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Identification of Five Embryonic Hemoglobins of Rat and Ontogeny of Their Constituent Globins during Fetal Development

Shin-ichiro Iwahara,[1] Yasuko Abe, and Taro Okazaki

Department of Biochemistry and Molecular Biology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113

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Hemoglobins of rats switch from an embryonic to an adult type during fetal development. However, very little is known about the structures and molecular species of hemoglobins occurring in the fetal life of rats. In the present study we isolated five embryonic hemoglobins, designated E1, E2, E3, E4, and E5, from the blood of rat fetuses on day 14 of gestation by ion exchange chromatography. Reverse-phase high performance liquid chromatography revealed that these hemoglobins each consist of two kinds of globins: E1($^{II}\alpha$:ε1), E2($^{I}\alpha$:ε1), E3(ζ:ε1), E4($^{I}\alpha$:ε3), and E5(ζ:ε3), respectively. The complete amino acid sequences of the ζ, ε1, and ε3 globins were determined. The ζ globin showed characteristic features common in α-type embryonic globins of known species in that the N-terminus is blocked and the amino acid at position 38 is Gln. ε1 and ε3 are β-type embryonic globins, sharing 73.7% amino acid homology. Interestingly, they are more similar to the corresponding mouse β-type embryonic globins, y and z, respectively, than to each other, implying that these globins have evolved orthologously from common ancestral proteins. It was also shown that the ζ, ε1, and ε3 globins are almost completely replaced by the adult type α and β globins in the blood of rat fetuses by day 18 of gestation.

Key words: embryonic hemoglobin, hemoglobin switching, primary structure, rat, zeta and epsilon globins.

Numerous studies have been carried out to elucidate the molecular mechanisms involved in hemoglobin switching by analyzing the structures of hemoglobins occurring in the embryonic to adult life of individual animals, and also the structures and linkages of the respective globin genes (*1-4*). Human hemoglobins are known to switch from an embryonic to a fetal and then an adult type, while those of the mouse and rat switch directly from an embryonic to an adult type (*1*). Rat hemoglobins are highly heterogeneous, as compared with those of other animals (*5-10*). In adult rats, at least ten hemoglobin fractions have been resolved on ion exchange chromatography (*9*), and three α and three to four β constituent globins have been identified based on their complete or partial amino acid sequences (*6, 7, 10*). In contrast to these findings in adults, hemoglobins of rat fetuses have not so far been described in detail. The occurrence of a few hemoglobins during fetal development of rats has been documented in earlier studies (*5, 11, 12*). Congote and Mulay have separated globins from rat fetuses by reverse-phase high performance liquid chromatography (HPLC), that were considered to be embryonic because they exist only in a limited period of gestation and disappear in adult rats (*13*). In spite of these studies, the structures and molecular species of rat embryonic hemoglobins remain entirely unclear. And nothing has yet been described on rat embryonic globin genes.

We have been investigating the structures of rat hemoglobins and globin genes, aiming to elucidate the molecular mechanism underlying their heterogeneity. From the results of these structural analyses at both the protein and gene levels, we are seeking to examine how their developmental stage- and tissue-specific expressions are regulated. In the present study we isolated five embryonic hemoglobins from the blood of rat fetuses and determined the complete primary structures of one α- and two β-like constituent globins. Changes in proportions of these globins during development of the rat fetus are also presented. We consider that this is the first report of a precise analysis of the structure and molecular species of rat embryonic hemoglobins.

EXPERIMENTAL PROCEDURES

Materials—Wistar rats used in this study were obtained from Nippon Bio-Supply Center (Tokyo). TPCK-treated trypsin and *Staphylococcus aureus* V8 protease were the products of Miles Labs (Elkhart, IN) and Worthington Biochem. (Freehold, NJ), respectively. Vydac C4 and C18 columns were purchased from Vydac (Hesperia, CA), and Mono Q columns were from Pharmacia Fine Chemicals (Uppsala). Acetonitrile and trifluoroacetic acid (TFA) used were of HPLC grade. All other chemicals were of reagent grade.

