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Authors: Wakahara, Masami, and Yamaguchi, Masahiro

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[REVIEW]

Erythropoiesis and Conversion of RBCs and Hemoglobins from Larval to Adult Type during Amphibian Development

Masami Wakahara* and Masahiro Yamaguchi†

Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan

ABSTRACT—In anuran amphibians transitions of hemoglobins (Hbs) and red blood cells (RBCs) from the larval to the adult type have been reported to occur at metamorphosis, depending on certain influence of thyroid hormones (THs). Contrary to this, transition of RBCs/Hbs from the larval to the adult type during the metamorphosis in a urodele, *Hynobius retardatus* occurs almost independently of thyroid activity, but dependent on certain pituitary factor(s). All findings reported so far support the idea that the Hb switching in *H. retardatus* occurs in a single RBC population ("Hb switching" model), rather than the concept that larval RBCs are replaced by new, adult RBCs ("RBC replacement" model) as is known to occur in many anurans. Erythropoiesis in vertebrates occurs with two distinct phases, termed primitive and definitive. Primitive erythropoiesis generally provides embryonic/larval erythroids, and definitive hematopoiesis contributes to adult RBCs. Primitive erythropoiesis in *Xenopus laevis* occurs in the ventral blood island (VBI), and the dorsolateral plate (DLP) cells remain undifferentiated until later for definitive hematopoiesis. *H. retardatus* embryos also have two distinct hematopoietic sites, the VBI and DLP. The DLP cells of *H. retardatus*, however, differentiate *in situ* to RBCs containing larval globin, suggesting that both the VBI and DLP contribute to "primitive" erythropoiesis. Some DLP cells may be set aside in an undifferentiated state during embryogenesis for future "definitive" erythropoiesis coming to express only adult globin during metamorphosis. A tentative model was proposed to explain similarities and dissimilarities in erythropoiesis and conversion of RBCs/Hbs between anurans and urodeles.

Key words: erythropoiesis, RBCs, hemoglobin transition, metamorphosis, amphibians.

INTRODUCTION

Hemoglobin (Hb) switching has long been one of the leading models for investigating the regulation of gene expression during animal development. In most species of vertebrates globin genes are organized in clusters in which different globin sequences are closely spaced. The expression of these genes is typically regulated both at a tissue-specific and at a stage-specific level (see Gilbert, 1994). The Hb transition may be physiologically important for inducing the change in oxygen affinity required for the adaptation from an embryonic or fetal environment to outdoor atmosphere in mammals and birds, or from aquatic environment to terrestrial life in amphibians (Hourdry, 1993a). In amphibians, Hb switching from the larval to the adult type has been investigated with special interest in metamorphosis (Cardellini and Sala, 1979; Ducibella, 1974a, b; Hosbach *et al.*, 1982; Hourdry, 1993b; MacLean and Jurd, 1972; Weber, 1996), which is a complete reconstruction of the body at the biochemical as well as the morphological level triggered by thyroid hormones (THs) (Weber, 1967; Yoshizato, 1989, 1992). The TH-dependent globin gene expression during amphibian metamorphosis is a useful model to investigate hormone-dependent gene expression (Widmer *et al.*, 1981; Hosbach *et al.*, 1982; Banville and Williams, 1985; Weber *et al.*, 1991).

We have investigated several phenotypic transitions from the larval to the adult type during the metamorphosis of a salamander *Hynobius retardatus* (Arai and Wakahara, 1993; Kanki and Wakahara, 1999, 2000; Kanki *et al.*, 2001; Ohmura and Wakahara, 1998; Satoh and Wakahara, 1997, 1999; Wakahara *et al.*, 1994; Wakahara and Yamaguchi, 1996; Yamaguchi *et al.*, 1996; Yamaguchi and Wakahara, 1997). Among these, Hb transition from the larval to the adult type in *H. retardatus* has been reported to be very unique, and somewhat different from other amphibians: 1) the Hb transition

* Corresponding author: Tel. +81-11-706-4455;
FAX. +81-11-706-4455.
E-mail: chami@sci.hokudai.ac.jp

† Present Address: Daiichi Pharmaceutical Co., Ltd., Tokyo R&D Center, 16-13, Kita-Kasai 1-Chome, Edogawa-ku, Tokyo 134-8630, Japan.

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occurs on almost the same time schedule in normally metamorphosing animals and in metamorphosis-arrested (goitrogen-treated) larvae (Arai and Wakahara, 1993; Wakahara and Yamaguchi, 1996). 2) The Hb transition is extraordinarily retarded in metamorphosis-arrested larvae whose pituitary gland has been surgically removed, whereas the transition in thyroidectomized larvae occurs on the same time schedule as normally metamorphosed controls (Satoh and Wakahara, 1997, 1999). These observations suggest that the Hb switching depends on the activity of pituitary gland, but not on that of the thyroid gland in this species. 3) Larval and adult Hbs are coexpressed in one RBC, suggesting that the Hb transition occurs within a single erythroid population (Yamaguchi and Wakahara, 1997), whereas that in other amphibians involves replacement of the larval red blood cells (RBCs) by adult ones (Hollyfield, 1966; Dorn and Broyles, 1982; Weber *et al.*, 1989; Just and Klaus-Just, 1996).

Here we describe recent progress in studies on erythropoiesis and conversion of RBCs/Hbs from the larval to the adult type during the metamorphosis in amphibians, at the level of molecular biology as well as cell biology, and discuss possible differences in the erythropoiesis and the conversion between anurans and urodeles. In this review, the erythropoiesis and conversion of RBCs/Hbs in *Xenopus* and *Rana* as representatives of anurans, and in *Hynobius* as of urodeles are reviewed, whereas we are not so confident at present, whether *H. retardatus* is eligible for a representative of urodeles.

AMPHIBIANS AS EXPERIMENTAL ANIMALS

Anurans vs. Urodeles

Modern amphibians can be grouped into three orders, such as Anura (Salientia), Urodela (Caudata) and Caecilia (Apoda and Gymnophiona) (Shi, 2000). Recent molecular studies indicate that the anuran group may have branched relatively early from the urodele/caecilian group, perhaps during the beginning of the Mesozoic period (240 million years ago), while the urodele and caecilian groups probably branched relatively late, in the late Mesozoic period (160-190 million years ago) (Feller and Hedges, 1998). It seems thus reasonable to assume that anurans and urodeles/caecilians are quite different animals, even though they constitute a Phylum Amphibia. Indeed, pattern of primordial gem cells formation in urodeles is completely different from that in anurans (Wakahara, 1996b). The urodele pattern is closer to mammals than to anurans. Furthermore, it has been recently reviewed that mechanisms of egg activation and polyspermy block at fertilization are considerably different between anurans and urodeles (Iwao, 2000).

Anuran metamorphosis is the most studied and the most dramatic metamorphosis. Almost all experiments on erythropoiesis and conversion of RBCs/Hbs from the larval to the adult type during metamorphosis are substantially limited to only three species of anurans: *Xenopus laevis*, *Rana catesbeiana* and *R. pipiens*. Contrary to the dramatic morphological changes in anuran metamorphosis, urodeles undergo fairly subtle morphological changes during the larva-to-adult transition. The most noticeable morphological changes are the resorption of the three sets of external gills and the tail fin at the final stage of metamorphosis (Weber, 1967). In spite of the subtle morphological changes in the urodele metamorphosis, it is controlled by THs, as in anurans. Experimental studies on conversion of RBCs/Hbs are very few in urodeles except for several neotenic salamanders such as the axolotl, a neotenic form of *Ambystoma mexicanum* and *Hynobius retardatus*, a Japanese salamander that has been recently used in our laboratory. Caecilian metamorphosis is the least studied among the three classes of amphibians, and thus erythropoiesis and possible conversion of RBCs/Hbs from larval to adult type in caecilians are entirely unknown.

Neoteny vs. Direct Development

Changes in developmental timing (heterochrony) are considered to be important in producing morphological changes during evolution (Gould, 1977; Akam *et al.*, 1994; Richardson, 1995). The heterochrony is also important in analyzing ontogeny of chronological expression of several phenotypes such as Hb transition. The heterochrony is conventionally categorized into neoteny (retardation in somatic development, and thus resulting in reproduction in larval form), progenesis (acceleration in germ cell development, and also resulting in sexual maturity in larval form), and direct development (acceleration in somatic development, and resulting in lack of larval stages).

