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Embryonic → fetal Hb switch in humans: Studies on erythroid bursts generated by embryonic progenitors from yolk sac and liver

(human embryos/ontogeny/erythropoiesis in culture)

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ABSTRACT **The synthesis of embryonic (ζ, ε), fetal (α, γ), and adult (β) globin was evaluated in human yolk sacs (YS) and livers at different ontogenic stages (i.e., from 6 through 10–12 wk of age) by means of analytical isoelectric focusing. Globin production was comparatively evaluated *in vivo* (i.e., in directly labeled erythroblasts from YS and liver) and *in vitro* [i.e., in erythroid bursts generated in culture by erythroid burst-forming units (BFU-E) from the same erythropoietic tissues]. Erythroid bursts generated *in vitro* by BFU-E from 6-wk livers and YS show essentially a "fetal" globin synthetic pattern: this is in sharp contrast to the "embryonic" pattern in corresponding liver and YS erythroblasts directly labeled *in vivo*. The *in vitro* phenomenon suggests that (*i*) 6-wk BFU-E constitute a new generation of progenitors, which have already switched from an embryonic to a fetal program, and/or (*ii*) expression of their fetal program is induced by unknown *in vitro* factor(s), which may underlie the *in vivo* switch at later ontogenic stages. It is emphasized that 6- to 7-wk BFU-E are endowed with the potential for *in vitro* synthesis of not only ε- and γ-chains but also some β-globin. In general, we observed an inverse correlation between the levels of ε- and β-chain synthesis. These results, together with previous studies on fetal, perinatal, and adult BFU-E, are compatible with models suggesting that in ontogeny the chromatin configuration is gradually modified at the level of the non-α gene cluster, thus leading to a 5′ → 3′ activation of globin genes in a balanced fashion.**

It is generally conceded that in humans the embryonic → fetal Hb switch (i.e., $\zeta \rightarrow \alpha$, $\varepsilon \rightarrow \gamma$) initiates at ≈6 wk and is gradually completed by 10–12 wk (1). In this regard, the smallest embryos reported so far synthesize Hb Gower I (ζ_2 ε_2) and II (α_2 ε_2) as well as Hb Portland (ζ_2 γ_2) and F (α_2 γ_2) (1–4). In addition, traces of adult Hb (α_2 β_2) may be detected at as early as ≈7 wk (5).

Little is known about the mechanism(s) underlying the switch. In particular, it is not established whether an embryonic erythropoietic lineage is gradually replaced by a fetal one. The site of production of embryonic and fetal chains has not been elucidated either. In this regard, the switch occurs at the same time as the liver replaces the yolk sac (YS) as the main site of erythropoiesis (6). Hence, it is generally assumed that the YS may represent the main site for synthesis of embryonic Hb, whereas the liver would be later involved for the production of Hb F and A.

We report here studies on the Hb synthesis pattern in YS and liver at different ontogenic stages (i.e., from 6 through 10–12 wk). Globin production was evaluated both *in vivo* (i.e., in directly labeled erythroblasts from YS and liver) and *in vitro* [i.e., in erythroid bursts generated in culture by erythroid burst-forming units (BFU-E) from the same erythropoietic tissues].

MATERIALS AND METHODS

Livers and YS. Human livers and YS from virtually intact embryos were obtained from legal curettage abortions, from 6 through 8 wk. Fetal livers were similarly obtained. The age, established by standard ecography criteria (7), was confirmed by evaluation of the crown-rump length of the embryo (see ref. 1). The studies included five 6-wk, thirteen 7- to 8-wk, and eight 9- to 12-wk embryos and fetuses. These were maintained in Iscove's modified Dulbecco's medium (IMD medium, GIBCO) at +4°C for 2–3 hr. A unicellular suspension from liver or YS (or both) was then prepared as in refs. 8 and 9, except that cells were passaged through progressively smaller needles (down to no. 25).

