Zebrafish Acetylcholinesterase Is Encoded by a Single Gene
Localized on Linkage Group 7

GENE STRUCTURE AND POLYMORPHISM; MOLECULAR FORMS AND EXPRESSION PATTERN
DURING DEVELOPMENT*

Received for publication, July 17, 2000, and in revised form, September 20, 2000
Published, JBC Papers in Press, October 2, 2000, DOI 10.1074/jbc.M006398200

Christelle Bertrand‡, Arnaud Chatonnet‡, Christina Takke‡, YiLin Yan§, John Postlethwait§,
Jean-Pierre Toutant‡, and Xavier Cousin‡‡

From the ¶Differenciation Cellulaire et Croissance, INRA, 2 Place Viala, 34060 Montpellier Cedex, France
and the §Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403

We cloned and sequenced the acetylcholinesterase gene and cDNA of zebrafish, Danio rerio. We found a single gene (ache) located on linkage group LG7. The relative organization of ache, eng2, and shh genes is conserved between zebrafish and mammals and defines a synteny. Restriction fragment length polymorphism analysis was allowed to identify several allelic variations. We also identified two transposable elements in non-coding regions of the gene. Compared with other vertebrate acetylcholinesterases genes, ache gene contains no alternative splicing at 5′ or 3′ ends where only a T exon is present. The translated sequence is 60–80% identical to acetylcholinesterases of the vertebrates and exhibits an extra loop specific to teleosts. Analysis of molecular forms showed a transition, at the time of hatching, from the globular G4 form to asymmetric A12 form that becomes prominent in adults. In situ hybridization and enzymatic activity detection on whole embryos confirmed early expression of the acetylcholinesterase gene in nervous and muscular tissues. We found no butyrylcholinesterase gene or activity in Danio. These findings make zebrafish a promising model to study function of acetylcholinesterase during development and regulation of molecular forms assembly in vivo.

The role of acetylcholinesterase (AChE, EC 3.1.1.7) in synaptic transmission is clearly demonstrated by the effects of inhibitors of this enzyme. In addition to hydrolysis of the neurotransmitter acetylcholine at synapses, AChE was shown to be involved in non-cholinergic functions, influencing differentiation and neuronal outgrowth (reviewed in Ref. 1). During development in vertebrates, AChE appears long before synapses are functional, and its role in this context is not clear (2, 3). Successive expression of butyrylcholinesterase (BChE, EC 3.1.1.8) and AChE was associated with transition from the proliferation state to differentiation in embryonic chick somites (2).

Zebrafish is a useful model for vertebrate early development and is amenable to experimental modulation of the cholinergic system, AChE activity, or ache gene expression. In this fish, AChE is expressed in neurons long before axons reach their target (4–7). When embryos were bathed with organophosphate (diisopropylfluorophosphate), AChE was totally inhibited, and somitogenesis was altered (8). It is to note, however, that effect on development may not be related to AChE inhibition since diisopropylfluorophosphate inhibits many hydrolases.

In vertebrates, AChE is characterized by a large set of molecular forms. During development and tissue differentiation their proportion varies. This polymorphism is due to alternative mRNA processing at the 3′ end of the gene resulting in proteins containing different C-terminal peptides. Exon H encodes a hydrophobic peptide that is cleaved upon glycolipid addition. Exon T encodes a peptide highly conserved among species. Soluble monomers (G1), dimers (G2), and tetramers (G4) are composed of T subunits. In addition these tetramers may be associated with structural subunits, a collagenic tail in neuromuscular junctions, or a membrane protein in brain (9). In some cases, the genomic sequence following the last common exon is retained into the mRNA. Physiological significance of this readthrough transcript is not known (9).

In order to study AChE functions during development of Danio rerio, we first characterized its early expression. In this study, we cloned and sequenced the zebrafish ache gene and cDNA. We showed that zebrafish has a single ache gene encoding only T subunits and has no bche gene. We located the ache gene in the zebrafish genome on LG7 near eng2 and shh. It contains transposable elements in non-coding regions and also presents allelic variations. We show that the fully mature molecular forms of AChE molecular forms is reached only after 1 week of development, in free swimming larvae, whereas the AChE expression pattern analysis showed an early expression from the 5- to 7-somite stage (12 h).

EXPERIMENTAL PROCEDURES

Materials—D. rerio adults and embryos were from our facility. Fish are maintained at 28 °C on a 13-h light/11-h dark cycle. The AB strain...
was a gift of Dr. Bricaud (Université des Sciences et Techniques du Languedoc, Montpellier, France), and the ABO strain was a gift of Dr. Strahle (Institut de Génétique et de Biologie moléculaire et Cellulaire, Strasbourg, France). Embryos were collected from spontaneous spawnings and staged according to Westerfield (10).

Cloning procedures using genomic DNA and RNA isolation, Northern and Southern blots, PCR amplification followed standard techniques (10, 11) or the manufacturer's protocols. Chemicals are from Sigma, Fluka, or Aldrich, and enzymes are from Promega, New England Biolabs, and Roche Molecular Biochemicals. Sequencing was performed with the Big Dye kit from PerkinElmer Life Sciences following the supplier's protocol.

cDNA Cloning, Library Screening, and Gene Isolation—Degenerate primers P1, P2, and P3 were designed from conserved sequences of AChEs. Reverse transcription was performed with Expand RT (Roche Molecular Biochemicals) using primer (P1) 5'CAGACCAAGATGCATCCATTTGCATCG-3' on total adult RNAs. A first cDNA fragment was isolated by PCR using primers (P1) 5'TTTCATCGGATCGTCGAAGTGTTCGAGGGTG-3' and (P2) 5'GGCGGCTTGCAGCAGACCTTCCTC-3'. Rapid amplification of cDNA ends experiments were performed to find the 5′- and 3′-untranslated regions and to locate the transcription start site.

