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Spatio-temporal distribution of fatty acid-binding protein 6 (*fabp6*) gene transcripts in the developing and adult zebrafish (*Danio rerio*)

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Database
Sequences for the six *fabp6* clones have been submitted to the DDBJ/EMBL/GenBank databases under the accession numbers EU665309, EU665310, EU665311, EU665312, EU665313 and EU665314

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We have determined the structure of the fatty acid-binding protein 6 (*fabp6*) gene and the tissue-specific distribution of its transcripts in embryos, larvae and adult zebrafish (*Danio rerio*). Like most members of the vertebrate *FABP* multigene family, the zebrafish *fabp6* gene contains four exons separated by three introns. The coding region of the gene and expressed sequence tags code for a polypeptide of 131 amino acids (14 kDa, pI 6.59). The putative zebrafish Fabp6 protein shared greatest sequence identity with human FABP6 (55.3%) compared to other orthologous mammalian FABPs and paralogous zebrafish Fabps. Phylogenetic analysis showed that the zebrafish Fabp6 formed a distinct clade with the mammalian FABP6s. The zebrafish *fabp6* gene was assigned to linkage group (chromosome) 21 by radiation hybrid mapping. Conserved gene synteny was evident between the zebrafish *fabp6* gene on chromosome 21 and the *FABP6/Fabp6* genes on human chromosome 5, rat chromosome 10 and mouse chromosome 11. Zebrafish *fabp6* transcripts were first detected in the distal region of the intestine of embryos at 72 h postfertilization. This spatial distribution remained constant to 7-day-old larvae, the last stage assayed during larval development. In adult zebrafish, *fabp6* transcripts were detected by RT-PCR in RNA extracted from liver, heart, intestine, ovary and kidney (most likely adrenal tissue), but not in RNA from skin, brain, gill, eye or muscle. *In situ* hybridization of a *fabp6* riboprobe to adult zebrafish sections revealed intense hybridization signals in the adrenal homolog of the kidney and the distal region of the intestine, and to a lesser extent in ovary and liver, a transcript distribution that is similar, but not identical, to that seen for the mammalian *FABP6/Fabp6* gene.

Intracellular lipid-binding proteins (iLBPs) are encoded by a highly conserved multigene family, and include fatty acid-binding proteins (FABP/Fabps), cellular retinol-binding proteins (CRBPs) and cellular retinoic acid-binding proteins (CRABPs) [1,2]. Currently, 16 paralogous iLBP genes have been identified in animals, but no member of this multigene family has thus far been identified in plants and fungi. Schaap *et al.* [2]

Abbreviations
EST, expressed sequence tag; FABP (mammals)/Fabp6 (zebrafish), fatty acid-binding protein 6; hpf, hours postfertilization; iLBP, intracellular lipid-binding protein; SNP, single nucleotide polymorphism.

have therefore suggested that the first iLBP gene emerged after the divergence of animals from plants and fungi approximately 930 million years ago. This ancestral iLBP gene presumably then underwent a series of duplication events followed by sequence divergence, giving rise to the extant iLBP multigene family.

To date, 11 isoforms of FABP/Fabps or their genes, or both, have been identified in vertebrate species [3]. Originally, these proteins were named according to the tissue from which they were initially isolated, e.g. liver-type fatty acid-binding protein (L-FABP), brain-type fatty acid-binding protein (B-FABP), intestinal-type fatty acid-binding protein (I-FABP), etc. However, this nomenclature has become confusing because different types of FABPs have been isolated from the same tissue, and some orthologous FABPs from different species exhibit distinctly different tissue-specific patterns of distribution [4,5]. Furthermore, two so-called liver-type *fabp* genes, *fabp1a* and *fabp1b* (based on phylogenetic analysis and conserved gene synteny), are not expressed in the liver of teleost fishes [6]. In this paper, we have used the alternative nomenclature proposed by Hertzel and Bernlohr [4], which uses numerals to distinguish the FABP proteins and genes corresponding to the chronological order of their discovery, e.g., FABP1 (liver-type FABP), FABP2 (intestinal-type FABP), FABP3 (heart-type FABP), etc. We have also followed the gene and protein designations for mammalian and teleost fish genes and proteins according to the recommendations of the Zebrafish Model Organism Database (http://zfin.org), in which zebrafish genes and proteins are represented as *fabp6* and Fabp6, respectively, human genes and proteins are given in upper-case letters, e.g. *FABP6* and FABP6, respectively, and the mouse gene is designated *Fabp6* and its protein FABP6.

Phylogenetic studies have identified three main groups for the FABPs: group 1 includes FABP1, FABP6 and Fabp10 (Fabp10 has only been found in non-mammalian vertebrates), group 2 consists of a single protein, FABP2, and group 3 consists of FABP4, FABP5, FABP8, FABP9 and Fabp11 (Fabp11 may be unique to teleost fishes [3]). Schaap *et al.* [2] estimate that the FABPs from group 1 diverged from the last common ancestral FABP gene approximately 679 million years ago.

Although the first FABP, FABP1, was described almost four decades ago [7], and extensive studies have focused on the tissue distribution and binding activities of FABPs and regulation of *FABP* genes, including FABP gene knock-out experiments [8], our understanding of the physiological function(s) of these proteins remains limited or, in many cases, unknown. However, sufficient evidence exists to strongly suggest the following roles for FABPs: (a) uptake of fatty acids across the plasma membrane and transport to various subcellular organelles, (b) modulation of the activity of enzymes involved in fatty acid metabolism, (c) protection of enzymes and membranes from the detergent effects of excess fatty acids by sequestering them, and (d) modulation of cell growth and differentiation by transport of fatty acids to the nucleus where they activate specific gene transcription [4,8,9].

