

Erythrocyte differentiation during the metamorphic hemoglobin switch of *Rana catesbeiana*

(cell separation/cellular differentiation/globin gene expression/erythrocyte removal)

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ABSTRACT Anurans (frogs and toads) switch from tadpole to adult hemoglobin synthesis during metamorphosis. A number of workers have attempted to determine whether tadpole and adult Hb types are expressed in the same or different erythroid cells during the switch. If the different Hb types are found in different cells during the transition, the switch in globin gene expression occurs at an early stage of cellular differentiation. Previous studies, in which immunocytochemical techniques were used to approach this question, are in conflict in regard to the metamorphic Hb switch of the North American bullfrog *Rana catesbeiana*. We have purified newly differentiating erythroid cells from the blood of metamorphosing tadpoles by using Percoll gradients. These new cells have an immature morphology, are very active in the synthesis of adult Hb, and contain no detectable tadpole Hb. The tadpole cells have no detectable adult Hb, are synthetically inactive, increase in density during the switch, and are then cleared from the circulation. Thus, only adult Hb expression is detected in newly differentiating erythroid cells during metamorphosis.

One of the most attractive models for studying the regulation of gene expression in eukaryotes is the synthesis of specific globins during erythrocyte differentiation. The gene products (hemoglobins) are well characterized for a number of animal species. Developmental changes or switches in Hb types occur in all classes of vertebrates. However, the molecular and cellular mechanisms involved in Hb switching are poorly understood, despite recent advances in our knowledge of the molecular fine structure of embryonic, fetal, and adult globin genes from several species (1–3).

The expression of pre- and post-switch Hbs in separate erythrocytes during a developmental transition would imply that the regulation of Hb switching operates at an early stage in hemopoietic cell differentiation (4). Thus, immunocytochemical studies using antisera specific to different Hb types have been done during Hb switching of humans (5), mice (6), chickens (4), and several amphibians (7–12). The degree to which pre- and post-switch Hbs coexist in the same cell during Hb switching seems to vary from species to species, even within the amphibia. There have been conflicting reports from different laboratories (7–10) as to the metamorphic Hb switch of *Rana catesbeiana*—the first studied and prototypic example of Hb switching in animals (13). The potentials and importance of this animal model have been discussed elsewhere (14).

We have resolved this matter for *R. catesbeiana* by taking a direct approach—by physically separating the newly differentiating erythroid cells during the metamorphic Hb switch. Shallow density gradients of Percoll (colloidal silica) have been shown to cleanly separate artificial mixtures of tadpole and adult frog erythrocytes (15). We have combined this approach with

electrophoretic separation of Hb types and specific radiolabeling of newly synthesized adult Hb. We conclude that the metamorphic Hb switch is accomplished through (i) the differentiation of a new erythrocyte population that synthesizes only adult Hb and (ii) the selective removal of tadpole Hb-containing erythrocytes from the circulation.

MATERIALS AND METHODS

Animals. Bullfrog tadpoles (*R. catesbeiana*) were purchased from Carolina Biological and maintained as described (16). Metamorphic stages were identified by using the criteria described by Taylor and Kollros (17). Animals were anesthetized in a solution of 0.1% of tricaine before being sacrificed (16).

Chemicals. Radioactive chemicals (L-[3,4,5-³H(N)]leucine, 110 Ci/mmol (1 Ci = 3.7×10^{10} becquerels); L-[³⁵S]methionine, 1,200 Ci/mmol) were purchased from New England Nuclear, as was the scintillation cocktail (Aquasol). The disodium salt of 2-naphthol-6,8-disulfonic acid (G acid) used in the cell maintenance medium (composition given below) was prepared by passing a hot solution (18%, wt/vol) of the dipotassium salt (Fisher) through a sodium-charged Dowex resin (Dowex-X8, 20–50 mesh; BioRad). The disodium salt was precipitated by adding 7 vol of *tert*-butanol to the eluate. The precipitate was collected by vacuum filtration and washed twice with diethyl ether. Bovine serum albumin (fraction V), dithiothreitol, and Hepes were purchased from Sigma; Percoll was obtained from Pharmacia.