A zebrafish genomic library cloned in Lambda Fix II vector was purchased from Stratagene. Approximately 1 × 10⁶ colonies were first screened using primers P1–P2 DNA fragments. Clone 1 was isolated and sequenced, and although it covered 8 kb of genomic AChE sequence, it did not contain non-coding exon 1. We performed a second screen with a 220-bp probe covering exon 1. Two additional clones 8N and 14N were isolated and sequenced, and they both contained the transcriptional start site and promoter region.

Sequence analysis using the blast algorithm (12) was performed at NCBI. For identification of transposable elements, blast (nucleotide search versus a nucleotide data base) was performed versus the "non-human, non-mouse EST" or versus the "non-redundant GenBank™ nucleotide data base. Blastx analysis (translated nucleotide sequence search versus a protein sequence data base) was performed versus the GenBank™ non-redundant protein data base. Cholinesterase sequence analysis was performed at ESTHER data base.

Genomic Mapping—The polymorphism found in the fourth intron (see "Results") in genomic clones was used to search for segregation in the MOP haploid mapping cross-panel (13). The allele-specific oligonucleotides, indicated in Fig. 1, (Forward) 5' GAGGAACTCATAGAAGAGTGAGAAATG 3' and (Reverse) 5' CGCAGCAAGGCGCTTGGCTTGA 3' amplified a fragment of 800 bp for one allele or 1100 bp for the other allele. The mapping panel was previously characterized over 800 PCR-based markers (13, 14). The strain distribution patterns were analyzed using MapManager. The sequences of zebrafish loci, including simple sequence length (14) were compared with sequences of human and mouse genes in GenBank™ using the blast algorithm (12). The map locations of human ACHE and mouse AChE genes were found in Online Mendelian Inheritance in Man, GeneMap 99, and Mouse Genome Database. Homologous gene mapping was accomplished at the NCBI HomoloGene site.

Gene Polymorphism Analysis—For Southern blot experiments, about 30 μg of DNA were digested with each restriction enzyme and loaded on 0.8% agarose gels. After migration, the DNA was transferred to nylon membranes (Hybond-N+, Amersham Pharmacia Biotech) overnight in 0.4 × NaOH, 1 × NaCl. Probes were synthesized with [β-32P]-dCTPs and manufacturer's instructions (Bio-Rad Protein Assay). C-banding was performed using recombinant AChE secreted in growth medium or native AChE from whole zebrafish extracts. Activity was assayed in Ellman reaction in vitro with reaction buffer containing 150 mM NaCl, 10 mM CaCl₂, and 50 mM Tris-HCl buffer, pH 8.0. Collagenase (Clostridiopeptidase A, Worthington) was added to a final concentration of 125 μg/ml and incubated at 30 °C. The reaction was stopped by addition of NaCl to 0.5 mM final and stored on ice prior to centrifugation on sucrose gradient.

Determination of Kinetic Parameters—Kinetic experiments were performed in 96-well plates containing the entire coding sequence of AChE was assembled from three genomic fragments and cloned in the expression vector pMT5-V-His (Invitrogen). The gene extends from 117 bases upstream of exon 2 (183 upstream of start codon) to the stop codon. The final construct was totally sequenced before transfection in Drosophila S2 cells. This mini-gene was also introduced in pcdNA3 vector and used in vivo overexpression in zebrafish embryos.

Transfection of 50 μg of plasmid and expression in Drosophila S2 cells (3 × 10⁶ cells/ml) was performed with the Drosophila Expression System (Invitrogen). After induction of expression with copper, no serum was added to the cell growth medium. AChE was recovered from the cell medium.

Microinjections into 1–2-cell stage embryos were performed with 200 ng/ml AChE-pcDNA plasmid in 0.5 M KCl, 2.5% rhodamine-B isothiocyanate dextran (Sigma). About 500 pl of solution were injected into each embryo using a Transjector 5246 (Eppendorf).

Determination of Kinetic Parameters—Kinetic experiments were performed in 96-well plates containing the entire coding sequence of AChE secreted in growth medium or native AChE from whole zebrafish extracts. Activity was assayed in Ellman reaction buffer containing 100 mM sodium phosphate, pH 7.4, 0.5 mM EDTA, 0.5 mM dithiothreitol, and 0.01% bovine serum albumin. AChE extracts were incubated for 15 min with buffer reaction before addition of various concentrations of acetylcholine iodide at 25 °C. The reaction was monitored in a spectrophotometer at 412 nm, Kₘ and Vₘₐₓ were determined according to the Haldan equation with 0.01–100 mM acetylcholine. The apparent first order rate constant, kₚ, was calculated as described previously (17). The constants of inhibition, Kᵢ and Kₘ. 1
Acetylcholinesterase of Zebrafish

**Fig. 1. Structure of zebrafish ache gene and genomic clones.** The *scheme* represents 12 kb of sequenced genomic DNA containing the zebrafish ache gene. The five ache exons are shown as *open boxes*, and the extra-coding region specific of teleosts is *hatched*. ATG and TAG point out initiation and stop codons. *H* and *E* indicate positions of polymorphic *HindIII* and *EcoRI* restriction sites (see also Fig. 4). Three isolated genomic clones (14, 14N, and 8N) are shown below the deduced gene structure of AB strain. *Shaded boxes* I, II, and III indicate repeated and transposable elements that are further described in Fig. 3. Insertions found in clone 14N are indicated. *Arrowheads* on both sides of clone 14N 3’ insertion indicate location of primers used for gene mapping. Probes used during this work are represented as *thick lines* above the gene structure.