Here we report studies on the *fabp6* gene from zebrafish, the first *fabp6* gene described for non-mammalian vertebrates. Previous work has reported the cloning and sequencing of mammalian *FABP6/Fabp6* genes and cDNAs, and their expression in mammalian species including human [10], rat [11–13], mouse [14,15] and pig [16]. Over the years, FABP6 has been given a variety of names, such as ileal lipid-binding protein, intestinal 15 kDa protein, ileal bile acid-binding protein and gastrotropin, reflecting the speculations of authors on its intracellular function(s). *In vitro* binding assays revealed a surprisingly low affinity of recombinant-derived human FABP6 and rat Fabp6 for long-chain fatty acids, such as palmitate and oleate, despite these proteins having a common three-dimensional structural motif with other FABP/Fabps known to bind long-chain fatty acids [10,17]. Work by Gong *et al.* [12] suggests that the ligands of FABP6 are bile salts and that FABP6 is involved in their uptake from the ileal epithelium. However, other studies have detected mammalian *FABP6/Fabp6* gene transcripts and encoded proteins in the ovary and steroid endocrine cells of the adrenal gland, leading to speculation that FABP6 may also function in steroid metabolism [11]. Comparative studies of *FABP6/Fabp6/fabp6* gene expression in mammals and teleost fishes may provide additional evidence for the role of this protein in cellular physiology. In this paper, we describe the structure of the zebrafish *fabp6* gene, its linkage group (chromosome) assignment, the conserved gene synteny with mammalian orthologs, and the tissue-specific distribution of *fabp6* gene transcripts in embryos, larvae and adults.

Results and Discussion

Identification of zebrafish cDNA and genomic *fabp6* sequences

Following BLAST searches of GenBank at the National Center for Biotechnology Information (NCBI), we identified an expressed sequence tag (EST) (GenBank accession number NM_001002076) [3] for which the deduced amino acid sequence showed highest percentage sequence identity to the amino acid

sequences of mammalian FABP6/Fabp6s (see below). Using this EST sequence as a query, we retrieved numerous other ESTs coding for zebrafish Fabp6 from NCBI and the sequence for the zebrafish *fabp6* gene from the genomic DNA assembly Zv7, scaffold 296.3 (ENSDARG00000044566), at the Wellcome Trust Sanger Institute (http://www.ensembl.org/Danio_rerio/index.html). In order to generate a hybridization probe for further study of the tissue-specific distribution of *fabp6* transcripts in zebrafish embryos, we amplified the *fabp6* transcript by RT-PCR of total RNA extracted from a whole adult zebrafish. The resulting DNA of the expected size was cloned, and six independent clones were sequenced and found to be identical (GenBank accession numbers EU665309–EU665314). Five single nucleotide polymorphisms (SNP) were seen between the sequence of the *fabp6* transcripts cloned by us and the coding sequence of the *fabp6* gene (Fig. 1). Only one SNP in the coding sequence, located at nucleotide (nt) position +1633 in Fig. 1, changed the deduced amino acid sequence of Fabp6, a change of valine (GTC) to isoleucine (ATC). We attribute the five SNPs to differences between established strains of zebrafish. The coding sequence derived from the *fabp6* gene at the Wellcome Trust Sanger Institute was derived from the Tübingen strain of zebrafish, while the sequences for the *fabp6* cDNA generated in this study were derived from the AB strain of zebrafish (see http://zfin.org for strain details).

The coding sequence of the *fabp6* gene contained an open reading frame of 393 bp (not including the stop codon), with 5′ and 3′ untranslated regions of 50 bp and 74 bp, respectively (Fig. 1). The open reading frame codes for a polypeptide of 131 amino acids, with a molecular mass of 14 406 Da and an isoelectric point of 6.59. With the exception of some Fabp10s, which have an isoelectric point of 8.8–9.0, all other FABPs have isoelectric points of approximately 6 [18].

The zebrafish *fabp6* gene consists of four exons of 112, 174, 89 and 141 bp, coding for 22, 59, 30 and 20 amino acids, respectively. The intron/exon structure of the *fabp6* gene from zebrafish is consistent with that of all other *fabp* genes studied to date [1], with the exception of the muscle-type FABP (M-FABP) gene from desert locust [19], which lacks intron II, and the *fabp1b* gene from zebrafish, which contains an additional intron in the 5′-untranslated region [6]. Each of the intron/exon splice junctions in the zebrafish *fabp6* gene conform to the GT/AG rule proposed by Breathnach and Chambon [20].

Alignment of the zebrafish Fabp6 sequence with mammalian FABP6 sequences (human, rat, mouse and pig) and to paralogs of other zebrafish Fabps and human FABPs showed that zebrafish Fabp6 shared greatest sequence identity with human (55.3%), mouse (50%), rat (50%) and pig (49.2%) FABP6 sequences (Fig. 2). Sequence identity of the zebrafish Fabp6 with paralogous zebrafish Fabps and human FABPs varied

