The switch from larval to adult globin gene expression in *Xenopus laevis* is mediated by erythroid cells from distinct compartments

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Summary

The transition of hemoglobins during metamorphosis of *Xenopus laevis* involves replacement of the larval erythrocytes by adult ones, suggesting that the developmental control of this event depends upon the growth characteristics of the precursor cells. To identify the erythroid precursor cells and to investigate their developmental fate, we analyzed the distribution of stage-specific globin mRNAs by northern blotting in dorsal and ventral fragments of stage 32 embryos after *in vitro* culture as well as presumptive erythropoietic tissues of tadpoles during metamorphosis.

The histological analysis shows that erythrocytes differentiate only in ventral fragments, suggesting that the ventral blood islands and most likely also the dorsolateral mesoderm are the primary sites of erythropoiesis. We also demonstrate that the first generations of erythrocytes, already express the predominating larval-specific α-globin mRNAs. The globin mRNA patterns obtained from presumptive erythropoietic tissues suggest an important role of circulating precursor cells in larval erythropoiesis, whereas the liver appears to be the main site of formation and maturation of the adult erythrocytes.

Tentatively we propose that anuran erythropoiesis is dependent upon a self-perpetuating stem-cell line and that the larval and the adult erythrocytes are derived from successive generations of erythroid precursors, whose commitment may be imposed by the erythropoietic sites.

Key words: hemoglobin transition, metamorphosis, *Xenopus laevis*, erythropoiesis, globin mRNA, northern blotting, *in situ* hybridization.

Introduction

In *Xenopus laevis*, a switch in hemoglobin synthesis occurs at metamorphosis (Just *et al.* 1977) resulting in the replacement of the larval globin subunits by a set of distinct adult ones (Hosbach *et al.* 1982; Sandmeier *et al.* 1988). This event, therefore, provides a useful model for investigating the developmental control of gene expression.

Analysis of cDNA clones, derived from poly(A)+ RNA of erythrocytes, revealed that different sets of α- and β-globin genes are expressed in the larval and the adult stage of *Xenopus laevis* (Hentschel *et al.* 1979; Widmer *et al.* 1981; Banville and Williams, 1985a,b). The corresponding genes were found to represent a unique arrangement, as shown by the linkage of α- and β-genes, as well as the occurrence of pairs of closely related genes that are located in the same order on two clusters (Jeffreys *et al.* 1980; Hosbach *et al.* 1983). Although differences in DNAase I-hypersensitivity in the upstream sequence between the transcriptionally active and silent adult α-globin genes could be demonstrated (Stalder *et al.* 1986), the regulatory elements for cell-specific expression of these genes remain to be identified.

Studies on cell morphology, hemoglobin phenotypes as well as DNA and protein synthesis in the circulating erythrocytes of various anuran species suggest that hemoglobin transition reflects the replacement of the larval erythrocytes by a new population of erythroid cells committed to adult hemoglobin synthesis (review by Broyles, 1981). In a recent study (Weber *et al.* 1989), we presented evidence for a similar mechanism in *Xenopus laevis*, for which it had been previously suggested that hemoglobin switching may occur within the same cell (Jurd and Maclean, 1970).

Extensive work on anuran erythropoiesis, involving grafting of genetically marked tissue primordia in embryos, led to the conclusion that the ventral blood islands give rise to a short-lived population of primitive erythrocytes and that the liver is the main source of the definitive larval as well as the adult erythrocytes (review by Broyles, 1981). From similar investigations on the origin of the hematopoietic stem cells in *Xenopus laevis*, it was suggested that the primitive erythrocytes arise from precursors located in the ventral blood
islands, whereas the definitive larval erythrocytes are derived from precursors in the dorsolateral mesoderm (review by Turpen et al. 1982; Katagiri et al. 1986). However, there is as yet no information on the origin of the adult erythrocytes in *Xenopus laevis*.