Preparation of Hemolysates—On days 12, 13, 14, 16, and 18 of gestation, pregnant Wistar rats were anesthetized with ether and fetuses were removed surgically from the amniotic sac. The umbilical cord was dissected and the

[1] To whom correspondence should be addressed. Tel: +81-3-3822-2131, Fax: 81-3-5685-3054

Abbreviations: HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; TPCK, L-1-tosylamido-2-phenylethyl-chloroethyl ketone.

blood was collected by immersing the fetuses in cold Krebs-Ringer phosphate solution. Circulating blood of the newborn rats was obtained by heart puncture. The collected blood was saturated with carbon monoxide, and the erythrocytes were washed three times with saline solution, followed by lysis in 10 mM Tris-HCl buffer containing 5 mM $MgCl_2$, pH 7.4.

Separation of Hemoglobin Fractions by Ion Exchange FPLC—Approximately 1 mg hemoglobin in hemolysates from rat fetuses prepared under carbon monoxide atmosphere was applied to a Mono Q HR5/5 column (0.5×5 cm) which had been equilibrated with 0.2 M glycine containing 0.01% KCN. The column was developed in 60 min with a linear gradient of 0–60 mM NaCl contained in the same buffer, at a flow rate of 1.0 ml/min. The peak fractions were collected, concentrated and stored at 4°C.

Isolation of Globin Chains by Reverse-Phase HPLC—Globin chains were separated by reverse-phase HPLC using a Vydac C4 column essentially according to the method described by Congote and Mulay (*13*). Approximately 100 μg of protein in hemolysates or hemoglobin fractions separated by ion exchange FPLC was applied to the column (0.46×15 cm), which was developed in 60 min with a linear gradient of 32–50% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. The separated globins were concentrated using a Speedvac concentrator (SAVANT Instruments, Flamingdale, NY).

Protein Digestion—The isolated globins were reduced and pyridylethylated with 4-vinylpyridine in 6 M guanidium HCl, then chromatographed by reverse-phase HPLC. *S*-Pyridylethylated globins were digested with TPCK-trypsin or V8 protease, in each case in 1% NH_4HCO_3, pH 8.5, at 37°C for 16 h. After digestion, peptides were purified by reverse-phase HPLC on a Vydac C18 column (0.46×25 cm), which was developed in 120 min with a linear gradient of 0–54% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min. Mild acid cleavage of aspartylproline bonds was accomplished by incubating the protein in 70% formic acid at 37°C for 36 h (*14*). Deblocking of the N-terminus of the ζ-globin was performed by treating the N-terminal peptide obtained by protease digestion with 25% TFA at 55°C for 2 h (*14*).

Amino Acid Sequence Analysis—The amino acid sequence analysis was performed by use of a model 477A protein sequencer on line with a model 120A PTH-amino acid analyzer (Applied Biosystems, Foster, CA).

RESULTS

Separation of Hemoglobins from the Rat Fetus on Day 14 of Gestation—Figure 1 shows the separation of globin chains in hemolysates from adult rats (A) and 14-day rat fetuses (B) by reverse-phase HPLC. The globins eluted were designated based on their primary structures, which were determined as described below. As seen, the globins in adult rat hemolysates were separated into two major peaks, α and β, of comparable amounts, indicating that hemoglobins of the adult rat are essentially composed of these two kinds of globins, although both globins are considered still to be heterogeneous. In contrast, the hemolysates from 14-day rat fetuses contained three additional globins, designated ε3, ε1, and ζ, which were eluted later than the adult globins (Fig. 1B). The figure also reveals that the β-globin peak is hardly detected in the 14-day fetuses, suggesting that the adult β globins have not yet appeared in the blood.

