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Characterization and Expression of Embryonic and Adult Globins of the Teleost *Oryzias latipes* (Medaka)[1]

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Using the teleost *Oryzias latipes* (medaka), we isolated three embryonic globin cDNAs (em.α-0, em.α-1, and em.β-1) from the embryos 5 days after fertilization (at 30°C) and two adult globin cDNAs (ad.α-1 and ad.β-1) from the kidney of the fully-grown adult fish, and predicted their amino acid sequences. Molecular phylogenetic analysis showed that the embryonic globins were highly homologous in amino acid sequence to the embryonic globins previously identified in rainbow trout and zebrafish, and that they formed a monophyletic group among the teleostean globin molecules. They were clearly discriminated from the adult globin of the medaka. RT-PCR analysis showed that the embryonic globin mRNAs were intensely expressed in stage 30 and 38 embryos and in young fish 30 days after hatching. The level of expression decreased drastically after the young fish stage, and was low in fully-grown adult fish. The adult α globin mRNA ad.α-1 was scarcely expressed in the embryos, and the level of expression gradually increased in young to fully-grown adult fish. Unexpectedly, the adult β globin mRNA ad.β-1 was expressed throughout life, from the early embryonic stage to the fully-grown adult stage. This expression profile was quite different from that of the rainbow trout previously investigated. Some globins of the medaka were expressed both in primitive hematopoiesis and in definitive hematopoiesis.

Key words: embryonic globin, globin switching, hemoglobin, medaka, teleost.

Hematopoiesis in vertebrates generally occurs in two distinct phases, termed primitive hematopoiesis and definitive hematopoiesis, based on difference in site, timing and potency of progenitor cell differentiation, morphology of differentiated blood cells, and on molecular species of expressed globins (*1*). Primitive erythroid cells, the only type of blood cells produced in early-stage embryos, circulate in the embryos for a short term, then disappear. Definitive erythrocytes emerge after the primitive and continue for the remainder of life. In the primitive hematopoiesis, erythroid cells having embryonic or larval hemoglobin differentiate, while in the definitive, erythrocytes having fetal or adult hemoglobin differentiate (*2*). Thus, hemoglobin switching occurs during the transition from primitive to definitive hematopoiesis.

Among teleosts, the switch from embryonic to adult hemoglobin during development has been well investigated in rainbow trout, *Oncorhynchus mykiss* (*3*). Hemoglobin of embryos immediately after hatching was distinctly different from that of 2-year-old adult fish in molecular properties such as stability, electrophoretic mobility and antigenicity, and physiological property such as oxygen affinity (*4–6*). The former was originally named larval hemoglobin, and recently renamed embryonic hemoglobin (*7*), while the latter is adult hemoglobin. Recently, reverse-phase HPLC analysis showed that the embryonic hemoglobin consisted of eight globins with different retention times from the eight globins constituting the adult hemoglobin. The primary structures of two α globins and two β globins of the embryonic hemoglobin were predicted from their cDNAs (*7*). They were clearly distinguishable from the previously characterized adult globins of rainbow trout (α globin of HbI, *8*; β globin of HbI, *9*; α globin of HbIV, *10*; β globin of HbIV, *11*). Recently, an embryonic β globin cDNA has been identified in zebrafish, *Danio rerio*, and investigated (*12*).

During the development of rainbow trout, the embryonic hemoglobin/globins appeared first in the intermediate cell mass beneath the notochord of the embryos 6–7 days after fertilization, and shortly after that, in the blood islands on the posterior part of the yolk sac (*7*). Circulating erythroid cells of the pre-hatching embryos had exclusively these embryonic globins. The adult globins did not appear until after hatching. Thus, the switching of globin expression in developing rainbow trout is characterized in terms of "post-hatching switching of expression from embryonic to adult globins." During this globin switching, round disc-like erythroid cells were replaced by elliptic disc-like cells (*13–15*). The former were termed embryonic erythrocytes, while the latter were termed adult erythrocytes. It is probable that they are closely associated to primitive and definitive hematopoiesis, respectively.

In the present study, we cloned globin cDNAs from embryos and fully-grown adults of another teleost, the medaka (*Oryzias latipes*), characterized them, and investigated their developmental expression by RT-PCR. Changes of globin mRNA expression during the development of meda-

[1] The nucleotide sequences of the cDNAs reported in the present paper will appear in the DDBL/EMBL/GenBank nucleotide sequence databases with accession number, AB026052, AB080117, AB080118, AB080119, and AB080120.

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ka were very complicated, and were different from those in rainbow trout and zebrafish.

MATERIALS AND METHODS

Animals—Embryos and fully-grown adults of commercially available medaka, *Oryzias latipes*, were employed. The adult fish were fed on Tetra Fin (Tetra Werke) as described elsewhere (*16*). The embryos were allowed to develop until hatching (day 6) in a shaking incubator at 30°C. The developmental stages of embryos were determined according to Iwamatsu (*17*).

Preparation of Hemoglobin—Occurrence of embryonic hemoglobin in medaka has not been investigated to date.