In neoteny, the reproductive system (and germ cells) mature, while the rest of the body remains its juvenile form throughout its life (Lynn, 1961; Dent, 1968; Gould, 1977; Armstrong and Malacinski, 1989; Wakahara, 1996a). The neotenic urodeles have been divided into three categories according to their ability of metamorphosis; permanent or obligate neoteny which cannot metamorphose at all in both natural and experimental conditions (*Necturus*, *Proteus*, *Siren*), “inducible” obligate neoteny which cannot metamorphose in nature but can metamorphose after treatment with THs (axolotl), and facultative neoteny which metamorphoses depending on the environmental conditions (*Ambystoma tigrinum*, *A. gracile*) (Frieden, 1981).

A particular population of *H. retardatus* has been reported to show neoteny in a specific environment of Lake Kuttara, a small volcanic lake in Hokkaido, Japan (Sasaki, 1924; Sasaki and Nakamura, 1937). Unfortunately, however, the neotenic population in Lake Kuttara is believed to be extinct at present. Since it was reported that the neotenic individuals of *H. retardatus* that had been captured at Lake Kuttara metamorphosed under the laboratory condition (Sakai and Nakamura, 1937), it is reasonable to assume that this neoteny in *H. retardatus* must be a facultative neoteny, in which animals metamorphose depending on the environments (Wakahara, 1996a). Because adaptation from aquatic environment to terrestrial life is not necessary in neotenic forms, the RBCs/Hbs conversion in neotenic urodeles is expected to be different

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from that in non-neotenic urodeles and anurans, and thus may provide unique experimental system.

The direct development is a widespread alternate reproductive mode in living amphibians that is characterized by evolutionary loss of the free-living, aquatic larval stage (Lynn, 1961; Dent, 1968; Wake and Hanken, 1996). The direct developing larvae have no gill slits because respiration in water is not required. Instead, they specialize other organs/tissues for respiration, abdominal respiratory folds in *Cornufer* and a balloon-like structure of a highly vascularized tail in *Eleutherodactylus* (Dent, 1968). The direct development in amphibians is quite similar to the oviparous development typical of birds and reptiles which allows the animal to withdraw from water (Shi, 2000). A few studies have been carried out to determine the hormonal requirements during the developmental process that leads to the formation of the miniature adult (Jennings and Hanken, 1996), and to know several phenotypic transitions from the larval to the adult type (Callery and Elinson, 1996). Despite their radically altered ontogeny, direct developers still undergo a TH-dependent metamorphosis, which occurs before hatching (Callery and Elinson, 2000).

CONVERSION OF RBC/HB DURING AMPHIBIAN METAMORPHOSIS

Conversion of RBCs/Hbs in Anurans

In an anuran amphibian, *Xenopus laevis*, a switch in Hb synthesis occurs at metamorphosis resulting from the replacement of the larval globin subunits by a set of distinct adult ones (Hosbach *et al*., 1982; Sadmeyer *et al*., 1988). The transition of Hbs during metamorphosis in *Xenopus* has been reported to involve replacement of the larval RBCs (i. e., RBCs containing only larval Hb) by adult RBCs (RBCs containing only adult Hb) (Weber *et al*., 1989). Similar replacement of the larval RBCs by adult ones has been reported in *Rana pipiens* (Hollyfield, 1966) and *R. catesbeiana* (Dorn and Broyles, 1982; Just and Klaus-Just, 1996; Moss and Ingram, 1968). In *X. laevis*, however, Jurd and MacLean (1970) reported that approximately 25% of the RBCs contained both larval and adult globins using larval- and adult-specific antibodies, suggesting that the Hb switching occurs within a single RBC population. Recently, Tamori and Wakahara (2000) have convincingly demonstrated the replacement of larval RBCs by adult ones using Hb immunostain in *X. laevis*. The larval RBCs in circulation gradually decreased in number during normal metamorphosis, and that adult ones conversely increased. Because the sum of the percentages of larval and adult RBCs relative to total RBCs did not exceed 100% at any time during the period of RBC conversion, it was concluded that no RBCs expressed both larval and adult Hbs concurrently.

To examine mechanisms of the conversion of RBCs in *Xenopus* apoptotic cell death in larval or adult RBCs was detected by means of double-staining with *in situ* DNA nick-end labeling (TUNEL) (Gabrieli *et al*., 1992) and Hb immunostain (Fig. 1). Using different fluorescent dyes conjugated with secondary antibodies, it was possible to identify the origins of the RBCs that underwent apoptosis (Tamori and Wakahara, 2000). Fig. 2 shows chronological changes in the proportions of RBCs of the larval and adult types and in the proportion of TUNEL-positive RBCs to total RBCs in the spleen of *X. laevis*. The conversion of RBCs was at the halfway point at stage 59 in the spleen. Even at the end of the metamorphosis (stage 66), 30% of the RBCs showed larval Hbs, basically identical to the case of RBCs in circulation (Tamori and Wakahara, 2000). Although larval RBCs that also stained with TUNEL were not observed before stage 57, a prometamorphic stage, they began to be observed at and after stage 58. During the stages 60 to 62, the climax stage of metamorphosis, 5% to 7% of larval RBCs showed TUNEL-positive staining, suggesting that a certain proportion of the larval RBCs underwent apoptosis. The proportion of double-stained larval RBCs gradually decreased thereafter until the end of metamorphosis. In contrast, no adult RBCs showing the TUNEL reaction were observed during any developmental stages examined so far, suggesting that adult RBCs were not subjected to apoptotic cell death during the conversion of RBCs in *X. laevis*.

Selective Removal of Larval RBCs during Metamorphosis

Recently, Hasebe *et al*. (1999) demonstrated that the larval RBCs were selectively removed from the systemic circulation at the time of the metamorphic climax in *R. catesbeiana*,

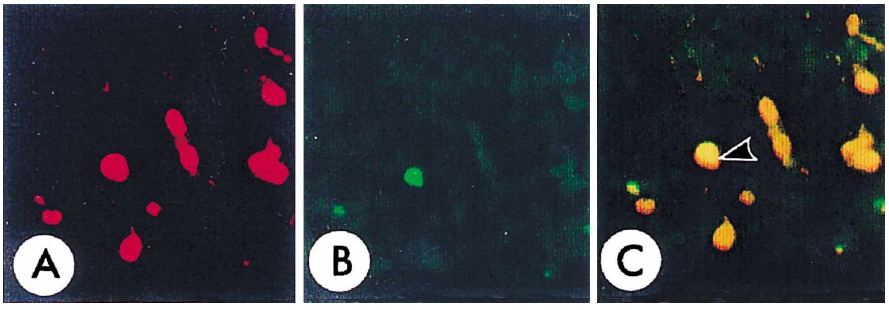

Fig. 1. RBCs double-stained with TUNEL and Hb immunohistochemistry. A section of the spleen from metamorphosing *Xenopus* was stained with antibody to larval Hb conjugated with Cy3 (red) and with TUNEL (fluorescein, green). The section was observed with different excitation filters for (A) Cy3 (larval Hb), (B) for fluorescein (TUNEL), and (C) for Cy3 and fluorescein (double-stained with larval Hb and TUNEL). A double-positive RBC is clearly shown, emitting bright yellow fluorescence mixed with red and green (C, arrowhead). From Tamori and Wakahara (2000).