Figure 2 presents the separation of hemoglobin fractions in the blood of rat fetuses on day 14 of gestation by ion exchange FPLC. Five hemoglobin fractions, indicated as E1, E2, E3, E4, and E5 in the order of elution, are resolved,

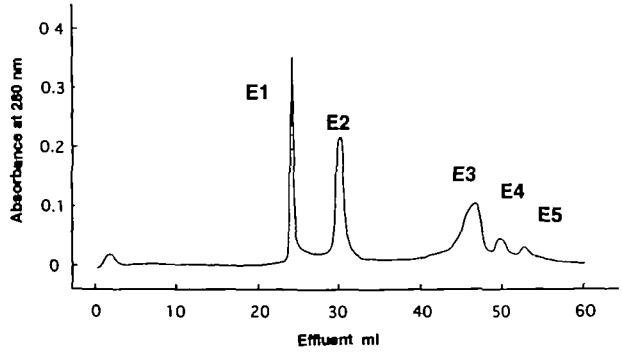

Fig. 2. **Separation of hemoglobin fractions in hemolysates from 14-day rat fetuses by ion exchange FPLC.** Hemolysates containing approximately 1 mg of hemoglobin were applied to a Mono Q column. Proteins were eluted with a linear gradient of 0–60 mM NaCl in 0.2 M glycine containing 0.01% KCN at a flow rate of 1.0 ml/min.

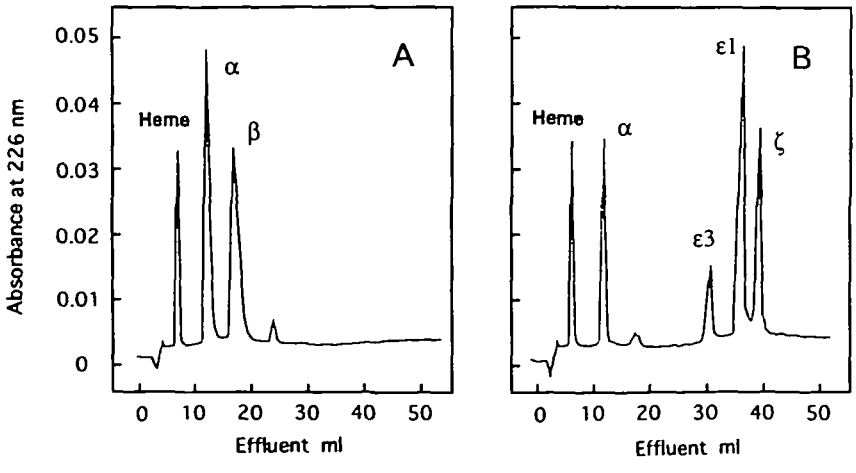

Fig. 1. **Separation of globins in hemolysates from adult rats (A) and 14-day rat fetuses (B) by reverse-phase HPLC.** Hemolysates containing approximately 100 μg of hemoglobin were applied to a Vydac C4 column, which was developed with a linear gradient of 32–50% acetonitrile in 0.1% TFA at a flow rate of 1.0 ml/min.

although some shoulder peaks are observable between E2 and E3.

The peak fractions were separated and each subjected to reverse-phase HPLC in order to identify their constituent globins. As shown in Fig. 3, it is apparent that each hemoglobin consists of comparable amounts of two kinds of globins, which fairly correspond in their elution positions to one of the globins occurring in the fetus hemolysates shown in Fig. 1B. It is also true that no globins other than the constituents of the isolated embryonic hemoglobins are found in the fetus hemolysates. The $^{I}\alpha$ and $^{II}\alpha$ globins in Fig. 3 were distinguished from one another by the differences in their N-terminal amino acid sequences, as described below.

Amino Acid Sequence Determination of the Rat Embryonic Globins—The complete amino acid sequences of the separated ζ, $\varepsilon 1$, and $\varepsilon 3$ globins that specifically exist in rat fetuses were then determined, and the results are summarized in Figs. 4 and 5.