Cell Separations. Blood cells were collected as previously described (16) and kept thereafter at 4°C in a cell maintenance medium having the following composition (per liter): 2.0 g of NaCl, 7.0 g of G acid (disodium form), 4.0 g of Hepes (sodium form), 1.13 g of Hepes (hydrogen form), 0.25 g of KCl, 0.67 g of glucose, 0.72 g of MgSO₄·7H₂O, and 10.0 g of bovine serum albumin (filtered). The pH (7.7) and osmolality (190 mOsm/kg) of this medium are the same as tadpole blood (18). G acid (19) and bovine serum albumin (20) used together prevent cell aggregation. The detergent G acid allows high concentrations of cells to be separated. Blood cells were washed twice and carefully suspended 1:10 (vol/vol) in this medium in a Wintrobe tube.

Shallow density gradients (11.4 ml each) were formed by centrifuging a Percoll mixture at $50,330 \times g_{max}$ in a Beckman JA-21 fixed-angle rotor at 4°C for 10 min. This mixture contained 7.0 ml of Percoll, 3.8 ml of triple-strength cell maintenance medium, and 0.6 ml of deionized distilled H₂O.

Approximately 2×10^7 cells were layered onto each preformed Percoll gradient, and the cell populations were separated by centrifuging the gradient tubes in a swinging bucket (JS 7.5) rotor in a Beckman J-21C centrifuge at 2,000 rpm and

Abbreviation: G acid, 2-naphthol-6,8-disulfonic acid.

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4°C for 20 min. Four identical gradients were used for each experiment (each animal): three tubes contained equal amounts of cells and the fourth contained density marker beads (Pharmacia) for determining the shape and reproducibility of the gradients.

Bands of cells (Fig. 1) were collected with a fraction collector interfaced to a gradient fractionator and a continuously recording column monitor (all ISCO). Corresponding fractions from three identical gradients for each animal were pooled, and the cells were washed twice with physiological saline. Cells from the peaks (Fig. 2) and other selected fractions were counted with a hemocytometer, photographed (Fig. 3), and lysed (16). Lysates were stored frozen (-20°C) and clarified prior to electrophoresis by centrifugation at $10,000 \times g$ for 5 min. Hb concentrations of lysates were determined spectrophotometrically with Drabkin's reagent (21).

In Vivo Hemoglobin Synthesis. Animals at each stage of metamorphosis were injected in the dorsal musculature with [³H]leucine and [³⁵S]methionine (each at 5 μ Ci/g of body weight). After 16 hr at 20°C, the animals were anesthetized and bled, and populations of erythrocytes were separated as described above. Of each lysate, aliquots containing 100 μ g of Hb and 0.25 mM dithiothreitol/1 mM Tris base, pH 8.0, were electrophoresed on 7% polyacrylamide disc gels as described (22),

except that 90-mm gels and a 1:10 (vol/vol) dilution of the Tris glycine buffer were used. The individual Hbs, whose relative migrations are known (15), were stained with benzidine/H₂O₂ and quantified by densitometry (22). The use of dithiothreitol is important in the analysis and detection of Hb types by electrophoresis. The dithiothreitol was added to each lysate to prevent polymerization of adult Hb C by disulfide bond formation among tetramers (23), allowing the adult Hb C to migrate as one tetrameric Hb band distinct from the tadpole Hbs. The migration of the tadpole Hbs is unaffected by dithiothreitol because they lack cysteine residues (24). The gels were frozen and sliced at 1-mm intervals, each gel slice was hydrolyzed with 0.1 ml of 30% H₂O₂ at 60°C for 24 hr, and the dpm of ³H and ³⁵S in each sample were determined with a liquid scintillation system. The [³⁵S]methionine label is specific for adult Hb because tadpole globins contain neither methionine nor cysteine (24); this was verified by amino acid analysis of purified adult and tadpole Hbs (data not shown).

