Two zebrafish (*Danio rerio*) antizymes with different expression and activities

Takatoshi SAITO^{*1}, Tomasz HASCILOWICZ^{*}, Ichiro OHKIDO^{*}, Yutaka KIKUCHI[†], Hitoshi OKAMOTO[†], Shin-ichi HAYASHI^{*}, Yasuko MURAKAMI^{*} and Senya MATSUFUJI^{*2}

^{*}Department of Biochemistry II, Jikei University School of Medicine, Minato-ku, Tokyo 105-8461, Japan, and [†]Laboratory for Developmental Gene Regulation, Brain Science Institute, The Institute of Physical and Chemical Research, Hirosawa, Wako-shi, Saitama 351-0198, Japan

Cellular polyamines are regulated by a unique feedback mechanism involving ornithine decarboxylase (ODC) antizyme. The synthesis of mammalian antizyme requires a programmed translational frameshift event induced by polyamines. Antizyme represses ODC, a key enzyme for polyamine synthesis, through accelerating enzyme degradation by the 26 S proteasome. Antizyme also inhibits the cellular uptake of polyamines. In the present study we isolated two distinct zebrafish (*Danio rerio*) antizyme cDNA clones (AZS and AZL) from an embryonic library. Their sequences revealed that both clones required translational frameshifting for expression. Taking account of +1 frameshifting, AZS and AZL products were 214 and 218 residues long respectively and shared 51.8% amino acid identity. In rabbit reticulocyte lysates, both mRNA species were translated through spermidine-induced frameshifting. The presence of the two antizyme mRNA species in embryos, adult fish and a cultured

cell line was confirmed by Northern blot analysis. The ratio of AZS mRNA to AZL mRNA in the adult fish was 1.8-fold higher than in the embryos. Whole-mount hybridization *in situ* demonstrated that both mRNA species are expressed in every tissue in embryo, but predominantly in the central nervous system and the eyes. Bacterial expression products of both cDNA species inhibited ODC activity, but only the AZS product accelerated ODC degradation *in vitro*. These results show that both zebrafish antizymes are induced by polyamines but their mRNA species are expressed differently during development. The difference in activities on ODC degradation suggests their functional divergence.

Key words: development, ornithine decarboxylase, 26 S proteasome, polyamines, translational frameshifting.

INTRODUCTION

Polyamines (putrescine, spermidine and spermine) are major cellular components essential for normal cell growth, differentiation and embryonic development [1]. In rapidly growing cells, polyamine levels are up-regulated by an increase in their synthesis and cellular uptake [2]. Inversely, excess polyamines cause a decrease in their synthesis [3] and uptake [4]. The feedback regulation is primarily mediated by a polyamine-induced protein, ornithine decarboxylase (ODC) antizyme, which binds to, and inhibits, ODC (EC 4.1.1.17), a key enzyme for polyamine synthesis [5,6]. Binding of antizyme is the signal for the ODC degradation catalysed by the 26 S proteasome [7]. Although most protein substrates for the proteasome are tagged by ubiquitin, antizyme is the only known non-ubiquitin signal for a substrate of the proteasome. Another function of antizyme is to inhibit the cellular uptake of polyamines [8,9]. With the ability to suppress both polyamine biosynthesis and transport, antizyme effectively attenuates cellular polyamine levels. Overproduction of antizyme in transfected cells results in the depletion of polyamines, leading to impaired cell growth [10] or cell death [11], depending on the system. Further, the antizyme function is interfered with by the product of an ODC-related gene, antizyme inhibitor, which binds to antizyme with a higher affinity than does ODC but is devoid of catalytic activity [12].

It is known that the induction of antizyme by polyamines is regulated at the translational level [13,14]. Sequencing of the cDNA and the gene for rat antizyme [15] revealed that antizyme mRNA does not have a complete open reading frame (ORF) and, as shown by Matsufuji and colleagues, the reading-frame change is required for the decoding of antizyme mRNA (reviewed in [16]). Translation experiments *in vitro* showed that the frame shifting is stimulated by polyamines [14,17]. This is the first example of the involvement of frameshifting in animal gene expression and is one of the only two known cases of regulatory frameshifting (the other is bacterial release factor 2) [18]. A proportion of ribosomes that start translating antizyme mRNA in the initiating frame (termed ORF1) change their reading phase to +1 to access a longer ORF (ORF2) overlapping ORF1 in the +1 frame. ORF2 does not have its own translational initiator but carries all the known set of antizyme activities [19–22]; the frameshifting is therefore mandatory for the synthesis of functional antizyme.