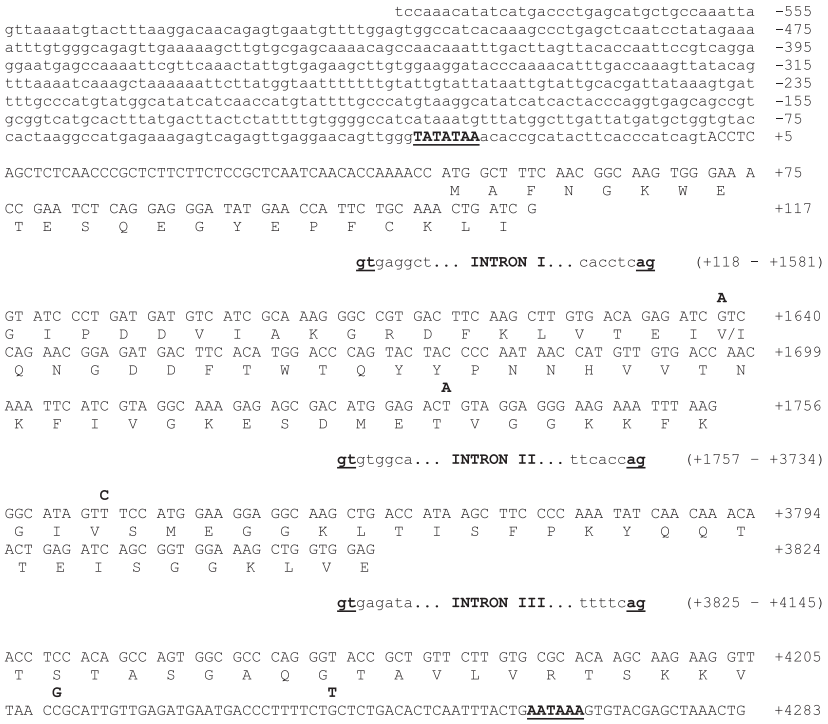

Fig. 1. The nucleotide sequence of the zebrafish *fabp6* gene. The coding sequence is shown in upper-case letters, with the deduced amino acid sequence below. The stop codon is indicated by an asterisk. The size of each intron is shown, with the exon/intron splice junctions (gt/ag) shown in bold and underlined. The 5′ upstream sequence of the *fabp6* gene is shown in lower-case letters, with a putative TATA box in upper-case letters, underlined and in bold. SNPs based on differences between the *fabp6* gene derived from the Tübingen strain and cDNA sequences from the AB strain of zebrafish are shown in bold above the genomic sequence. The polyadenylation signal sequence AATAAA is underlined and in bold.

```
ZF FABP6    M--AFNGKWETESQEGYEPFCKLIGIPDDVIAKGRDFKLVTEIVQNGDDFTWTQYYPNNHVVTNKFIVGK
HU FABP6    .--..T..F.M..EKN.DE.M..L..SS...E.A.N..I...VQ.D.Q....S.H.SGG.TM....T...
MO FABP6    .--..S..Y.F..EKN.DE.M.RL.L.G...ER..N..II..VQ.D.Q....S.S.SGGNIMS...TI..
RA FABP6    .--..T..Y.F..EKN.DE.M.RL.L.E...ER..N..II..VQ.D.EN...S.S.SGGNIMS...TI..
PI FABP6    .--..T..Y.I..EKN.DE.M.RLAL.S.A.D.A.NL.IIS.VK.D.QN...S.Q..GG.SI..T.TI..
ZF FABP1B   .--S.T..YQL.....F.E.M.AV.L...M.E..K.I.S.S..EE..NQ.KV.VT-TGSK.L..S.TI.Q
HU FABP1    .--S.S..YQLQ...NF.A.M.A..L.EEL.Q..K.I.G.S......KH.KF.IT-AGSK.IQ.E.T..E
ZF FABP1A   .--..T..YQL..H.NF.A.M.AV.V...EVE..K.I.SIS..H.D.K..KV.VT-AGTK.ILYS.T..E
HU FABP3    .VD..L.T.KLVDSKNFDDYM.SL.VGFATRQVASMT.PT.I.EK...IL.LKTH-STFKNTEIS.KL.V
ZF FABP7A   .VD..CAT.KLVDSQNFDEYM.SL.VGFATRQV.NVT.PTIV.SHE..KVVIKTL-STFKNTEIS.KL.E
HU FABP2    .--..DST.KVDRS.N.DK.MEKM.VNIVKRKLAAHDN.KLT.T.E.NK..VKES-SAFRNIEVV.EL.V
HU FABP7    .VE..CAT.KLTNSQNFDEYM.AL.VGFATRQV.NVT.PTVI.S.E..KVVIRTL-STFKNTEIS.QL.E
ZF FABP3    .AD..I.T.NLKESKNFDEYM.G..VGFATRQVANMT.PT.I.SKE..V..LKTV-STFKSTEIN.KL.E
HU FABP4    .CD..V.T.KLV.S.NFDDYM.EV.VGFATRKVAGMA.PNMI.SV...VI.IKSE-STFKNTEIS..L.Q
ZF FABP2    .--T...T.KVDRN.N..K.MEQM.VNMVKRKLAAHDN.KITLE.T..K.NVKEV-STFRTLEIN.TL.V
ZF FABP7B   .VD..C.T.KLV.SDNFDEYM.SL..GFATRQV.NVT.PTLV.SKE.EKVVIKTQ-STFKNTEIS.TL.E

ZF FABP6    ESDMETVGGKKFKGIVSMEG-GKLTISFPK------YQQTTEISG-GKLVETSTASGAQGTAVLVRTSKKV-    100.0%
HU FABP6    ..NIQ.M...T..AT.Q...-...VVN..N------.H..S..V.-D....V..IG.VT----YE.V..RLA    55.3%
MO FABP6    .CE.Q.M......AT.K...-..VVAE..N------.H..S.VV.-D....I..IGDVT----YE.V..RLA    50.0%
RA FABP6    .CE.Q.M......AT.K...-..VVAD..N------.H..S.VV.-D....I..IGDVT----YE.V...R.A    50.0%
PI FABP6    .C.I..I......AT.Q...-..VVVNS.N------.HH.A..VD-.....V..VG.VT----YE.V...LA    49.2%
ZF FABP1B   .A.I..LT.E.V.TT.NR..-N..KVVLNR------ITSI..LVDTNT..N.L.LG.LV----YK.I..R.A    42.1%
HU FABP1    .CEL..MT.E.V.TV.QL..DN..VTT.KN------IKSV..LN.-DIITN.M.LGDIV----FK.I..RI-    39.3%
ZF FABP1A   .CEL..FT.DRA.TV.Q.D.-N...AFVKG------IESV..LD.-DTISN.LSFN.IV----YK.I.RRIS    36.3%
HU FABP3    .F.ET.ADDR.V.S..TLD.-...VHLQKWD--GQETTLVR.LID-...IL.L.HGT.V----CT..YE.EA    26.8%
ZF FABP7A   .F.ET.ADDRHV.ST..L..-DN.VQVQRWD--GKETKFVR..KD-..M.M.L.FE.V.----A...YE.A-    25.5%
HU FABP2    TFNYNLAD.TELR.TW.L..-N..IGK.KRTDNGNELNTVR..I.-DE..Q.YVYE.VE----AK.IF..D-    24.8%
HU FABP7    .F.ET.ADDRNC.SV..LD.-D..VHIQKWD--GKETNFVR..KD-..M.M.L.FGDVV----A..HYE.A-    24.0%
ZF FABP3    .F.ET.ADDR.V.SVITLD.-...LHVQKWD--GKETTLLR.V.D-NN.TL.L.LGDIV----ST.HYV.AE    23.9%
HU FABP4    .F.EV.ADDR.V.STITLD.-.V.VHVQKWD--GKSTTIKRKRED-D...VECVMK.VT----ST.VYERA-    22.6%
ZF FABP2    TF.YSLAD.TELT.SWVI..-DT.KGT.TRKDNGKVLTTVRT.VN-.E..QSYSYD.VE----AK.IF.RA-    22.6%
ZF FABP7B   .FEET.ADDRHC.ST.LLK.-NQ.VHVQKWD--GKETTFIR..KD-..M.MKL.FGDVE----AL..YE.A-    21.8%
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Fig. 2. Sequence alignment and amino acid sequence identity of zebrafish Fabp6 and FABP6s from various species, and paralogs of other zebrafish Fabps and human FABPs. The deduced amino acid sequence of the zebrafish Fabp6 (Ensembl peptide ID ENSDARP00000065447) was compared to sequences of FABP6s from human (HU FABP6; GenBank accession number U19869), mouse (MO FABP6; CAI24826), rat (RA FABP6; NP_058794), pig (PI FABP6; P10289), and to zebrafish Fabp paralogs FABP1A (ZF FABP1A; DQ062095), FABP1B (ZF FABP1B; DQ062096), FABP2 (ZF FABP2; AAH75970), FABP3 (ZF FABP3; NP_694493), FABP7A (ZF FABP7A; NP_571680), FABP7B (ZF FABP7B; AAQ92970), and human FABPs FABP1 (Hu FABP1; M10617), FABP2 (HU FABP2; M18079), FABP3 (HU FABP3; X56549), FABP4 (HU FABP4; NP_00133) and FABP7 (HU FABP7; CAI15449). Dots indicate amino acid identity. Gaps (dashes) have been introduced to maximize alignment. The percentage amino acid sequence identities between the zebrafish Fabp6 and other FABPs are shown at the end of each sequence.