RESULTS

Cell Separations. Separation of erythrocyte populations of *R. catesbeiana* (Figs. 1 and 2) by the techniques described above is rapid (20 min) and gentle (cells are subjected to centrifugal forces of 197–466 g). The isolated cells are neither damaged nor

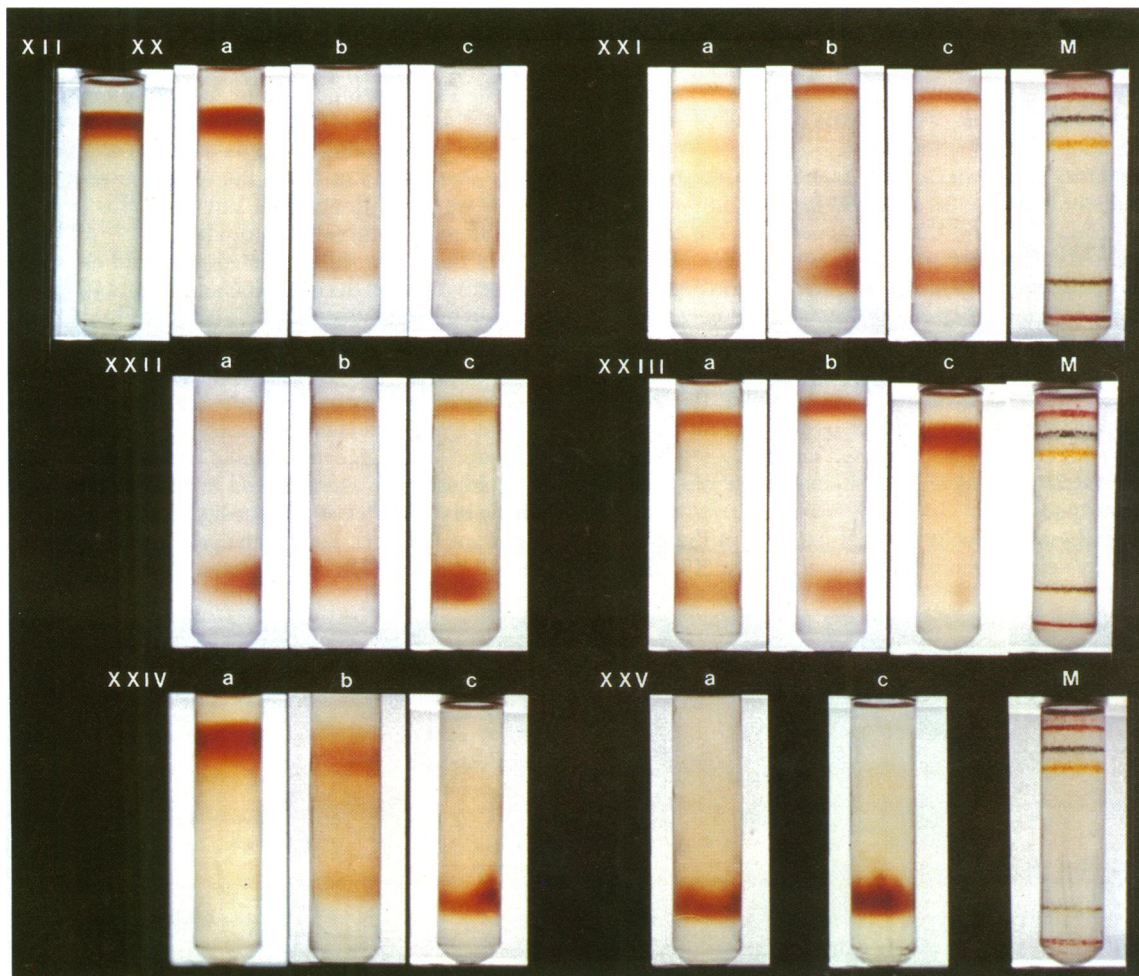


FIG. 1. Separation of erythrocyte populations on shallow density gradients of Percoll. Roman numerals indicate developmental stages (17); a, b, and c refer to the early, middle, and late part of each stage, respectively. Each tube is one of a triplicate set and shows the erythrocyte population from the blood of an individual animal. Bands in the tubes on the right (M) are density marker beads; densities, in g/ml, from top to bottom are 1.060, 1.070, 1.085, 1.095, and 1.100. Note the appearance of a new band of cells of density 1.060 at stage XXIIa, the increase in density (from 1.070 to 1.095) of tadpole erythrocytes between stages XXIIa and XXIIIb, the disappearance of tadpole erythrocytes (stage XXIIIc), and the subsequent increase in density of adult cells (stages XXIVb to XXV).