Antizyme mRNA has been detected in all vertebrate tissues examined [13]. Antizyme cDNA species and genes have been cloned from various vertebrate sources [13–15,23–28]. All these antizymes require translational frame-shifting for their synthesis. In invertebrates, *Drosophila melanogaster* antizyme expressed through polyamine-dependent frameshifting has been isolated [29,30]. Recently, Ivanov et al. [31] found a second mammalian

Abbreviations used: GST, glutathione S-transferase; ODC, ornithine decarboxylase; ORF, open reading frame; poly(A)⁺, polyadenylated; UTR, untranslated region.

¹ Present address: Medical and Molecular Research Laboratory of Hypertension and Vascular Disease, Department of Medicine and Clinical Science, Kyoto University Graduate School of Medicine, Sakyo-ku, Kyoto 606-8507, Japan.

² To whom correspondence should be addressed (e-mail senya@jikei.ac.jp).

The nucleotide sequence data reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession numbers AB017117 (AZS) and AB017118 (AZL).

antizyme (antizyme 2, AZ2). Human AZ2 shares 54% amino-acid sequence identity with the previously reported human antizyme (AZ1). It requires polyamine-inducible translational frameshifting for its expression. *AZ2* mRNA is detected approx. 1/16 of the amount of *AZ1* mRNA throughout human tissues; the *AZ1/AZ2* mRNA ratio remains unchanged during development.

Interested in the molecular evolution and biological significance of antizyme, we intended to isolate antizyme cDNA from zebrafish *Danio rerio*, a model vertebrate suitable for the study of development. Our results indicate the presence of two distinctive antizymes in this organism too. Both antizymes were synthesized through polyamine-dependent translational frameshifting. Unlike the mammalian counterparts, the mRNA species of the two zebrafish antizymes were differentially expressed from embryos to adult fish. In addition, their products exhibited different activities in regulating ODC degradation *in vitro*.

MATERIALS AND METHODS

Materials and cell line

Young adult zebrafish (male and female) were purchased from Nippon Bio-Supp. Center. Enzymes for DNA manipulation were obtained from either Takara Suzo Co. Ltd. (Shiga, Japan) or New England Biolabs. [α - 32 P]dCTP (3000 Ci/mmol) and [35 S]methionine (1200 Ci/mmol) were from NEN. A fibroblast-like zebrafish cell line, BRF41, was established by Dr. H. Mitani and provided by Riken Cell Bank with the agreement of the establisher. The cells were maintained in Leibovitz's L-15 medium containing 10% (v/v) fetal bovine serum, 10 mM Hepes, 1 mg/l fungizone, 0.1 g/l kanamycin and 25 i.u./ml penicillin G at 33 °C under air.

cDNA cloning and sequence analysis

Polyadenylated [Poly(A)⁺] RNA was isolated from whole zebrafish embryos collected 18–20 h after fertilization and used to construct a cDNA library in λ gt10 vector as described previously [32]. Plaques (10^4 plaque-forming units per 90 mm plate) were grown on lawns of *Escherichia coli* C600/hfl and transferred to nylon membranes (Hybond N⁺, Amersham). The hybridization probe was a full-length *Xenopus laevis* antizyme cDNA [24] labelled with [α - 32 P]dCTP and Random Primer Labelling kit (Takara). The hybridization and subsequent washing were performed at 42 °C in accordance with the instructions for the membrane. Positive plaques were purified with repeated plaque hybridization. The cDNA inserts were excised with *NotI* and subcloned into the *NotI* site of pBluescript SK(–) (Stratagene). Sequencing was performed with a dye-terminator cycle sequencing kit and an ABI 373A sequencer (Perkin Elmer). Phylogenetic trees were constructed with the Genetyx-Mac program (Software Development).

Plasmid construction

A plasmid vector pGEM-N was derived from pGEM-4Z (Promega) by replacing the *HindIII*–*SalI* fragment by a pair of oligonucleotides, 5'-AGCTTGCGGCCGCCATGG-3' and 5'-TCGACCATGGCGGCCGCA-3'. Fragments of the cDNA species including the entire coding region and 3' untranslated region (UTR) were amplified by PCR on the subcloned cDNA species with the T3 promoter primer and 5'-CCCCATGGT-TAAATCCAACCTTCA-3' for *AZS* or 5'-CCCCATGGTAA-AATCCAACCTCCA-3' for *AZL*. They were digested with *NcoI* and *BamHI* (the *BamHI* site being at the 3' end of each cDNA

