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Hemoglobin synthesis in cultures of hepatic erythroid cells from the human fetus

(differentiation/erythropoietin/cell culture/abortus/gene expression)

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ABSTRACT **A recent theory of the control of human fetal hemoglobin synthesis, based on studies in cultured adult marrow, proposes that the phenotypic expression of fetal hemoglobin is largely dependent on the level of differentiation of the parental stem cells; that is, the earlier the progenitor, the greater the ability of its progeny to express fetal hemoglobin [Papayannopoulou, Th., Brice, M. & Stamatoyannopoulos, G. (1977) *Proc. Natl. Acad. Sci. USA* 74, 2923–2927]. To test this relationship with fetal tissue, we have studied hemoglobin synthesis in cultured human fetal liver, comparing γ chain synthesis in the descendants of the early progenitors ("bursts") with that in the descendants of the later progenitors ("colonies"). Cells from the livers of midtrimester fetuses were cultured in methylcellulose with erythropoietin. The $\beta/(\beta + \gamma)$ globin synthetic ratio on days 5 to 7, when colonies predominated, was 0.09–0.11, a value characteristic of fetal reticulocytes, and on days 11 and 12, when bursts predominated, was 0.15–0.17. Thus, in fetal liver, the descendants of the earlier progenitor, the burst-forming unit, may be making more β chains rather than more γ chains, compared to descendants of the later progenitor, the colony-forming unit. Our data on fetal liver, taken together with the data on adult marrow by others, suggest that the erythroid colonies express the gene characteristic of the age of the organism to a greater degree than bursts, which express β and γ genes less specifically. Thus, the capacity for highly selective gene expression characteristic of differentiated cells appears to be less well developed in the burst-forming unit than in the colony-forming unit.**

In man, the principal hemoglobin synthesized in fetal life is fetal hemoglobin (Hb F = $\alpha_2\gamma_2$), whereas the principal hemoglobin synthesized in adult life is adult hemoglobin (Hb A = $\alpha_2\beta_2$). Since both hemoglobins contain α chains, the switch from the former to the latter represents a switch from γ to β chain synthesis (1).

When hematopoietic cells are cultured in semisolid medium with erythropoietin, individual erythroblast clusters ("colonies") appear first and groups of erythroid clusters ("bursts") appear later. A colony is regarded as a clone arising from a later erythroid progenitor (colony-forming unit) and a burst, as a clone arising from an earlier erythroid progenitor (burst-forming unit) (2).

When marrow cells from adults are cultured in semisolid medium with erythropoietin, the proportion of fetal hemoglobin synthesized by their progeny is greater than the proportion synthesized by the cells prior to culture (3, 4). The proportion of colonies that contain fetal hemoglobin is highest among the late-appearing colonies (bursts) and among those cultured at a high erythropoietin concentration (5). These findings have prompted the hypothesis that the phenotypic expression of fetal hemoglobin is largely dependent on the level of differentiation of the stem cells from which the clones are derived; that the earlier the progenitor, the greater the ability of its progeny to express fetal hemoglobin; and that regulatory decisions about γ chain synthesis in erythroblasts are made in progenitor cells with the *in vitro* proliferative characteristics of burst-forming units (5).

To elucidate these relationships, we have studied hemoglobin synthesis in cultured human fetal liver. On the basis of the adult marrow data, one might expect that in fetal hepatic erythroid cells the proportion of fetal hemoglobin synthesis might be higher in bursts than in colonies.

Our laboratory has recently reported that the liver of the human fetus induced to abort by the intrauterine instillation of prostaglandin or hypertonic saline contains cells that form colonies in methylcellulose *in vitro* (6). We have recently had an opportunity to study midtrimester fetuses delivered in a more native state, those delivered by a surgical procedure called dilatation and evacuation. This operating room procedure provides fetal tissue without intrauterine drug instillation. As will be reported elsewhere, whereas cultures of livers from saline and prostaglandin fetuses appear to yield only erythroid colonies, cultures from dilatation and evacuation fetuses yielded not only erythroid colonies but also erythroid bursts.