TABLE I. **Globin sequences used in the present analysis.**

Taxon and species	Hemoglobin and subunit	Abbreviation	Accession numbers or references
Non-teleost adult hemoglobin (outgroup):			
Homo sapiens	HbA; α, β	Human	NP_000549, AAA16334
Gallus gallus	α	Chick	HACH2
Chrysemys picta bellii	α	Turtle	P13273
Rana catesbeiana	α	Bullfrog	B49296
Squalus acanthias	α	Dogfish	P07408
Latimeria chalumnae	α, β	Coelacanth	S15411, P23741
Adult hemoglobin in teleosts:			
Anguilla anguilla	HbA; α, β	*A. anguilla*	P80945, P80946
	HbC; α, β		P80726, P80727
Muraena helena	HbI; β	*M. helena*	S67981
	HbIII; β		S67982
Electrophorus electricus	α, β	*E. electricus*	P14520, P14521
Carassius auratus	α, β	*C. auratus*	P02018, P02140
Cyprinus carpio	β	*C. carpio*	P02139
	α		BAA20510
Danio rerio	aA1	*D. rerio*	AAB05404
	bA1		AAB05403
	bA2		AAB05402
Catostomus clarki	α	*C. clarki*	P02017
Hoplosternum littorale	HbC; α, β	*H. littorale*	P82315, P82316
Oncorhynchus mykiss	HbI; α, β	*O. mykiss*	P02019, P02142
	HbIV; α, β		S03995, 1009195A
	β		BAA11632
Salmo salar	β	*S. salar*	Q91473
	Clone 3α		CAA65949
	Clone 4β		CAA65950
Oryzias latipes	ad.α-1	*O. latipes*	AB080119
	ad.β-1		AB080120
Chrysophrys auratus	α, β	*Chr. auratus*	Stam *et al.* (1997) (*23*)
Seriola quinqueradiata	HbA; α, β	*S. quinqueradiata*	BAA86218, BAA86220
Thunnus thynnus	α, β	*T. thynnus*	P11748, P11749
Sparus aurata	β	*S. aurata*	CAB83257
Decapterus maruadsi	β	*D. maruadsi*	AAB50798
[Major hemoglobin in antarctic fishes]			
Pseudaphritis urvillii	Hb1; α, β	*P. urvillii*	Stam *et al.* (1997) (*23*)
Pleuragramma antarcticum	Hb1+3; α, β	*P. antarcticum*	Stam *et al.* (1997) (*23*)
Notothenia coriiceps	α, β	*N. coriiceps*	I51011, AAC60372
Notothenia angustata	Hb1+2; α, β	*N. angustata*	AAB24974, P29628
Trematomus newnesi	Hb1+2; α, β	*T. newnesi*	P45718, P45720
Trematomus hansoni	β	*T. hansoni*	AAC41389
Cygnodraco mawsoni	Hb1; α, β	*C. mawsoni*	P23016, P23017
Racovitzia glacialis	α, β	*R. glacialis*	Stam *et al.* (1997) (*23*)
Bathydraco marri	α, β	*B. marri*	A56898, B56898
Gymnodraco acuticeps	α, β	*G. acuticeps*	P29623, AAC41386
[Minor hemoglobin in antarctic fishes]			
Pseudaphritis urvillii	Hb2; β	*P. urvillii*	Stam *et al.* (1997) (*23*)
Pleuragramma antarcticum	Hb2; β	*P. antarcticum*	Stam *et al.* (1997) (*23*)
	Hb3; α		Stam *et al.* (1997) (*23*)
Trematomus newnesi	HbC; β	*T. newnesi*	P45721
	Hb2; α		P45719
Trematomus bernacchii (=*Pagothenia bernacchii*)	HbC; β	*T. bernacchii*	P45722
Cygnodraco mawsoni	Hb2; β	*C. mawsoni*	P23018
Embryonic hemoglobin in teleosts:			
Danio rerio	E1; β	*D. rerio*	AAC62069
Oncorhynchus mykiss	E1; α, β	*O. mykiss*	BAA34948, BAA34950
	E2; α, β		BAA34949, BAA34951
Oryzias latipes	em.α-1	*O. latipes*	BAA85018
	em.α-1		AB080117
	em.β-1		AB080118

Therefore, based on a preceding finding that rainbow trout embryos immediately after hatch have embryonic hemoglobin different from that of adult fish (*4, 5*). About 2,000–3,000 embryos of medaka immediately after hatching were used for the preparation of embryonic hemoglobin, while adult hemoglobin was prepared from fully-grown adult medaka. After cutting the tails in isotonic saline (0.143 M NaCl) containing 0.5% sodium citrate, blood cells were collected by centrifugation at 3,000 ×*g* for 5 min, and washed several times with the isotonic saline. The cells were suspended in 2 volumes of distilled water by vigorous vortexing, then centrifuged at 28,000 ×*g* for 15 min. The supernatant, hemolysate, was used for the analysis by high performance liquid chromatography (HPLC), or for the preparation of antibody. The amount of hemoglobin was determined by the method of Suzuki *et al.* (*18*).

Fractionation of Hemolysate by Reverse-Phase HPLC, and Determination of Partial Amino Acid Sequences—Hemolysate containing about 50 μg of hemoglobin was applied to a Senshu Pak VP-304 column (Senshu Scientific, Tokyo) equilibrated with 28% acetonitrile–0.15% trifluoroacetic acid (TFA). Elution was performed with a gradient of 28 to 80% acetonitrile containing 0.15% TFA under the HPLC system. Protein was monitored by measuring absorbance at 215 nm. Fractions were pooled, lyophilized, and subjected to SDS-PAGE, immunoblotting, and protein sequencing with an automatic gas-phase protein sequencer, PSQ-1 (Shimadzu, Kyoto).

Preparation of Antibody against Embryonic or Adult Hemoglobin—Male mice (BALB/c, 8-week-old) were immunized with hemolysate containing 250 μg of medaka adult hemoglobin essentially according to the method of Tung *et al.* (*19*). The immunogen emulsified with 9 volumes of complete Freund's adjuvant (Difco, Detroit) was intraperitoneally injected once a week. About 5 months later, ascitic fluid was collected and stored at 4°C. The ascites containing an adequate amount of antibody was employed for screening cDNAs for adult globins.

Because of the difficulty in obtaining a sufficient amount of pure erythroid cells from pre-hatching embryos, we could not prepare an antibody against medaka embryonic hemoglobin. Therefore, we used a previously prepared antibody against rainbow trout embryonic hemoglobin to screen cDNAs for embryonic globins (*7*). Molecular phylogenetic analysis showed that embryonic globins of medaka were highly homologous to embryonic globins of rainbow trout (Figs. 3 and 4).

SDS-PAGE and Western Blot Analysis—Hemolysate or globin fractionated by HPLC was added to a buffer consisting of 1% SDS, 0.03 M Tris-HCl (pH 6.8), 3% mercaptoethanol, and 0.5% glycerol, and boiled for 5 min. The sample was loaded on a 15% SDS-PAGE gel and electrophoresed according to the method of Laemmli (*32*). After the electrophoresis, protein bands were electrotransferred onto a nitrocellulose filter. The filters were treated with 1% BSA dis-