insert) and cloned into the *NotI* and *BamHI* sites of pGEM-N vector. Most of the 3' UTR in the *AZL* insert was then truncated at nt 765 by digestion with *BglII* and *BamHI* followed by self-ligation. The resultant plasmids were designated pGEM-N-AZS and pGEM-N-AZL. In-frame controls, pGEM-N-AZS-IN and pGEM-N-AZL-IN, were prepared by deleting the T base at the stop codon of ORF1, as described previously [14]. For bacterial expression, pGEM-N-AZS-IN and pGEM-N-AZL-IN were digested with *NcoI* and *EcoRI*; the inserts were then ligated into the corresponding restriction sites of plasmid vectors pTV118N (Takara) or pGEX-N. pGEX-N is a derivative of pGEX-2T (Pharmacia), of which the *BamHI*–*EcoRI* fragment was replaced by a pair of oligonucleotides, 5'-GATCCATGGTACCAAGCTTG-3' and 5'-AATTCAAGCTTGGTACCATG-3'. A construct for the fusion of glutathione S-transferase (GST) with rat antizyme, pGEX-N-Z1, was prepared by inserting the *NcoI*–*EcoRI* fragment of Δ T205 [14] into pGEX-N, followed by replacement of its 275 nt *NcoI*–*NheI* fragment by the corresponding fragment of pTV-Z1NN2 [13]. All the plasmids were verified by sequencing.

Transcription and translation *in vitro*

The pGEM-N plasmids containing the cDNA inserts were linearized by digestion with *EcoRI* and used for the transcription of capped mRNA species *in vitro* by T7 RNA polymerase with mCAP kit (Stratagene). Translation *in vitro* in nuclease-treated rabbit reticulocyte lysates (Promega) and detection of [35 S]methionine-labelled products were performed as described previously [14]. The intensity of bands was measured with a BAS2000 analyser (Fuji). Relative frameshift efficiency was determined from the ratio of the product of the wild-type mRNA to that of the in-frame control. Polyamine concentrations in the lysate were determined by fluorimetry on HPLC [33].

Northern blot analysis

Poly(A)⁺ RNA was isolated from whole bodies of young adult zebrafish, 24 h zebrafish embryos or BRF41 cells with the use of a kit (Pharmacia). Northern blot analysis was performed as described previously [12]. The *AZS*- and *AZL*-specific hybridization probes were prepared by PCR amplification of fragments corresponding to nt 523–959 for *AZS* cDNA and nt 463–837 for *AZL* cDNA respectively (as numbered in Figure 1). The specificities of the probes were checked by a parallel run of the T7 transcripts from the pGEM-N-AZS and pGEM-N-AZL (2 ng per lane); no cross-hybridization was observed under the conditions used. A control hybridization probe, a cDNA fragment for elongation factor 1 α ('EF-1 α ') corresponding to the entire protein-coding region [34], was prepared by reverse-transcriptase-mediated PCR on poly(A)⁺ RNA from human liver.

Whole-mount RNA hybridization *in situ*

Embryos were fixed in 0.1 M sodium phosphate, pH 7.4, containing 4% (w/v) paraformaldehyde, 4% (w/v) sucrose and 0.12 mM CaCl₂ (fixation solution) at 4 °C overnight, and stored in methanol at –20 °C until use. Digoxigenin-labelled anti-sense RNA probes were prepared from *NcoI*-linearized pGEM-N-AZS and pGEM-N-AZL by transcription with SP6 RNA polymerase with a DIG RNA Labelling Kit (Boehringer-Mannheim). For sense-strand probes, *EcoRI* linearization and T7 RNA polymerase were used. Hybridization *in situ*, binding of the alkaline phosphatase-labelled antibody, and enzyme de-

© 2000 Biochemical Society

Figure 2 Comparison of mRNA sequences around the frameshift site

(A) Alignment of nucleotide sequences in the 5' vicinity of the frameshift site of various antizyme cDNA species [14,23,24,30,31]. The 7 nt frameshift site is indicated with an overbar. Conserved bases are highlighted. (B) Comparison of the potential pseudo-knots 3' adjacent to the shift site of rat, frog, AZS, AZL and human AZ2 mRNA species. Only one of the two pseudo-knot structures of mammalian AZ2 mRNA proposed by Ivanov et al. [31] is shown. Bases different from those in the rat sequence are highlighted in the frog and zebrafish pseudo-knots. The UGA termination codons of ORF1 are boxed.

RESULTS

Two distinct antizymes exist in zebrafish embryo

A cDNA library of 18–20 h zebrafish embryos was screened by cross-hybridization with *X. laevis* antizyme cDNA. In all, 17 positive clones were isolated out of 3.2×10^5 plaques screened. These were divided into two groups: 15 clones carried similar-sized inserts (approx. 1.2 kb) with identical restriction maps; the

other two clones had inserts of 1.8 and 2.1 kb respectively, sharing identical 3'-end sequences. One of the 1.2 kb clones (AZS) and the clone with the 2.1 kb insert (AZL) were sequenced entirely and used for further analyses.