Culture Methods. Erythroid bursts were grown in methylcellulose cultures according to a slight modification of previously described methods (10, 11). Each 1-ml plate contained the following components in IMD medium: methylcellulose (0.8%, final concentration), α-thioglycerol (0.1 mM), fetal calf serum (30%), 0.5–1 × 10^5 nucleated cells, and semipurified erythropoietin (2 international units), kindly provided by the National Heart, Lung and Blood Institute (1140 international units/mg of protein). The plates were incubated in a humidified 5% CO_2 in air atmosphere at 37°C. Bursts, comprising 1–3 × 10^4 cells, were identified *in situ* on the basis of their red color. The bursts were scored or incubated (or both) with radioactive amino acid on day 8 (see ref. 9).

Radioactive Labeling of Globin Chains. Individual well-hemoglobinized bursts were visualized through an inverted microscope, randomly picked up by means of a fine Pasteur pipette in a 2- to 3-μl volume, transferred into 10 μl of culture medium containing 25–50 μCi (1 Ci = 37 GBq) of [^{3}H]leucine (specific activity, 100–150 Ci/mM) (Amersham), further incubated for 16–24 hr, washed in 1.2 ml of cold (+4°C) saline (containing 2.5 × 10^5 carrier erythrocytes), and stored at −30°C. Liver and YS cells were similarly labeled (3 × 10^6 cells in 0.1 ml; 100 μCi; 18–24 hr of incubation).

Globin Preparation and Analysis. Globin, prepared from stroma-free lysates by the acid acetone procedure, was analyzed by means of isoelectric focusing (IEF) in the presence

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Abbreviations: BFU-E, erythroid burst-forming unit(s); YS, yolk sac(s); IEF, isoelectric focusing.

of Nonidet P-40 (10) according to the modified method in refs. 12, 13. Gel fluorography and scanning of the exposed film were performed as described (10).

RESULTS

Fetal Livers at 9–12 Wk. Previous observations showed that individual bursts from 9- to 12-wk livers are characterized by a homogeneous globin chain synthesis pattern: non-α-globin is represented largely by γ-chains and to a small extent by β-globin (refs. 8, 14; see Fig. 1 *Top*). Particularly, γ-chain production represents >95% of non-α synthesis in bursts composed of immature erythroblasts versus ≈80–90% in colonies containing more mature erythroid cells (8). A pattern similar to that observed in bursts has been documented *in vivo* (i.e., at the level of erythroblasts directly derived from the livers) (ref. 15 and results not shown).

Livers at 7–8 Wk. We have analyzed a series of thirteen 7- to 8-wk livers (representative studies on two specimens are shown in Fig. 1 *Middle* and *Bottom*). In 8 livers, globin chain synthesis was evaluated in single bursts (≈10 colonies per liver). In the other ones, production was evaluated *in vivo* as well as in pooled bursts (5–10 pools per liver). In colonies from 7- to 8-wk livers β-chain synthesis is low, varying from barely detectable levels (Fig. 1 *Middle*) up to ≈10% (Fig. 1 *Bottom*). In some livers an additional band is observed in all colonies, which is focused in the position expected for ε-globin chains (Fig. 1 *Middle* and *Bottom*). This band corresponds to the ε-chain from K562 leukemic cells (which had been purified by means of haptoglobin-Sepharose affinity chromatography of Hb; see ref. 12), when focused in the presence of Nonidet P-40 (12) (Fig. 1 *Middle*). It also corresponds to the ε band observed by means of IEF analysis of Hb Gower I and II purified from human embryos (13). Furthermore, if IEF is carried out in the absence of Nonidet P-40, the band is placed at the top of the gel, very close to the α-chain: in this particular condition, the migration of the putative ε-chain from livers corresponds again to the ε band from K562 leukemic cells (results not shown). The level of ε-chain synthesis is homogeneously low [e.g., ≈1–3% of non-α synthesis (Fig. 1 *Middle* and *Bottom*), down to barely detectable levels (Fig. 1 *Middle* and *Bottom*)], in single colonies (Fig. 1 *Middle*) or different burst pools (Fig. 1 *Bottom*).

These results, confirmed in numerous experiments on 13 different livers, indicate that ε-chain synthesis may still occur during hepatic erythropoiesis. More important, they suggest that fetal BFU-E might be endowed simultaneously with the program for both embryonic and fetal globin chain synthesis. Furthermore, the potential for residual ε-chain production may be associated with an initial program for β-globin synthesis. These aspects are further analyzed below on the basis of single colony studies.