from 42.1% to 21.8%. Phylogenetic analysis revealed the inclusion of zebrafish Fabp6 with human, rat, mouse and pig FABP6s in a distinct clade with a robust bootstrap value of 99/100 (Fig. 3). The phylogenetic tree indicates a closer evolutionary relationship between FABP6s and FABP1s, and a more distant relationship between FABP6s and FABP7s, a finding consistent with early phylogenetic studies of rat and human FABP6 and FABP1 [10,12]. The sequence alignment (Fig. 2) and phylogenetic analysis (Fig. 3) strongly suggests that the ESTs and genomic sequence retrieved from DNA assembly Zv7, scaffold 296.3 (Wellcome Trust Sanger Institute zebrafish genome sequence) described above, code for Fabp6 in zebrafish.

Linkage group assignment of the zebrafish *fabp6* gene by radiation hybrid mapping and its conserved gene synteny with mammalian *FABP6/Fabp6* genes

To provide additional evidence that the gene located on the DNA assembly Zv.7, scaffold 296.3, indeed codes for zebrafish Fabp6, we determined the linkage group (chromosome) assignment of the zebrafish *fabp6* gene and examined its conserved gene synteny with the human, rat and mouse *FABP6/Fabp6* genes. Using the LN54 panel of radiation hybrids [21] and specific primers to exon 2 and intron 2, repectively (see Fig. 1 and Experimental procedures), the zebrafish *fabp6* gene was mapped to linkage group (chromosome) 21 at a distance of 26.79 cR from the marker fc08c06, with an LOD (logarithm of the odds [to the base 10]) of 10.8 (mapping data available at http://dir.nichd.nih.gov/Img/devb.htm). This result is consistent with the chromosomal location of *fabp6* on Zv6 in the Wellcome Trust Sanger Institute database, but not with the latest version, Zv7, which places the zebrafish *fabp6* gene on chromosome 3. We have previously observed incompatibilities between radiation hybrid mapping data for other zebrafish *fabp* genes and their chromosomal assignment in the Wellcome Trust Sanger Institute genome sequence database for zebrafish. Later, versions of the zebrafish genome sequence have been corrected in agreement with the chromosomal assignment of *fabp* genes by radiation hybrid mapping.

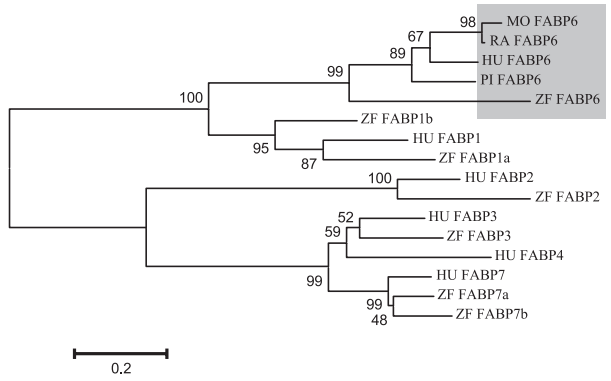

Fig. 3. A neighbor-joining tree showing the phylogenetic relationship of zebrafish Fabp6 with selected paralogous and orthologous Fabp/FABPs from zebrafish and mammals. The bootstrap values, as percentage (based on 100 replicates), are indicated at the nodes. The sequences used were zebrafish FABP6 (Ensembl peptide ID ENSDARP00000065447), mammalian sequences for FABP6 from human (HU FABP6, GenBank accession number U19869), mouse (MO FABP6, CAI24826), rat (RA FABP6, NP_058794) and pig (PI FABP6, P10289), and sequences for zebrafish FABP1A (ZF Fabp1A, DQ062095), FABP1B (ZF Fabp1B, DQ062096), FABP2 (ZF Fabp2, AAH75970), FABP3 (ZF Fabp3, NP_694493), FABP7A (ZF Fabp7A, NP_571680) and FABP7B (ZF Fabp7B, AAQ92970) and human FABP1 (Hu FABP1, M10617), FABP2 (HU FABP2, M18079), FABP3 (HU FABP3, X56549), FABP4 (HU FABP4, NP_00133) and FABP7 (HU FABP7, CAI15449). The distinct clade of FABP6/Fabp6s is shaded in gray. Scale bar = 0.2 substitutions per site.

The conserved gene synteny between the zebrafish *fabp6* gene on chromosome 21 and human *FABP6* gene on chromosome 5 is extensive (Table 1). Conserved gene synteny was also evident between the zebrafish *fabp6* gene and the *Fabp6* genes on rat chromosome 10 and mouse chromosome 11. Not all the genes that show conserved gene synteny between zebrafish chromosome 21 and human chromosome 5 are located on rat chromosome 10 and mouse chromosome 11. Other genes are located on rat chromosomes 2, 17, 18 and 20, and mouse chromosomes 13, 15 and 18, suggesting chromosomal rearrangements or translocations in these regions after divergence of the human and rodent lineages. Despite these chromosomal rearrangements, the conserved gene synteny shown in Table 1 strongly indicates that a common linkage group containing the *FABP6/Fabp6/fabp6* gene was inherited from a common ancestor of fishes and mammals. The conserved gene synteny (Table 1), sequence identity (Fig. 2) and phylogenetic analysis (Fig. 3) provide compelling evidence that the putative zebrafish *fabp6* gene described here and the mammalian *FABP6/Fabp6* genes are orthologs.

Distribution of *fabp6* gene transcripts in zebrafish embryos and larvae

To determine the spatio-temporal distribution of *fabp6* transcripts during zebrafish embryonic and larval development, we performed whole-mount *in situ* hybridization to zebrafish embryos and larvae at various developmental stages (Fig. 4). *fabp6* transcripts were not detected in embryos at 48 h postfertilization (hpf), but a very strong hybridization signal was detected in the distal region of the zebrafish intestine at 72 hpf (Fig. 4A), indicating that initiation of *fapb6* gene transcription occurred between 48 and 72 hfp. The distribution of *fabp6* transcripts remained constant in the distal region of the intestine of zebrafish larvae from 3 to 7 days postfertilization (Fig. 4A–C). A transverse section of a 4-day-old larva showed the presence of *fabp6* transcripts located predominately in epithelial cells of the intestine (Fig. 4B).