The nucleotide sequences of AZS and AZL are shown in Figure 1. In both mRNA species, the 5'-most AUG codon is followed by an ORF with approx. 50 codons (ORF1), which overlaps a longer ORF (ORF2). ORF2 is in the +1 frame with

Figure 3 Alignment of amino acid sequences of various antizymes

Residues are numbered at the top on the basis of the rat sequence. Residues conserved in more than half of the sequences are highlighted.

Figure 4 Translation of T7 transcripts in a rabbit reticulocyte lysate

The wild-type (WT) and mutant (ΔT) mRNAs of both *AZS* and *AZL* were translated with or without the addition of 0.4 mM spermidine (Spd). ΔT mRNA is the in-frame construct with a single T base deletion at the stop codon of ORF1. The products were separated by 15% (w/v) PAGE and detected by autoradiography. Two major products are indicated by arrowheads at the right. The positions of molecular mass markers are indicated at the left.

respect to ORF1. No potential initiator AUG codon is found in the 5' region of ORF2. Thus the two mRNA species share the frame configuration common to the previously reported antizyme mRNA species from various organisms. The nucleotide sequences at both the 5' side and the 3' side of the UGA terminator codon of ORF1 are highly conserved (Figure 2). This region is known to contain the signals for translational frameshifting [14,30,35]. The 7 nt frameshift site is identical in all the reported antizymes. The 3' vicinity of the frameshift site of each zebrafish antizyme mRNA can be folded into a pseudo-knot structure, which is found in all vertebrate antizymes but not in fly antizyme [30]. These findings strongly suggest that +1 translational frame shifting at the end of the initiating frame is involved in the expression of the two zebrafish antizymes. Taking account of the +1 frameshifting at the last codon of ORF1, *AZS* mRNA encodes a protein of 214 residues with molecular mass of 24339 Da, and *AZL* mRNA encodes 218 residues with a molecular mass of 24229 Da (*AZL* protein is relatively rich in small amino acid residues). The amino acid identity of the two zebrafish antizymes is 51.8%. A strong sequence similarity is found in the 20 residues at the N-terminus of ORF1, the region corresponding to the frameshift signals, and approx. 90 residues in the C-terminal half of ORF2 (Figure 3). The latter two regions are also conserved in other vertebrate antizymes. The coding regions of *AZS* and *AZL* mRNA species share 64.2% nucleotide identity. Each mRNA contains the consensus signal for polyadenylation (AAUAAA) and the poly(A) tail. The greater size of the *AZL* cDNA is mainly attributed to its longer 3' UTR, which contains a monotonous region comprising approx. 200 repeats of a tetranucleotide motif, 5'-GATT-3', and its degenerate forms (Figure 1).

Translation *in vitro*

Frameshifting of the two zebrafish antizyme mRNA species was directly tested with cell-free translation in a rabbit reticulocyte lysate. The mRNA species transcribed from the wild-type *AZS* and *AZL* cDNA species were translated into two major products of approximate molecular masses 26 and 22 kDa in the presence of exogenously added spermidine (Figure 4). The 26 kDa band was less abundant than the 22 kDa band in the products from both mRNA species. Both of the bands were assigned as frame

Figure 5 Northern blot analysis of antizyme mRNA species

Poly(A)⁺ RNA (5 μ g) was separated by electrophoresis on a 1% (w/v) agarose/3.5% (v/v) formaldehyde gel and transferred to a nylon membrane. The blot was hybridized with specific probes for either *AZS* or *AZL* and autoradiography was performed. Subsequently the *AZS* probe was split from the blot, which was used for rehybridization with the elongation factor 1 α (*EF1 α*) probe. The positions of size markers are indicated (in kb). Abbreviations: A, adult fish; E, embryos; C, cultured cells.

shift products: their sizes were identical with those of the products from the in-frame control mRNA species in which the two ORFs were connected in the same frame by a single nucleotide deletion. The 22 kDa products probably originated from initiation at the internal AUG codons in ORF1, which is downstream of the 5'-most AUG, as previously observed for mammalian antizyme [14,39]. Potential internal initiators are located at codon 26 of *AZS* mRNA and at codons 30 and 41 of *AZL* mRNA (Figure 1). However, the second AUG of *AZS* mRNA does not match the preferred context for a translational initiator, whereas both the second and third AUGs of *AZL* mRNA do so [40]. The addition of spermidine to the lysate increased the products of wild-type mRNA species but not of their in-frame controls. Thus spermidine increases the frameshift efficiencies of both mRNA species as observed in the mammalian and *Drosophila melanogaster* antizyme mRNA species [14,17,30,31]. Without the addition of spermidine, the translation reaction mixtures contained 2.7 μ M putrescine, 504 μ M spermidine and 36 μ M spermine; under these condition the frameshift efficiencies of *AZS* and *AZL* mRNA species were 1.9% and 1.7% respectively. After the addition of 0.4 mM spermidine to the mixture, they increased to 14.8% and 12.2% respectively.