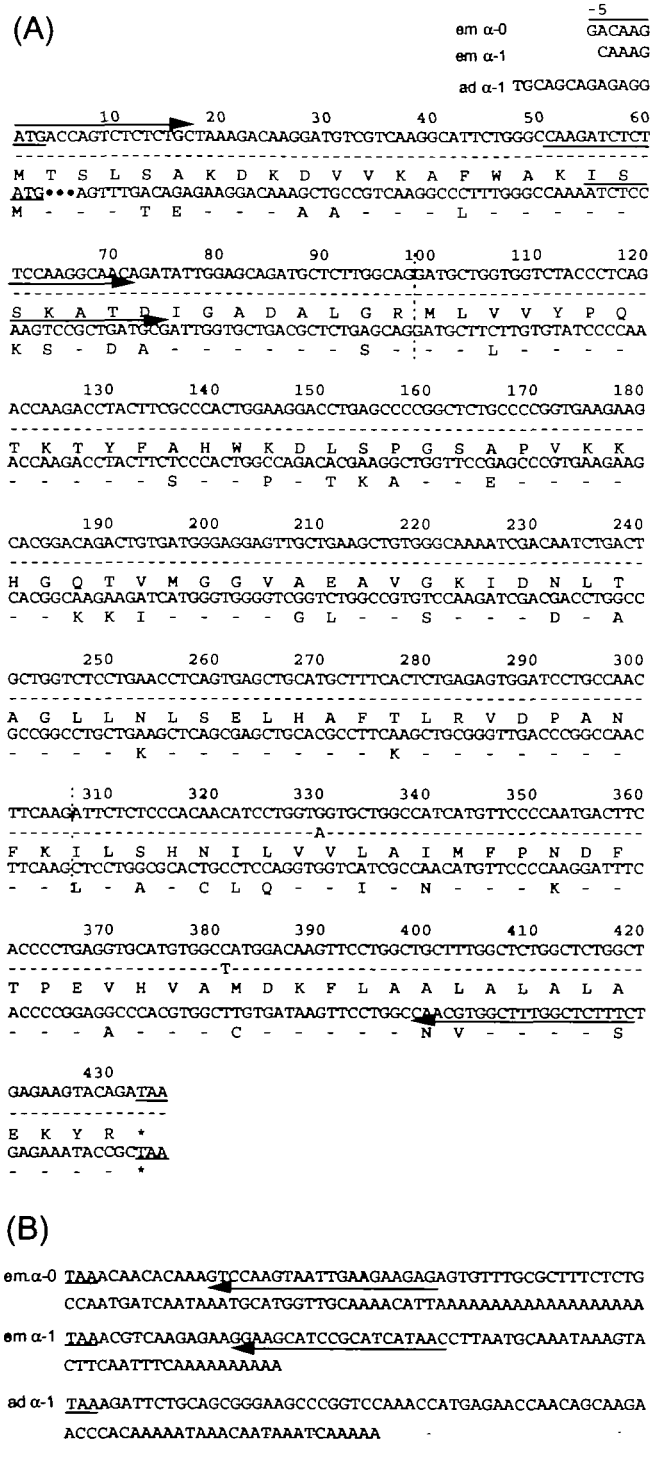

Fig. 1. **Nucleotide sequences of cDNAs for α globins em.α-0, em.α-1, and ad.α-1, and amino acid sequences predicted from them.** (A) Nucleotide sequences of em.α-0, em.α-1, and ad.α-1 from 5′-non-coding regions to stop codons, and the predicted amino acid sequences. The cDNAs of em.α-0 (first line) and em.α-1 (second line) differed in only two nucleotides, while their amino acid sequences were identical (third line). The fourth and fifth lines are the nucleotide sequence of ad.α-1 and its predicted amino acid sequence, respectively. Identical nucleotides and amino acids are shown by hyphens. The initiation codon and stop codon are underlined. Vertical broken lines indicate the positions of exon-intron junctions deduced from many other globin genes. Arrows show the positions of forward primers for the RT-PCR analysis of em.α-0, or em.α-1, and the positions of forward or reverse primer of ad.α-1. (B) Nucleotide sequences of 3′-non-coding regions from stop codon to poly A tail. Stop codons and poly A additional signals are underlined. Arrows show the positions of reverse primers for the RT-PCR analysis for em.α-0 or em.α-1.

solved in TBST (50 mM Tris-HCl, pH 7.0, 0.15 M NaCl, 0.05% Tween-20) overnight at 4°C, incubated with antibody against medaka adult hemoglobin (1:200 diluted in TBST and 1% BSA) at room temperature for 1 h, and washed three times with TBST. Protein bands on the filters were visualized with an ABC alkaline phosphatase system (Vectastain ABC kit, Vector Lab., Burlingame). In some cases, the protein bands on the SDS-PAGE gels were detected according to the silver staining method.

Isolation and Sequencing of cDNA—Whole embryos 5 days after fertilization or adult kidneys were homogenized with 3 volumes of Isogen (Nippon Gene, Toyama) using a blender. Total RNA was extracted from the homogenate according to an instruction manual. A cDNA library was constructed using λgt-11 phage as a vector. We screened embryonic and adult globin cDNAs with the antibody against embryonic hemoglobin of rainbow trout and the antibody against adult hemoglobin of medaka, respectively. The inserted cDNAs in the phage vector were sequenced and their amino acid sequences were deduced.

Molecular Phylogenetic Analysis of Globin—As shown in Table I, we obtained amino acid sequences of α or β globins of various animals from the DDBJ/EMBL/GenBank database, and aligned them using the program CLUSTAL W version 1.7 (*20*). Distances based on amino acid substitutions were determined with a transition probability matrix for the JTT-F model (*21*), then the phylogenetic relationship between embryonic or adult globins of medaka and those of other teleost globins was analyzed with the RELL algorithm packaged in the program MOLPHY version 2.3 (*22*). The analysis was performed according to the neighbor-joining method, and the maximum likelihood method.

Reverse Transcription-PCR (RT-PCR) Assay—To examine globin gene expression, we designed specific primers for each cDNA and performed an RT-PCR assay. Blood cells were collected from stage 30 embryos, stage 38 embryos, young fish 30 days after hatching and fully-grown adult fish, and counted with a counting chamber (Erma, Tokyo). One hundred red blood cells were directly added to the reaction mixture of a Qiagen One Step RT-PCR kit (Qiagen), and the RT-PCR was performed. The forward (f) and the reverse (r) primers for cDNAs, em.α-0, em.α-1, em.β-1, ad.α-1, or ad.β-1, were as follows:

em.α-0(f): 5′-GACAAGATGACCAGTCTCTCTGC-3′
em.α-0(r): 5′-CTCTTCTTCAATGACTTGGAC-3′
em.α-1(f): 5′-CAAGATCTCTTCCAAGGCAACA-3′
em.α-1(r): 5′-GTAATGATGCGGATGCTTCC-3′
em.β-1(f): 5′-GGATGTTGTTGGACCTGCTGCTCTCT-3′
em.β-1(r): 5′-TCTGGAAAGCTGCCTGGACGTCGCA-3′
ad.α-1(f): 5′-ATCTCCAAGTCCGCTGATGCG-3′
ad.α-1(r): 5′-GAAAGAGCCAAAGCCACGTTG-3′
ad.β-1(f): 5′-TCATCACCAACATCTTCGGCAACC-3′
ad.β-1(r): 5′-AGAACTTCTGGAAGGTTGCCTGAATC-3′

The positions of these primers in the cDNAs are shown in Figs. 1 and 2.