In all these studies, the erythrocytes were characterized by cytological criteria. Yet, to get a precise picture of the mechanism of hemoglobin transition, an earlier and more stringent identification of the committed erythroid cells is needed. Our cDNA clones, which allow detection of stage-specific globin mRNAs (Widmer et al. 1981), provide very sensitive tools for identification of erythroid cells even at an early stage of differentiation.

The purpose of this investigation was to trace the origin of the larval and the adult erythrocytes of *Xenopus laevis* by analyzing globin mRNA expression in embryonic and larval tissues. Embryonic fragments were cultured *in vitro* and analyzed with respect to erythropoietic potency and expression of larval-specific globin mRNA. Putative erythropoietic tissues and blood cells of tadpoles undergoing metamorphosis were screened for the appearance of adult-specific globin mRNA. Our results suggest that the ventral blood islands and possibly also the dorsolateral mesoderm represent the primary source of erythropoietic cells. Furthermore, they indicate that the early progeny of the erythroid primordia express larval-specific globin mRNA, whereas later derivatives, located in the liver, give rise to the adult erythrocytes.

**Materials and methods**

**Animals**

Embryos and larval stages were obtained by induced breeding and raised under standard conditions (New, 1966). Stages were designated according to Nieuwkoop and Faber (1967).

**In vitro culture of embryonic fragments**

Embryos of stage 32 were removed from the jelly coat and cut into dorsal and ventral halves with tungsten needles. Operations were performed in NAM (= normal amphibian medium, Slack and Forman, 1980) containing 100 IU ml⁻¹ penicillin and 10 μg ml⁻¹ streptomycin. The fragments were cultured in 50% NAM for 3 days at 25°C.

**Histological procedures**

Embryonic fragments were fixed in Romeis fixative (25 ml saturated HgCl₂, 20 ml 5% trichloroacetic acid, 15 ml 37% formaldehyde), dehydrated in ethanol and after passage through n-butylacetate embedded in paraffin. Sections were stained with acid hemalum and 1% aqueous erythrosine. For *in situ* hybridization small pieces of liver from stage 59 tadpoles were fixed in Carnoy solution (60 ml ethanol, 30 ml chloroform and 10 ml acetic acid) for 12 h at 0°C. The specimens were dehydrated in ethanol, transferred to n-butylacetate and embedded in paraffin.

**In situ hybridization**

After removal of paraffin the liver sections (3 μm) were taken through decreasing concentrations of ethanol and rinsed in distilled water. After treatment with 0.4 μg ml⁻¹ proteinase K (Merck) for 15 min at 37°C in 20 mM Tris–HCl, pH 7.5, 2 mM CaCl₂ the sections were incubated in 50% formamide, 2×SSC for 60 min at 40°C. For hybridization an aliquot of 100 000 cts min⁻¹ of H-labelled adult α-globin antisense or sense mRNA μl⁻¹ was diluted to 2500 cts min⁻¹ μl⁻¹ with hybridization buffer containing 50% formamide, 0.3 mM NaCl, 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 100 mg ml⁻¹ dextran sulfate (Pharmacia), 0.2 mg ml⁻¹ polyvinylpyrrolidone (Merck), 0.2 mg ml⁻¹ Ficoll (Pharmacia), 0.2 mg ml⁻¹ bovine serum albumin (Boehringer), and 0.5 mg ml⁻¹ yeast tRNA (Sigma). The hybridization probes were denatured for 5 min in boiling water and quickly chilled on ice. 20 μl of the probe was placed on the slides after removal of the liquid around the tissue sections and covered with a 21×26 mm siliconized coverslip. Incubation was done in a moist chamber for 16 h at 40°C. After flushing off the coverslips with 4×SSC, the slides were washed twice for 30 min in 2×SSC followed by 0.1×SSC at room temperature. The slides were dehydrated in 70% ethanol followed by 90% ethanol containing 0.3 mM sodium acetate and then air-dried.

For autoradiography the slides were processed according to standard techniques using Kodak NTB-2 emulsion, exposed for 10 days at 4°C and stained with acid hemalum and 1% aqueous erythrosine.