The sequence of the ζ globin was established from peptides isolated from trypsin and V8 protease digests as indicated (Fig. 4). No N-terminal residue was detected in the direct sequencing of the intact chain, suggesting that the N-terminus was blocked. Deblocking was achieved by the method of Petruzzelli *et al.* (*14*). Mild acid hydrolysis, on the other hand, produced a specific cleavage at an aspartic acid-proline bond, which enabled us to determine the sequence from residues 95 to 141 directly, with no interference from the N-terminal fragment, that remained blocked. The nature of the blocking group was not determined.

The sequences of $\varepsilon 1$ and $\varepsilon 3$ were defined from peptides obtained on trypsin digestion (Fig. 5), which were arranged by sequence analogy with the human and mouse embryonic β-like globins (see also Fig. 6B).

Figure 6 (A and B) compares the sequences of the three rat embryonic globins with those of the corresponding human and mouse α- and β-like embryonic globins. The rat ζ globin differs from human ζ (*15, 16*) by 29 and from mouse ζ (*17*) by 12 of 141 residues. The rat $\varepsilon 1$ globin differs from $\varepsilon 3$ by 37 of 146 residues, while it differs from the human ε (*18*), and mouse y (*19*) and z globins (*20, 21*) by 25, and 3 and 34 residues, respectively. Table I summarizes the amino acid sequence homologies among these β-like embryonic globins. It is of interest that, when the rat $\varepsilon 1$ and $\varepsilon 3$ globins are compared with the mouse y and z globins, there are more similarities between globins of different species than between those of the same species.

Table II presents the globin chain assembly of the five

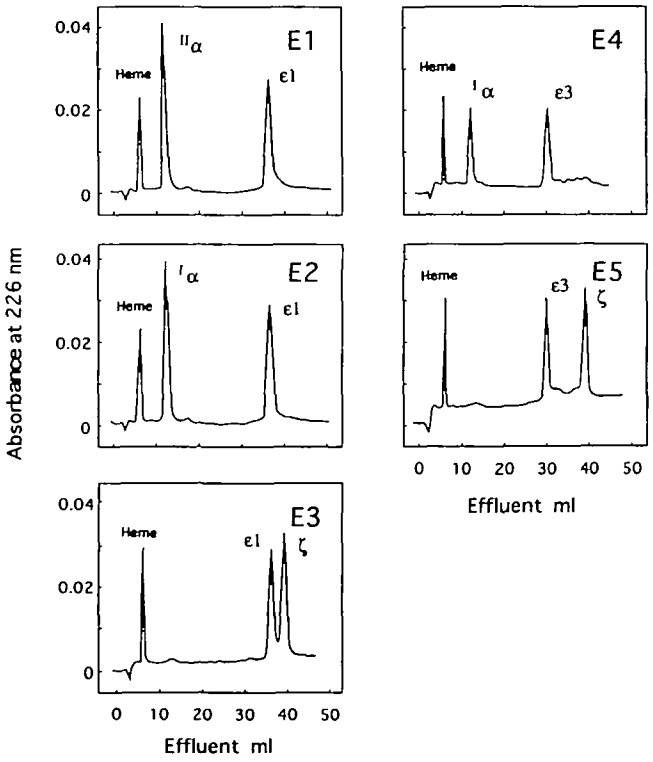

Fig. 3. **Separation of globin components of the isolated hemoglobins by reverse-phase HPLC.** Approximately 30 to 100 μg of each hemoglobin fraction was applied to a Vydac C4 column, which was developed under the same conditions as described in Fig. 1.

TABLE I. **Amino acid sequence homologies of the β-like embryonic globins.** Values in parenthesis are the number of mismatch residues.