It should be emphasized that a synthetic pattern equivalent to that observed in bursts is shown in erythroblasts derived from the liver (a representative experiment is shown in Fig. 1 *Bottom*).

Livers and YS at 6 Wk. We next analyzed the globin synthesis pattern in bursts from 6-wk livers and YS (Figs. 2–4). As the usual control, erythroblasts from the same tissues were directly labeled with radioactive amino acid and subjected to IEF analysis. A striking difference was observed when comparing globin synthesis *in vivo* and *in vitro*.

In vivo studies. Directly labeled erythroblasts exhibit an embryonic pattern, characterized by elevated levels of ε- and ζ-chain synthesis†† (Figs. 2–4 and results to be presented elsewhere). Particularly, the $\varepsilon/\varepsilon + \gamma$ synthetic ratio is ≈50–70% in three 6-wk livers (Figs. 2 and 3 *Upper* and *Lower*) but is distinctly lower (10–15%) in a 7-wk one (Fig. 4). The $\zeta/\zeta + \alpha$ quotient is markedly elevated in the three former cases (>70%), whereas ζ-chain production is apparently absent in the last one. It is emphasized that the synthetic pattern in YS is equivalent to that in liver from the same embryo (Fig. 3 *Upper* and results not shown). In this regard,

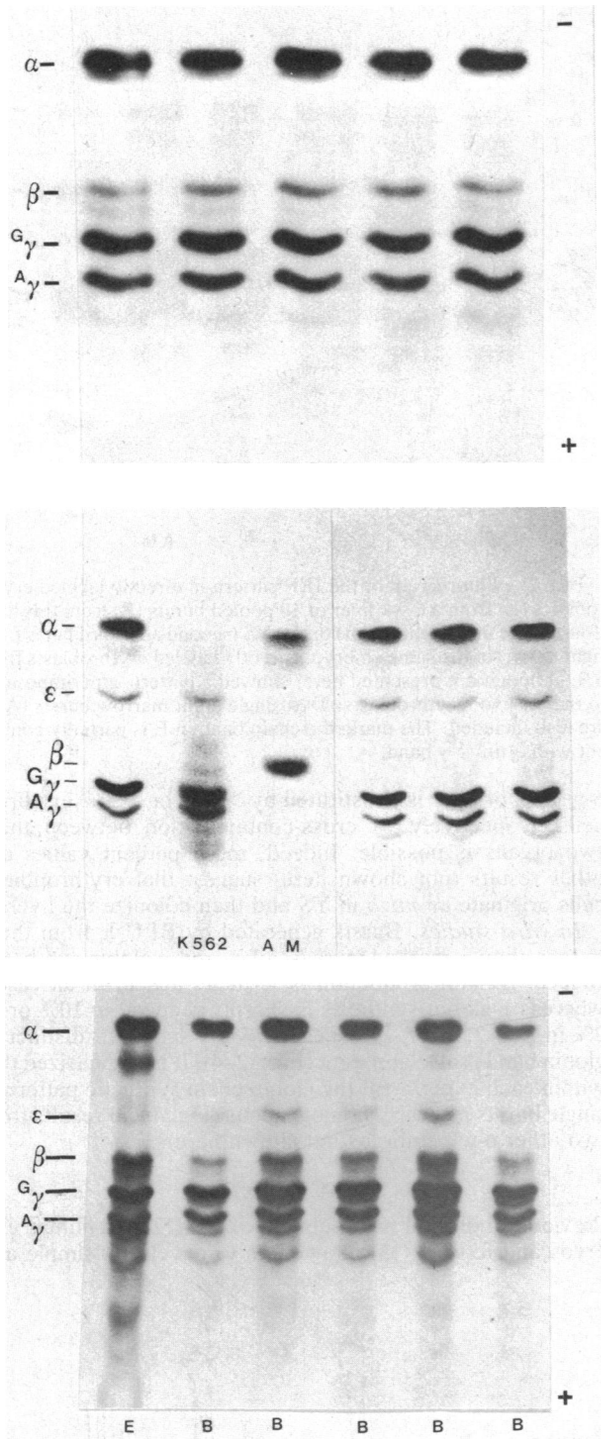