To discriminate between PCR products amplified from mRNAs and those from genomic genes, we designed a pair of forward and reverse primers from the sequences encompassing the position of exon-intron junction deduced from globin genes of other animals. The positions are highly conserved and are marked in Figs. 1 and 2. This was confirmed by a preliminary study on globin gene structure in medaka genome (Maruyama *et al.*, unpublished data). In addition, to distinguish em.α-0 mRNA from em.α-1 mRNA, we constructed the RT-PCR primers from 5′- and 3′-non-coding regions as shown in Fig. 1, A and B. The expected sizes of the PCR products amplified from the globin mRNAs em.α-0, em.α-1, em.β-1, ad.α-1, and ad.β-1 were 472, 417, 334, 365, and 372 bp, respectively. Some of these products were confirmed to correspond to the respective cDNAs by nucleotide sequencing or by restriction enzyme digestion. These products are shorter than the PCR products amplified from genomic globin genes, as shown in Fig. 5.

The reactions were performed according to the instruction manual for the Qiagen One Step RT-PCR kit; first, 50°C for 30 min and 94°C for 15 min; then, 25 or 35 cycles of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. Five micro-

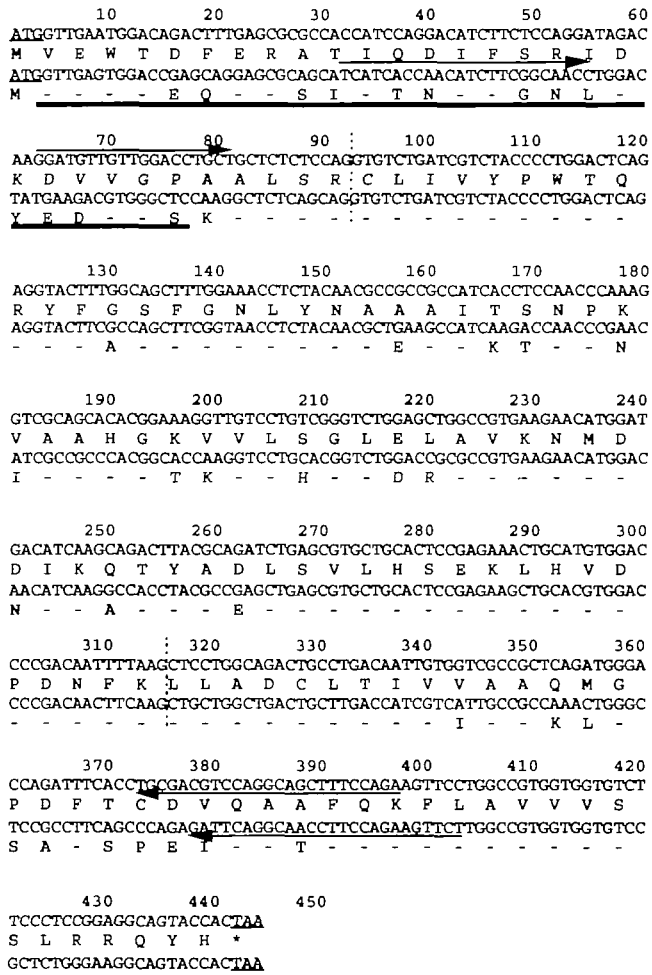

Fig. 2. **Nucleotide sequences of cDNAs for β globins em.β-1 or ad.β-1, and amino acid sequences predicted from them.** The first and the second lines are nucleotide sequence of em.β-1 and amino acid sequence predicted from it, respectively. The third and the fourth lines are nucleotide sequence of ad.β-1 and its predicted amino acid sequence, respectively. Identical amino acids are shown by hyphens. Initiation and stop codons are underlined. The bold line under the amino acid sequence of ad.β-1 shows a region identical to the sequence of the globin e6 or a4, which was fractionated by HPLC and determined by protein sequencing (Fig. 6). Vertical broken lines indicate the positions of exon–intron junctions deduced from many other globin genes. Arrows show the positions of forward and reverse primer for the RT-PCR analysis of em.β-1 or ad.β-1.

liters of the PCR product was electrophoresed on 2% agarose gel containing ethidium bromide (50 μg/liter).

RESULTS

Characterization of cDNAs for Embryonic and Adult Globin of Medaka—cDNA for embryonic globin of medaka was immunologically screened and isolated from the cDNA library of whole 5-day embryos (30°C), while adult globin cDNA was from the cDNA library of adult kidney. We cloned two α globin cDNAs (em.α-0, em.α-1) and one β globin cDNA (em.β-1) from the former library, and one α globin cDNA (ad.α-1) and one β globin cDNA (ad.β-1) from the latter library.

Nucleotide sequences of the α and β globin cDNAs and amino acid sequences predicted from them are indicated in Figs. 1 and 2, respectively. Only two nucleotide substitutions were observed in coding regions of the em.α-0 and em.α-1 cDNAs, and amino acid sequences predicted from the cDNAs were identical to each other (Fig. 1A). Their 5′-non-coding sequences and 3′-non-coding sequences from stop codon to poly (A) tail were different from each other (Fig. 1, A and B).

Amino acid sequences of the globins were highly congruent in frame with those of other teleostean and human globins. One or two deletions/insertions were found in the sequences. As shown by bold type in Fig. 3, the embryonic α and β globins of medaka closely resemble those of rainbow trout and zebrafish (amino acid identity, 73.3–84.5%). The adult globins of medaka were more similar to the embryonic globins of medaka itself than they were to the adult globins of rainbow trout and zebrafish. For example, the amino acid identities of medaka adult β globin ad.β-1 to embryonic globins of medaka em.β-1, zebrafish β_{E1}, rainbow trout em.β-1 and em.β-2 were 72.6, 72.6, 72.6, and 76.7%, respectively, while its identities to adult globins of zebrafish β_{A1}, rainbow trout ad.β-1 and rainbow trout ad.β-4 were 63.9, 60.0, and 63.9%, respectively. With one exception (medaka ad.α-1 *versus* zebrafish ad.α), this tendency was also found among α globin molecules of these teleost species. The identities of teleost to human globins were considerably low (44.8 to 55.9%). Such similarity or difference was further investigated by molecular phylogenetic analysis.