	Human ε	Mouse y	Mouse z	Rat $\varepsilon 1$	Rat $\varepsilon 3$
Human ε		82.2%	77.4	82.9	75.3
Mouse y	(26)		76.0	97.9	74.7
Mouse z	(33)	(35)		76.7	94.5
Rat $\varepsilon 1$	(25)	(3)	(34)		74.7
Rat $\varepsilon 3$	(36)	(37)	(8)	(37)	

TABLE II. **Subunit composition of the rat embryonic hemoglobins.**

Hb	E1	E2	E3	E4	E5
α-like globins	$^{II}\alpha$	$^{I}\alpha$	ζ	$^{I}\alpha$	ζ
β-like globins	$\varepsilon 1$	$\varepsilon 1$	$\varepsilon 1$	$\varepsilon 3$	$\varepsilon 3$

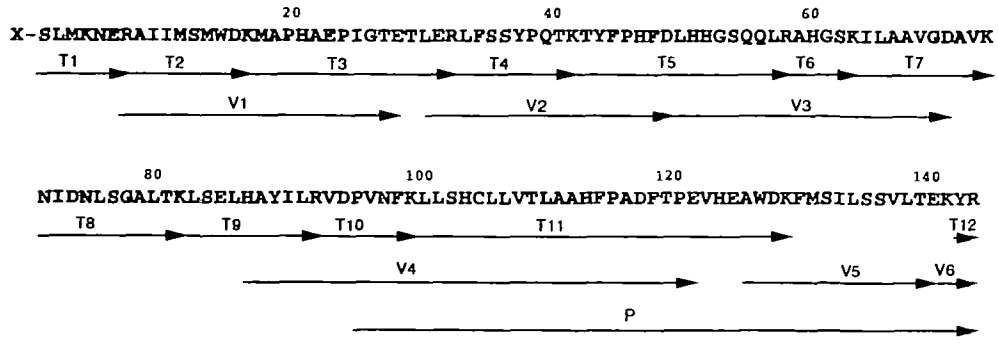

Fig. 4. **Amino acid sequence analysis of the rat ζ globin.** T and V denote the peptides obtained by trypsin and V8 protease digestion, respectively. P denotes the peptides obtained by mild acid cleavage at an Asp-Pro bond. The sequence of the N-terminal peptide was determined after deblocking with 25% TFA as described in the text.

Fig 5 **Amino acid sequence analysis of the rat ε1 and ε3 globins.** T denotes the peptides obtained by trypsin digestion The sequences of N-terminal 30 and 45 amino acid residues in ε1 and ε3, respectively, were also determined by direct sequencing of the intact peptides.