Expression of antizyme mRNA species in embryos, adult fish and cultured cells

The expression of antizyme mRNA species was examined by Northern blot analysis with probes specific for the two mRNA sequences. Figure 5 shows a typical result of the use of mRNA species from a pool of adult zebrafish ($n = 10$), a pool of 24 h embryos ($n > 50$) and cultures of a transformed zebrafish cell line. *AZS* and *AZL* mRNA species were detected as 1.3 kb and 2.1–2.2 kb bands respectively, in agreement with their cDNA sizes. The ratio of *AZS* mRNA to *AZL* mRNA in the adult fish was 1.8-fold that in the embryos. A similar result was obtained in an independent experiment. The two antizyme mRNA species were also detected in the cell line. The *AZS*-to-*AZL* mRNA ratio in the cells was intermediate between those in adults and embryos.

Figure 6 Detection of antizyme mRNA species in 24-h zebrafish embryos with hybridization *in situ*

(A) Lateral view with AZS sense probe (negative control). (B) Lateral view with AZL sense probe (negative control). (C) Lateral view with AZS anti-sense probe. (D) Lateral view with AZL anti-sense probe. (E) Dorsal view with AZS anti-sense probe, at higher magnification. (F) Dorsal view with AZL anti-sense probe, at higher magnification.

The *AZL* mRNA in the cells was slightly larger than that in the adult and embryo fish; this is likely to have been due to polymorphism involving the repeated sequences at the 3' UTR of *AZL* mRNA.

We further examined the expression patterns of the two mRNA species in zebrafish embryos by whole-mount RNA

Figure 7 Activities of GST-antizyme fusion proteins

(A) ODC-inhibitory activity of fusion proteins of GST with zebrafish antizyme. Rat ODC (3.6 units) was mixed with various quantities of the fusion proteins and assayed for activities. (B) Effects of zebrafish antizymes on ODC degradation *in vitro*. Labelled ODC (1.5 units; 2500 d.p.m. of ^{35}S) was added to the cell extract together with an equal activity of the GST-fused zebrafish antizymes or rat antizyme. In control reactions, either vehicle (None) or a non-fused GST protein was used. The amount added was 1.4 μg for GST-AZS, and 22.3 μg for GST-AZL and GST. Values are averages for two measurements.

hybridization *in situ*. In contrast with the negative control with sense probes (Figures 6A and 6B), the hybridization signals with anti-sense RNA probes of *AZS* and *AZL* were distributed in every tissue in embryos at 24 h after fertilization (Figures 6C and 6D). Both anti-sense probes revealed stronger signals in telencephalon, lens, retina, cerebellum and hindbrain primordia than in the other parts of the body. The only noticeable difference between *AZS* and *AZL* expression was in the rhombomere primordia, in which the *AZS* mRNA, but not the *AZL* mRNA, exhibited a segmental pattern in most of the embryos examined (Figures 6E and 6F).

Both antizymes inhibit ODC but only AZS accelerates ODC degradation *in vitro*

To test the activity of the two zebrafish antizymes, we expressed the antizyme cDNA species in *E. coli*. First, the cDNA species were expressed as non-fused proteins. Both *AZS* and *AZL*

products in the bacterial extracts showed ODC-inhibitory activities; the inhibition was reversed by the addition of rat antizyme inhibitor to the extract (results not shown). This confirms the specificity of the inhibition and indicates that the two antizymes bind not only to rat ODC but also to rat antizyme inhibitor.