α*K*, were determined for several inhibitors with acetylthiocholine from 0.06 to 0.7 mM. Each experiment was repeated 3–5 times using 5 concentrations of ASCh and 4–5 concentrations of inhibitor. Results were identical for recombinant or native AChE, with a standard error less than 10%.

**RESULTS**

A *Single Acetylcholinesterase in Zebrafish—Cholinesterase activity* was first detected, in whole embryos, using a histochemical reaction adapted from Karnovsky and Roots (15). In 24-h embryos, strong appearance appeared with acetylthiocholine (ASCh) within a few hours of staining, whereas there was no staining with butryrylthiocholine (BSCh). Eserine (10^-6 M) inhibited all ASCh hydrolysis and staining of embryos.

Cholinesterase activity was also measured in protein extracts. The level of BSCh hydrolysis in 24-, 48-, and 72-h embryo extracts was less than 1% of the rate of ASCh hydrolysis. This value is similar to the ratio determined for AChE of lamprey and hagfish, which have only an AChE (18). A low V_max/BSCh/V_max/ASCh ratio is consistent with the properties of AChE (19). We also analyzed cholinesterase activity in adult in total extracts and in brain and heart extracts. In all tissues there was no residual ASCh or BSCh hydrolysis activity when a specific inhibitor of AChE, BW284c51, was used at 10^-6 M. We conclude that a single AChE was responsible for ASCh and BSCh hydrolysis in zebrafish. Cholinesterase activity in zebrafish is sensitive to BW284c51, contrary to the cholinesterase activity found in the heart of *Torpedo*, mainly originating from BChE (20). In addition, an excess of the substrate ASCh inhibits activity of the enzyme, showing a specific property of AChEs versus BChEs.

We failed to detect *bche* gene by RT-PCR with degenerated oligonucleotides specific for BChEs. We did find, however, the 3’-coding region of an esterase (GenBank™ accession number AF003943) 67% homologous to carboxyl ester lipase from *Salmo salar* (GenBank™ accession number L23929). This indicates that we used conditions that were relaxed enough to allow us to detect hybridization of oligonucleotides to a putative zebrafish *bche* gene if it had been present.

**Sequence and Structure of AChE Gene and cDNA—**By using primers P1 and P2, we first isolated a cDNA fragment that was used to screen a genomic library. We isolated three independent genomic clones (see “Experimental Procedures”). They overlap as shown in Fig. 1. The total sequence covered 12 kb of genomic sequence including 3.3 kb of 5’ potential promoter region. The *ache* gene contains 5 exons; the first is non-coding and is followed by a large intron of 5 kb. Compared with other vertebrate *ache* gene sequences, the zebrafish *ache* gene contains an insertion interrupting the large exon 2. This 226-nt insertion encodes 30 amino acids (Fig. 2) followed by a small intron of 136 bp (Fig. 1). The inserted amino acids were located on the protein surface as determined by three-dimensional modeling (21). The last exon is a T exon. We fully sequenced all intronic sequences upstream of the T exon where the H exon is located in other vertebrate *ache* genes. We did not find any sequence in genomic DNA that could correspond to a hydrophobic H exon. The start of transcription, a cytosine, was found 303 bp upstream from the Met initiation codon. A canonical TATA box is found 28 bp upstream of the transcription start site. Coding sequence covers 1905 bp, and at the 3’ end, a poly(A) site identified with 3’-rapid amplification of cDNA ends defined a non-coding sequence of 340 bp. An additional downstream poly(A) site may be also used as PCR performed with an oligonucleotide located after the first poly(A) signal also amplified AChE cDNA. The total length of sequenced cDNA is 2548 bp, and a unique mRNA was detected on Northern blots (data not shown). We never found 3’ alternative splicing or readthrough cDNA as seen in some vertebrate AChEs (Massoulie et al. (9)). Only T exon is present. No cryptic alternative splicing could be found similar to what was described for *Electrophorus electricus AChE* (21).

The 634-amino acid sequence of zebrafish AChE is 62% identical to mammalian AChE, 64% identical to *Torpedo*, and 80% identical to *Electrophorus*. We found at conserved positions all the elements specific of AChEs, detailed in Fig. 2. The protein contains 6 potential N-glycosylation sites, all identical in position to glycosylation sites in a ray-finned fish, the electric eel *Electrophorus*, but only two of them were conserved in the AChE of a cartilagenous fish, the electric ray *Torpedo*.

**Transposable Elements in the Ache Gene—**In the non-coding region of the zebrafish *ache* gene (Fig. 1 and 3), we identified three domains presenting high similarity to some otherwise unrelated fish genes and ESTs.

Domain I, located upstream from non-coding exon 1, is 1162 bp long. It is flanked by two inverted repeats of 35 nt showing...
80% of identity (see Fig. 8, in “Discussion”). Blastx analysis with the protein data base showed that almost all the sequence inside the inverted repeats was highly homologous to the Tc1-like transposase family. A translated sequence of transposase could be assembled by removing frameshifts and stop codons. We identified 3 boxes corresponding to the catalytic domain (for a review see Ref. 22). The second D box is shortened compared with the canonical sequence, and therein aspartic acid is changed to an asparagine. We also identified a G-rich region as well as the nuclear localization signal. The DNA binding domain is difficult to recognize in this sequence as such domains in transposases are structural elements with low sequence conservation (22). Blast analysis showed that more than 50 zebrafish ESTs showed homology with the transposase region. This is also the case of 7 non-coding regions of cloned genes from fish (4 from zebrafish and one from either medaka fish *Oryzias latipes*, tilapia *Haplochromis burtoni* or pufferfish *Fugu rubripes*). A 443-bp internal segment of this domain I is deleted.
in the AB line and in one of the isolated genomic clones (Fig. 1).