FIG. 1. Fluorogram of the IEF pattern in single erythroid bursts from an 11- (*Top*) and a 7-wk (*Middle*) liver and in directly labeled erythroblasts (E) or 10 pooled bursts (B) from an 8-wk liver (*Bottom*). In *Middle* an ε-chain K562 line marker (13) and a single adult marrow burst (AM) are shown, whereas the faint β band in burst lanes is scarcely visible. A total of 14 or 11 single bursts and 10 pooled colonies was evaluated in experiments presented in *Top*, *Middle*, and *Bottom*, respectively.

††Here again, ε and ζ bands were identified on the basis of studies on ε- and ζ-chains from K562 leukemic cells (12) and Hb Gower I and II purified from human embryos (13).

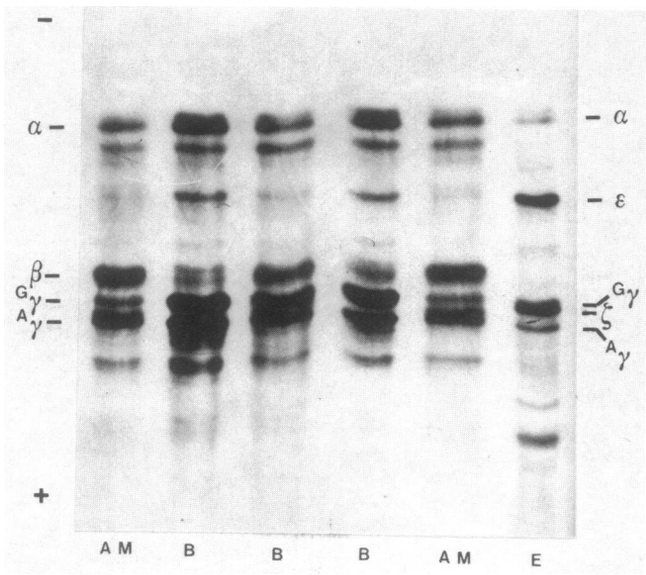
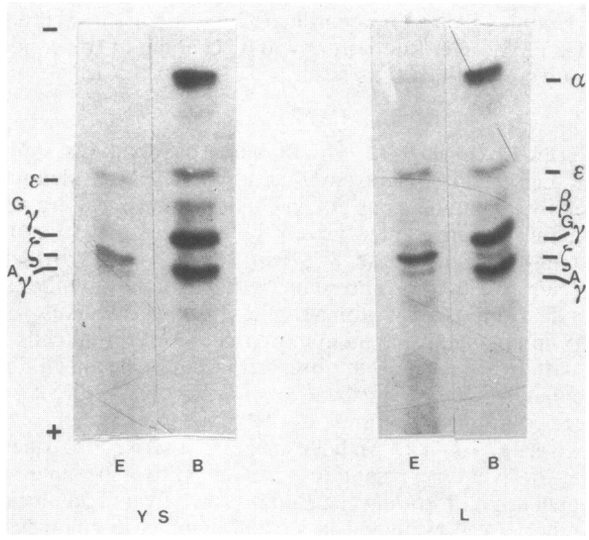


FIG. 2. Fluorogram of the IEF pattern in directly labeled erythroblasts (E) from a 6-wk liver or 10 pooled bursts (B) from this liver (fourth lane from right to left) or the YS (second and third lanes from right to left) of the same embryo. Directly labeled erythroblasts from YS, although not presented here, showed a pattern superimposable to that of liver erythroblasts. Two single adult marrow bursts (AM) are also included. The marked ζ-chain band in E is partially confluent with a tiny $^{G}\gamma$ band.