We have found that in the phase of culture in which erythroid colonies predominate the relative synthesis of β and γ chains is similar to that in fetal reticulocytes. In the later phase of culture, during which bursts predominate, β-chain synthesis may actually be relatively increased. Thus, the descendants of the earlier progenitors do not express the γ gene to a greater degree than those of the later progenitors.

These data on fetal liver, taken together with the data cited above on adult marrow, suggest that, whereas erythroid colonies express predominantly the gene characteristic of the age of the organism, erythroid bursts express β and γ genes more equally. The capacity for highly selective gene expression characteristic of differentiating cells appears to be less well developed in the burst-forming unit than in the colony-forming unit.

METHODS AND MATERIALS

Methylcellulose A4M premium grade was obtained from Dow (Midland, MI), alpha medium (powder form) was from Flow Laboratories (Rockville, MD), fetal calf serum was from GIBCO, and α-thioglycerol and diisopropyl fluorophosphate were from Sigma. Human urinary erythropoietin (Pool H-16-Ta LSL, 13.3 units/mg) was generously provided by the Division of Blood Diseases and Resources, National Heart, Lung, and Blood Institute, Bethesda, MD. Petri dishes and tissue culture flasks were obtained from Falcon.

After abortion by dilatation and evacuation, the products of conception, normally discarded after pathologic examination, were transported from the operating room to the laboratory in a sterile container. Fetal age was estimated by foot length (7), when available, and was expressed as weeks from the first day

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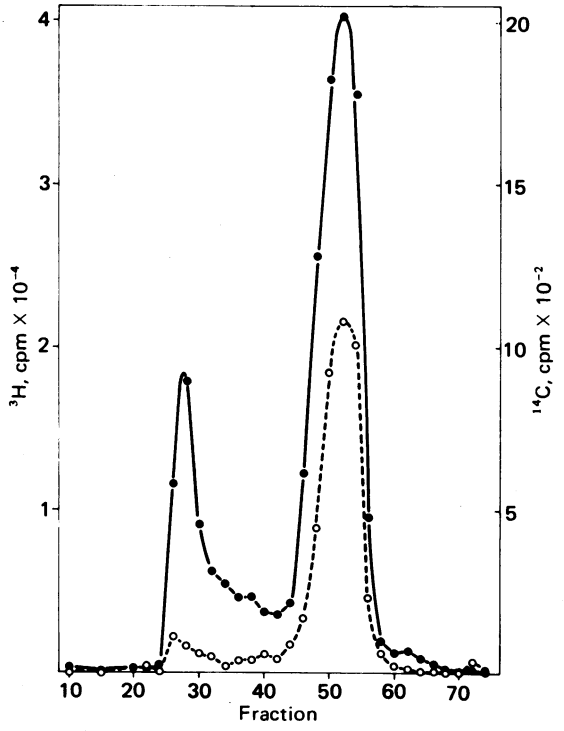

FIG. 1. Purification of [^{3}H]globins from lysates of cultures of human fetal hepatic erythroid cells by gel filtration. From the culture of hepatic erythroid cells from a 16.5-wk fetus, 2.5 X 10^6 nucleated cells were harvested on the 7th day and incubated with [^{3}H]leucine. After preparation of the lysate, addition of ^{14}C-labeled adult and cord globins, and acid/acetone precipitation, the material was added to a 50 X 2 cm column of Sephadex G-100 in 5% formic acid. Seventy-five fractions (1.6 ml) were collected for 30 hr. ●, ^{3}H; ○, ^{14}C.