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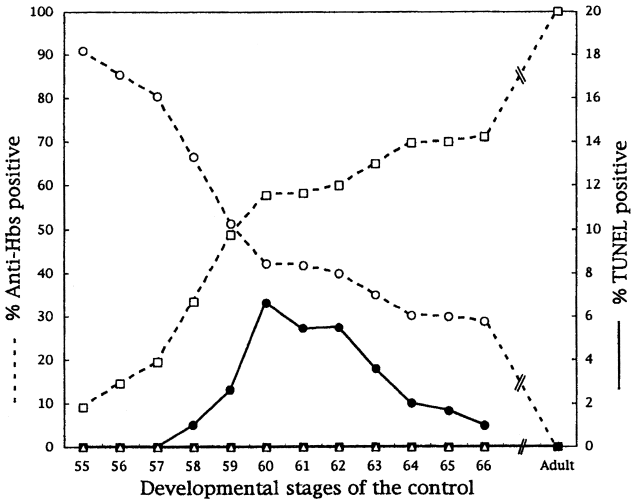

Fig. 2. Chronological changes in the proportion of larval and adult RBCs to total RBCs in the spleen and in TUNEL-positive RBCs during *Xenopus* metamorphosis. Sections of the spleen were double-stained with TUNEL and Hb immunohistochemistry. Numbers of RBCs stained with antibodies (open symbols) to larval (circle) and adult (square) Hbs, and with TUNEL (closed symbols) were counted. Double-positive RBCs were detected during and after the metamorphic climax, although adult Hb-positive and TUNEL-positive RBCs (closed square) were not detected at all throughout metamorphosis. Open triangles indicate the percentage of adult RBCs in goitrogen-treated, metamorphosis-arrested larvae. From Tamori and Wakahara (2000).

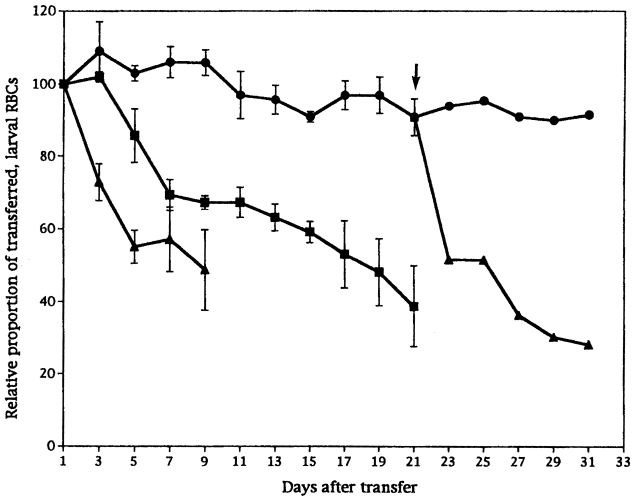

Fig. 3. Fates of transferred, larval RBCs in adult recipients of the histocompatible J strain of *Xenopus laevis*. Larval RBCs were injected directly into the heart of either control or T_3-treated adults, and then RBCs were collected from recipients every other day. Although the transferred larval RBCs survived for a long time in control adults, and thus did not decrease in number (closed circle), they gradually decreased in number in T_3-treated adults (closed triangle, 10^{-7} M T_3; closed square, 10^{-9} M T_3). When adult recipients were treated with T_3 21 days after the transfer (arrow), the population of the transferred larval RBCs to total RBCs drastically decreased. From Tamori and Wakahara (2000).

by means of *in vitro* fluorescence labeling of RBCs and injection of the labeled cells into animals at various metamorphic stages. The labeled larval RBCs were ingested by hepatic and splenic macrophages, indicating that macrophages are involved in the splenic elimination of larval cells. In this respect, Nishikawa and Hayashi (1999) showed that larval erythroblasts decreased through the apoptotic process in *X. laevis*, by means of double-staining experiments with TUNEL and Hb immunostaining. Their results indicated that the erythropoietic system is converted during metamorphosis effectively by two distinct hormonal mechanisms, T_3-hydrocortisone (HC) synergism on adult erythroblast proliferation and T_3-mediated programmed death of larval erythroblasts.

Selective removal of larval RBCs from circulation of metamorphosing and metamorphosed animals was thus examined more simply by using histocompatible J strain of *Xenopus* (Nakamura *et al.*, 1985; 1987) than using wild animals, without considering factors such as immunological rejection of transferred cells (Tamori and Wakahara, 2000). The results of our experiments with this strain showed that transferred larval RBCs cannot survive in T_3-treated adults while those can survive in control adults (Fig. 3), suggesting that the mature, larval RBCs are selectively removed from circulation under the influences of THs. Double-staining experiments with TUNEL and Hb immunostain convincingly demonstrate that mature, larval RBCs of *X. laevis* are subjected to the apoptosis in the spleen and liver of the recipients under the influence of THs (Tamori and Wakahara, 2000). It is thus concluded that the larval RBCs are specifically removed by apoptotic cell death from the circulation during the metamorphic climax. Nishikawa and Hayashi (1999) have shown that larval-type erythroblasts are subjected to the apoptosis in the liver, suggesting the larval-adult conversion of RBCs is conducted by T_3-mediated programmed death of larval precursor cells. In this model, however, it is difficult to explain how the mature, larval RBCs circulating in metamorphosing tadpoles are selectively removed from the circulation. Because selective apoptosis of mature, larval RBCs in the spleen was demonstrated *in vivo* at the metamorphosis climax, and in recipient adults treated with T_3 in RBC-transfer experiments (Tamori and Wakahara, 2000), it can be concluded the larval-adult conversion of RBCs in *X. laevis* is conducted by replacement of RBC populations, which is similar to the mechanism known in general transformation in anuran metamorphosis, selective removal of mature, larval specific cells (Hasebe *et al.*, 1999; Izutsu *et al.*, 1996; Nishikawa and Hayashi, 1995; Ohmura and Wakahara, 1998; Yoshizato, 1992).

Conversion of RBCs/Hbs in Urodeles

Adult RBCs in *H. retardatus* were readily distinguished from larval RBCs not only by their globin subunits and density on a Percoll gradient, but also by their cell shape and behavior in a hypertonic solution (Yamaguchi and Wakahara, 1997). The RBCs from metamorphosing animals could not be separated into two distinctive populations, but showed a single density intermediate between typical larval and adult densities on a Percoll density gradient (Fig. 4), in contrast to what has been reported in *R. catesbeiana* (Dorn and Broyles, 1982).

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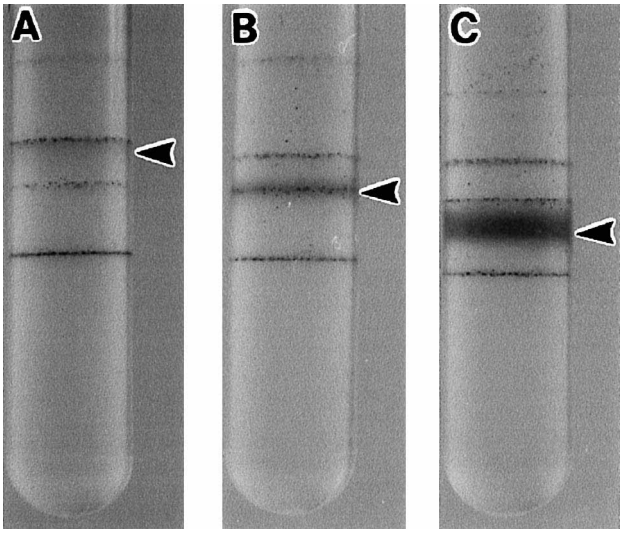

Fig. 4. Changes in a buoyant density of RBCs during the metamorphosis of *Hynobius retardatus*, on Percoll gradient density with color density markers. A, Typical larval RBCs. B, RBCs from metamorphosing larvae. C, Typical adult RBCs. During the metamorphosis, RBC fraction cannot be separated into two distinctive populations, but show a single density, suggesting that the Hb switching occurs in a single RBC population ("Hb switching" model") rather than the "RBC replacement" model. Arrowheads indicate the position of RBCs. Modified from Yamaguchi and Wakahara (1997).