Next, AZS and AZL were expressed in *E. coli* as GST fusion proteins and purified by affinity chromatography so that they could be tested in a degradation assay system *in vitro*. Both fusion proteins inhibited rat ODC in a dose-dependent manner, although the AZS fusion protein was much more active than the AZL fusion protein (Figure 7A). More than 90% of the ODC was inhibited with an excess amount of the fusion proteins in both cases. The effects of the fusion proteins on the ODC degradation were then tested by adding the same activity (to cause an inhibition of 0.9 unit of ODC) of GST-antizyme fusion proteins to a Chinese hamster ovary cell extract together with labelled ODC and ATP. As shown in Figure 7(B), when rat antizyme or zebrafish AZS was added to the extract, ODC degradation was accelerated from the basal level of approx. 15%/h to 60%/h. In contrast, the addition of AZL did not change the basal-level ODC degradation. To exclude the possibility that AZL was quickly degraded in the extract, the remaining AZL activity was measured as described in the Materials and methods section. It turned out that 66% of the initial amount of AZL still remained in the extract, even at the end of the reaction, indicating that the apparent lack of acceleration of ODC degradation *in vitro* by AZL is not explained by its instability. Thus the two zebrafish antizymes show different activities on ODC degradation *in vitro*.

DISCUSSION

We isolated two distinct, but related, antizyme cDNA species from zebrafish. A mammalian second antizyme has also been found in human and mouse [31]; their relationships to zebrafish antizymes are intriguing. Only AZS, but not AZL, has the activity to accelerate ODC degradation *in vitro*, which is common to mammalian AZ1 (Figure 7). Figure 8 shows an evolutionary tree drawn by the neighbour-joining method. It is suggested that AZ2 and AZL comprise one phylogenetic group and that all the other vertebrate antizymes belong to another. It should be noted that this is based only on ORF2, which carries the protein functions. In contrast, the amino-acid sequences encoded by ORF1 of both AZS and AZL are similar to that of AZ1: there

is more than one AUG codon and the first AUG is followed by a highly conserved region of approx. 20 residues (Figure 2). Mammalian AZ2 mRNA has a much shorter ORF1, with only one AUG codon and without the conserved N-terminal region. The putative pseudo-knot structures 3' of the frameshift site of the two zebrafish antizymes are also more similar to those of the AZ1 than to AZ2 pseudo-knot (Figure 2B). In fact, the phylogenetic analysis with the entire sequences (ORF1 and ORF2) of the vertebrate antizymes could not clearly show the evolutionary relationship between the isoforms (results not shown). These findings suggest that AZL is not a simple orthologue of mammalian AZ2. We have therefore used the terms AZS and AZL rather than AZ1 and AZ2 in this paper.

It is known that mammalian antizyme (AZ1) has at least three distinguishable activities. Those are: (1) binding to and inhibiting ODC, (2) the acceleration of ODC degradation by the 26 S proteasome, and (3) the inhibition of cellular polyamine uptake. Although both AZS and AZL inhibit ODC, only AZS accelerates ODC degradation *in vitro*. In these experiments we used rat ODC because a cDNA for zebrafish ODC that could be used in the degradation assay has not been cloned. The assay was performed in a crude cell extract and the observed basal level degradation might have been due to the endogenous antizyme in the extract. We cannot exclude the possibility that the added AZL had replaced the endogenous antizyme in the extract and weakly stimulated ODC degradation to the same basal level. Nevertheless, with the amount that caused the same ODC inhibition, the degradation activity of AZL was far less than that of AZS. The assay of ODC degradation *in vitro* has been proved to reflect the specificity *in vivo* [37]. Although the observation must be confirmed in experiments *in vivo*, we conclude that AZL lacks the activity to accelerate ODC degradation that is detected *in vitro* for mammalian AZ1 and zebrafish AZS.

Previous mutational analyses on mammalian antizyme suggested that distinct domains of ORF2 are involved in ODC regulation [19–21]. The proposed ODC-binding domain is encoded by a relatively distal part of ORF2, which is also essential for regulating the polyamine transporter [22]. A region of rat antizyme necessary for the acceleration of ODC degradation has been located in the N-terminal half of ORF2, although the precise residues are still contentious. This region, particularly residues 89–139 (as in the rat sequence), is one of the most poorly conserved segments of the various antizymes, and particularly diverges in AZL (see Figure 3). A comparison of the degradation-stimulating antizymes including AZS and degradation-non-stimulating AZL would provide useful information for identifying a critical element for the activity to accelerate ODC degradation.

What biological role(s) does AZL have? It might not function as an effective negative regulator of ODC. In mammalian cells the repression of ODC depends mainly on the acceleration of ODC degradation by antizyme in a recycling manner, but not on the inhibition of ODC, because the antizyme level is usually much lower than that of ODC [6]. Meanwhile, the observation that the synthesis of AZL is also induced by polyamines through translational frameshifting suggests that AZL might be involved in the regulation of some other polyamine-dependent gene expression or directly in the regulation of cellular polyamine levels. For these reasons the polyamine transporter might be a good candidate as the target for AZL.