of the last menstrual period. In a sterile hood, the liver was identified by its characteristic size, shape, color, and consistency; the identification was confirmed when necessary by microscopic examination after touch preparation and staining by hematoxylin and eosin. The liver was minced with a scalpel and suspended in alpha medium containing heparin, 10 units/ml, without preservatives. The mince was mixed in a 25-ml spinner flask for 10 min, and the dispersed cells were poured through stainless steel meshes of 100 and 200 perforations per cm^2. The residual material was mixed for two additional 10-min periods and strained. The strained suspensions were combined and aspirated 20 times into a 10-ml pipette to produce a single-cell suspension as judged microscopically. The concentration of nucleated cells was determined in 2% acetic acid in a hemocytometer. The suspended cells were allowed to adhere by diluting to 5 X 10^6 cells per ml in alpha medium/30% fetal calf serum and placing them in a Falcon tissue culture flask at 10^6 nucleated cells per cm^2 at 37°C in 5% CO_2/95% air. After 16 hr the nonadherent cells were harvested by swirling and decanting. Recovery was about 50%.

The nonadherent cells were plated at 5 X 10^5 nucleated cells per ml in alpha medium/0.8% methylcellulose/30% fetal calf serum/0.1 mM α-thioglycerol containing 1 μg of $Fe(NO_3)_3 \cdot 9H_2O$ per ml, 100 units of penicillin per ml, 100 μg of streptomycin per ml, and 1 unit of erythropoietin per ml except where otherwise indicated. The final mixture was added from a syringe without a needle at 1.0 ml per 35-mm petri dish. Plates were incubated in 95% air/5% CO_2 at 100% relative humidity at 37°C. Colonies were designated as erythroid only if red. Colony counts were based on counts in 10 reticles selected at random along each of six diameters in each of two plates at X40 magnification. The reticle, 1 cm^2, was in the eyepiece. The day of plating was designated day 0.

For each analysis of globin synthesis, cells from 2–20 plates were washed with alpha medium to reduce the methylcellulose concentration and incubated with [^{3}H]leucine (118 Ci/mmol, 8.4 μM) (1 Ci = 3.7 X 10^{10} becquerels) in the above medium without the methylcellulose and erythropoietin at 5 X 10^5 cells per ml at 37°C for 24 hr. After the incubation, cells were collected by centrifugation and suspended in 1 ml of 5 mM diisopropyl fluorophosphate to inhibit destruction of globins by proteases, as will be described elsewhere by P. Pan and P. T. Rowley. After centrifugation at 18,000 X *g*, the supernate was stored in liquid nitrogen until analyzed.

Prior to analysis of [^{3}H]globin chains, about 6 mg of [^{14}C]-hemoglobin from [^{14}C]leucine-labeled cord and adult blood cells was added to each sample. Globins were purified by acid/acetone precipitation (8) and subsequent Sephadex G-100 chromatography in formic acid as described by Chalevelakis *et al.* (9); globin acid/acetone precipitate was dissolved in 20% formic acid and chromatographed on a 50 X 2 (inside diameter) cm column in 5% formic acid at 25°C for 30 hr. The ^{3}H-labeled peak containing [^{14}C]globin was pooled and dialyzed against 2.6 mM sodium phosphate, pH 6.8/8 M urea/50 mM 2-mercaptoethanol, and the globins were separated on carboxymethylcellulose columns, 12 X 0.9 (inside diameter) cm (10), with a four-chamber gradient (11), modified by using sodium phosphate buffer, pH 6.8, at 2.6, 10, 15, and 32 mM, respectively, for 4 hr. Fractions (2 ml) were collected and 1 ml of each was added to 20 ml of Bray's solution and assayed for radioactivity in a liquid scintillation counter.

This project was approved by the Committee on Investigation Involving Human Subjects at the University of Rochester.

Table 1. Effect of medium and method of cell lysis on globin synthesis in cultured fetal hepatic erythroid cells

Medium*	Method of cell lysis†	[^{3}H]Leucine incorporation into globin, cpm X 10^{-3}			
		Total	γ	β	α
Alpha	Freeze–thaw	421	196	37	189
Alpha	$NaDodSO_4$	298	141	26	130
NCTC-109	Freeze–thaw	243	117	23	103
NCTC-109	$NaDodSO_4$	132	59	14	59

* Nucleated cells (2 X 10^7) from the liver of a 16-wk fetus were incubated in 2 ml of either alpha medium/30% fetal calf serum/0.1 mM α-thioglycerol/1 μg of $Fe(NO_3)_3 \cdot 9H_2O$ per ml or NCTC-109/10% fetal calf serum/25% human type AB Rh(+) plasma/1 μg of $Fe(NO_3)_3 \cdot 9H_2O$ per ml. After 24 hr, 20 μCi of [^{3}H]leucine was added and incubation continued for an additional 24 hr.