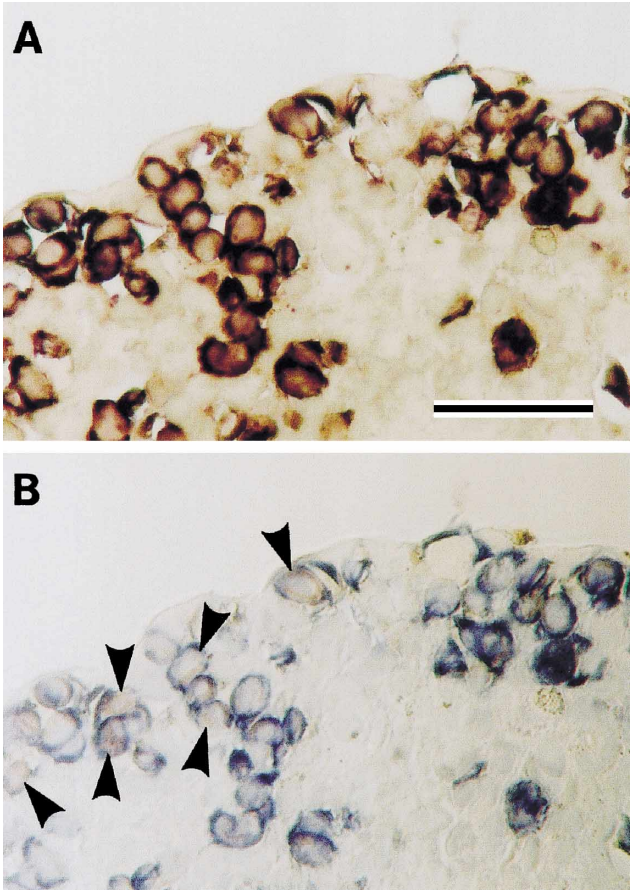

Fig. 5. Double *in situ* hybridization showing coexpression of larval and adult globin mRNAs in the same erythroid cells in *Hynobius retardatus*. Sections of the spleen were hybridized with DIG-labeled adult β-globin probe (A) and fluorescein-labeled larval β-globin probe on A (B). Several erythroid cells express both larval and adult β-globin mRNAs (arrowheads), demonstrating that the Hb transition occurs within single erythroid cells. Bar, 50 μm. From Yamaguchi *et al.* (2000).

These observations favor the idea that the Hb switching occurs in a single RBC population, rather than the concept that larval RBCs are replaced by new, adult RBCs (see Broyles, 1981). Continuous changes in the RBC morphology during their transition from the larval to the adult type in 30% PBS are also consistent with the "Hb switching" model in this species. This model was supported by the fact that RBCs from early larvae to metamorphosed juveniles in *H. retardatus* expressed, more or less, concurrently both larval and adult phenotypes of Hbs (Yamaguchi and Wakahara, 1997). Therefore, the selective removal of larval-specific RBCs from the circulation of metamorphosing animals is not expected during the conversion of RBCs/Hbs in *H. retardatus.*

"RBC Replacement" vs. "Hb Switching" Models

There are two concepts explaining the Hbs/RBCs transition from the larval to the adult type in amphibians: either the transition involves replacement of the larval RBCs by adult ones ("RBC replacement" model, postulated in *Rana*, Hollyfield, 1966; Dorn and Broyles, 1982; in *Xenopus*, Weber *et al*., 1989, 1991; Just and Klaus-Just, 1996; Tamori and Wakahara, 2000), or the Hb transition occurs within a single erythroid population and thus no replacement of RBCs occurs ("Hb switching" model, postulated in *Hynobius*, Yamaguchi and Wakahara, 1997; Yamaguchi *et al*., 2000). According to the "RBC replacement" model, which asserts that the Hb transition from the larval to the adult type involves replacement of the larval RBCs by adult ones, different classes of Hbs are expected to be expressed in different erythroid precursor cells which differentiate in the specific erythropoietic organs (Weber *et al*., 1991). In contrast, in the "Hb switching" model, as postulated in *H. retardatus* (Yamaguchi and Wakahara, 1997), the Hb switching may occur in the same erythroid precursor cells. Yamaguchi *et al*. (2000) conclusively demonstrated that the single erythroid cells expressed both, larval and adult globin mRNAs concurrently (Fig. 5), in favor of the "Hb switching" model at the level of globin gene expression. Similar Hb switching is known in mouse as well: coexpression of embryonic and adult globin mRNAs and globin subunits within single erythroid cells originated from extraembryonic yolk sac have been reported (Brotherton *et al*., 1979; Leder *et al*., 1992). Thus, the mode of RBCs/Hbs conversion in *H. retardatus* or urodeles in general, seems similar to that in mammals rather than in anurans.

HORMONAL REGULATION OF THE RBC/HB CONVERSION

Thyroid Hormones

Morphological metamorphosis in amphibians is known to

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be controlled by thyroid hormones (THs); thyroxine (T_4) and triiodotyronine (T_3) (Dodd and Dodd, 1976; Frieden, 1981; Hourdry, 1993b; Kaltenbach, 1996; Weber, 1967). In anurans the conversion of RBCs/Hbs from the larval to the adult type occurs depending on the THs. Even in the axolotl the Hb transition has been reported to occur depending on a very low concentration of THs (Ducibella, 1974b; Jurd, 1985). In *Hynobius retardatus*, however, T_4 was reported to have little accelerating potency on the Hb transition from the larval to the adult type, when exogeneously applied to larvae, even though the hormone stimulated a premature metamorphosis in external morphology (Wakahara *et al*., 1994). This observation is consistent with the fact that the Hb transition in both thyroidectomized larvae (Satoh and Wakahara, 1997) and goitrogen-treated larvae (Arai and Wakahara, 1993; Wakahara and Yamaguchi, 1996), is almost identical to that in the normally developing larvae, suggesting an independence of the Hb transition from the thyroid activity in this species (Satoh and Wakahara, 1997). In aged larvae of *H. retardatus*, which are living in cold ponds at high altitude, and thus spend 2 winter seasons before completion of the metamorphosis, the transition to adult Hb occurs in larval forms (Iwasaki and Wakahara, 1999), suggesting further the separation of morphological metamorphosis and transition of RBCs/Hbs in *H. retardatus*. Independence of the Hb conversion from THs in *H. retardatus* was also demonstrated at the transcriptional level of globin genes (Yamaguchi *et al*., 2000). The expression of adult β-globin gene was detected 19 days after hatching, much earlier than an initiation of the morphological metamorphosis (Yamaguchi *et al*., 2000). Although the plasma concentration of THs was not determined, it must be practically zero 19 days after hatching. Together with the fact that adult globin gene expression in the metamorphosis-arrested larvae was detected almost the same time schedule as in the controls (Yamaguchi *et al*., 2000), the earlier expression of adult globin gene supports the concept that the globin transition is independent of the thyroid activity in *H. retardatus.* Persistent expression of larval globin genes and subunits as long as 2 years after the hatching (Yamaguchi *et al*., 2000) is also in favor of the concept that the Hb transition in *H. retardatus* is independent of THs.

Contrary to this, it was reported that T_3 and hydrocortisone (HC) had an accelerating potency on the Hb transition when applied to hypophysectomized (Hx) *H. retardatus* larvae (Satoh and Wakahara, 1999). Furthermore, an inhibitor of corticoid, metyrapone (MTP), was demonstrated to have an inhibitory effect on the Hb transition when applied to intact larvae in combination with goitrogen (Satoh and Wakahara, 1999). Because the Hb transition in goitrogen-treated larvae was repeatedly demonstrated to occur on the same time schedule as in normally metamorphosing animals (Arai and Wakahara, 1993; Wakahara and Yamaguchi, 1996; Satoh and Wakahara, 1997), the retardation of the Hb transition in goitrogen-MTP treated larvae was attributable to combined effects of goitrogen and MTP. These results from two experiments described above suggest a positive involvement of T_3 and HC in some phase of adult Hb expression in *H. retardatus*.