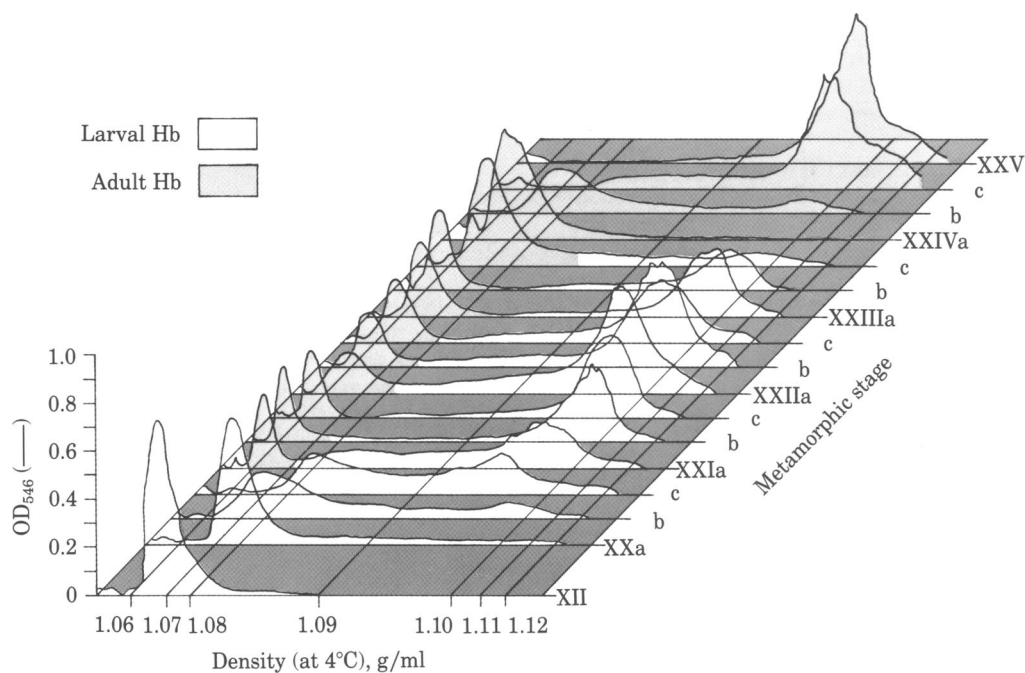


FIG. 2. Changes in erythrocyte populations as a function of density and development. Note that the two main cell populations contain different Hbs as detected by gel electrophoresis and selective radiolabeling of adult Hb (see Fig. 4).

distorted (Fig. 3). The separations are apparently isopycnic (based on density) since bands of cells retain constant positions when centrifugation time is prolonged.

Cell Populations. As shown in Figs. 1 and 2, during metamorphosis, tadpole erythrocytes (Fig. 3a) increase in density from 1.075 to 1.095 g/ml and they disappear from the circulation by stage XXIV. Concurrently, beginning at stage XXI, a population of erythroid cells of immature morphology (Fig. 3b) appears, initially with a density of 1.060 g/ml. This new population of cells increases in number (note the increase in area under the shaded peaks, Fig. 2) as metamorphosis progresses. When the tadpole erythrocytes are no longer present, the new adult cells mature (Fig. 3c) and increase in density to a maximum of 1.095 g/ml. Cells of these different morphologies were completely separated (no detectable cross-contamination) at all stages of metamorphosis.

Hemoglobins. Lysates of cells from all fractions of the gradients (Fig. 2) were electrophoresed and analyzed for Hb, ^3H ,

and ^{35}S . The benzidine staining method allowed the detection of as little as 0.1 μg of Hb in a single band (22), and incorporation of the ^{35}S label facilitated the detection of adult Hb. As shown by the shading in Fig. 2 and the representative gel tracings in Fig. 4, we found the tadpole and adult Hbs to be segregated into different erythroid cell populations.