**Extraction of nucleic acids**

Liver, kidney and blood cells were collected from 2–3 larvae. Tissue samples and blood cells were washed in isotonic phosphate-buffered saline (PBS=102.7 mM NaCl, 2.7 mM KCl, 0.7 mM phosphate buffer, pH 7.5) and 1.9% sodium citrate, respectively. Homogenization was done at 4°C in 400 μl of a solution containing 50 mM Tris–HCl, pH 8.0, 100 mM NaCl, 5 mM EDTA and 0.5% sodium dodecylsulfate, using a loosely fitting micro-homogenizer. To prevent degradation of RNA 0.1% diethylpyrocarbonate was added to the medium.

Nucleic acids were extracted in three steps, using 400 μl of phenol saturated with 0.3 M NaCl, followed by the same volume of phenol-chloroform (1:1) and chloroform, respectively. Nucleic acids were precipitated by addition of 1/10 volume of 0.3 M sodium acetate (pH 5.5) and 2.5 volumes of ethanol. The precipitate of crude nucleic acids was washed with 70% ethanol, dried and dissolved in 20 μl of distilled water. The solutions were stored at -20°C.

**Electrophoresis and northern blotting**

Aliquots of nucleic acid extracts representing the same amount of DNA, were separated by electrophoresis on 1% agarose gels with glyoxal as denaturing agent (Thomas, 1980). DNA was determined by the modified diphenylamine procedure of Giles and Myers (1965). The gels were stained with acridine orange (McMaster and Carmichael, 1977) and photographed under UV-illumination. Northern transfer and filter hybridization followed the standard protocol (Maniatis et al. 1982). As hybridization probes, we used 32P nick-translated inserts of the cDNA clones pXGL9 and pXGA5 which, as shown by Widmer et al. (1981), allow specific detection of larval and adult α-globin mRNAs, respectively.

**Results**

The larval erythroid cells are mainly derived from the ventral mesoderm. To establish the origin of the larval erythrocytes, we
investigated the potential of dorsal and ventral fragments of prehatching embryos to form erythrocytes. As shown in Fig. 1A, stage 32 embryos were cut into dorsal and ventral halves, which were cultivated in 50% NAM for three days, when control embryos of the same batch reached stage 42. From Fig. 1B we see that dorsal fragments develop into somewhat distorted larvae, which are capable of swimming, but lack the ventral derivatives of endoderm. However, ventral fragments, shown in Fig. 1C, consistently formed transparent vesicles containing opaque masses of endodermal cells and occasionally also aggregates of contracting muscle cells.

The histological analysis revealed a marked difference with respect to erythropoietic potency of the dorsal and the ventral fragments. Fig. 2A shows that defective larvae, obtained from dorsal fragments, display more or less normal morphology of the axial structures including a well-developed heart, which, as seen from Fig. 2B, is, however, devoid of erythrocytes. This is in contrast to normal larvae, which apart from their larger size, show intense erythropoietic activity, as is noted in Fig. 2C and D, by the abundance of mature erythrocytes in blood vessels and the heart.

The internal organization of a vesicle, derived from a ventral fragment, is shown in Fig. 3A. It comprises fully differentiated derivatives of the mesoderm and undifferentiated aggregates of yolk-rich endodermal cells. As shown by Fig. 3B, the formation of blood vessels and mature erythrocytes is a characteristic feature of these fragments, suggesting that the ventral mesoderm plays a key role in anuran erythropoiesis.

The early derivatives of the ventral blood island express larval specific globin mRNA