Goitrogen-Treatment

In goitrogen-treated, metamorphosis-arrested larvae of *Xenopus laevis*, selective removal of larval RBCs is not expected to occur, because there are no THs enough to elicit the morphological metamorphosis as well as the apoptosis in RBCs. In spite of this, larval Hbs as well as RBCs finally disappeared even in the metamorphosis-arrested tadpoles though it took as long as 6 months (Tamori and Wakahara, 2000). It is thus assumed that larval RBCs in the metamorphosis-arrested tadpoles disappear through other mechanisms than by an active removal of them. Probably, larval RBCs in the metamorphosis-arrested tadpoles passively disappear by exhausting the source of them. This assumption is consistent with previous observations that the primitive erythropoiesis for the source of larval RBCs has a limited potentiality to differentiate and proliferate (Flores and Frieden, 1972; Widmer *et al*., 1983). An appearance of adult RBCs in the metamorphosis-arrested larvae even though it takes a long time, suggests that there is at least a certain process independent of THs in RBC conversion in *X. laevis* (MacLean and Turner, 1976). Although it must be true during the normal metamorphosis in *Xenopus* that specific removal of mature, larval RBCs is conducted by apoptotic cell death under influences of T_3 (Tamori and Wakahara, 2000), and that specific proliferation of adult erythroblasts is elicited by T_3-HC synergism (Nishikawa and Hayashi, 1999), possible contribution of the TH-independent event(s) to the RBC conversion, e.g., chronological ages, or size, or some other independent factors such as pituitary factor(s) (Satoh and Wakahara, 1999), should be in mind to clarify comprehensive mechanisms regulating the conversion.

Pituitary Function in Hb Transition

Satoh and Wakahara (1997) originally found in *H. retardatus* that the Hb transition in hypophysectomized (Hx) larvae was extraordinarily retarded, and thus was not completed within a year, whereas the transition in thyroidectomized (Tx) larvae occurred on almost the identical time schedule to the normal controls. It was thus suggested that the transition can proceed regardless of the morphological metamorphosis, and that the pituitary gland is involved in the transition. When the pituitary gland from *H. retardatus* was transplanted to the Hx *H. retardatus* larvae, the Hb transition in the transplanted Hx larvae showed apparently accelerated pattern compared with the Hx larvae that had not been transplanted with the pituitary gland (Satoh and Wakahara, 1999). From these results, it was tentatively concluded that the pituitary gland had some potency to accelerate the Hb transition.

Fig. 6 shows a tentative model for the hormonal control mechanism in the Hb transition during the metamorphosis in *H. retardatus*. Almost all studies have shown that metamorphic changes from the larval to the adult phenotype in amphibians are regulated by complicated hormonal controls including THs, prolactin (PRL), adrenocorticotropic hormone (ACTH) and corticoids (Dodd and Dodd, 1976; Rosenkilde,

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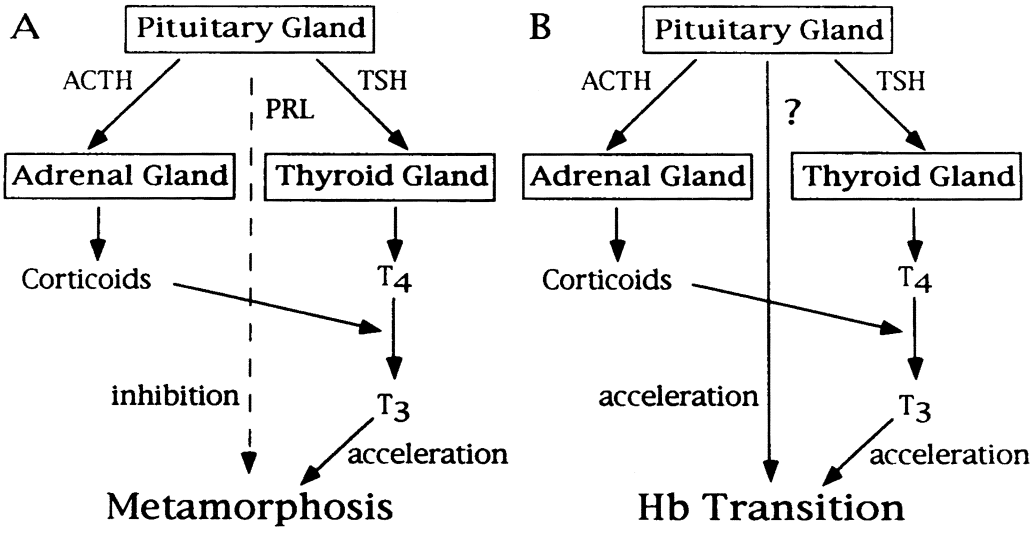

Fig. 6. Tentative model for hormonal control mechanisms of the Hb transition during the metamorphosis in *Hynobius retardatus*. A, General scheme for hormonal control mechanism of the metamorphosis in amphibians. B, Specific scheme for hormonal control mechanism of Hb transition during the metamorphosis in *H. retardatus*. The Hb transition proceeds autonomously within each erythroid cell at very low speed. Both T_3 and unknown pituitary factor(s) are assumed to have an accelerating effect on Hb transition. See text for detail. From Satoh and Wakahara (1999).

1985). Major hormones involved in the amphibian metamorphosis are (1) T_3, an active metamorphic hormone, which is converted from T_4 secreted from the thyroid gland that is stimulated by TSH secreted from the pituitary gland, (2) PRL secreted from the pituitary gland, and (3) corticoids which are secreted from the adrenal cortex following stimulation by ACTH secreted from the pituitary gland (Fig. 6A). Corticoids stimulate the conversion of T_4 to T_3 by inducing the conversion enzyme, and T_3 elicits metamorphic changes in somatic tissues, whereas PRL inhibits the metamorphic changes. In the Hb transition in *H. retardatus*, however, no inhibitory factor(s) are demonstrated, such as PRL in the general metamorphosis in amphibians. Both T_3 and unknown pituitary factor(s) (Satoh and Wakahara, 1999) are assumed to have accelerating potency on the Hb transition in *H. retardatus* (Fig. 6B).

ERYTHROPOIESIS IN AMPHIBIANS

Primitive and Definitive Erythropoiesis

Hematopoiesis in vertebrates occurs with two distinct phases, termed primitive and definitive, based on the timing and site of their development, morphology, and their potentialities to differentiate (Zon, 1995). In general the primitive erythropoiesis provides embryonic and/or larval erythroids, and the definitive hematopoiesis supplies adult RBCs. In mammals and birds, primitive erythropoiesis occurs in the extraembryonic yolk sac and consists primarily of erythrocytes. On the other hand, definitive hematopoietic cells are derived from the intraembryonic region such as dorsal mesentery, para-aortic foci, anterior region of the mesonephros in association with the post cardinal vein, and dorsal aorta in birds (Martin *et al.*, 1978; Dieterlen-Lievre and Martin, 1981; Dieterlen-Lievre, 1993) and aorta-gonads-mesonephros (AGM) region in mammals (Medvinsky *et al.*, 1993; Medvinsky and Dzierzak, 1996), although there are contrary reports claiming that yolk sac stem cells provide all lineages of blood cells (Yoder *et al.*, 1997a, b). In amphibians, the hematopoiesis also occurs in two waves, primitive and definitive similarly to the mammals and birds as described below. The erythropoietic organs change dramatically during ontogeny, from embryonic organ (the ventral blood island, VBI), which produces the primitive blood cells to the dorsolateral plate (DLP) mainly contributing to the definitive cells. In many fishes, however, both primitive and definitive blood cells arise from intraembryonic intermediate cell mass (ICM) (Detrich *et al.*, 1995).

Erythropoietic Organs in Anurans

In *Xenopus* transplantation experiments demonstrate that hematopoiesis occurs in two waves: primitive RBCs which exclusively produce larval Hb are derived from the VBI, while definitive ones (expressing adult Hb) originate mainly from the DLP (Turpen *et al.*, 1997), although cells of the VBI contribute to adult erythropoiesis to some extent (Maeno *et al.*, 1985a, b: Rollins-Smith and Blair, 1990). Primitive erythropoiesis in *X. laevis* occurs in the VBI during early embryogenesis, and the DLP cells remain undifferentiated until later for definitive hematopoiesis. A switch from larval to adult globin gene expression in *Xenopus* has been reported to be mediated by erythroid cells from distinct, two compartments (Weber *et al.*, 1991), in agreement with the "RBC replacement" model. The erythropoietic transition from the VBI to the DLP is dependent on the THs (Rollins-Smith and Blair, 1990). The expressions of globin and GATA-1 genes, terminal differentiation markers of erythroid cells, are detected exclusively in the VBI of tailbud embryos (Kelley *et al.*, 1994; Bertwistle *et al.*, 1996), suggesting further that the VBI is the sole source of primitive erythroid cells in *X. laevis*. The different responses in larval and adult RBCs to THs, apoptotic cell death in larval

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but not in adult RBCs in X. laevis (Figs. 2, 3), might reflect the different origins of these two RBC populations. It has also been reported in *Rana pipiens* that primitive erythroid cells originate in the VBI and definitive cells in the DLP (Zon, 1995). In *R. catesbeiana*, however, it has been suggested that the DLP (mesonephric kidney) has both primitive and definitive hematopoietic cells (Broyles *et al*., 1981; Maples *et al*., 1983).