Only the newly differentiated erythroid cells are synthetically active *in vivo* during metamorphosis, and these cells contain, and incorporate radioactive precursors into, only adult Hb (Fig. 4). As these adult cells mature (Fig. 3b and c) and increase in density (Fig. 2), their Hb content increases and the amount of radioactivity incorporated into Hb decreases (Table 1).

Although prometamorphic (stage XII) tadpoles readily incorporate [^3H]leucine into tadpole Hbs *in vivo* (data not shown), little radioactivity was detected in tadpole Hbs from purified tadpole erythrocytes at any density or stage of metamorphosis (Fig. 4 and Table 1). Purified tadpole erythrocytes of all densities had the same Hb concentration (Table 1).

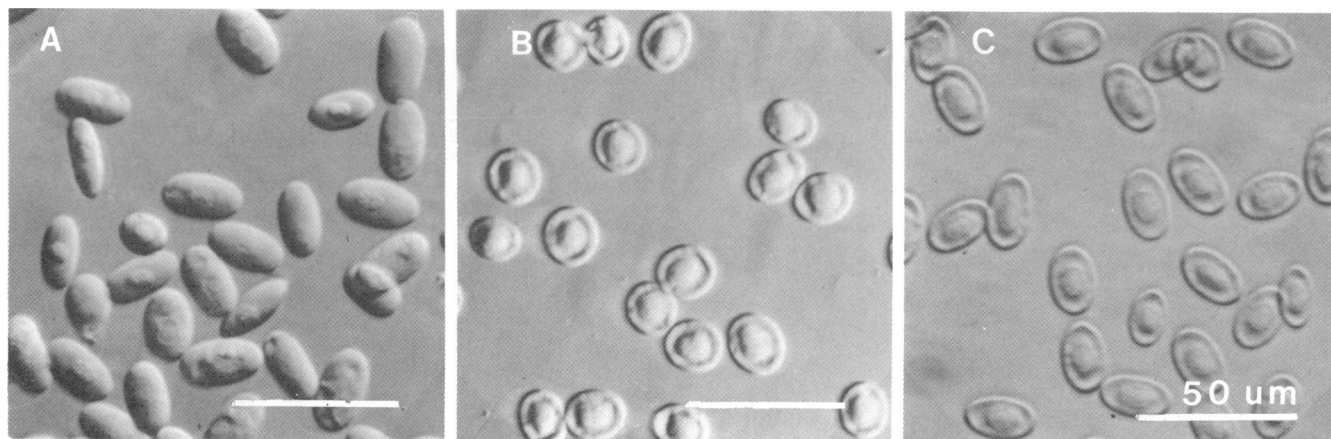


FIG. 3. Erythroid cell populations purified during metamorphosis (representative fractions). (a) Tadpole erythrocytes, stage XXI (density, 1.095 g/ml). (b) Immature adult erythroid cells, stage XXII (density, 1.065 g/ml). (c) Mature adult erythrocytes, stage XXIV (density, 1.095 g/ml). Bar = 50 μm .