To characterize further the early larval erythrocytes, we analyzed the nucleic acid extracts from dorsal and ventral fragments by northern blotting. As hybridization probe we used a mixture of the inserts of the cDNA clones pXGL9 and 19, which represent the closely related larval-specific α- and α₁-mRNAs (Widmer et al. 1981; Sandmeier et al. 1988), respectively. As control for specificity of the hybridization, we used a nucleic acid extract from erythrocytes of stage 54 tadpoles, in which the larval α-globin mRNAs are most abundant. The result of this experiment is shown in Fig. 4B. It demonstrates that the larval-specific α-globin mRNAs occur already in prehatching embryos at stage 32, and are apparently restricted to the ventral fragment. In the vesicles, obtained from ventral fragments after three days of culture, a higher content in larval α-globin mRNAs is found, whereas no such RNA is detectable in partial larvae, derived from dorsal fragments. In contrast, some larval α-globin mRNA is detected in partial larvae obtained from dorsal fragments, cut such as to include the dorsolateral mesoderm, as indicated in Fig. 4A. This suggests that the erythropoietic potential is not restricted to the ventral mesoderm. Furthermore, it should be noted that the mRNAs from both the embryonic fragments and the mature larval erythrocytes hybridize under highly stringent conditions with the larval-specific α-globin cDNAs. Therefore, we conclude that the early generations of erythrocytes express sequences that must be closely related, if not identical to the α-globin mRNAs,
which are abundant in the erythrocytes of stage 54 tadpoles.

Erythrocytes, expressing adult-specific globin genes are formed and undergo maturation in the liver

To localize the origin of the adult erythrocyte population, we compared the distribution of larval- and adult-specific globin mRNAs in putative erythropoietic sites of tadpoles during metamorphosis. Nucleic acid extracts, representing equivalent samples of liver and kidney tissue as well as blood cells from the same animals, were analyzed on northern blots. To monitor larval- and adult-specific globin mRNAs, filters were hybridized with nick-translated DNA from the cDNA clones pXGL19 and pXGA5 (Widmer et al. 1981), respectively. The vector DNA sequence was eliminated by restriction and gel isolation.

In the experiment, shown in Fig. 5A, we found highest levels of larval and adult globin mRNAs in liver
Fig. 3. Internal structure of vesicle obtained from a ventral fragment. Overall view of section in A and blood vessel in B. ep, epidermis; en, endoderm; m, muscle cells; bv, blood vessel; e, erythrocytes. Bar in A=100 μm, in B=20 μm.

Fig. 4. Distribution of the major larval α-globin mRNAs in dorsal and ventral fragments and the corresponding differentiation products. (A) Dissection of embryos at level 1 resulted in dorsal and ventral fragments, whereas dissection at level 2 yielded dorsal fragments including the dorsolateral mesoderm and fragments containing only the ventral mesoderm. (B) Autoradiogram of northern transfer analysis of nucleic acid extracts from erythrocytes of stage 54 tadpoles, dorsal (d) and ventral (v) fragments of stage 32 embryos (day 0) and the corresponding partial larvae (d) and vesicles (v) obtained in culture (day 3) as well as from partial larvae (dlp) and vesicles (vm) that developed from dorsal fragments with dorsolateral mesoderm and ventral fragments with ventral mesoderm only. The samples correspond to two of each embryonic fragments, partial larvae or vesicles. For hybridization a mixture of 32P-nick-translated inserts of pXGL9 and pXGL19, specific for the most abundant larval α-globin mRNAs, were used.
Fig. 5. Distribution of larval- and adult-specific α-globin mRNA in erythropoietic tissues of tadpoles undergoing metamorphosis. Autoradiograms of northern transfer blots from an erythropoietic screening experiment (A) and a fine timing experiment (B). For these experiments, two different batches of tadpoles were used. Nucleic acid extracts from liver (L), kidney (K) and blood cells (B) were analyzed in aliquots, representing tissue equivalents of 1 µg DNA. 32P-nick-translated inserts of pXGL19 and pXGA5 were used for detection of the major larval- and adult-specific α-globin mRNA, respectively. 57–58 = premetamorphic, 59–66 = metamorphic stages, F = adult.

and blood cells of metamorphosing tadpoles. In contrast, only trace amounts, presumably reflecting capillary blood contamination, were detected in the mesonephros. Larval globin mRNA was abundant at the onset but declined gradually until the end of metamorphosis. However, adult globin mRNA was already detected at the beginning of metamorphosis. It was most abundant during early metamorphosis and decreased during later stages, presumably indicating progressive maturation of the adult erythrocytes.