The exact erythropoietic organ(s) in larvae and adults in anurans have been controversial: the kidney (Turner, 1988), liver (Ohinata and Enami, 1991), or peripheral to the liver (Weber *et al*., 1991) in *Xenopus*, and the liver and kidney in *Rana* (Broyles *et al*., 1981).

Erythropoietic Organs in Urodeles

The transition in the erythropoietic organs in *Hynobius retardatus* has been studied on the basis of immunohistochemical studies using specific antibodies to larval and adult Hbs (Yamaguchi and Wakahara, 1997). Thereafter, the scheme of the transition was substantially confirmed by molecular biological studies using cDNA probes for globin genes (Yamaguchi *et al*., 2000; Yamaguchi and Wakahara, 2001). In *H. retardatus*, the VBI at a tailbud stage (stage 34, according to the developmental stages by Iwasawa and Yamashita, 1991) is intensely stained *in situ* hybridization with the larval β-globin RNA probe. At stage 37/38, the VBI is still recognizable with the larval globin antibody and β-globin RNA probe. In addition, cells of the DLP also became intensely stained with the larval antibody as well as with the larval RNA probe (Fig. 7), suggesting that cells of the VBI as well as the DLP differentiate *in situ* to erythroid cells that contain larval globin subunit and larval globin mRNA. In other words, both the VBI and DLP contribute to primitive erythropoiesis (Yamaguchi *et al*., 2000). The contribution of the DLP to the primitive erythropoiesis probably characterizes the erythropoiesis in *Hynobius* or in urodeles, in general. There are two, possible explanations for the contribution of the VBI and DLP to the primitive and/or definitive erythropoiesis: both the VBI and DLP supply common erythroid precursor cells which differentiate to every type of RBCs, such as larval, larval-adult and adult RBCs, or the VBI gives rise to only larval erythroid cells but the DLP supplies common erythroid cells which differentiate to every type of RBCs (Yamaguchi *et al*., 2000). Unfortunately, however, it was difficult to determine conclusively the developmental fates of the erythroid stem cells from the VBI and the DLP by examining only the expression of globin genes. To obtain molecular probes such as GATA genes, which sequentially regulate vertebrate hematopoiesis (Kulessa *et al*., 1995; Visvader *et al*., 1995), GATA-3 was cloned to elucidate further mechanisms controlling the hematopoiesis in *H. retardatus* (Yamaguchi and Wakahara, 2001).

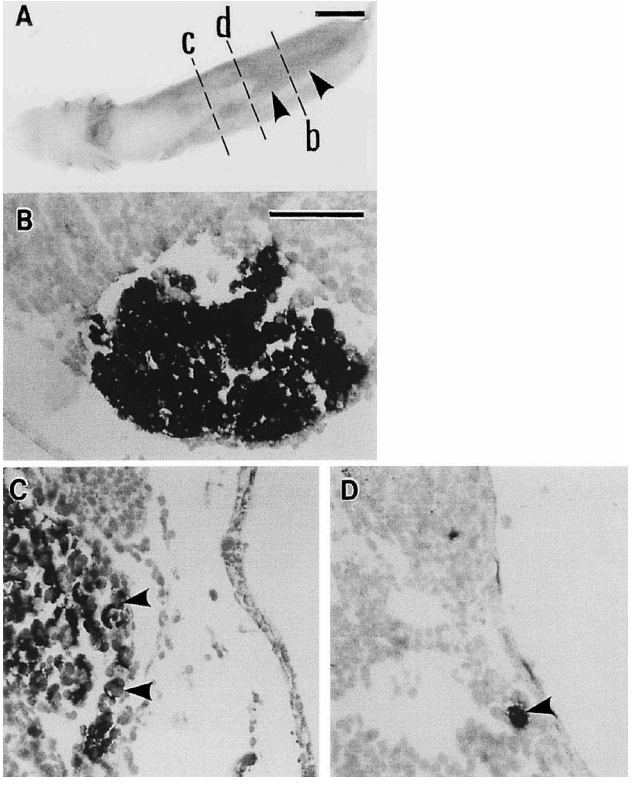

Fig. 7. *In situ* hybridization of an embryo of *Hynobius retardatus* at stage 37/38 with antisense larva β-globin probe, performed to the whole mount embryo (A) or sectioned specimens (B-D) made of different embryos from A. (A) Ventral view of the whole mount embryo. V-shaped VBI is stained clearly (arrowheads). (B) Sectioned specimens of the VBI, at the level corresponding to the dashed line b in A. Erythroid cells expressing larval β-globin gene are nested in the VBI. (C) Anterior region of the DLP, at the level corresponding to the dashed line c in A. Erythroid cells stained positively with larval β-globin probe are detected (arrowheads). (D) Posterior region of the DLP, at the level corresponding to the dashed line d in A. Erythroid cells expressing larval β-globin gene are observed, but only a few (arrowhead). Bars, 1mm (A), 100 μm (B). Modified from Yamaguchi and Wakahara (2001).

GATA-3 Expression during Erythropoiesis

The GATA-3 clone possessed highly conserved zinc-finger domains, suggesting that the *Hynobius* GATA-3 genes have similar regulatory roles in hematopoiesis as do those of other vertebrates. We tentatively regarded GATA-3 as a marker of definitive hematopoietic cells for the reason described below. First, GATA-3 knock-out mice have a defect affecting definitive hematopoiesis, although primitive hematopoiesis occurs normally even in such mice (Pandolfi *et al*., 1995; Ting *et al*., 1996). Second, GATA-3 gene expression in RBCs increases at the time of the Hb transition in chicken (Leonard *et al*., 1993). Third, GATA-3 is expressed in the DLP, but not in the VBI, at tailbud stage in *X. laevis* (Bertwistle *et al*., 1996). In *H. retardatus*, the GATA-3 gene was expressed as early as stage 39 embryos (Fig. 8), but it was not expressed in RBCs of larvae at 20 days after the hatching (Yamaguchi and Wakahara, 2001). These findings suggest that the GATA-3 gene works at the early stages of development but not at later stages. *In situ* hybridization convincingly demonstrated that the GATA-3 gene was expressed in the DLP (Fig. 8C) but not in the VBI (Fig. 8D) or peripheral blood at stage 39. These facts may imply that some DLP cells contribute to erythropoiesis at early stages of development, while others are set aside in an undifferentiated state for hematopoiesis at later

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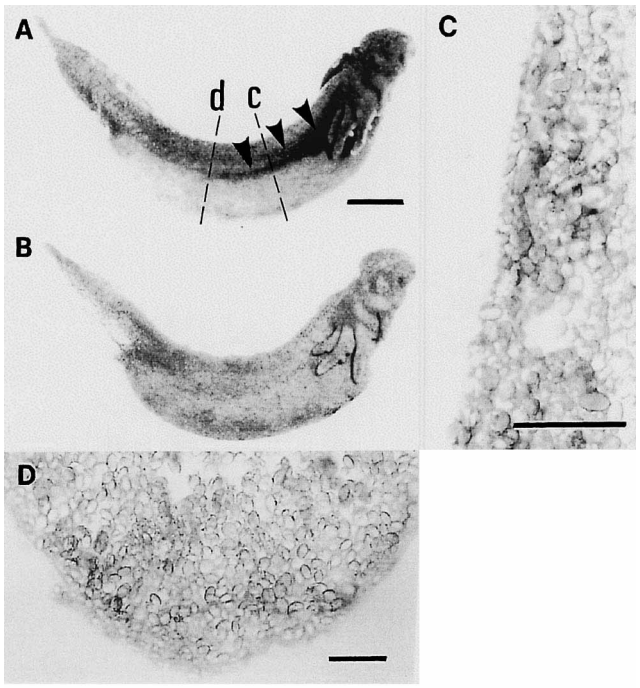

Fig. 8. *In situ* hybridization of *Hynobius retardatus* embryos at stage 39 treated with GATA-3 probe. A, Lateral view of the whole embryo treated with antisense probe. The DLP region is clearly stained (arrowheads), but not the VBI. B, Lateral view of the whole embryo treated with sense GATA-3 probe. No signals are detected at all. C, Cross section of the specimen shown in A at the level indicated by a dashed line c. Cells of the DLP are positively stained. D, Cross section through the VBI region of the specimen shown in A at the level indicated by dashed line d. Cells of the ventral region are not stained. Bars, 1 mm (A, B); 100 μm (C, D). Modified from Yamaguchi and Wakahara (2001).

stages. In other words, the DLP cells set aside in an undifferentiated state may have more "definitive" characteristics. These explanations are consistent with the concept that primitive erythropoiesis occurs in cells of the VBI and in some cells of the DLP, but that other DLP cells are reserved during embryogenesis for future definitive erythropoiesis.