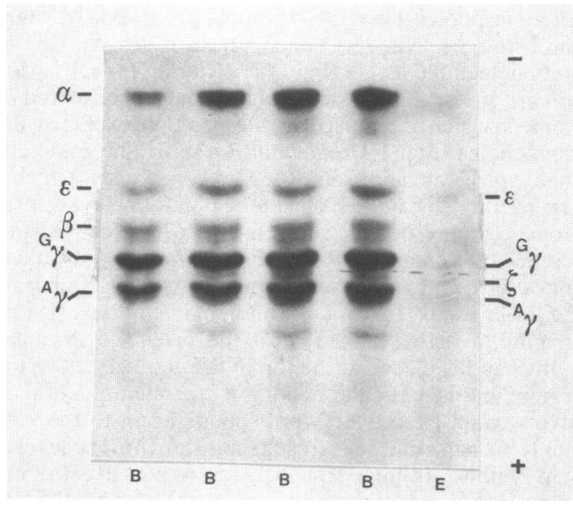


FIG. 3. Fluorogram of the IEF pattern in directly labeled erythroblasts (E) or 10 pooled bursts (B) from YS and liver (L) of a 6-wk embryo (*Upper*); E and B from liver of a 6-wk embryo (*Lower*). A total of 10 or 5 pools of FL or YS bursts was analyzed in this experiment.

6-wk YS or liver is constituted by >70% or ≈20% erythroid cells, respectively. A cross-contamination between these two organs is possible. Indeed, these percent values and other results (not shown here) suggest that erythropoietic cells originate *de novo* in YS and then colonize the liver.

In vitro studies. Bursts generated by BFU-E from these tissues show a marked increase of α- and γ-globin synthesis. Indeed, ζ-chain production is virtually absent in all cases, whereas ε-globin synthesis is sharply reduced (≈10% or 1–2% in Fig. 2, 3, or 4, respectively). A small but distinct β-globin band is also apparent (Figs. 2–4). It is emphasized that within each experiment the globin chain synthetic pattern in single bursts is clearly homogeneous (Fig. 4 and results from two other 6-wk embryos not shown here).

DISCUSSION

Previous studies on K562 leukemic cells (12) and human embryos and fetuses (13) allowed us to develop a simple and sensitive method for IEF analysis of embryonic globin chains. This technique made it possible to investigate the synthesis of individual embryonic, fetal, and adult globin

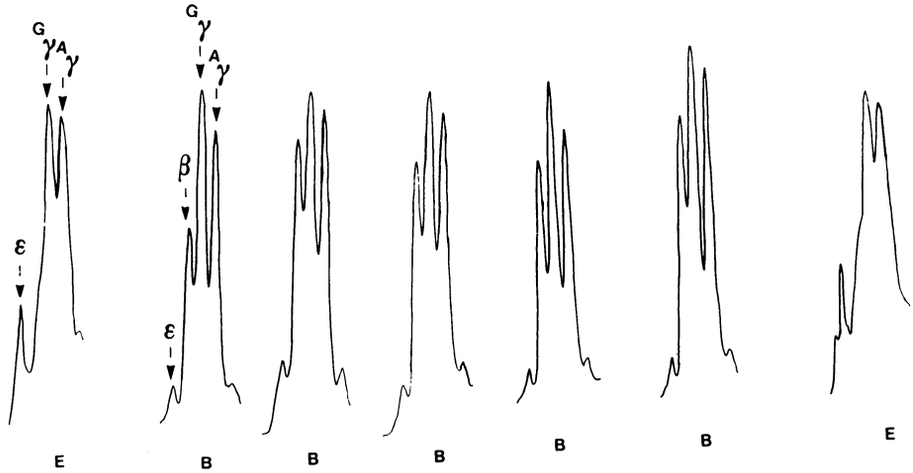


FIG. 4. Densitometric tracing of the IEF pattern in directly labeled erythroblasts (E) and *single* bursts (B) from a 7-wk embryo. A total of 10 single bursts was analyzed in this experiment.

chains in human embryos, as comparatively evaluated *in vivo* and *in vitro*.