The experiment shown in Fig. 5B, which refers to a more delayed globin transition, demonstrates that larval globin mRNA is most abundant in the blood cells until the onset of metamorphosis (stage 59), but decreases thereafter. In contrast, adult globin mRNA is not detected in premetamorphic tadpoles, but appears during early metamorphosis (stage 62) and is exclusively found in the liver. With the progress of metamorphosis the adult-specific globin mRNA increases in the liver and becomes also abundant in the circulating blood cells. Despite apparent degradation, the level of adult-specific globin mRNA is highest in stage 66 tadpoles. Steady state levels of adult globin mRNA in mature frogs are rather low, which is consistent with the small number of erythroblasts in the circulating blood (Müller, unpublished data).

These results strongly suggest that the erythrocytes, which express adult-specific α-globin mRNA, originate and apparently also undergo maturation in the liver.

This conclusion is further supported by in situ hybridization of liver sections of stage 59 tadpoles with 3H-labelled adult α-globin antisense mRNA. In fact, Fig. 6A shows that erythroblasts in liver sinusoids are labelled, thus indicating again the role of the liver as origin of erythrocytes, expressing the adult-specific α-globin mRNA. The specificity of the reaction is documented by the absence of labelling after hybridization with the corresponding sense mRNA probe shown in Fig. 6B.

Discussion

The early progeny of the erythropoietic primordia expresses larval-specific globin mRNA

In vitro culture of embryonic fragments provides evidence that the emerging erythrocytes in embryos of Xenopus laevis originate mainly from the ventral, and most likely also from the dorsolateral mesoderm. This is in agreement with the results of grafting experiments of Maeno et al. (1985), who showed that erythropoietic stem cells of Xenopus laevis predominantly originate from the ventral mesoderm (=ventral blood islands) and in part also from the dorsolateral mesoderm, which includes the prospective mesonephric region.

In addition, northern analysis of nucleic acid extracts with larval-specific cDNA probes revealed that the erythroid cells of prehatching embryos contain mRNA sequences very similar if not identical to the α-globin mRNAs of mature larval erythrocytes. This finding is consistent with the study of Banville and Williams (1985) who showed that two closely related α-like globin mRNAs (αT3, αT4) are expressed in prefeeding tadpoles and represent the most abundant α-globin mRNA in premetamorphic tadpoles. Although our results do not rule out the possibility of differential expression of globin genes, the presence of the major larval-specific α-globin mRNAs in both the emerging erythrocytes of embryos and those of premetamorphic tadpoles, suggests that the larval erythrocytes represent a uniform cell population. This is at variance to the current concept on the larval erythropoiesis in anurans, which – in analogy to mammalian and avian systems – assumes a population of primitive erythrocytes, derived from the ventral blood islands, to be replaced by the definitive larval erythrocytes, originating from the liver.
Globin gene expression in Xenopus

Fig. 6. Localization of adult globin-mRNA in the tadpole liver at metamorphosis.
Autoradiogram of liver section of stage 59 tadpole after in situ hybridization with $^3$H-labelled anti-adult $\alpha$-globin mRNA (A) and the corresponding sense-mRNA (B). eb, erythroblasts; e, mature erythrocytes. Bar=20 $\mu$m.

(review by Broyles, 1981). This concept is based on morphological and biochemical criteria (Dorn and Broyles, 1982) as well as on grafting experiments involving exchange of genetically marked erythroid primordia and subsequent analysis of their progeny by purely cytological criteria (reviews by Turpen et al. 1982; Katagiri et al. 1986). However, it is not known whether these apparently distinct erythrocyte populations represent the same or distinct cell phenotypes. In this context, it should be noted that very recent grafting experiments with genetically marked ventral blood islands by Rollins-Smith and Blair (1990) indicate that erythrocytes, apparently derived from the grafts, even persist in postmetamorphic froglets of Xenopus laevis. It would be of great interest to know, if the graft-derived erythrocytes in froglets express larval- or adult-specific globin genes. The latter situation might be taken to indicate that the graft contains self-perpetuating hematopoietic stem cells which during metamorphosis give rise to erythroid precursor cells that are committed to adult globin gene expression.