During early developmental stages, the spleen and liver must be major erythropoietic organs in *H. retardatus*. In the liver, however, erythropoietic activity ceases and thus RBC-precursor cells are hardly observed in metamorphosing larvae and metamorphosed juveniles (Yamaguchi and Wakahara, 1997). This suggests that the liver functions as an erythropoietic organ only at early larval stages, from which RBCs containing only larval globins differentiate (Yamaguchi *et al*., 2000). The spleen becomes the major erythropoietic organ before the Hb transition in *H. retardatus* like in *Triturus* (Tournefier, 1973). It is thus suggested that the spleen functions as a major erythropoietic organ throughout life in this species, in contrast to *Rana* and *Xenopus* (Broyles, 1981; Weber *et al*., 1991).

Induction of Anemia

Induction of anemia by phenylhydrazine (PHZ), resulting in a rapid regeneration of the circulating RBCs, has been frequently used to explore the origin of the developmental properties of the larval RBCs (Flores and Frieden, 1972; Widmer *et al*., 1983). These authors have shown a precocious appearance of adult Hbs or adult globin mRNAs in tadpoles recovering from anemia of *R. catesbeiana* and *X. laevis*. These findings suggest that the larval erythropoietic system has a limited capacity for self-renewal, and by its decline may trigger the outgrowth of the adult erythroid cell population, and support the "RBC replacement" model. Contrary to this, in pre- and prometamorphic larvae of *H. retardatus* recovering from anemia induced by PHZ-treatment, a precocious Hb transition did not occur (Yamaguchi *et al*., 2000), suggesting that the RBC replacement during ontogeny could not be expected in this species.

On the other hand, when metamorphosing larvae of *H. retardatus* were treated with PHZ to induce anemia and then bled at a postmetamorphic stage after they recovered from the anemia, a precocious Hb transition was observed in these animals (Yamaguchi and Wakahara, 2001). This finding suggests that RBCs expressing only adult Hb, i. e., "definitive" RBCs, proliferate and differentiate during and after metamorphosis. We consider that PHZ treatment reduces the number of mature "primitive" RBCs (expressing larval and adult globins) in metamorphosing larvae, and that after the treatment, only "definitive" but not "primitive" erythroid cells proliferate in response to THs. Contrary to this, the number of "primitive" RBCs in the controls does not decrease. As a result, the Hb transition in PHZ-treated animals might progress faster than in the controls. If blood is collected before metamorphosis, "definitive" RBCs will not have emerged yet, and no precocious transition occurs (Yamaguchi *et al*., 2000). These observations suggest that there are two distinctive RBC populations which express and/or come to express adult Hb in *H. retardatus*: the "primitive" and "definitive" erythroids (Yamaguchi and Wakahara, 2001). The former initially expresses larval Hb and comes to express finally adult Hb by switching globin genes within every single cell, and the latter which is reserved for later ontogenesis proliferates and differentiates to express only adult Hb during metamorphosis in response to THs (Fig. 9). This model can explain the facts (1) that exogeneously applied T_3 accelerate the Hb transition in *H. retardatus* (Satoh and Wakahara, 1999) even though the transition is not affected by goitrogen-treatment (Arai and Wakahara, 1993; Wakahara and Yamaguchi, 1996), (2) that the precocious Hb transition was not observed in *H. retardatus* recovering from anemia before metamorphosis (Yamaguchi *et al*., 2000), but was observed in *H. retardatus* recovering from anemia after the metamorphosis (Yamaguchi and Wakahara 2001), and (3) that the number of RBCs expressing only adult Hb, which must be definitive, was significantly larger in controls than in the goitrogen-treated, metamorphosis-arrested animals (Yamaguchi and Wakahara, 2001), even though there were no differences in the degree of completion of the Hb transition between controls and goitrogen-treated animals (Arai and Wakahara, 1993; Wakahara and Yamaguchi, 1996).

In mammals, it has generally been accepted that the primi-

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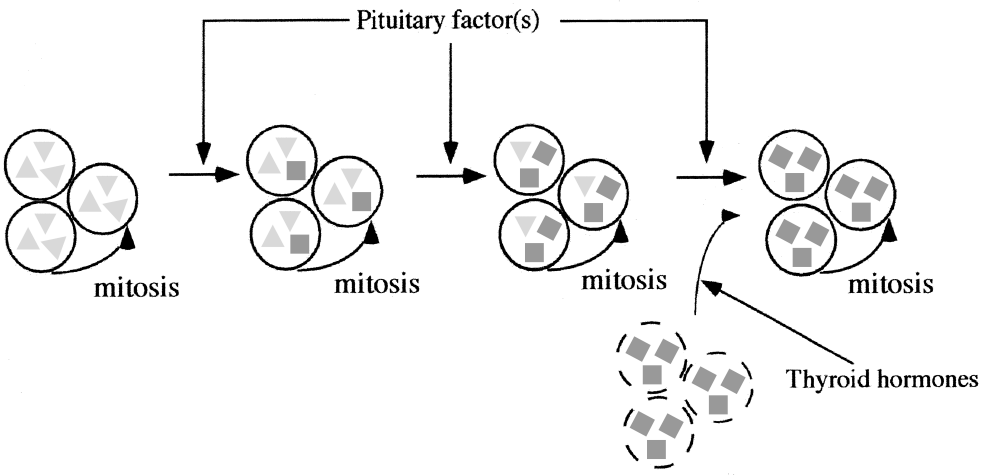

Fig. 9. Tentative model showing cellular mechanism of RBCs/Hbs transition from the larval to the adult type in *Hynobius retardatus*. Both VBI and DLP cells differentiate into RBCs containing larval Hb. In other words, both contribute to the "primitive" erythropoiesis. Much earlier than the morphological metamorphosis, Hb transition initiates within a single erythroid cell population originated from the VBI as well as the DLP, depending on pituitary factor(s). During the metamorphosis, RBCs of another lineage which have only adult Hb (i. e., "definitive" erythroids from the DLP which are set aside in an undifferentiated state for later hematopoiesis) come to proliferate and differentiate responding to thyroid hormones. Triangles indicate larval Hb and squares indicate adult Hb.