Domain II (822 bp) is located inside intron 1. As in the case of the first domain, it is flanked by two inverted repeats (58 nt with 57% of identity). Domain II has no homology with coding sequences when compared with protein data base. It includes two sub-domains (Fig. 3). A first sub-domain shows a large number of blast homology hits with ESTs (>100), with non-coding region of 11 zebrafish genes, and contains several large direct repeats. A second sub-domain is characterized by two large inverted repeats separated by an A-rich domain. This second sub-domain is homologous to Danio-specific transposable element called angel (23). We identified only one of the T2 domains that form short inverted repeats at both ends of angel elements (23).

Domain III is located in intron 4 and was only found in genomic clone 14N (Fig. 1). A 16-nucleotide sequence (AGCCCCCTTCACACAG) is found in inverted orientation at both ends of the insertion. The central 0.5-kb domain was flanked by direct long repeats of about 60 bp. As for domains I and II, this sequence shows homology with a large number of zebrafish ESTs (Fig. 3).

Evidence for Polymorphism in Ache Gene—A commercial genomic library made with DNA from several adults was screened. Three independent clones were isolated. They differed from one another by two large insertions and multiple point mutations (Fig. 1).

The whole domain III, previously described in intron 4, was only found in the clone 14N. This insertional polymorphism was used to map ache on zebrafish genetic map (see below). In addition, a 443-bp segment located inside domain I is deleted in fish from our stock, in AB strain, and in one genomic clone.

Southern analysis of genomic DNA was first performed on fish from our stock using probes 1 and 2 (see Fig. 1). Fifteen restriction enzymes were used, and some results are presented on Fig. 4A. For most restriction enzymes, or combinations, the sizes of the bands matched the expected size derived from the genome restriction map, but an extra high molecular weight band was detected when EcoRI or HindIII were used. Sizes of these second bands correspond to a genomic sequence in which one HindIII and one EcoRI site, previously located in intron 1 and 2, are missing (Fig. 1). The same experiment performed on AB and ABO fish showed that AB was polymorphic, whereas ABO genomic DNA had only the short HindIII and EcoRI fragments.

To ensure that the extra bands were due to allelic polymorphism rather than the presence of a gene duplicate, we analyzed genetic transmission of the two forms. We performed PCRs on genomic DNA with primers surrounding the alternative EcoRI allelic site, on adult breeding pairs, and their progeny. Amplified fragments were digested with EcoRI to determine the genotype of each fish. When one parent was homozygous for one variant and the other parent was homozygous for the alternative variant (Fig. 4B), all F1 progeny were heterozygotes and presented bands both resistant and sensitive to EcoRI digestion. We also observed mendelian transmission of this variation of restriction site by crossing one homozygote and one heterozygote (not shown). By using the restriction polymorphism, we have cloned and sequenced the HindIII-EcoRI-resistant allele from the end of intron 1 to the 3’-untranslated regions. Most of the 20 nucleotide variations found in the whole gene are located in introns, and the four polymorphisms occurring in the coding sequence are silent. Sequences confirmed that the HindIII site and the EcoRI site located in introns 1 and 2, respectively, are lost. Mendelian transmission of the restriction polymorphism and conservation of the coding sequence of the two forms allowed us to conclude that these differences correspond to two different alleles of a unique ache gene.

Mapping of Ache on the Zebrafish Genetic Map—we took advantage of the length of polymorphism in the fourth intron to

---

**Fig. 3. Description of transposable and repeated elements interspersed in the zebrafish ache gene.** Domains are numbered as in Fig. 1. Below each shaded box, representing the whole domain, lines indicate regions homologous with zebrafish EST, nucleotide, and protein data bases, respectively (details under “Experimental Procedures”). Values at right of each line indicate number of hits in blast analysis (E value < 2·10⁻¹⁴) and no indicates no homologue. Pairs of arrowheads in the 4th line show localization of repeated elements. Transposon specific features are indicated in last line. In domain I, letters indicate identified domains of transposase, nuclear localization signal (N) and G-rich domain (G). The three boxes defined by Plaster et al. (22), corresponding to catalytic domains of Tc1 transposase, are also indicated (D and E). D′ corresponds to the second mutated D box. For domain II arrowheads show the repeated elements of angel transposable element separated by a conserved motif (GAAA GCCAAA marked by a line). No homology with transposable element has been identified in domain III.
locate the ache gene with the specific allelic amplification method and segregation on the MOP cross-haploid mapping panel (13). According to usual nomenclature, symbols used are ache for zebrafish, AChE for human, and Ache for murine genes.

On the MOP panel, ache mapped to LG07 between z4706 and z3445 (Table I). Intercalation between these markers places ache at about LG07_39.5 on the MGH sex-averaged diploid map (14). As shown in Table I, a few centimorgans away on LG07 reside shh and eng2, co-localizing with x1059 at LG07_52.3 on the MGH map. In human, ACHE is located at Hsa7q22, and SHH and EN2, the orthologues of shh and eng2 (24, 25), reside in Hsa7q36. The mouse orthologues of these three loci are also syntenic, located on chromosome 5 (Ache, Mmu5_80.0; Shh, Mmu5_12.0; En2, Mmu5_15.0). Interspersed between ache and shh in zebrafish is islet3, whose human orthologue is uncertain, and cyclin E, whose human orthologue is located at Hsa19q12-13. These results suggest that ache belongs to a conserved chromosome segment that originally included ache, shh, and eng2 in the last common ancestor of zebrafish, human, and mouse.