We observed that the AZS-to-AZL mRNA ratio was significantly higher in the adult fish than in the 24 h embryos (Figure 5). This observation suggests distinctive roles for the two antizymes during development that are probably related to cellular proliferation or differentiation. The mechanism for the

Figure 8 Phylogenetic tree for antizymes from various sources

The tree was drawn by the neighbour-joining method on the peptide sequences at ORF2 of various antizymes from *Homo sapiens*, *Mus musculus*, *Drosophila melanogaster*, *Danio rerio*, *X. laevis*, *Gallus gallus* and *Rattus rattus* [14,23,24,26–28,30,31]. The numbers at the nodes are the bootstrap values with 10000 trials.

difference in the mRNA levels is also intriguing. It has been reported that certain growth- or differentiation-related stimuli alter the level of mammalian antizyme transcript [41,42]. Whole-mount hybridization *in situ* revealed that the two antizyme mRNA species are expressed ubiquitously in embryonic tissues and that the central nervous system and the eyes are particularly rich in both mRNA species. We found a slight but reproducible difference in the distribution of the two mRNA species in the rhombomere region (Figures 6E and 6F). Previous reports showed the presence of a large amount of antizyme in the brain of mammals [43]. Mammalian AZ2 was identified as being induced in mouse brain by pentyleneetetrazol, which causes epileptic seizure [44]. Further analyses might reveal specific roles for antizymes and the polyamines in brain.

During the revision of this manuscript, Zhu et al. [45] reported that human AZ2 is a negative regulator of ODC and polyamine transport but lacks an ODC-destabilizing effect in rabbit reticulocyte lysate.

We thank Dr. J. F. Atkins and I. P. Ivanov for discussion, for sharing unpublished results and for a critical reading of the manuscript, and also Dr. H. van Steeg for rat ODC cDNA, Dr. H. Mitani for BRF41 cells, and Dr. M. K. Chattopadhyay for comments on the manuscript. This work was supported by Grants-in-Aid from the Ministry of Education Science and Culture of Japan, and by the Human Frontier Science Program.