To our knowledge, only two studies have investigated the tissue-specific distribution of *FABP6/Fabp6* transcripts during embryogenesis [14,15]. In the mouse, Sacchettini *et al.* [14] showed by dot-blot hybridization that no *Fabp6* transcripts were detected in any tissues during fetal life, or throughout the suckling period of 1–12 postnatal days. Mouse *Fabp6* transcripts were first detected and restricted to the ileum at the beginning of the suckling/weaning transition at postnatal days 12–14. In contrast, Crossman *et al.* [15] did detect *Fabp6* transcripts in mouse embryos. They used Northern blot analysis and quantified mRNA steady-state levels by scanning autoradiograms of RNA extracted from total intestine and sections along the entire length of the intestine (i.e. from the gastroduodenal junction to the rectum). *Fabp6* transcripts were first detected in RNA from total intestine at E18, which is the stage at which the 'proximal-to-distal wave of cytodifferentiation of the pseudo-stratified gut epithelium to a monolayer had reached the ileum' [14]. During postnatal development, *Fabp6* transcripts were restricted to the distal third of the small intestine and cecum. No *Fabp6* transcripts were detected in the duodenum, jejunum or 12 other extraintestinal tissues (the latter tissues were not specified). The transcriptional initiation of the zebrafish *fabp6* gene in the distal region of the intestine at around 72 hpf, prior to hatching (Fig. 5), occurs at approximately the same developmental stage as the transcriptional initiation of the mouse *Fabp6* gene in the ileum at E18 [14].

Table 1. Conserved gene synteny between zebrafish linkage group (chromosome) 21 and human chromosome 5, rat chromosome 10 and mouse chromosome 11.

Chromosomal position										
	Zebrafish	Human	Rat				Mouse			
Genes	Linkage group	5	2	10	17/20	18	11	13	15	18
c6	21	5p13	2q16						3.0 cM	
c7	21	5p13	2q16						3.0 cM	
rpl37	21	5p13	2q16						A1	
fgf10	21	5p13-p12	2q15-q16					75.0 cM		
taf9	21	5q11.2-q13.1	2q12					D1		
f2rl1	21	5q13	2q12					75.0 cM		
thbs4	21	5q13	2q12					46.99 cM		
bhmt	21	5q13.1-q15	2q12					D1		
glrx	21	5q14	2q11					44.0 cM		
ell2	21	5q15	2q11					C1		
pcsk1	21	5q15-q21	2q11-q12					44.0 cM		
rnf14	21	5q23.3-q31				18p11				17.0 cM
cdc23	21	5q31				18p12				17.0 cM
pou4f3	21	5q31				18p11				24.0 cM
sept8	21	5q31		10q22			28.5 cM			
skp1a	21	5q31		10q22			31.0 cM			
vdac1	21	5q31		10q22			29.0 cM			
cnot8	21	5q31-q33		10q22			B1.3			
ddx46	21	5q31.1			17p14			B2		
pdlim4	21	5q31.1	–	–	–	–	28.5 cM			
rapgef6	21	5q31.1		10q22			B1.3			
sara2	21	5q31.1		10q22			B1.3			
tcf7	21	5q31.1		10q22			28.0 cM			
zcchc10	21	5q31.1		10q22			28.5 cM			
spry4	21	5q31.3				18p11				18.0 cM
zmat2	21	5q31.3				18p11				B2
rbm22	21	5q33.1				18q12.1				D2
larp1	21	5q33.2		10q22			B2			
sap301	21	5q33.2		10q22			B2			
rnf145	21	5q33.3	–	–	–	–	B1.1			
fabp6	**21**	**5q33.3-q34**		**10q21**			**24.0 cM**			
sgcd	21	5q33.3-q34		10q21			B1.2			
mat2b	21	5q34-q35		10q12			A5			
drd1	21	5q35.1			20p12			32.0 cM		
fgfr4	21	5q35.1			17p14			33.0 cM		
rars	21	5q35.1		10q12			A4			
ubtd2	21	5q35.1	–	–	–	–	A4			
cnot6	21	5q35.3		10q22			B1.2			
nola2	21	5q35.3		10q22			28.5 cM			

Tissue-specific distribution of the *fabp6* gene transcript in adult zebrafish

We explored the tissue-specific distribution of *fabp6* transcripts in adult zebrafish by RT-PCR amplification from total RNA extracted from various tissues and by *in situ* hybridization of a *fabp6*-specific antisense oligonucleotide probe to sections of adult zebrafish. A *fabp6*-specific RT-PCR product of expected size was amplified from total RNA extracted from liver, heart, intestine, ovary and kidney (Fig. 5, top panel). No *fabp6*-specific RT-PCR product was amplified from total RNA extracted from the skin, brain, gill, eye or muscle. As a positive control to determine the integrity of the RNA samples used in these assays, transcripts for the constitutively expressed elongation factor 1α (*ef1α*) gene were amplified by RT-PCR. A product of the expected size was generated from RNA extracted from all tissues assayed (Fig. 5, bottom panel).

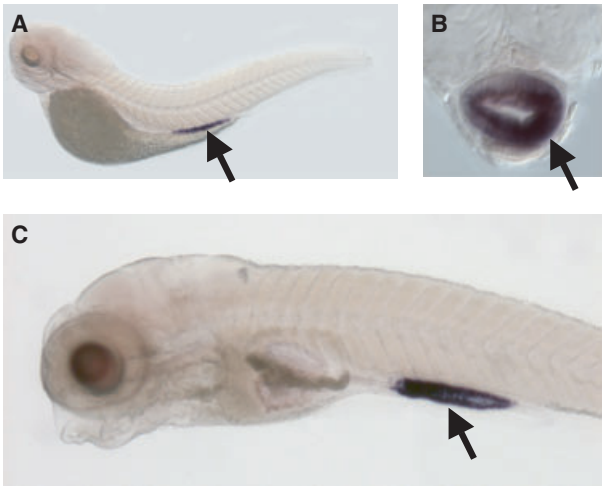

Fig. 4. The spatio-temporal distribution of *fabp6* transcripts during zebrafish embryonic and larval development was determined by whole-mount *in situ* hybridization. *fabp6* transcripts were first detected at 72 h postfertilization in the distal region of the intestine (A) and remained confined to this region of the intestine up to 7 days postfertilization (C), the last time point assayed. (B) Distribution of *fabp6* transcripts throughout the enterocytes of the intestine in a transverse section at 4 days postfertilization.

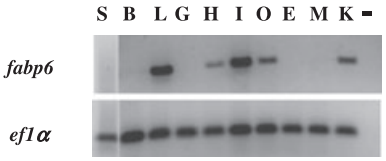

Fig. 5. RT-PCR detection of *fabp6* transcripts in RNA extracted from tissues of adult zebrafish. RT-PCR generated a *fabp*6 mRNA-specific product from RNA extracted from adult zebrafish liver (L), heart (H), intestine (I), ovary (O) and kidney (K). No *fabp*6 mRNA-specific product was generated by RT-PCR of RNA extracted from adult zebrafish skin (S), brain (B), gills (G), eyes (E), muscle (M), or the negative control (–) lacking RNA template. As a positive control for the integrity of each RNA template, an *ef1α* mRNA-specific product was generated from all the adult zebrafish tissues analyzed.