Pattern of Expression of AChE mRNA and Protein in Embryos—In situ hybridization with labeled antisense mRNAs was performed at several developmental stages. In all cases, overnight or longer incubations are required to detect staining of AChE mRNAs in embryos. Controls performed with sense RNA probe showed only staining of notochord (not shown).

ache mRNA was first detected in the trunk, in discrete regions of paraxial mesodermal segmental plate at 12 h of development (6-somite stage). At this stage there was usually no signal in the two or three most recently formed somites. In contrast, in older embryos a weak diffused staining was always found, even in unsegmented presomatic mesoderm (Fig. 5A). Expression, probably located in myoblasts, proceeds in a rostro-caudal sequence according to the state of differentiation of the somites. Initial narrow staining progressively enlarged in the differentiating somite. At all stages, the ventral part of anterior somites showed more intense staining than the dorsal part (Fig. 5A). This could be due to a higher rate of development of ventral myotome as accurately described in mouse embryos (26). In the spinal cord, mRNAs are also present in primary motoneurones as seen in Fig. 5B, in a 24-h embryo.

In the brain, mRNAs first appear in a symmetrical cluster at presumptive midbrain-hindbrain boundary from the 10-somite stage (14 h) (not shown). Two hours later, additional stainings are detected in three large bilateral clusters, in anterior telencephalon, on the floor of the diencephalon and of the mesencephalon. At 24 h, these clusters co-locate with the axonal tract of the anterior commissure, the postoptic commissure, and the ventral longitudinal tract (6, 7, 27). We can also detect messengers in hindbrain, in ventrolateral clusters at regularly repeated intervals (not shown). Small groups of cells, segmentally reiterated, expand in longitudinal columns. This corresponds to the location of differentiating reticulospinal neurons in the seven hindbrain rhombomeres as previously shown with zn1-antibody staining (4).

The method of Karnovsky and Roots (15) was used to detect AChE activity in whole embryos, in order to complete the pattern of expression described for neuronal (5, 6) and brain (7) differentiation in zebrafish and also to correlate this pattern with the mRNA expression.

In the trunk the spatio-temporal expression of AChE protein matches mRNA expression in peripheral nervous system and in muscles, along differentiation of myoblasts. AChE activity is first detected in small clusters of cells on both sides near the spinal cord (Fig. 6A). No activity is detectable before 5–7 somites, at this stage staining appears in few cells in each segment. This staining should correspond to muscle precursors because it is found in myoblasts than in myofibers (8). The enzyme activity appears more intense in somites following a rostro-caudal sequence in a pattern similar to mRNA, and all somites show activity (Fig. 6, B and E). Shortly after 18 h, myocommata, the borders between somites, start to express AChE (see also at 24 h, Fig. 6E). This coincides with clustering of acetylcholine receptors at neuromuscular junctions (28) and the first spontaneous twitches of embryos. In addition, in the peripheral nervous system, AChE protein initially appears in presumptive cell bodies of primary sensory and motoneurons from 14 h (9–10-somite stage). By 24 h (Fig. 6E), AChE is found in the spinal cord in motoneurons, located in each hemisegment, in sensory neurons (Rohon-Beard cells), and in reticulospinal interneurons. As a general feature, AChE activity is strongly detected in cell bodies of neurons but only slightly in axons.

In the posterior brain activity appeared after 14 h. A bilateral large cluster, just anterior to hindbrain rudiment, indicates position of forming trigeminal ganglia. In the embryo at 16 h, three new symmetrically bilateral clusters are present as follows: dorso-rostral, ventro-rostral, and ventrocaudal clusters are shown on Fig. 6B. Shortly after new clusters appear in the anlage of the epiphysis, in the posterior commissure, and in differentiating rhombomeres of hindbrain. At 24 h (Fig. 6C) AChE is detected in all primary neurons in the brain (see also Ref. 5). Expression in cranial ganglia can be detected in whole embryo until about 36 h. We found additional stainings, yet undescribed, in a sensory system in anterior and posterior lateral line ganglia as seen in the embryo at 24 h (Fig. 6D). In addition the heart shows strong AChE activity from its early morphogenesis.

Identification and Localization of Molecular Forms of AChE—AChE protein in vertebrates exists in numerous molecular forms that can be identified by their sedimentation and hydrophobic properties after centrifugation on sucrose gradi-
ents with or without non-denaturing detergent.

We first followed the evolution of different molecular forms repartition during zebrafish embryonic development (Fig. 7A). Until 48 h of development, in an HST protein extract, AChE sediments as soluble globular G4 (12 S) and a minor G2 form (6 S). The asymmetric form A12 (17.5 S) that contains a collagenic subunit appears after 48 h and becomes prominent after the 1st week of development.

In total extracts of adults (Fig. 7B), sequential extractions of proteins in LS, HS, and LST showed that asymmetric A12 and A8 (14.5 S), the major forms of HS extract (Fig. 7B2), were extremely predominant as 70% of total activity was found in HS extracts.

To ensure these 17.5 S forms correspond to collagen-tailed asymmetric forms, we performed collagenase digestion of HS extract (inset in Fig. 7B2). After treatment, two forms appear. In the heavier (19.5 S), the C-terminal domain of collagen tail which slows migration of A12 forms is removed and migration accelerated. In the lighter (15.5 S) digestion probably removes most of the collagen domain. We also observed appearance of tetramers (not shown). We conclude that 17.5 S corresponds to true A12 asymmetric forms. Tetramers and dimers extracted with LS or LST were found in similar amounts (Fig. 7, B1 and B3). The sedimentation coefficient of dimers was reduced in the presence of non-denaturing detergent indicating their amphiphilic nature (Fig. 7, B1 and B3). In addition, G2 forms were insensitive to phosphatidylinositol phospholipase C treatment, but their migration was slightly modified on non-denaturing electrophoresis when proteinase K was used (not shown). We conclude that these dimers have no glycolipid anchors and are type II amphiphilic dimers made of T subunits (9).