REFERENCES

- Cohen, S. S. (1998) in *A Guide to the Polyamines* (Cohen, S. S., ed.), pp. 184–207, Oxford University Press, New York.
- Heby, O. and Persson, L. (1990) *Trends Biochem. Sci.* **15**, 153–158.
- Hayashi, S. (1989) in *Ornithine Decarboxylase: Biology, Enzymology, and Molecular Genetics* (Hayashi, S., ed.), pp. 35–45, Pergamon Press, New York.
- Rinehart, Jr., C. A. and Chen, K. Y. (1984) *J. Biol. Chem.* **259**, 4750–4756.
- Heller, J. S., Fong, W. F. and Canellakis, E. S. (1976) *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1858–1862.
- Hayashi, S., Murakami, Y. and Matsufuji, S. (1996) *Trends Biochem. Sci.* **21**, 27–30.
- Murakami, Y., Matsufuji, S., Kameji, T., Hayashi, S., Igarashi, K., Tamura, T., Tanaka, K. and Ichihara, A. (1992) *Nature (London)* **360**, 597–599.
- Mitchell, J. L. A., Judd, G. G., Bareyal-Leyser, A. and Ling, S. Y. (1994) *Biochem. J.* **299**, 19–22.
- Suzuki, T., He, Y., Kashiwagi, K., Murakami, Y., Hayashi, S. and Igarashi, K. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 8930–8934.
- Murakami, Y., Matsufuji, S., Miyazaki, Y. and Hayashi, S. (1994) *Biochem. J.* **304**, 183–187.
- Iwata, S., Sato, Y., Asada, M., Takagi, M., Tsujimoto, A., Inaba, T., Yamada, T., Sakamoto, S., Yata, J., Shimogori, T. et al. (1998) *Oncogene* **18**, 165–172.
- Murakami, Y., Matsufuji, S., Ichiba, T. and Hayashi, S. (1996) *J. Biol. Chem.* **271**, 3340–3342.
- Matsufuji, S., Miyazaki, Y., Kanamoto, R., Kameji, T., Murakami, Y., Baby, T. G., Fujita, K., Ohno, T. and Hayashi, S. (1990) *J. Biochem. (Tokyo)* **108**, 365–371.
- Matsufuji, S., Matsufuji, T., Miyazaki, Y., Murakami, Y., Atkins, J. F., Gesteland, R. F. and Hayashi, S. (1995) *Cell* **80**, 51–60.
- Miyazaki, Y., Matsufuji, S. and Hayashi, S. (1992) *Gene* **113**, 191–197.
- Gesteland, R. F., Weiss, R. B. and Atkins, J. F. (1992) *Science* **257**, 1640–1641.
- Rom, E. and Kahana, C. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 3959–3963 [erratum (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9195].
- Gesteland, R. F. and Atkins, J. F. (1996) *Annu. Rev. Biochem.* **65**, 741–768.
- Li, X. and Coffino, P. (1994) *Mol. Cell. Biol.* **14**, 87–92.
- Ichiba, T., Matsufuji, S., Miyazaki, Y., Murakami, Y., Tanaka, K., Ichihara, A. and Hayashi, S. (1994) *Biochem. Biophys. Res. Commun.* **200**, 1721–1727.
- Mamroud-Kidron, E., Omer-Itsicovich, M., Bercovich, Z., Tobias, K. E., Rom, E. and Kahana, C. (1994) *Eur. J. Biochem.* **226**, 547–554.
- Sakata, K., Fukuchi-Shimogori, T., Kashiwagi, K. and Igarashi, K. (1997) *Biochem. Biophys. Res. Commun.* **238**, 415–419.
- Tewari, D. S., Qian, Y., Thornton, R. D., Pieringer, J., Taub, R., Mochan, E. and Tewari, M. (1994) *Biochim. Biophys. Acta* **1209**, 293–295.
- Ichiba, T., Matsufuji, S., Miyazaki, Y. and Hayashi, S. (1995) *Biochim. Biophys. Acta* **1262**, 83–86.
- Hayashi, S., Matsufuji, S. and Hayashi, S. (1997) *Gene* **203**, 131–139.
- Kankare, K., Mikko, U. and Jänne, O. A. (1997) *Biochem. J.* **324**, 807–813.
- Nilsson, J., Koskineemi, S., Persson, K., Grahn, B. and Holm, I. (1997) *Eur. J. Biochem.* **250**, 223–231.
- Drozdowski, B., Gong, W. and Lomax, M. I. (1998) *Biochim. Biophys. Acta* **1396**, 21–26.
- Salzberg, A., Golden, K., Bodmer, R. and Bellen, H. J. (1996) *Genetics (Princeton)* **144**, 183–196.
- Ivanov, I. P., Simin, K., Lestou, A., Atkins, J. F. and Gesteland, R. F. (1998) *Mol. Cell. Biol.* **18**, 1553–1561.
- Ivanov, I. P., Gesteland, R. F. and Atkins, J. F. (1998) *Genomics* **52**, 119–129.
- Inoue, A., Takahashi, M., Hata, K., Hotta, Y. and Okamoto, H. (1994) *Dev. Dyn.* **199**, 1–11.
- Murakami, Y., Nishiyama, M. and Hayashi, S. (1989) *Eur. J. Biochem.* **180**, 181–184.
- Brands, J. H. G. M., Maassen, J. A., van Hemert, F. J., Amons, R. and Moeller, W. (1986) *Eur. J. Biochem.* **155**, 167–171.
- Matsufuji, S., Matsufuji, T., Wills, N. M., Gesteland, R. F. and Atkins, J. F. (1996) *EMBO J.* **15**, 1360–1370.
- Murakami, Y., Marumo, M. and Hayashi, S. (1988) *Biochem. J.* **254**, 367–372.
- Murakami, Y., Tanaka, K., Matsufuji, S., Miyazaki, Y. and Hayashi, S. (1992) *Biochem. J.* **283**, 661–664.
- Van Steeg, H., Van Oostrom, C. T. M., Hodemaekers, H. M., Peters, L. and Thomas, A. A. M. (1991) *Biochem. J.* **274**, 521–526.
- Mitchell, J. L. A., Judd, G. G., Leyser, A. and Choe, C.-Y. (1998) *Biochem. J.* **329**, 453–459.
- Kozak, M. (1989) *J. Cell. Biol.* **108**, 229–241.
- Pujic, Z., Matsumoto, I., Yamataka, A., Miyano, T. and Wilce, P. (1996) *Life Sci.* **58**, 2289–2296.
- Yang, D., Hayashi, H., Takii, T., Mizutani, Y., Inukai, Y. and Onozaki, K. (1997) *J. Biol. Chem.* **272**, 3376–3383.
- Hietala, O. A. (1983) *J. Neurochem.* **40**, 1174–1177.
- Kajiura, K., Nagawawa, H., Shimizu-Nishikawa, K., Ookura, T., Kimura, M. and Sugaya, E. (1996) *Biochem. Biophys. Res. Commun.* **219**, 795–799.
- Zhu, C., Lang, D. W. and Coffino, P. (1999) *J. Biol. Chem.* **274**, 26425–26430.

Received 23 July 1999/14 October 1999; accepted 12 November 1999