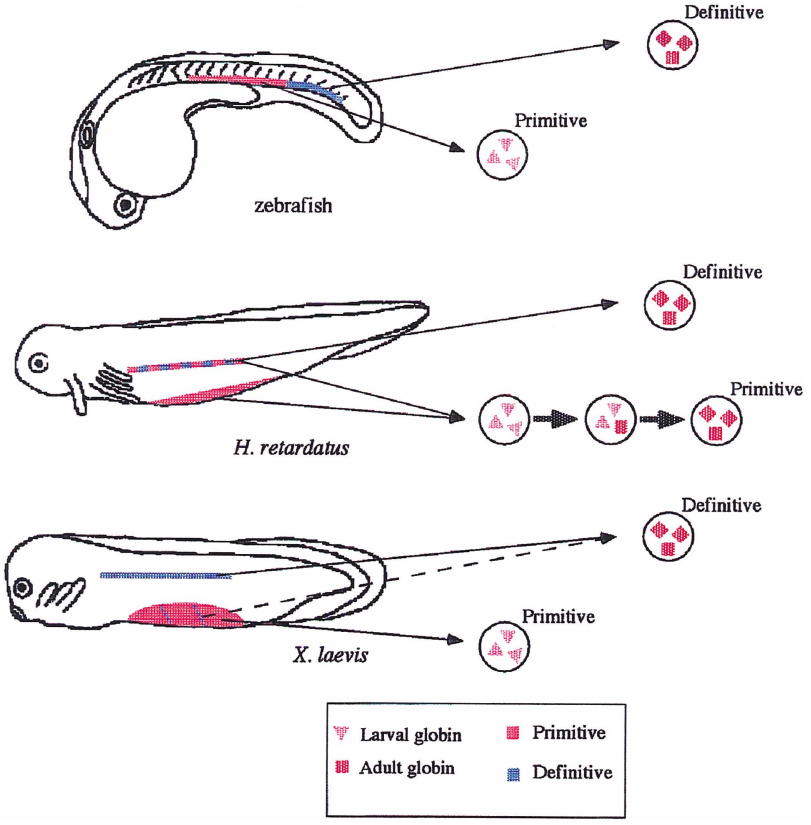

Fig. 10. Tentative model showing the hematopoietic sites that contribute to primitive and definitive erythropoiesis and the cellular mechanism of Hb transition from the larval to the adult type in lower vertebrates, compared with a bony fish (zebrafish), a urodele (*Hynobius retardatus*), and an anuran amphibian (*Xenopus laevis*). The sites from which primitive blood arises are shown by red, and those from which definitive blood arises are shown by blue. Pink triangles indicate larval Hb and orange squares indicate adult Hb. In the zebrafish embryo, the intraembryonic ICM is a source of primitive blood, and the cells of the posterior ICM may contribute to definitive hematopoiesis. On the other hand, primitive erythropoiesis in *X. laevis* occurs in the VBI contributing to the larval RBCs, and the DLP (intraembryonic) cells remain undifferentiated until later for definitive hematopoiesis. During the metamorphosis, the larval RBCs are replaced by adult ones under the influences of thyroid hormones. The *H. retardatus* embryo also has two distinct hematopoietic sties, the VBI and DLP. The DLP cells of *H. retardatus*, however, differentiate in situ to RBCs containing larval globin (i. e., "primitive"). During metamorphosis, RBCs of another lineage from the DLP, which come to express only adult Hb (i. e., "definitive"), begin to proliferate and differentiate in response to thyroid hormones. From Yamaguchi and Wakahara (2001).

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tive erythropoiesis generates embryonic RBCs containing embryonic Hbs and that definitive one generates adult RBCs containing adult Hbs (Zon, 1995). Contrary to this, Palis *et al*. (1999) recently proposed a model that RBCs express only embryonic globins in earlier developmental stages but come to express adult globins during later stages. This mammalian model in the Hb transition proposed by them is very similar to our model explaining the Hb transition in *Hynobius* proposed here.

Tentative Models of Erythropoiesis in Lower Vertebrates

Tentative models of erythropoiesis in lower vertebrates such as a fish (zebrafish), urodele (*H. retardatus*) and anura (*X. laevis*) are illustrated in Fig. 10. Contrary to the erythropoiesis in amphibians, there are no extraembryonic hematopoietic sites in many fishes: both primitive and definitive blood cells arise from intermediate cell mass (ICM) (Al-Adhami and Kunz, 1977; Detrich *et al*., 1995; Thompson *et al*., 1998; Maruyama *et al*., 1999). In zebrafish embryo GATA-2 is expressed throughout the ICM region while GATA-1 is not expressed in posterior ICM (Detrich *et al*., 1995; Thompson *et al*., 1998). This implies that the hematopoietic cells of the posterior ICM remain in undifferentiating phase and contribute to definitive hematopoiesis in this species.

In *Xenopus*, primitive erythropoiesis occurs in the VBI, and the DLP (intraembryonic) cells remain undifferentiated until later for definitive hematopoiesis (Turpen *et al*., 1997), although some cells of the VBI are reported to contribute to adult erythropoiesis (Maeno *et al*., 1985a, b; Rollins-Smith and Blair, 1990). In *H. retardatus* we have shown that cells of the DLP as well as the VBI expressed larval globin mRNA and globin subunits in tailbud embryos and early larvae (Yamaguchi *et al*., 2000; Yamaguchi and Wakahara, 2001). This means that hematopoietic cells of the DLP differentiate *in situ* to RBCs, indicating that not only the VBI but also the DLP is source of “primitive” erythroid cells. Some DLP cells are set aside in an undifferentiated state for “definitive” hematopoiesis at later stages. From these it is assumed that *H. retardatus*, or urodeles in general, may occupy phylogenetically an intermediate position between fishes and anurans (Yamaguchi and Wakahara, 2001) (Fig. 10). Because there are little studies on erythropoiesis in urodeles (Grasso, 1973), it remains to be clarified whether the model proposed in *H. retardatus* can explain the erythropoiesis in urodeles, in general.

PERSPECTIVES

Hematopoietic induction has been studied in explants of blastula animal cap in *Xenopus* (Ariizumi *et al*., 1991; Green *et al*., 1990). In response to basic fibroblast growth factor (bFGF), bone morphogenetic proteins (BMPs), or activin A, a lot of “blood-like cells” were induced. Many studies indicate that erythropoiesis may be activated when inducers are present in combination (Miyanaga *et al*., 1998, 1999; Nishimatsu and Thomsen, 1998; Huber *et al*., 1998). Because no *in vitro* studies have been done in urodeles, it is not possible to know effects of different growth factors on animal cap explants. Differences and similarities in the contribution of VBI and DLP to the primitive and/or definitive erythropoiesis between anurans and urodeles must be clarified in the *in vitro* systems.

Although many transcription factors are expected to regulate erythropoiesis and differentiation of RBCs, the cascade is not fully clarified in amphibians. Members of the GATA family of zinc-finger transcription factors are required for proliferation and differentiation of several hematopoietic lineages. Recently GATA cofactors (friend of GATA, FOG) have been identified, and *Xenopus* homologue of FOG was cloned (Deconinck *et al*., 2000). FOG may regulate the differentiation of RBCs by modulating expression and activity of GATA-1 and GATA-2, and participate in the switch from progenitor proliferation to RBC maturation and differentiation. Contrary to this, PU.1, a hematopoietic-specific transcription factor has been reported to interact GATA-1 showing functional antagonism in erythroid cells: ectopic expression of PU.1 in *Xenopus* embryos is sufficient to block erythropoiesis during normal development (Rekhtman *et al*., 1999). Misexpression of the dorsal mesodermal patterning factor goosecoid on the ventral side of *Xenopus* embryos results in inhibition of blood formation in early embryognesis, suggesting that goosecoid protein may interact with PU.1 (Konishi *et al*., 1999). The Ikaros family of zinc-finger transcription factors also plays an essential role as a master switch in hematopoiesis (Hansen *et al*., 1997; Durand *et al*., 1999). The most important is to analyze these transcription factors, which have been known mainly in mammalian erythropoiesis and Hb conversion, in amphibian system.

Positive involvement of the pituitary factor(s) in Hb transition during metamorphosis was firstly observed in *Hynobius retardatus* (Satoh and Wakahara, 1997, 1999), but not in anurans. The Hb transition proceeds very slowly even in Hx larvae of *H. retardatus* whose metamorphosis is completely arrested (Satoh and Wakahara, 1997), and even in normal larvae reared at 4°C (Arai and Wakahara, 1993), at which temperature the thyroid glands have been reported to be completely inactive (Moriya, 1983a) and the THs have been insensitive at eliciting the metamorphic changes in *H. retardatus* (Moriya, 1983b). Therefore, it seems true that the Hb transition from the larval to the adult type proceeds autonomously at a certain degree without any stimulation of hormones, whatever the speed of transition is very low. This is similar to the autonomous transition known to occur in chicken and mammalian globin genes (Choi and Engel, 1988; Engel, 1993). The Hbs/RBCs change in *H. retaratus* is completely different from general metamorphic changes in amphibians, in which no autonomous changes from the larval to the adult phenotype occur without THs, thus is remained unsolved.

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