In isolated tissues of the adult (Fig. 7C), we found asymmetric forms prominent in muscles and a few G4 and G2. In contrast, amphiphilic tetramers, which aggregate in absence of detergent, were the major form in the brain (not shown). In the heart, we found G2 and G4 and also some A12 in HST extracts (Fig. 7C). We never identified glycolipid-anchored dimers. This is in agreement with the absence of H exon in cDNA and confirm what is observed in the other teleost, E. electricus (21).

In Vitro and in Vivo Expression of AChE, Catalytic Properties—Activity and molecular forms of expression of a recombinant AChE were checked in vitro and in vivo. Enzymatic assays indicated that AChE was produced in transfected Drosophila S2 cells and highly secreted into the growth medium. At 36 h, zebrafish embryos injected with the AChE mini-gene construct under control of cytomegalovirus promoter expressed recombinant AChE in all tissues as detected by in situ hybridization and activity staining in whole embryos (not shown). Four independent injection experiments were performed, and for each, two batches of 10–15 embryos were assayed for AChE activity in whole protein extracts. Depending on injections, AChE was overexpressed 5–25-fold compared with control embryos. This result was consistent with a variable amount of mRNAs injected in overexpression experiments and observed by in situ hybridization.

Fig. 7D shows that overexpressed AChE in embryos was composed of dimers and tetramers in similar amounts, whereas in non-injected embryos, prominent G4 and few G2 forms were detected. Recombinant AChE in S2 cell extracts was only found as G2 forms (Fig. 7D). These results suggest a regulation of tetramers assembly, different in Drosophila cells and in embryos.

The AChE of zebrafish has all residues characteristic of AChEs (Fig. 2). Kinetics parameters of AChE from zebrafish extracts or recombinant enzyme are close to values measured for ASch with other vertebrates AChE, $K_m = 230 \mu M$ and $K_e = 20 \mu M$. Zebrafish AChE $k_{cat}$ (1300/s) is lower than mammalian AChE $k_{cat}$ (about 3000/s) and 10 times lower than the E. electricus $k_{cat}$ despite the very high sequence homology between these two enzymes. Zebrafish enzyme is not sensitive to specific BChE inhibitor tetrakispropylphosphoramide. On the contrary, high inhibition is observed in the presence of active site inhibitors, serine ($K_i = 14.4 \mu M$) and edrophonium ($K_i = 0.53 \mu M$), or bis-quaternary inhibitor BW284c51 ($K_i = 66 \mu M$), $K_m$ for propidium (16 $\mu M$), a peripheral site inhibitor, is 5–10-fold higher than $K$ for mammalian or Torpedo AChEs. This could be due to the change of tyrosine 70 in phenylalanine since this residue is part of the propidium-binding site, located at the periphery of AChE.

**DISCUSSION**

**AChE Is Encoded by a Unique Gene**—The insertion polymorphism observed in intron 4 (see Fig. 1) allowed us to locate ake gene on zebrafish genome by segregation on MOP cross-haploid mapping panel (13). A unique ake gene was mapped in linkage group 7 within a few centimorgans of shh and eng2 loci.
The synteny observed for these genes in zebrafish, human, and mouse could indicate relative organization of the genes in the common ancestor of teleosts and mammals living approximately 450 million years ago according to molecular time scale (29).

Many genes have been duplicated during evolution in zebrafish lineage (13, 30, 31). For example, zebrafish has two copies of the \textit{EN2} and \textit{SHH} genes of human, and they are called \textit{eng2a} and \textit{eng2b} (formerly called \textit{eng3} (25)) and \textit{shh} and \textit{twhh} (32), respectively. The \textit{eng2a} and \textit{shh} loci are closely linked on LG7, and the \textit{eng2b} and \textit{twhh} loci are syntenic on LG19 (13). These are duplications of a portion of Hsa7q as shown by genetic mapping and phylogenetic analysis of HOX clusters (30). We hypothesize that subsequent to the divergence of the human and fish lineages, a chromosome inversion event in the zebrafish lineage rearranged this chromosome segment and separated \textit{ache} from \textit{shh} and \textit{eng2}. \textit{ache} and \textit{Shh/En2} are also separated by apparent translocations and inversions on mouse chromosome 5: pieces orthologous to human chromosomes 4, 1, 12, and 22 intervertebrate between them. In teleost lineage, the segment of chromosome containing \textit{ache} was probably duplicated, and then the duplicate \textit{ache} was lost in zebrafish. Loss of one or more copies of duplicated genes occurred frequently during evolution as also demonstrated by phylogenetic analysis in neurotrophin and Trk receptor families (33).