Molecular Phylogenetic Analysis of Medaka Globins—Using the maximum likelihood method (MOLPHY version 2.3), we examined molecular phylogenetic relationship between medaka globins sequenced in the present study and other teleostean globins previously investigated. The distances based on amino acid substitutions were determined with transition probability matrix for the JTT-F model. An approximate tree topology was constructed with the neighbor-joining method, and the final topology was determined by the RELL method. Bootstrap values in the trees were higher than 50%.

As shown in Fig. 4, A and B, embryonic α and β globins of rainbow trout and zebrafish were positioned within one branch of an α or β phylogenetic tree, and they formed a monophyletic group. It is interesting that α or β globins constituting minor hemoglobin of Antarctic fishes (Notothenioidei) were positioned in this branch. We tentatively named these branches "embryonic globin branches." In addition, embryonic α and β globins of the medaka, em.α-0/1 and em.β-1, belonged to these respective embryonic globin branches. The embryonic globin branches were clearly discriminated from the adult globin groups. Teleostean embryonic globin molecules may be orthologous to each other.

Of the α or β globins constituting adult hemoglobin of various teleosts, at least three branches were discriminated: the first group included α or β globins of *A. anguilla* HbC, *H. littorale* HbC, and *O. mykiss* HbI; the second group, *A. anguilla* HbA, *O. mykiss* HbIV, *C. carpio*, *C. auratus*, and *D. rerio*; and the third group, major hemoglobin of Antarctic fishes. The first group consists of molecules having little or no Bohr effect and migrating toward the cathode on electrophoresis. The second comprises molecules having high Bohr effect and migrating toward the anode on electrophoresis. The major hemoglobin of Antarctic fishes in the third branch is characterized by molecules diversifying under the cold (subzero temperature) adaptation.

Adult α globin of the medaka ad.α-1 was placed in the group consisting mainly of α globins of major adult hemoglobin of Antarctic fishes (Fig. 4A), and was clearly distinguishable from α globins in the embryonic globin branch. On the other hand, the position of adult β globin of medaka ad.β-1 in the phylogeny was closely related to that of the

	medaka em.α-0/1	r. trout em α-1	r. trout em.α-2	medaka ad.α-1	zebrafish α_{A1}	r. trout ad.α-1	r. trout ad.α-4	human ad.α	
medaka em.β-1		**76.1**	**78.2**	71.8	72.7	70.8	64.1	51.8	medaka em.α-0/1
zebrafish β_{E1}	**81.5**		**84.5**	66.9	62.7	64.6	60.6	50.4	r. trout em α-1
r. trout em.β-1	**82.2**	**80.8**		68.3	66.2	66.7	63.4	50 4	r. trout em.α-2
r. trout em.β-2	**74.0**	**73.3**	**75.3**		75.4	63.2	63.4	51.8	medaka ad.α-1
medaka ad.β-1	72.6	72.6	72.6	76 7		63 9	59.9	55 3	zebrafish α_{A1}
zebrafish β_{A1}	66.0	68.7	67.3	63 3	63.9		61 8	55.9	r. trout ad.α-1
r. trout ad.β-1	58.6	63.4	60.0	76.7	60.0	63.0		50.4	r trout ad.α-4
r. trout ad.β-4	64.6	66.0	67.3	74.0	63.9	78.2	59.6		human ad.α
human ad.β	47.1	49.3	46.6	44.8	46.6	51.0	53.1	51.7	
	medaka em.β-1	zebrafish β_{E1}	r. trout em.β-1	r. trout em.β-2	medaka ad.β-1	zebrafish β_{A1}	r. trout ad.β-1	r. trout ad.β-4	human ad.β

Fig. 3. **Homology of embryonic and adult globins in some teleosts.** Amino acid sequences of embryonic globins em.α-0/1 and em.β-1 and adult globins ad.α-1 and ad.β-1 of medaka determined in the present study were compared with those of embryonic or adult globins of rainbow trout (r. trout) and zebrafish previously determined. Percentages of identical amino acids in the full-length sequences are represented. Upper and lower triangle matrices are α globins and β globins, respectively. Human α and β globins are included as references. The abbreviations "em." and "ad." indicate "embryonic" and "adult," respectively. The amino acid identities between embryonic globins of the three teleostean species are represented in bold type.

embryonic branch (Fig. 4B), *i.e.*, both groups mainly consisting of major adult hemoglobin of Antarctic fishes and the embryonic β globin group formed two sub-groups of the medaka ad.β-1. However, it is reasonable to conclude that the ad.β-1 was clearly discriminated from β globins of the embryonic globin branch.

Although further investigations are necessary, the phylogenetic trees of α or β globins suggest that at least four paralogous groups of globin molecules diversified during the evolution of teleosts. This has in part been evidenced by analysis using maximum parsimony (*23*).

Expression of Embryonic or Adult Globin mRNAs during the Development of Medaka—The developmental expression of globin mRNA was investigated by RT-PCR. It was difficult to obtain a sufficient amount of blood cells for the purification of RNA, especially from embryos at the stage 30 or 38. Therefore, one hundred blood cells were directly added to the reaction mixture for RT-PCR. PCR fragments having the expected sizes were obtained in each reaction (Fig. 5).

A preliminary study suggested that during the development of medaka, globin mRNAs first appeared in the intermediate cell mass (corresponding to "blood island" in Iwamatsu's terminology) beneath the notochord at the embryonic stage 23 (Maruyama *et al.*, unpublished data). The embryonic α globin mRNAs em.α-0 and em.α-1 were strongly expressed in embryos at the stage 30 and the pre-hatching stage 38. In the stage 30 embryos, blood cells circulate in main blood vessel on the yolk sac, ducts of Cuvier, and in the trunk region having about 35 somites, while in the pre-hatching stage 38 embryos, the spleen becomes well developed (*17*). The expression of the 2 mRNAs persisted to 30 days after hatch, and drastically decreased after that. The em.α-0 was slightly detected in fully-grown adult fish, while the em.α-1 was not. The embryonic β-globin mRNA em.β-1 was strongly expressed in the stage 30 and 38 embryos, like the embryonic α-globin mRNAs, and the expression persisted to the young fish stage. However, the expression was not observed in fully-grown adult fish. Although further investigations are necessary, the expression