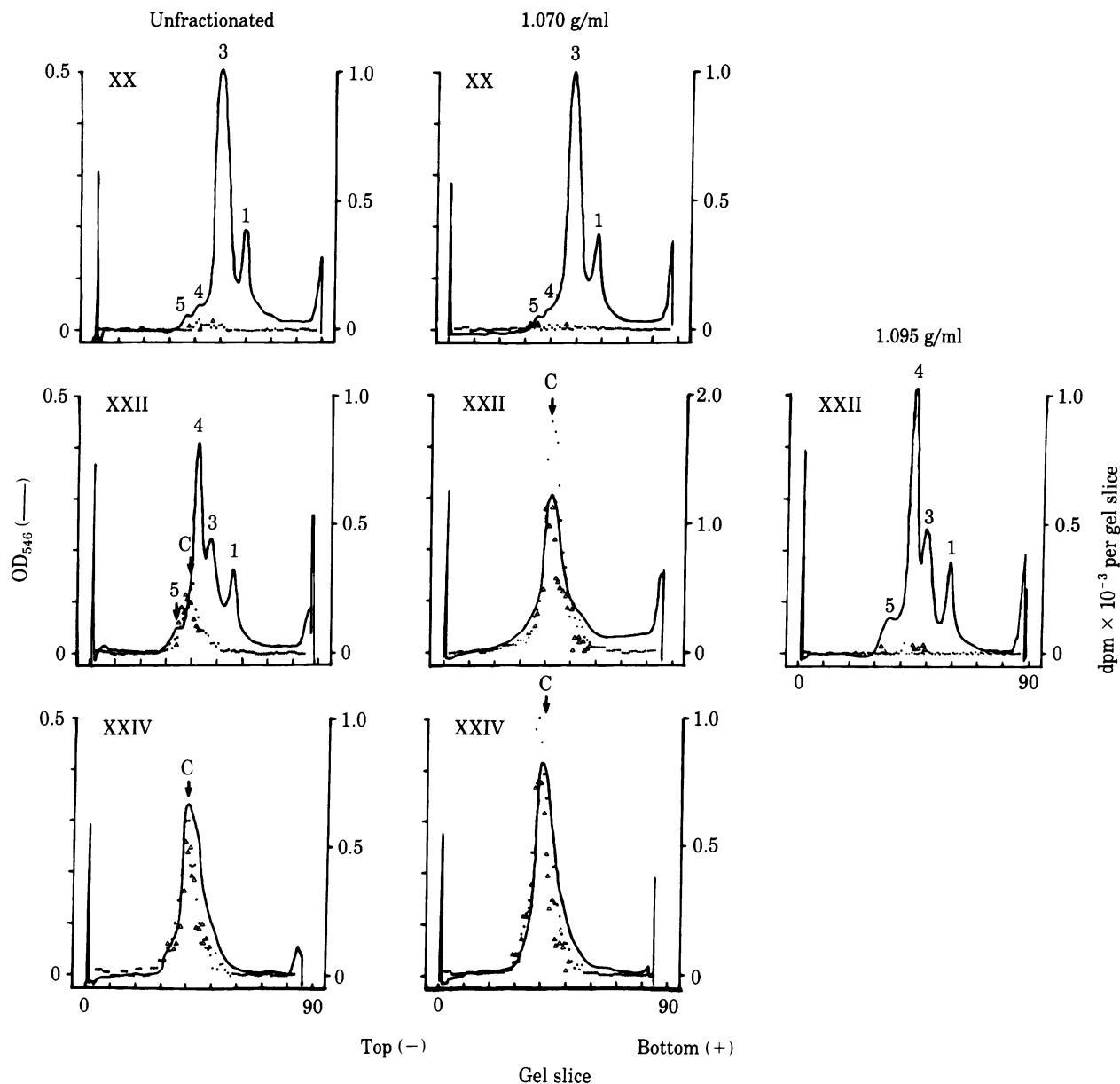


FIG. 4. Hemoglobins separated by polyacrylamide gel electrophoresis of lysates of isolated cell populations. Densitometer tracings of and radioactivity (Δ , ^{35}S ; \dots , ^3H) in gel slices are shown for representative samples. The [^{35}S]methionine label is specific for adult globins. Addition of the appropriate amounts of dithiothreitol to the samples prevents aggregation of adult Hb C tetramers (23); thus treated, adult Hb C migrates as a single band distinct from the tadpole Hbs (major Hbs 1–4, corresponding to bands labeled Td-1 through 4 in ref. 16 and minor tadpole Hb 5). The numbering of the Hbs is codified with the terminology of other workers in ref. 15, in which a review of the chemical and functional properties of the Hbs can be found. Note the absence of any detectable adult Hb at stage XX and the absence of tadpole Hbs at stage XXIV. At stage XXII (mid-transition), adult and tadpole Hbs are found in separate cell populations (densities of 1.070 and 1.095 g/ml, respectively). Tadpole Hbs showed no *in vivo* incorporation of radiolabels at any stage (XX–XXIII) of metamorphic climax. (Left) Lysates of cells not fractionated. (Middle) Density of cells, 1.070 g/ml. (Right) Density of cells, 1.095 g/ml.