Zebrafish Have No BChE—In protein extracts of 24 and 48-h and 3-day embryos, or whole adult or heart, BSCh hydrolysis was always less than 1% of ASCh activity. Catalytic parameters measured in the presence of specific inhibitors of BChE (tetrakis(isopropylphosphoramide) or AChE (BW284c51) correspond to a classical AChE. All attempts to identify BChE activity or BChE cDNA failed, showing that there is no BChE in zebrafish. Several duplications of cholinesterase genes occurred independently in different phylogenetic lineages as follows: 4 AChE genes in \textit{Caenorhabditis elegans} (34) and 2 genes in \textit{Amphioxus} (18). The duplication of an ancestral gene, giving
rise to AChE and BChE, probably occurred before the split of cartilaginous fish, but after divergence of jawless fish lineage as BChE is found in hagfish and lamprey (reviewed in Ref. 18). The situation is less clear in bony fish because a BChE or pseudocholinesterase is found at the nucleotide level with these sequences. However, the cholinesterase gene have also associated in domain II with repeated sequences. In Drosophila, the cholinesterase domain is composed by association of a mini-gene in zebrafish embryos or after transfection in Drosophila S2 cells were analyzed in GST extracts. In S2 cells there is no activity in controls, and AChE is overexpressed as G2 form. In 36-h injected embryos two peaks of identical height correspond to overexpressed G4 and G2. In control embryos, at the same developmental stage, tetramers G4 are the major form.

The Zebrafish aChE Gene Contains Several Transposable Elements—Analysis of the non-coding region of the aChE gene revealed several domains containing repeats and showing homology with transposable elements (Fig. 5).

Upstream of aChE, we identified an inactive transposon of the Tc1-like family (domain I, Fig. 1). Many mutations accumulated in transposase sequence since transposition occurred. Despite several frameshifts and stop codons, it is still possible to identify the original amino acid sequence. The transposase in aChE shares less than 30% identity with tes1 from hagfish (38) or other zebrafish transposases (39). This is in agreement with the fact that transposition is thought to have taken place 10 million years ago (22). As can be expected, no homology was found at the nucleotide level with these sequences. However, we identified several fish genes containing domains homologous to the aChE transposon-like (80–95% identity in nucleotides). For three of these genes, the homologous domain has a different size, but the same inverted repeat flanks the central part (Fig. 8). This suggests that aChE transposon member of a new Tc1-like transposon family in zebrafish.

Domains II and III are made of a different set of repeats, associated in domain II with angel transposable elements. This could indicate that insertion occurred by combining different repeated sequences. In Danio a retroelement inserted in the intron of the elf-4E gene is also composed by association of a DANA-transposable element with other repetitive sequences (40).

Since large numbers of ESTs present high similarity (>80%) with all three domains, it appears that repeated and transposable elements are very frequent in the zebrafish genome.

Repeated elements are also found in other cholinesterase genes. Two tandem repeats have been described in the non-coding region of the two alternative last exons of Torpedo californica aChE gene (41). Two Alu sequences probably integrated by retrotransposition in human BChE gene have also.
Acetylcholinesterase of Zebrafish

been described: one in the promoter region (42) and the other one occurred in exon 2 leading to gene inactivation and a silent mutation may be the result of an intron capture as recently described in zebrafish apolipoprotein E gene (45). When compared with the teleost ache gene (reviewed in Ref. 9). Sequence alignment with the Torpedo gene showed a similar organization of exon-intron junctions in Danio with the exception of an insertion that splits the large coding exon 2 into two parts. This additional sequence (Fig. 2), also found in Electrophorus ache gene (21), introduces 30 residues in the protein and a small insertion that splits the large coding exon 2 into two parts. This additional sequence is found in tetramers, and then excess dimers accumulated in embryos. It is possible that such assembler elements are missing in S2 cells. It should be noted that in Drosophila, ache gene has no T exon and tetramers never form. These findings indicate that zebrafish could be a suitable model to study regulation of molecular forms homo- and hetero-oligomerization.

**Early Expression of mRNAs and Protein in Embryos—** Tissue specificity and early expression of cholinesterases in mammals are due to specific transcription factor binding sites (50), alternative promoter usage (51), and intronic enhancers (52, 53). In the zebrafish ache 5’ non-coding region and first intron, no homology was detected with other ache genes. We identified a canonical TATA box that contrasts with other cholinesterase genes that are devoid of this regulating element. However, a TATA box has been identified in the recently described murine AChE neuronal promoter (51). A TATA repeat was also found 32 bp upstream from the Torpedo AChE transcription start site. Putative binding sites for AP1, Egr-1, MyoD, and TTF-2 transcription factors are also present. AChE mRNA and activity are undetectable before the 5-somite stage, after which expression begins in recently formed somites. After about the 10-somite stage, AChE transcripts and proteins are present in every somite primordium, preceding boundary formation, and their expression increases along with differentiation.

Current knowledge of somitogenesis suggests that the first few rostral somites could be patterned by a different mechanism than the more caudal ones (54). Appearance of AChE simultaneously in the 5–7 early somites, and progressively in the later ones, is consistent with this hypothesis. It suggests that AChE expression could depend on factors controlling the formation of homotetramers (48).

Repartition of molecular forms in adult, shown in Fig. 7, B and C, is consistent with what is observed in other vertebrates, asymmetric forms being major forms in muscle and amphipod tetrathers the major form in brain. As shown by sedimentation analyses, globular forms present at early developmental stages and asymmetric forms appearing later are both composed of T subunits (Fig. 7). Mechanisms driving molecular oligomerization of forms depend mainly on non-catalytic subunits. This has been shown by mRNA injections in Xenopus oocytes. Assembly of asymmetric forms is strictly correlated to collagenic tail amount (49). It is very likely that the same mechanism occurs in vivo. In zebrafish RT-PCRs performed on RNAs during embryonic development indicate that the cDNA encoding the collagenic tail appears around 48 h, shortly before the appearance of asymmetric forms (Fig. 7A).

AChE produced by S2 cells transfected with the AChE mini-gene are dimers, whereas the injection of the same construct in embryos resulted in overexpression of equal amounts of tetramers and dimers (Fig. 7D). Molecular forms in control embryos and in AChE-overexpressing embryos differ widely. In the latter, dimers and tetramers are present in equal amounts, whereas in non-injected embryos we found only tetramers. It has been shown that oligomerization of tetramers could depend on proline-rich factors (9). We suggest that in the case of AChE overexpression the "assembler proline-rich protein" may be present in too low a concentration to assemble all AChE dimers in tetramers, and then excess dimers accumulated in embryos. It is possible that such assembler elements are missing in S2 cells.