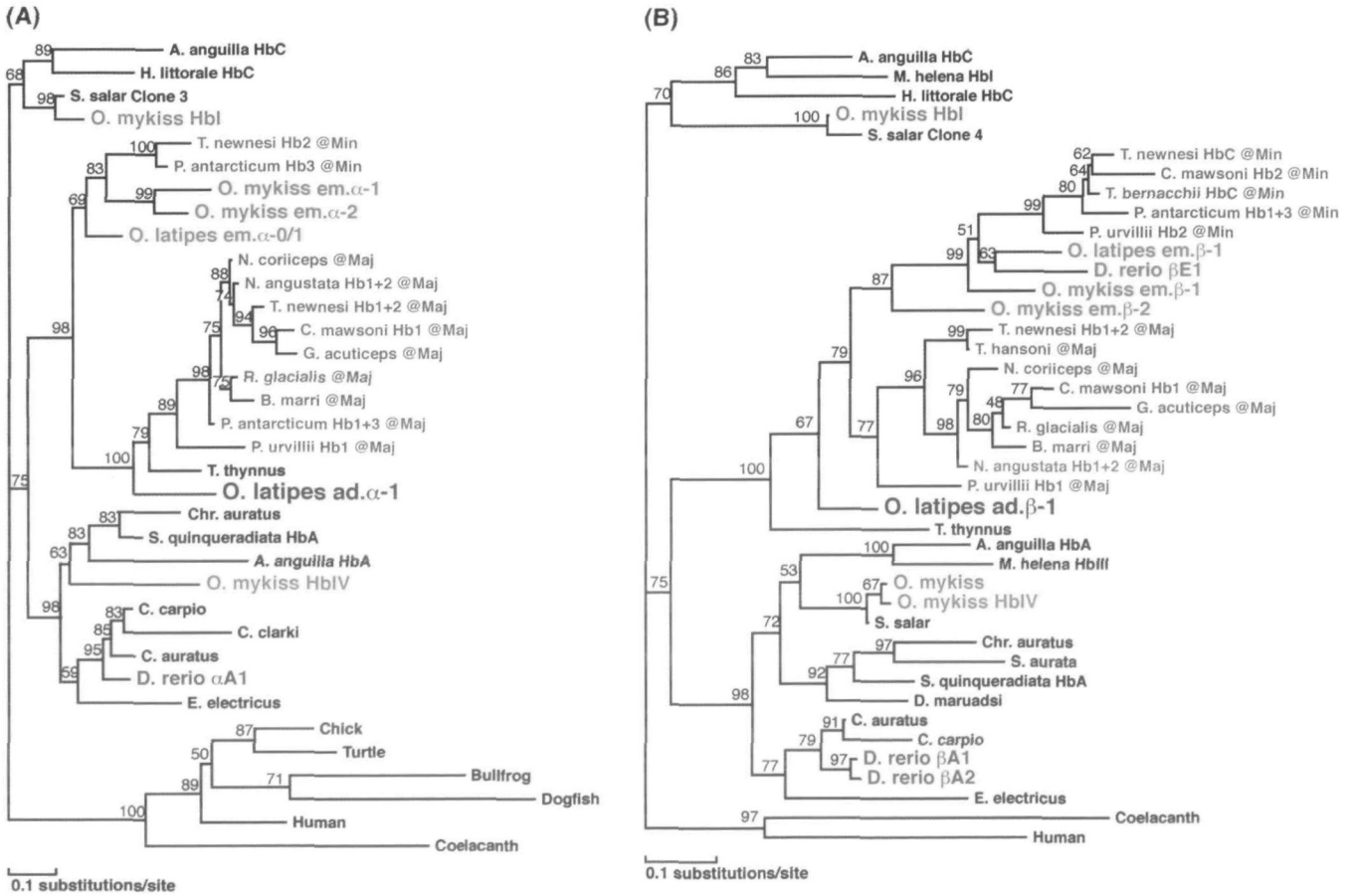

Fig. 4. **Molecular phylogenetic analysis of teleostean globins.** Amino acid sequences listed in Table I were aligned with CLUSTAL W ver. 1.7 (*20*). The distances based on amino acid substitutions were determined with the transition probability matrix for the JTT-F model, then analysis was performed with the RELL method in the program MOLPHY ver. 2.3 (*32*). An approximate tree topology of the branches was primarily determined by the neighbor-joining method, and that of subtrees was corrected with the RELL algorithm (*32*). Adult globins of medaka (*O. latipes*), rainbow trout (*O. mykiss*), and zebrafish (*D. rerio*) are shown in blue, light blue, and green, respectively, while embryonic globins of the three fishes are shown in red. The globins of major (@Maj) and minor (@Min) hemoglobins of Antarctic fishes are depicted in gray. Numbers above the branches are bootstrap values, and a scale shows a distance based on amino acid substitutions per site. (A) α Globins. α Gobins of adult hemoglobin of human (Mammalia), chick (Aves), turtle (Reptilia), bullfrog (Amphibia), dogfish (Chondrichthyes, Elasmobrachii), and coelacanth (Euteleostomi, Coelacanthiformes) were used as outgroups. (B) β Globins. β Globins of human and coelacanth adult hemoglobin were used as outgroups.

of embryonic β globin seemed to synchronize with the expression of embryonic α globin.

Two adult globin cDNAs, ad.α-1 and ad.β-1, were cloned from the kidney of fully-grown adult fish. The adult α-globin mRNA ad.α-1 was little expressed in stage 30 and 38 embryos. However, the expression drastically increased in 30-day young fish, and continued to the fully-grown adult stage. This is similar to the appearance of adult hemoglobin of the rainbow trout. Unexpectedly, a large amount of the ad.β-1 mRNA was expressed in the embryos at the stages 30 and 38. Such high expression level persisted from 30 days after hatching to the fully-grown adult stage. Thus, successive expression of adult globin ad.β-1 mRNA from the early embryonic stage to the fully-grown adult stage was characteristic in the teleost medaka. High expression of adult globin ad.β-1 in embryos may reflect a fact that its phylogenetic position is relatively closely related to the embryonic globin branch.

HPLC Analysis of Embryonic and Adult Hemoglobins in Medaka—To confirm the expression pattern of globin mRNAs investigated by RT-PCR, we prepared embryonic hemoglobin from embryos immediately after hatching and adult hemoglobin from fully-grown adult fish, and fractionated them by reverse-phase HPLC using a Senshu Pak VP-304 column.