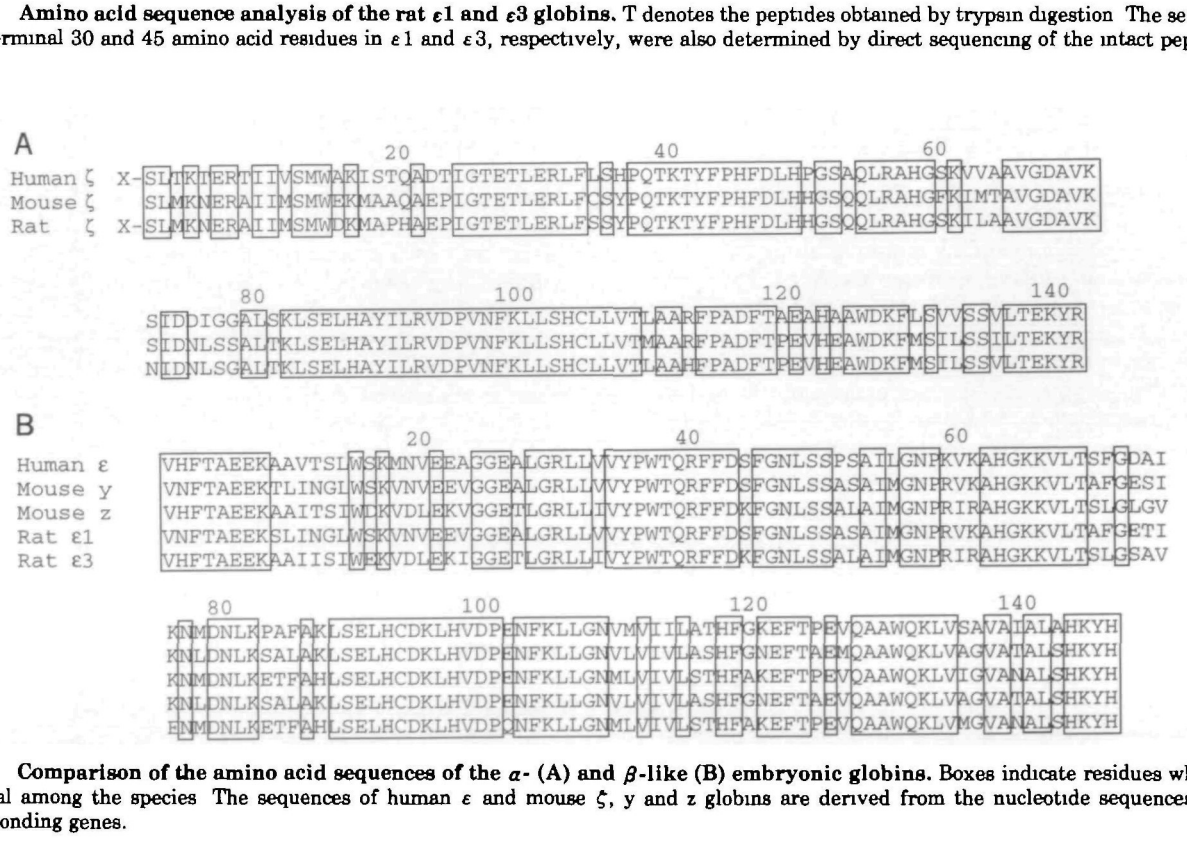

Fig. 6 **Comparison of the amino acid sequences of the α- (A) and β-like (B) embryonic globins.** Boxes indicate residues which are identical among the species The sequences of human ε and mouse ζ, y and z globins are derived from the nucleotide sequences of the corresponding genes.

embryonic hemoglobins of rats that we presently isolated. Of these, the $^{I}\alpha$ and $^{II}\alpha$ globins were identified by direct sequencing of the N-terminal 30 amino acid residues (data not shown), based on the known fact that the three α-globins $^{I}\alpha$, $^{II}\alpha$, and $^{0}\alpha$ differ from one another at least by the amino acids at positions 5 and 20, having respectively Asp, Ala, and Asp at position 5, and His, His, and Pro at position 20 (*10*).

It is plausible that one more embryonic hemoglobin, probably consisting of $^{II}\alpha$ and ε3, might have been present but have escaped from the present separation because of its minor quantity in the blood of rat fetus at this time of gestation.

Changes in Proportion of Globins during Fetal Development of Rat—Figure 7 shows changes in the relative amounts of the α- and β-like globins in the blood of rat fetuses during development. Amounts of respective globins were estimated from the elution profiles on reverse-phase HPLC of hemolysates from fetuses on days 12 to 18 of gestation. The haplotypes of adult rat globins were not determined, types being merely shown as α or β. From the results, it can be deduced that ε3 appears in the blood prior to ε1, which emerges maximally near day 14 of gestation; and that the switch from ζ to α occurs slightly earlier (day 14) than that from ε1 to β (day 16). It is also shown that the ζ, ε1, and ε3 globins disappear almost entirely from the