DISCUSSION

This direct solution to the question of the cellular distribution of Hb types is important because, as discussed in detail elsewhere (15), it has not been possible to decide which of the conflicting reports based on immunocytochemical approaches (7–10) is correct. Our approach has several advantages: (i) the different cell populations were purified before analysis; (ii) all Hbs present were identified and quantified; and (iii) Hb synthesis, rather than only Hb presence, was determined. It is possible that a few undetected cells were present that contained both Hb types or that all cells examined contained a small amount of the alternative Hb type (or both). However, in terms

of a meaningful phenotype, it is evident that adult and tadpole Hbs are essentially expressed in separate cell populations.

More important is the fact that our approach is a preparative as well as an analytical one. A major stumbling block to deciphering the regulation of Hb switching has been overcome by actually isolating the different cell populations involved in the Hb switch. The task of isolating intracellular regulators that may be specific for different genes should be easier with pure cell populations.

Practically nothing is known about the molecular mechanisms that control changes in globin gene expression during development of any animal. It seems to us that two factors are important. One is the structural organization of multiple globin

Table 1. Purified adult and tadpole* erythroid cells of *R. catesbeiana*: Cell densities, Hb content, and Hb synthesized *in vivo*

Cell density, g/ml	Adult cells		
	Hb, pg per cell	dpm/ μ g of Hb	
		^3H	^{35}S
1.060	73 \pm 6	183 \pm 56	196 \pm 5
1.065	70 \pm 5	120	220
1.070	93 \pm 11	120 \pm 60	78 \pm 32
1.075	116 \pm 8	55 \pm 31	41 \pm 2
1.080	159 \pm 10	6.1 \pm 3.2	11 \pm 7
1.085	186		
1.090	221 \pm 12	5.6 \pm 0.8	9.8 \pm 0.2
1.095	161 \pm 27	2.1 \pm 0.7	2.6 \pm 1.2

Results are mean \pm SEM for one to nine measurements of each parameter.

*Tadpole erythrocytes of all densities and all metamorphic stages (XX–XXIII) had the same Hb content (193 \pm 17 pg per cell) and low incorporation of isotopes into Hbs (1.8 \pm 0.4 dpm/ μ g of Hb for [^3H]leucine and 2.5 \pm 0.8 dpm/ μ g of Hb for [^{35}S]methionine). Note that these values are similar to those for adult erythrocytes of density 1.095 g/ml (near the end of metamorphosis).

genes. The other is that differences in the erythropoietic microenvironments of hemopoietic organs induce different programs of globin gene expression (16, 25).

In *R. catesbeiana*, adult and tadpole Hbs share no globin chains (26). Thus, the metamorphic transition involves both α - and β -globin switching. Part of the reason for both α and β switching may be that some or all of the globin genes of *R. catesbeiana* are arranged in α - β pairs as for adult globin in *Xenopus* (27) rather than as separate α and β clusters as in other species examined (1–3).

The separate and more important question of what factors actually stimulate the change in globin gene expression remains. Regulation of Hb type by different erythropoietic microenvironments has been supported by previous experiments (16, 25, 28). Before metamorphic climax, the mesonephric kidneys and the liver are erythropoietically active (25, 28) and produce erythrocytes of different morphologies containing predominantly different tadpole Hbs (16). During metamorphosis, the liver switches to adult Hb production (7–9) and undergoes other marked cytological (29) and biochemical (30) changes. We postulate that changes in the liver microenvironment mediate a reprogramming of the differentiating erythroid cells.

Animals with different ratios of the two tadpole erythrocyte types and of the four major tadpole Hbs (16) enter metamorphic climax. The metamorphic Hb switch is unaffected by the preexisting tadpole Hb pattern (Fig. 4). Both types of tadpole erythrocytes, isolated as one major band in the Percoll gradients, were found to be synthetically inactive and to increase in density during metamorphosis. Although we do not know the cause of the density increase, we propose the testable hypothesis that an increase in iron or ferritin content (or both) (31) is responsible. Since the life-span of tadpole erythrocytes has been reported to be about 100 days (32), the rapidity of the Hb and cell

switch (about 10 days) indicates, but does not prove, a decrease in the life-span of tadpole erythrocytes.

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