As mentioned above, differentiation of larval-specific erythrocytes occurs prior to the onset of blood circulation. However, we showed that larval-specific globin mRNA is most abundant in the blood cells, but relatively scarce in the liver and the mesonephros of premetamorphic tadpoles (cf. Fig. 5A). This is consistent with the abundance of immature erythroblasts, also capable of DNA synthesis, in the circulating blood at these stages (Weber et al. 1989). We therefore conclude that the circulating erythroid cells may play a prominent role in larval erythropoiesis.

The adult erythrocytes emerge and undergo maturation in the liver

Stage-specific cDNA probes for globin mRNAs allowed us to monitor the appearance of larval or adult erythroid cells with great sensitivity and precision at an
early stage of differentiation. Northern blots of nucleic acid extracts from liver and blood cells revealed some variation in the appearance of the major adult α-globin mRNA, which typically is present at the beginning of metamorphosis (stage 59). In the screening experiment of erythropoietic tissues (cf. Fig. 5A), adult-specific globin mRNA was first detected simultaneously in the liver and the blood cells of metamorphosing tadpoles, whereas in the fine timing experiment (cf. Fig. 5B), it was found first exclusively in the liver and at a later stage also in the blood cells. Since globin transition may vary considerably even among tadpoles of the same developmental stage (Weber, unpublished data) and different batches were used in these experiments, the discrepancy in appearance of adult-specific globin mRNA most likely reflects differential progress of globin transition. Furthermore, it should be noted that pooled tissue samples were used for RNA extraction. Therefore, differences in progress and synchrony of globin transition within each stage may account for the discrepancy in the distribution of adult-specific globin mRNA among liver and blood cells.

The first appearance of adult-specific globin mRNA in the liver together with our previous finding of a decline in DNA synthesis in the circulating blood cells during metamorphosis, suggests that the adult erythrocytes emerge and undergo functional maturation in the liver.

**A tentative model of anuran erythropoiesis**

The absence of erythropoiesis in partial larvae from embryos, operated prior to the onset of blood circulation, suggests that the ventral blood islands including the dorsolateral mesoderm comprise hematopoietic stem cells. Since erythrocytes derived from the grafted hematopoietic primordia, were found in froglets well after metamorphosis (Rollins-Smith and Blair, 1990; Turpen, unpublished data), it is conceivable that these stem cells are self-perpetuating. The recent data on early erythropoiesis and the more stringent identification of the emerging erythroid cells, call for some modification of the existing models on anuran erythropoiesis (Ingram, 1972; Broyles, 1981).

As shown in Fig. 7, we propose the view that the ventral blood islands and the dorsolateral mesoderm may contain self-perpetuating hematopoietic stem cells and also produce the first generation of erythroid precursors, whose progeny, as judged from the expression of the larval-specific α-globin mRNAs, represent an apparently uniform population of larval erythrocytes. The abundance of erythroblasts, showing DNA synthesis and cell division (Müller, unpublished data), indicates that the circulating erythroid cells are an important source of larval erythropoiesis. In addition, the precocious disappearance of larval erythrocytes after induction of anemia (Blum et al., unpublished data) indicates that the growth potential of the larval erythroid population is limited.

From the distribution of the adult-specific α-globin mRNA in the liver and the circulating blood cells of tadpoles during metamorphosis, it is evident that the precursors for adult erythrocytes originate in the liver, where they divide and undergo terminal differentiation.

This model assumes that the switch from larval to adult globin gene expression is mediated by the development of the corresponding erythroid precursor cells within two distinct compartments. At the present time, it is not known how growth of the adult erythroid precursor cells is regulated and how commitment of the larval and the adult erythroid precursor cells is achieved. Elucidation of these questions should be crucial to our understanding of the developmental control of gene expression.

The authors are grateful to Professor D. Schümpeleri for kindly providing laboratory facilities and critical comments on the manuscript. They are also indebted to Mr T. Wyler for technical support. This work was supported by the Swiss National Science Foundation, grant No. 3.409.86.

**References**


(Received 16 May 1991)