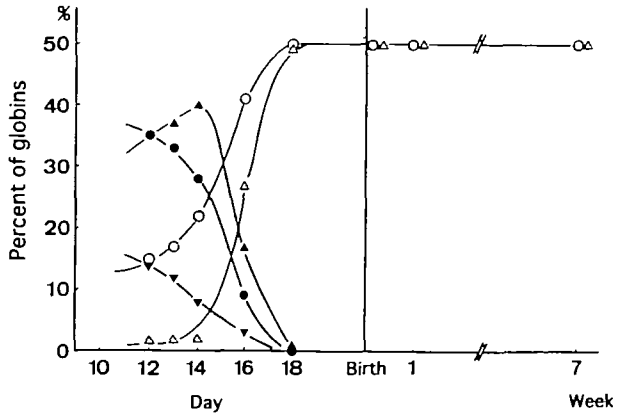

Fig. 7. **Changes in the proportion of the α- and β-like globins in peripheral blood during fetal and newborn development of the rat.** The amounts of respective globins were estimated by integrating the peak areas in the elution profiles of reverse-phase HPLC. ●, ζ; ▲, $\varepsilon 1$; ▼, $\varepsilon 3$; ○, α; △, β.

blood by day 18 of gestation.

DISCUSSION

The existence of embryonic and/or fetal hemoglobins during fetal development has long been known in a wide variety of animals including human and mouse, and their constituent globins have been characterized. Three kinds of embryonic hemoglobins, Hbs Gower 1 ($\zeta_2\varepsilon_2$) and Gower 2 ($\alpha_2\varepsilon_2$) (*22-24*) and Hb Portland 1 ($\zeta_2\gamma_2$) (*25, 26*), are found during the human embryonic development. Among their constituent globins, ζ and ε are embryonic α- and β-type globins, respectively, which are synthesized exclusively in the primitive nucleated erythrocytes formed in the yolk sac. On the other hand, γ and α chains are fetal β- and adult α-type globins, because they play major roles as the constituents of hemoglobins appearing in the fetal and adult periods of human development, respectively. The entire structures of all human globins and those of the corresponding genes have been worked out (*27, 28*). In mice, on the other hand, three embryonic hemoglobins, Hbs EI, EII, and EIII, have been described. Hb EI has the structure x_2y_2, EII has α_2y_2, and EIII has α_2z_2 (*29*). The x chain is an α-type and the y and z chains are β-type embryonic globins. The complete primary structure of the x globin has been derived from the nucleotide sequence of the embryonic ζ-gene (*17*), by which this protein is encoded. The entire structure of the mouse β-globin gene cluster that extends 56 kb has been established (*30*), in which the individual genes are arranged in the order 5′-y-βh0-βh1-βh2-βh3-βmaj-βmin-3′. The y gene codes for y globin (*19*), whereas the βh1 gene encodes z globin (*20, 21*). βh0 is also known to be transcribed in early mouse embryos and may code for a minor embryonic β-globin that appears in 10-day-old but not in 14-day-old embryos (*21*). These studies on the human and mouse globins and their coding genes, as well as those in several other animals, have made a great contribution to the recent progress in studies on molecular and cellular regulation of hemoglobin switching.

Contrary to these achievements in the human and mouse hemoglobins, hemoglobins of rats have long remained to be explored both on the protein and gene levels. Adult rat hemoglobins have been known to be very heterogeneous. Up to ten hemoglobins have been distinguished on ion exchange chromatography (*9*) and, as their constituents, three α ($^0\alpha$, $^I\alpha$, $^{II}\alpha$) and four β ($^0\beta$, $^I\beta$, $^{II}\beta$, $^{III}\beta$) globins have been identified based on the complete and/or partial amino acid sequences (*6, 7, 10*). They are regarded as non-allelic haplotype globins, except for $^I\beta$, which is now considered to be derived from $^{II}\beta$ by a post-translational modification (*31*), though the nature of the modification is still unknown. The genomic and/or complementary DNAs coding for some of these globins have also been isolated (*32-37*), but the entire linkage structure of rat globin genes is still far from established. In contrast to such complexities in adult rats, the occurrence of a few embryonic hemoglobins in rat fetuses has been described in earlier studies (*1, 11, 12*). However, the structures of these hemoglobins have not been investigated to even a limited extent, except that Congote and Mulay (*13*) have separated globins by HPLC from the liver of rat fetuses and given data on their partial amino acid contents. The gene structure of rat embryonic globins has not been described at all.