In 6-wk embryos (crown-rump length, ≈1.0–1.5 cm), erythroblasts are characterized by predominant or almost exclusive embryonic (ε and ζ) globin synthesis, in both YS and liver. Previous studies (1) were based on analysis of Hb content in circulating erythroid cells from later embryos (crown-rump length, ≥1.6 cm); thus, they did not clarify whether embryonic and fetal hemoglobins are produced by separate cell populations originating from specific organs (i.e., YS and liver, respectively) rather than by a single erythroid lineage. Our *in vivo* experiments may shed light on this issue, in that they demonstrate a superimposable globin synthesis pattern in both YS and liver from the same embryo. This indicates that the primitive erythroid lineage is present in both organs, where it is not influenced, at least at this ontogenic stage, by differential microenvironmental influences.

Interestingly, *in vitro* analysis of BFU-E from 6-wk embryos shows that the progenitors from both YS and liver are endowed with a potential for synthesis of not only ε- and γ-chains but also some β-globin. Similarly, large amounts of α-chains are produced in these bursts. This multiple program is also occasionally expressed in colonies from 7- to 8-wk livers. In later ontogenic phases, the potential for *in vitro* synthesis of ε-chains is obliterated, concomitantly with the increased ability to synthesize β-globin. This explains previous failures to demonstrate embryonic chains in fetal liver bursts (8, 14–16).

These results, in conjunction with data obtained on fetal, cord blood, and adult BFU-E (8, 10–11, 14–19), suggest the existence of a single BFU-E clone, which is progressively modulated through the various ontogenic stages to switch from embryonic (ζ, ε) to fetal (α, γ) and adult (δ, β) globin synthesis. In the course of ζ → α and ε → γ (+ some β) switches, the transcription of embryonic and fetal–adult globin genes in erythroid colonies may not occur simultaneously or not even in the same cell. However, our studies clarify that an inverse correlation exists between the levels of ε- and β-chain synthesis (i.e., bursts with elevated β-globin production exhibit low or absent ε-chain synthesis and vice versa). These observations, together with studies on fetal, perinatal, and adult BFU-E (8, 10–11, 14–19), are compatible with models suggesting that in ontogeny the chromatin configuration is gradually modified at the level of the non-α gene cluster, thus leading to 5′ → 3′ activation of globin genes in a balanced fashion (19–22). In addition the methylation pattern of embryonic and fetal globin gene domains is directly correlated with their expression (23).

An unexpected result emerged from the comparative analysis of globin synthesis in *in vivo* vs. *in vitro* erythroblasts from ≈6-wk embryos. Indeed, the typically embryonic pattern in erythroblasts directly labeled *in vivo* is strikingly different from the essentially fetal pattern observed in erythroblasts generated *in vitro*. This discrepancy can be explained by two alternative hypotheses. (*i*) Directly labeled erythroblasts derive from an early population of progenitors, still committed to embryonic globin synthesis, which is no longer present in YS and liver at the time of the experiment. The *in vitro* labeled erythroblasts are instead the progeny of a new generation of progenitors, already committed to a fetal synthetic pattern. (*ii*) Embryonic progenitors in 6-wk YS and liver have the potential for a fetal globin synthesis program. Expression of this program, induced *in vivo* by unknown factors at later ontogenic stages, is caused *in vitro* by the culture conditions.

These two models are not mutually exclusive, but their relative impact is totally unknown. The former mechanism would predict that *in vivo* ζ → α and ε → γ switches should occur fairly rapidly (i.e., within 2–3 wk at most). This should be verified by extensive *in vivo* analysis of globin chain synthesis at 6–8 wk of age. The latter might be verified obviously by identification of the putative physical (pO_2, pCO_2, etc.) or chemical [fetal calf serum, hemopoietin(s), etc.] factor(s) modulating *in vitro* the BFU-E program.

Murine embryonic circulating blood BFU-E grown in culture give rise to a progeny synthesizing adult Hb (24). These results are clearly in line with our observations and may be similarly interpreted on the basis of the models proposed above. On the other hand, Cudennec *et al.* (25) suggested that murine fetal liver releases a diffusible factor(s), which apparently induced embryonic YS cells to produce adult globin chains. The role of putative, time-programed "extrinsic" factor(s) in the embryonic → fetal (25) or fetal → adult (26) Hb switch is however still undetermined.

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