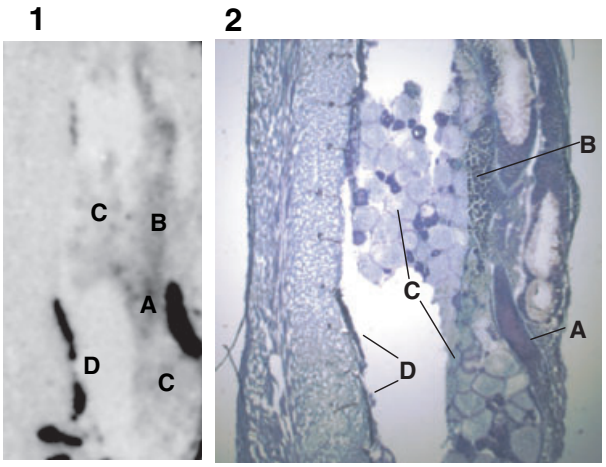

Fig. 6. Tissue-specific detection of *fabp6* gene transcripts by *in situ* hybridization of an antisense riboprobe to sections of adult zebrafish. Panel 1 shows the distribution of *fapb6* transcripts in the distal region of the intestine (A), liver (B), ovary (C) and the adrenal homolog of the fish kidney (D). Panel 2 shows the locations of the distal region of the intestine (A), liver (B), ovary (C) and the adrenal homolog of the fish kidney (D) in an adjacent tissue section stained with cresyl violet.

In situ hybridization of an antisense *fabp6* oligonucleotide probe to adult zebrafish sections revealed an intense hybridization signal in the distal region of the intestine (Fig. 6, 1A) and in the adrenal homolog of the kidney (Fig. 6, 1D). Less-intense hybridization signals were observed in the liver (Fig. 6, 1B) and the ovary (Fig. 6, 1C). Despite the difference in sensitivity of the two methods employed, the tissue distribution of *fabp6* transcripts in adult zebrafish assayed by RT-PCR and by *in situ* hybridization was identical.

In adult mammals, the reported tissue distributions of *FABP6/Fabp6* gene transcripts and its protein have varied, probably due to the assay techniques used. For example, Fujita *et al.* [10] used Northern blot analysis to detect a single-sized *FABP6* transcript in RNA extracted from the terminal region of the human ileum, whereas RT-PCR generated an abundant *FABP6*-specific product from total RNA extracted from the ileum, and to a much lesser extent from RNA extracted from the human ovary and placenta. Unfortunately, the authors do not state whether other tissues were assayed by RT-PCR in which *FABP6* transcripts were not detected. Rat *Fabp6* transcripts were detected by Northern blot analysis of RNA extracted from the ileum and ovary, but not in RNA extracted from the stomach, jejunum, colon, adrenal, brain, heart or liver [12]. Iseki *et al.* [11] used immunocytochemistry to localize the rat Fabp6 protein and *in situ* hybridization to localize *Fabp6* transcripts to the enterocytes of the ileum, luteal cells of the ovary and a subpopulation of steroid endocrine cells of the adrenal gland. Sato *et al.* [13] also detected rat FABP6 in the adrenal gland and ovary. In adult mouse, *Fabp6* transcripts were only detected by blot hybridization in the intestine, and not in the liver, stomach, pancreas, kidney, spleen, testis, skeletal muscle, heart or lung [14].

With the exception of one report [12], these studies consistently show that the *FABP6/Fabp6* gene transcripts are expressed at high levels in the ileum and to a lesser extent in the ovary and adrenal gland of adult mammals. In zebrafish, we showed by RT-PCR and

in situ hybridization that *fabp6* transcripts were detected at high levels in the distal region of the intestine, the tissue homologous to the mammalian ileum. The presence of *fabp6* transcripts suggests that Fabp6 may well play a role in the uptake of lipids from the distal region of the zebrafish intestine, which is similar to the suggested role for FABP6 in the uptake of bile salts from the mammalian ileum [11,12]. Zebrafish *fabp6* transcripts were shown by RT-PCR assay (Fig. 5) and *in situ* hybridization (Fig. 6) to be abundant in the ovary and kidney of adult zebrafish, similar to the distribution of mammalian *FABP6/Fabp6* transcripts, which are also generally found in the ovary and adrenal gland. In fishes, the adrenal homolog is not as compact as the adrenal gland found in mammals. In fishes, adrenal tissue exists as aminergic chromaffin and inter-regnal cells, mostly inside the head kidney, with the two tissues being either mixed, adjacent, or completely separated [22]. The distribution of the hybridization signal for zebrafish *fabp6* transcripts in the adrenal homolog of the kidney (Fig. 6, 1D) is consistent with the structure of the adrenal homolog in teleost fishes. With the exception of the zebrafish liver and heart, the overall pattern of adult tissue distribution of zebrafish *fapb6* and mammalian *FABP6/-Fabp6* gene transcripts, and the transcriptional initiation of these genes at similar embryonic stages of development, is surprisingly concordant, in contrast to some other members of the multigene family of iLBP genes (e.g., *fabp1a/b*, *fabp10*, *fabp11*, *rbp2*) [3,6,16,23,24]. As FABP6 has been implicated in human colorectal cancer [25] and type 2 diabetes [26], zebrafish may serve as a useful model experimental system to investigate the role of FABP6 in these disease states.

Experimental procedures

Husbandry of zebrafish