† Cells were lysed either by freeze–thaw or by addition of 1 ml of 1% sodium dodecyl sulfate ($NaDodSO_4$) to the cell pellet.

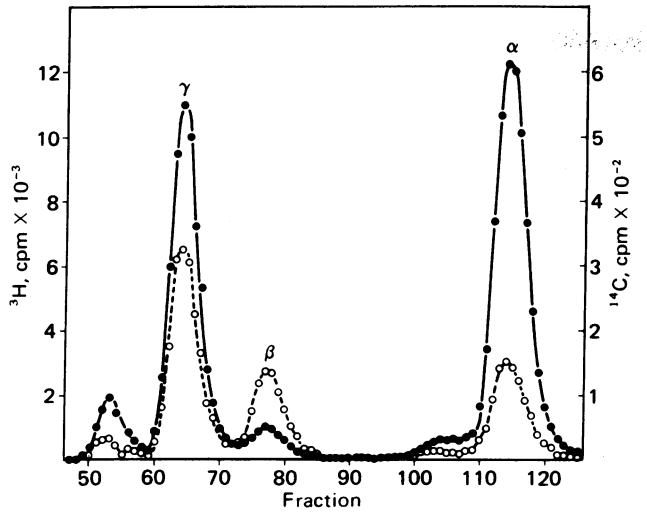

FIG. 2. Separation of [^{3}H]globins from lysate of human fetal hepatic erythroid cells by carboxymethylcellulose chromatography in urea. The fractions containing the [^{14}C]globin as shown in Fig. 1 were pooled, dialyzed against 2.6 mM sodium phosphate, pH 6.8/8 M urea/50 mM 2-mercaptoethanol, and chromatographed on a 12 × 0.9 cm carboxymethylcellulose column at 25°C for 4 hr. ●, ^{3}H; ○, ^{14}C.

RESULTS

Fig. 1 shows the partial purification of [^{3}H]globins from [^{3}H]-leucine-labeled lysates of cultured human fetal hepatic erythroid cells by gel filtration. Two ^{3}H-labeled peaks are evident. The globins elute in the second peak, as identified by the [^{14}C]globin marker.

Fig. 2 illustrates the separation by carboxymethylcellulose/urea chromatography of individual [^{3}H]globins from human fetal hepatic erythroid cells after partial purification by gel filtration. The γ, β, and α [^{3}H]globins are identified by the corresponding ^{14}C-labeled markers.

Several pilot experiments were performed to identify efficient labeling conditions. Table 1 demonstrates that, in terms of yield of [^{3}H]globin, alpha medium is superior to NCTC-109 medium despite the latter's having a richer composition and a lower leucine concentration than the former. It also shows that freeze–thaw is preferable to sodium dodecyl sulfate treatment for cell lysis. Other experiments (data not shown) suggested that labeling was more efficient when [^{3}H]leucine was added to harvested cells than when it was added to the cells in methylcellulose. This result may reflect incomplete cell harvesting or poor diffusion of label in the methylcellulose. Finally, 5 × 10^5 cells per ml yields more incorporation per plate (and per cell) than does a higher cell concentration whether during growth or during labeling.