In the present study we isolated five embryonic hemoglobins from the blood of rat fetuses on day 14 of gestation. The subunit compositions of these hemoglobins were resolved. The complete amino acid sequences of one α- and two β-like constituent globins, ζ, $\varepsilon 1$, and $\varepsilon 3$, were presented. This may be the first report to describe the detailed structures of rat embryonic hemoglobins. These three embryonic globins occur in the blood on day 14 of gestation but entirely disappear by day 18.

The rat α-like embryonic globin presently isolated was defined to be ζ by analogy with the human and mouse ζ globins, its amino acid sequence showing 80.1 and 92.9% homologies, respectively. It differs substantially from the adult rat major α-globin, $^I\alpha$, by 69 of 141 residues (*6, 32*).

The rat ζ globin was found to possess characteristic structural features common to the α-like embryonic globins, such as human ζ (*15*) and chicken π' (*38*): the blocked N-terminus and glutamine at position 38. Both features have been claimed to be responsible for a diminished Bohr effect and a high oxygen affinity of embryonic hemoglobins carrying a ζ chain, respectively (*15*). These features are also common in bullfrog tadpole α (*39*), carp α (*40*), and trout α (*14*).

The amino acid sequences of the rat $\varepsilon 1$ and $\varepsilon 3$ globins showed a high homology with human ε and mouse y and z globins, respectively. As stated above, the mouse y and z globins are encoded by the y and βh1 genes, which are located in the β-like globin gene cluster, and their complete primary structures have been derived from the respective gene structures (*19, 20*). It should be emphasized that $\varepsilon 1$ and $\varepsilon 3$ have extremely high amino acid homologies to the mouse y and z, showing that rat $\varepsilon 1$ corresponds to mouse y, and rat $\varepsilon 3$ to mouse z. The homology between the corresponding globins of different species is found to be higher than that between the globins of same animal species. This fact strongly suggests that the individual embryonic globins of each species have evolved orthologously from their respective ancestral proteins.

In a separate experiment, we isolated a set of overlapping DNA clones of about 30 kb from the rat genome that harbors a number of gene loci for rat embryonic β-globins arranged in the order 5′-ε1-ε2-ε3-ε4-3′ (Satoh, H., Inoku-

chi, N., Nagae, Y., and Okazaki, T., manuscript in preparation). It is surprising that the organization of each gene in this cluster exhibits a close correspondence to that in the β-globin gene cluster of the BALB/c mouse (*30*). Sequence analysis revealed that the rat genes other than $\varepsilon 4$ exhibit canonical structures for the stable expression. Notably, the amino acid sequences of the $\varepsilon 1$ and $\varepsilon 3$ globins presently determined are found to be completely identical to those derived from the nucleotide sequences of the corresponding gene loci, $\varepsilon 1$ and $\varepsilon 3$, respectively.

With respect to the globin switching during fetal development, the present study revealed that there is asynchrony between the switch from ζ to α and that from $\varepsilon 1$ to β, the former occurring earlier than the latter. It was also indicated that $\varepsilon 3$ is an early embryonic globin and $\varepsilon 1$ a late one, in analogy with the mouse y and z globins, respectively. Whitelaw *et al.* (*41*) have measured the switching of globins in the developing mouse fetus at the mRNA level, which is very comparable with that observed in the rat, although the latter was estimated at the protein level. Boussios and Bertles (*42*) have described the switching of the hamster embryonic globins in yolk sac erythroid cells, in which the ontogenic processes of three embryonic and three adult globins are shown to closely resemble those reported in mice. These previous and present observations indicate that the patterns of globin switching in these three animals are quite analogous, at least in the latter half of gestation, implying that the molecular mechanism involved in regulating the process is essentially similar in rodents. Analyses of the structures and regulation of multiple globin genes in rats are now in progress in our laboratory.

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