-AChE and snake genes (47). In ze-
Acetylcholinesterase of Zebrafish

pathway of somite anteroposterior determination (55) among which transcription factors Mesp, MyoD, or other members of basic helix-loop-helix proteins, and the Eph family of receptor tyrosine kinases and their ligand.

Such a regionalization of cholinesterases was also found during somitogenesis in other species. In chick embryo a rostrocaudal asymmetry is established with differential expression of BChE and then AChE in the rostral part of the somite (2). This suggests a conserved regulation of expression of AChEs in myotomes. Early expression could indicate that in zebrafish AChE covers both domains of expression of BChE and AChE found in chick somites.

In the nervous system, mRNA and then protein were found in several brain clusters and in spinal cord. Our results are consistent with previous studies showing that, in zebrafish, all primary neurons, including reticulospinal interneurons, sensorv neurons, and primary motoneurons, expressed AChE before axonal outgrowth and neurite arborization (4–7). The pattern of AChE mRNAs expression in brain is very similar to expression of the growth cone component GAP-43 (56). The presence of AChE, before expression of GAP-43 (starting at 17–18 h) and growth of primary neurons, adds strength to the hypothesis of AChE involvement in the control of neuronal differentiation as recently suggested by in vitro experiments (57). However, in the mouse, neither collagenic tail gene inactivation (58), which prevented accumulation of all AChE at the synapse, nor ache gene inactivation (59) prevented embryonic development. In these mutant animals, showing delayed postnatal growth, BChE or other carboxylesterases may supply AChE function (58, 59). In Drosophila, which has no other cholinesterase than AChE, a mutation in AChE induced abnormal neuronal development (60). Similarly in zebrafish no bche gene was found, and there is no close relative of the active AChE. Identification of ache mutants in D. rerio will thus be a valuable tool to investigate the implication of AChE in the development of vertebrates and especially during regulation of morphogenesis of the nervous system.

Acknowledgments—We thank Dr. Bricaud (Université des Sciences et Techniques du Languedoc, Montpellier, France) for the AB strain and Dr. Strähle (Institut de Genétique et de Biologie moléculaire et Génétique, Strasbourg, France) for the ABO strain. Dr. Westerfield and Dr. Rosa are acknowledged for constant support.

REFERENCES

1. Brimijoin, S., and Koenigsberger, C. (1999) Environ. Health Perspect. 1, 59–64
2. Layer, P. G., Alber, R., and Rathjen, F. G. (1988) Development 102, 387-396
3. Layer, P. G. (1990) BioEssays 12, 415–420
4. Hanneman, E., Trevorrow, B., Metcalfe, W. K., Kimmel, C. B., and Westerfield, M. (1988) Development 103, 49–58
5. Hanneman, E., and Westerfield, M. (1989) J. Comp. Neurol. 284, 350–361
6. Wilson, S. W., Ross, L. S., Parrett, T., and Easter, S. S., Jr. (1990) Development 108, 121–145
7. Ross, L. S., Parrett, T., and Easter, S. S., Jr. (1992) J. Neurosci. 12, 467-482
8. Haneman, E. H. (1992) J. Exp. Zool. 263, 41–53
9. Massoulie, J., Anselmet, A., Bon, S., Krejci, E., Legay, C., Morel, N., and Simon, S. (1998) J. Physiol. (Paris) 92, 183–190
10. Westerfield, M. (1995) The Zebrafish Book, 3rd ed., University of Oregon Press, Eugene, OR
11. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: a laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
12. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
13. Postlethwait, J. H., Yan, Y.-L., Gates, M., Horne, S., Amores, A., Brownlee, A., Donovan, A., Egan, E., Gekker, M., Force, A., Furuyama, J., Goutel, C., Fritz, A., Kimmel, C. B., Sanders, M., Soong, W., Milner, D., McClellan, J. S., Sapp, M., Cohn, C. J., Watabe, H., Watabe, M., Wurzburger, S., and Yelick, P. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 7418–7423
14. Xie, W., Stribley, J., Chatonnet, A., Wilder, P. J., Rizzino, A., McComb, R. D., Farrell, M. A., and Simon, S. (1999) Development 126, 1703–1713
15. Brennan, C., Green, A., Wilson, S., and Holder, N. (2000) Development 127, 21078–21084
16. Luo, Z. D., Camp, S., Mutero, A., and Taylor, P. (1995) J. Biol. Chem. 270, 1866–1872
17. Andersson, E., Chiappa, S., Wieben, E., and Brimijoin, S. (1999) Development 126, 21078–21084
18. Luo, Z. D., Camp, S., Mutero, A., and Taylor, P. (1995) J. Biol. Chem. 270, 1866–1872
19. Andersson, E., Chiappa, S., Wieben, E., and Brimijoin, S. (1999) Development 126, 21078–21084
20. Luo, Z. D., Camp, S., Mutero, A., and Taylor, P. (1995) J. Biol. Chem. 270, 1866–1872
21. Andersson, E., Chiappa, S., Wieben, E., and Brimijoin, S. (1999) Development 126, 21078–21084
22. Luo, Z. D., Camp, S., Mutero, A., and Taylor, P. (1995) J. Biol. Chem. 270, 1866–1872
23. Andersson, E., Chiappa, S., Wieben, E., and Brimijoin, S. (1999) Development 126, 21078–21084