As illustrated in Fig. 6, the HPLC pattern showed that the two hemoglobins comprised some common and some specific globin components. The embryonic hemoglobin consisted of two major globins (e5 and e6) and three minor ones (e4, e7, and e9). Further analysis by SDS-PAGE failed to provide evidence that the fractions e1, e2, e3, and e8 contained globin components (data not shown). Judged from its retention time, peak e9 was specific to embryonic hemoglobin. A partial amino acid sequence from the N-terminus of e9 was VKWSDFERATIQDIF-, which was highly homologous to other β globins. However, it was different from both embryonic globin em.β-1 and adult globin ad.β-1 determined in the present study. The partial N-terminal sequence of e6 in embryonic hemoglobin was VEWTEQERSI-ITNIFGNLDYEDVGS-, which coincided with the sequence of the N-terminal region predicted from adult globin cDNA ad.β-1. This result confirmed the above finding that adult globin mRNA ad.β-1 was expressed at the embryonic stage. SDS-PAGE (Fig. 7A) and Western blot analysis (Fig. 7B) revealed that the other major fraction, e5, contained a globin with an estimated molecular mass of 15 kDa, highly

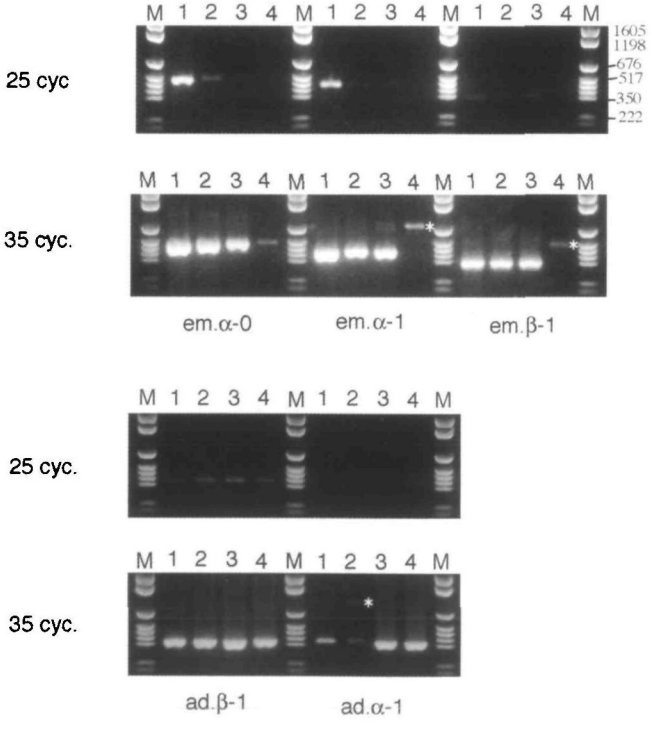

Fig. 5. **Developmental expression patterns of embryonic and adult globin mRNAs.** One hundred red blood cells collected from stage 30 embryos (lane 1), stage 38 embryos (lane 2), young fish 30 days after hatch (lane 3), or fully-grown adult fish (lane 4) were directly added to the reaction mixture for RT-PCR, and the RT-PCR analysis was performed using the primers for em.α-0, em.α-1, em.β-1, ad.α-1, and ad.β-1 shown in Figs. 1 and 2. Results from 25 cycles (25 cyc.) and 35 cycles (35 cyc.) are shown. Sizes of the PCR products amplified from the globin mRNAs for em.α-0, em.α-1, em.β-1, ad.α-1, and ad.β-1 are 472, 417, 334, 365, and 372 bp, respectively, and the bands having the expected sizes were obtained. * shows the PCR products that would be amplified from genomic globin genes. Lane M indicates molecular markers, the lengths of which are shown at the right side of the upper-most panel.

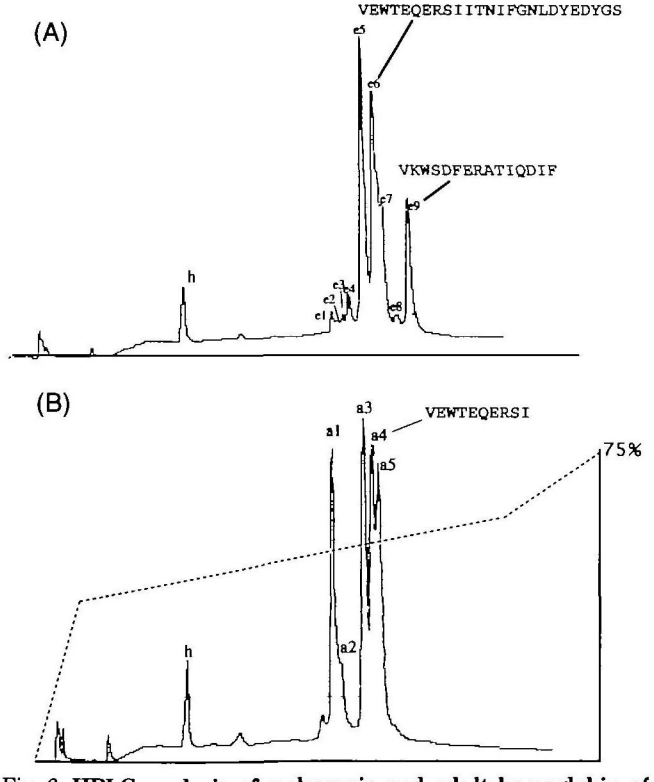

Fig. 6. **HPLC analysis of embryonic and adult hemoglobin of medaka.** Hemolysates containing embryonic and adult hemoglobin were prepared from embryos immediately after hatching and fully-grown adult fish, respectively. The hemolysate containing 50 μg of hemoglobin was fractionated by reverse-phase HPLC using a Senshu Pak VP-304 column. Elution profiles of the embryonic hemoglobin and the adult hemoglobin are shown in (A) and (B), respectively. Concentration of acetonitrile is shown by the broken line. h indicates heme molecule. Partial N-terminal amino acid sequences determined by automatic gas phase protein sequencer PSQ-1 are shown in the figure. After the fractionation, each fraction was applied to SDS-PAGE.

similar to that of other globins. The N-terminal sequence of e5 could not be determined by Edman degradation, suggesting that the e5 is an α globin molecule. In general, the terminal amino groups of teleostean α globins are masked with atomic groups such as an acetyl group (*23*).