The AB strain of zebrafish was used throughout this work and maintained according to established procedures [27]. Experimental protocols were reviewed by the Animal Care Committee of Dalhousie University in accordance with guidelines set down by the Canadian Committee on Animal Care.

Nucleotide sequence of the zebrafish *fabp6* cDNA and gene

We retrieved a previously uncharacterized Ensembl gene (ENSDAR00000044566) by a BLASTn search of the zebrafish genome sequence database at the Wellcome Trust Sanger Institute (version Zv7, scaffold 296.3, http://www.ensembl.org/Danio_rerio/index.html), using NM_001002076 (GenBank accession number) as the query sequence. This sequence was also used in BLASTn searches for other ESTs coding for zebrafish Fabp6. Based on the NM_001002076 sequence, primers were designed for RT-PCR amplification of this transcript from total RNA extracted from a whole adult zebrafish of strain AB (forward primer, 5′-CTC TTCTTCTCCGCTCAA-3′; reverse primer, 5′-ATCAGTT TAGCTCGTACA-3′). The resulting product of expected size as estimated by agarose gel electrophoresis was cloned into the pGEM-T vector (Promega, Madison, WI, USA) and six clones were sequenced. To identify SNPs, the cDNA sequences obtained by us were compared to the coding sequence of the zebrafish *fabp6* gene retrieved from the Zebrafish Genome Sequence Database at the Wellcome Trust Sanger Institute by alignment using CLUSTALW [28]. The molecular mass and isoelectric point of the Fabp6 polypeptide encoded by clone NM_001002076 was determined using the program at http://ca.expasy.org/tools/pi_tool.html.

Phylogenetic analysis

Sequence alignment and determination of percentage amino acid sequence identity of FABP/Fabp sequences from zebrafish and other vertebrates was performed using BIOEDIT (version 7.0.9) [29]. Phylogenetic analysis was performed using CLUSTALW [28] to generate a neighbor-joining tree. Bootstrap values were based on 100 replicates.

Linkage group (chromosome) assignment by radiation hybrid mapping of the zebrafish *fabp6* gene

Radiation hybrids of the LN54 panel were used to assign the *fabp6* gene to a specific zebrafish linkage group according to the protocol described by Hukriede *et al*. [21]. Two primers were designed (forward primer, 5′-TAGGCAAAGAGAG CCACATGCAGA-3′; reverse primer, 5′-TGCTCAAATCC TGACACCATGGAC-3′) to PCR-amplify a portion of the zebrafish *fabp6* gene from genomic DNA samples isolated from the LN54 hybrid panel using Platinum PCR Super Mix (Invitrogen, Burlington, Canada).

Whole-mount *in situ* hybridization to zebrafish embryos and larvae

Whole-mount *in situ* hybridization using a cloned *fabp6* cDNA to generate an antisense riboprobe was performed according to the methods described previously [30].

Detection of *fabp6* transcripts in adult zebrafish tissues by RT-PCR

RT-PCR was used to determine the tissue distribution of *fabp6* transcripts in RNA extracted from tissues of adult

zebrafish. RNA was extracted from tissue using Trizol reagent (Invitrogen). Following synthesis of cDNA using the Omniscript RT kit (Qiagen, Mississauga, Canada), the zebrafish *fabp6* transcripts were amplified by PCR from total RNA extracted from various tissues using the forward primer, 5′-TAGGCAAAGAGAGCCACATGGAGA-3′, and the reverse primer, 5′-GCGGTTAAACCTTCTTGCTTGT GC-3′, according to the protocol described by Liu *et al*. [23]. The constitutively expressed gene for elongation factor 1α (*ef1α*) was used as a positive control to assay the integrity of RNA extracted from each tissue. The primers and RT-PCR conditions employed have been described previously [31].

Detection of *fabp6* transcript in adult sections of zebrafish by *in situ* hybridization

A synthetic antisense probe, 5′-GTACTGGGTCCATGT GAAGTCATCTCCGTTC-3′, was used for *in situ* hybridization to detect *fabp6* transcripts in sections of adult zebrafish according to the method described by Denovan-Wright *et al*. [32].

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References

1 Bernlohr DA, Simpson MA, Hertzel AV & Banaszak LJ (1997) Intracellular lipid-binding proteins and their genes. *Annu Rev Nutr* **17**, 277–303.
2 Schaap FG, Van der Vusse GJ & Glatz JFC (2002) Evolution of the family of intracellular lipid binding proteins in vertebrates. *Mol Cell Biochem* **239**, 69–77.
3 Agulleiro MJ, André M, Morais S, Cedrà J & Babin PJ (2007) High transcript level of fatty acid-binding protein 11 but not of very low-density lipoprotein receptor is correlated to ovarian follicle atresia in a teleost fish (*Solea senegalensis*). *Biol Reprod* **77**, 504–516.
4 Hertzel AV & Bernlohr DA (2000) The mammalian fatty acid-binding protein multigene family: molecular and genetic insights into function. *Trends Endocrine Metab* **11**, 175–180.
5 Zimmerman AW & Veerkamp JH (2002) New insights into the structure and function of fatty acid-binding proteins. *Cell Mol Life Sci* **11**, 1096–1116.
6 Sharma MK, Liu R–Z, Thisse C, Thisse B, Denovan-Wright EM & Wright JM (2006) Hierarchical subfunctionalization of *fabp1a*, *fabp1b*, and *fabp10* tissue-specific expression may account for retention of these duplicated genes in the zebrafish (*Danio rerio*) genome. *FEBS J* **273**, 3216–3229.
