FABP gene in transgenic zebrafish. This is the first demonstration of transgenic zebrafish in which a reporter gene is driven by a liver-specific promoter.

To demonstrate the usefulness of L-FABP transgenic zebrafish in screening liver mutants, we microinjected the transgenic zebrafish embryos with morpholinos targeting zebrafish Hex or Xbp-1 and showed that liver phenotypes can very easily be observed in live embryos. Thus, these lines can be used as a rapid and efficient *in vivo* system for screening new genes required for liver development and as a valuable tool for the direct identification of liver mutants in expression-based mutagenesis screens where disruptions in GFP expression patterns can be observed in live embryos. In addition, there is increasing awareness and concern regarding the environmental effects of industrial chemicals such as those involved in water pollution. Many of these chemical compounds may become potential hepatotoxins, causing tumors or having other toxic effects in liver [45–49]. Our transgenic lines will be a useful tool in applied *in vivo* studies on toxic chemicals or carcinogens which cause liver toxicity, tumor, or disease.

Eventually, zebrafish models of human liver disease such as cirrhosis or even hepatocarcinogenesis may be established.

Acknowledgements: The authors are grateful to Dr. R.E. Hill (MRC Human Genetics Unit, UK) for his comments on the manuscript, to Mr. T.-L. Lin for his assistance on laser scanning confocal microscopy. This work was supported by the National Science Council grant 91-3112-P-001-018-y, Taiwan.

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