The adult hemoglobin comprised three major globins (a1, a3, and a4). Although fraction a5 was detected as one of the major peaks, SDS-PAGE showed that it scarcely contained a protein band (data not shown). It is not clear whether a5 really contains α globin molecule. The a4 fraction contained one major 15-kDa protein, probably globin (Fig. 7A), and was reactive to anti-medaka adult hemoglobin antibody (Fig. 7B). The partial N-terminal sequence of a4 was determined as VEWTEQERSI-, which matched that of the adult globin ad.β-1, confirming the expression of this mRNA in fully-grown adult fish. Although the sequences of a1 and a3 could not be determined, SDS-PAGE (Fig. 7A) and Western blot analysis (Fig. 7B) revealed that these fraction a1 or a3 really contained a globin molecule. The two may be α globins. The retention time suggests that a1 was highly specific to adult hemoglobin, while a3 was nearly similar to the e5 of embryonic hemoglobin.

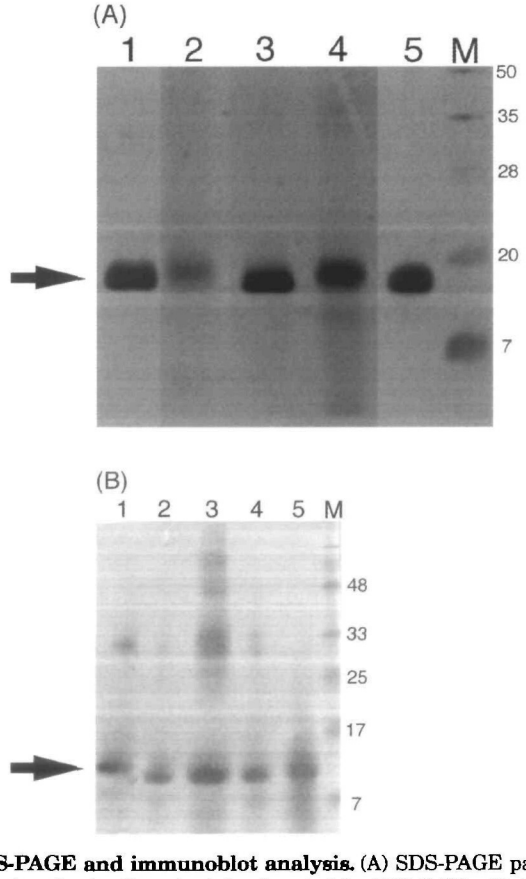

Fig. 7. **SDS-PAGE and immunoblot analysis.** (A) SDS-PAGE patterns of peaks e5, a1, a3, and a4 fractionated by HPLC (Fig. 6). (B) Western blot analysis with the antibody against medaka adult hemoglobin. Lanes 1, 2, 3, 4, and 5 show e5, a1, a3, a4, and medaka adult hemoglobin, respectively. Arrows indicate globin molecules. M, molecular mass markers (kDa).

DISCUSSION

Definition and Terminology of Embryonic and Adult Globin in Medaka—In the rainbow trout, the definitions of embryonic and adult hemoglobin/globin are based on the difference in molecular character and specificity in developmental expression profile. However, the definition in the medaka is somewhat different. The developmental wave of globin expression in the medaka is not the same as in the rainbow trout. In particular, ad.β-1 was expressed throughout life, from the early embryonic stage to the fully-grown adult stage.

Molecular phylogenetic comparison of medaka globins with other teleostean globins suggested that the medaka globins deduced from em.α-0, em.α-1, and em.β-1 cDNAs belonged to a monophyletic group consisting of embryonic globins of rainbow trout and zebrafish. This embryonic globin branch was quite different from the three branches of teleostean adult globins, including the globins deduced from ad.α-1 or ad.β-1 cDNAs of medaka. In respect of similarity or difference of its primary structure, the embryonic globin of the medaka was clearly discriminated from the adult globin. Therefore, on the basis of such molecular phylogenetic relationship, we defined the medaka globins em.α-0/1 and em.β-1 as "embryonic globins," and the medaka globins ad.α-1 and ad.β-1 as "adult globins."

Molecular Evolution of Teleostean Globins—In addition to cDNA analysis, HPLC analysis suggested that medaka globin genes were considerably polymorphic. As frequently found in genomic globin genes of other teleostean species (carp, *24*; Atlantic salmon, *25*), the multiple duplication of an ancestral globin gene occurred during the speciation of an ancestral teleost to the medaka. In a preliminary study on characterization and determination of the full-length structure of the globin gene and its locus in the medaka genome, we have identified total 12 globin genes including the genes corresponding to em.α-0, em.α-1, em.β-1, ad.α-1, and ad.β-1 cDNAs. The molecular phylogenetic position of all the medaka globin genes will be further investigated with special reference to evolution of teleostean globin genes.

Hematopoiesis and the Switching of Globin Gene Expression in Medaka—In rainbow trout, embryonic hemoglobin/globin is expressed in embryonic erythoid cells of round disc-like shape, while adult globin is expressed only in the elliptic disc-like cells appearing after hatching. The switch from embryonic to adult globin is due to the replacement of cell types. Such cell-type transition has also been found during the post-hatching development of zebrafish (*26*). In rainbow trout and zebrafish, as in other vertebrates such as mammals (*2*), birds (*27*), and amphibians (*28*), it is acceptable to assume that the expression of embryonic globins is closely related to the primitive hematopoiesis, and that of adult globins is to the definitive. In the medaka, however, we could not discriminate morphologically between erythroid cells in embryo and those in adult, and therefore, we could not determine the timing of disappearance of embryonic-type cells and appearance of adult-type cells. Thus, the switching of globin gene expression in medaka does not seem to simply reflect the difference of hematopoiesis. A globin molecule such as ad.β-1 was expressed both in primitive hematopoiesis and in definitive

hematopoiesis. Alternatively, it is possible that the medaka does not have two hematopoietic systems. The correlation of changes of globin types with changes from primitive to definitive hematopoiesis will be investigated using hematopoiesis-specific markers such as GATA-3 (*29*), c-*myb* (*30*) and AML-1 (*31*).

The difference in hematopoiesis-related globin gene expression between rainbow trout and medaka may be in part ascribable to the difference of function of *cis*-regulatory regions controlling the globin gene expression as well as of the structure of the globin gene locus. In the course of a preliminary study on the medaka globin locus, we have found some sequences rich in transcription factor–binding sites, such as GATA- and CACCC-boxes, in the 5′-upstream region of em.α-0 gene or in the inter-genic region between ad.α-1 gene and ad.β-1 gene. We will further investigate the role of such regions in controlling the globin gene expression.

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