7 Ockner RK, Manning JA, Poppenhausen RB & Ho WK (1972) A binding protein for fatty acids in cytosol of intestinal mucosa, liver, myocardium, and other tissues. *Science* **177**, 56–58.
8 Haunerland NH & Spener F (2004) Fatty acid-binding proteins – insights from genetic manipulations. *Prog Lipid Res* **43**, 328–349.
9 Storch J & Thumser AEA (2000) The fatty acid transport function of fatty acid-binding proteins. *Biochim Biophys Acta* **1486**, 28–44.
10 Fujita M, Fujii H, Kanda T, Sato E, Hatakeyama K & Ono T (1995) Molecular cloning, expression and characterization of a human intestinal 15-kDa protein. *Eur J Biochem* **233**, 406–413.
11 Iseki S, Amano O, Kanda T, Fujii H & Ono T (1993) Expression and localization of intestinal 15 kDa protein in the rat. *Mol Cell Biochem* **123**, 113–120.
12 Gong Y-Z, Everett ET, Schwartz DA, Norris JS & Wilson FA (1994) Molecular cloning, tissue distribution, and expression of a 14_kDa bile acid-binding protein from rat ileal cytosol. *Proc Natl Acad Sci USA* **91**, 4741–4745.
13 Sato E, Fujii H, Fujita M, Kanda T, Iseki S, Hatakeyama K, Tanaka T & Ono T (1995) Tissue-specific regulation of the expression of rat intestinal bile acid-binding protein. *FEBS Lett* **374**, 184–186.
14 Sacchettini JC, Hauft SM, Van Camp SL, Cistola DP & Gordon JI (1990) Developmental and structural studies of an intracellular lipid binding protein expressed in the ileal epithelium. *J Biol Chem* **265**, 19199–19207.
15 Crossman MW, Hauft SM & Gordon JI (1994) The mouse ileal lipid-binding protein gene: a model for studying axial patterning during gut morphogenesis. *J Cell Biol* **126**, 1547–1564.
16 Walz DA, Wider MD, Snow JW, Dass C & Desiderio DM (1988) The complete amino acid sequence of porcine gastrotropin, an ileal protein which stimulates gastric acid and pepsinogen secretion. *J Biol Chem* **28**, 14189–14195.
17 Kanda T, Odani S, Tomoi M, Matsubara Y & Ono T (1991) Primary structure of the 15-kDa protein

from rat intestinal epithelium. *Eur J Biochem* **197**, 759–768.
18 Denovan-Wright EM, Pierce M, Sharma MK & Wright JM (2000) cDNA sequence and tissue-specific expression of a basic liver-type fatty acid binding protein in adult zebrafish (*Danio rerio*). *Biochim Biophys Acta* **1492**, 227–232.
19 Wu Q, Andolfatto P & Haunerland NH (2001) Cloning and sequence of the gene encoding the muscle fatty acid binding protein from desert locus, *Schistocerca gregaria*. *Insect Biochem Mol Biol* **31**, 553–563.
20 Breathnach R & Chambon P (1981) Organization and expression of eukaryotic split genes coding for proteins. *Annu Rev Biochem* **31**, 349–383.
21 Hukriede NA, Joly L, Tsang M, Miles J, Tellis P, Epstein JA, Barbazuk WB, Li FN, Paw B, Postlethwait JH *et al.* (1999) Radiation hybrid mapping of the zebrafish genome. *Proc Natl Acad Sci USA* **96**, 9745–9750.
22 Gallo VP & Civinini A (2003) Survey of the adrenal homolog in teleosts. *Int Rev Cytol* **230**, 89–187.
23 Liu R-Z, Denovan-Wright EM & Wright JM (2003) Structure, linkage mapping and expression of the heart-type fatty acid-binding protein gene (*fabp3*) from zebrafish (*Danio rerio*). *Eur J Biochem* **270**, 3223–3234.
24 Liu R-Z, Denovan-Wright EM, Degrave A, Thisse C, Thisse B & Wright JM (2004) Spatio-temporal distribution of cellular retinol-binding protein gene transcripts (*CRBPI* and *CRBPII*) in the developing and adult zebrafish (*Danio rerio*). *Eur J Biochem* **271**, 339–348.
25 Ohmachi T, Inoue H, Mimori K, Tanaka F, Sasaki A, Kanda T, Fujii H, Yanaga K & Mori M (2006) Fatty acid binding protein 6 is overexpressed in colorectal cancer. *Clin Cancer Res* **12**, 5090–5095.
26 Fisher E, Nitz I, Lindner I, Rubin D, Boeing H, Möhlig M, Hampe J, Schreiber S, Schrezenmeir J & Döring F (2007) Candidate gene association study of type 2 diabetes in a nested case-control study of the EPIC-Potsdam cohort – role of fat assimilation. *Mol Nutr Food Res* **51**, 185–191.
27 Westerfield M (1995) *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*, 3rd edn. University of Oregon Press, Eugene, OR.
28 Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F & Higgins HG (1997) The CLUSTALW windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **24**, 4876–4882.
29 Hall T (1999) BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp Ser* **41**, 95–98.
30 Thisse C & Thisse B (2008) High-resolution *in situ* hybridization to whole-mount zebrafish embryos. *Nat Protoc* **3**, 59–69.
31 Pattyn F, Robbrecht P, Speleman F, De Paepe A & Vandesompele J (2006) RTPrimerDB: the real-time PCR primer and probe database, major update 2006. *Nucleic Acids Res* **34**, D684–D688.
32 Denovan-Wright EM, Newton RA, Armstrong JM, Babity JM & Robertson HA (1998) Acute administration of cocaine, but not amphetamine, increases the level of synaptotgmin IV mRNA in the dorsal striatum of rat. *Mol Brain Res* **55**, 350–354.