Table 2 illustrates that, as judged by synthesis on day 11/12, erythropoietin at 1–4 units/ml does not greatly influence the

Table 2. Effect of erythropoietin concentration on globin synthesis in cultured fetal hepatic erythroid cells*

Erythropoietin, units/ml	[^{3}H]Leucine incorp. into globin, cpm γ	β	α	$\frac{\beta+\gamma}{\alpha}$	$\frac{\gamma}{\beta+\gamma}$	$\frac{\beta}{\beta+\gamma}$
1	2353	557	2572	1.13	0.809	0.191
2	2498	701	3503	0.91	0.781	0.219
4	7492	1536	8810	1.02	0.830	0.170

* Nucleated cells (5 × 10^6) from the liver of a 16.8-wk fetus were cultured for 11 days in methylcellulose at each concentration of erythropoietin shown.

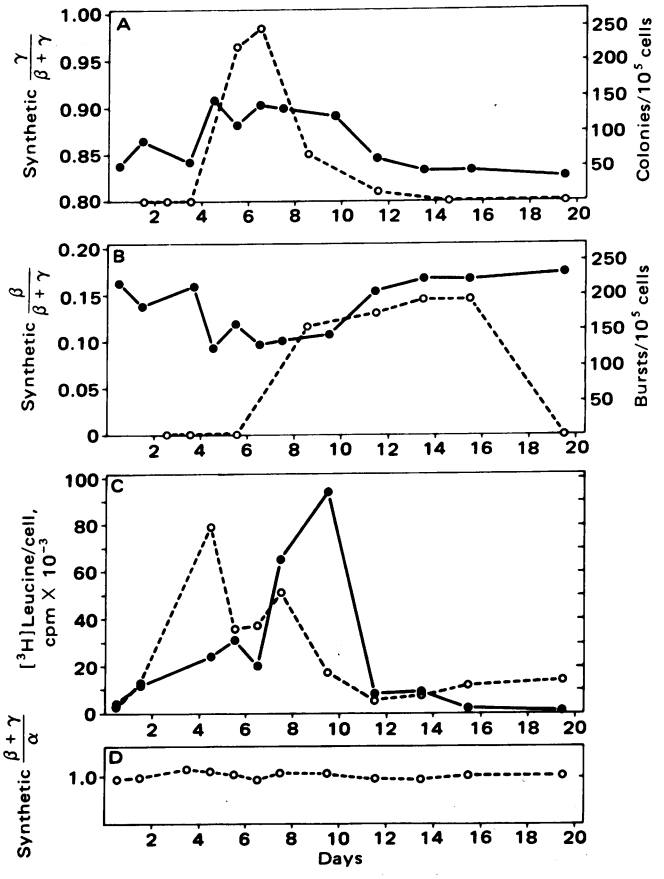

FIG. 3. Time course of globin synthesis in culture of hepatic erythroid cells from an 18-wk fetus. (*A*) Time course of $\gamma/(\beta+\gamma)$ synthetic ratio (●) and erythroid colonies per 10^5 nucleated cells plated (○). (*B*) Time course of $\beta/(\beta+\gamma)$ synthetic ratio (●) and erythroid bursts per 10^5 nucleated cells plated (○). (*C*) [^{3}H]Leucine incorporation into globin per cell (●) and into nonglobin protein per cell (○). Nonglobin protein radioactivity is estimated from the first ^{3}H-labeled peak to elute from Sephadex G-100. Cell number refers to number of cells incubated with [^{3}H]leucine. (*D*) Time course of $(\beta+\gamma)/\alpha$ synthetic ratio.

$(\beta+\gamma)/\alpha$ synthetic ratio or the relative synthesis of β and γ chains. For subsequent studies we chose 1 unit/ml as a compromise between a physiological concentration (12) and production of maximal numbers of erythroid bursts.

Table 3 shows the changes in these synthetic ratios as a function of time. The $(\beta+\gamma)/\alpha$ synthetic ratio averaged 1.06 ± 0.031 SEM and hence was relatively balanced throughout. In contrast, the relative synthesis of β and γ chains changed with time in culture.

The more detailed data are available from the 18-wk fetus and are depicted in Fig. 3. Fig. 3*A* shows that the $\gamma/(\beta+\gamma)$ synthetic ratio rises on about the 4th day and falls on about the 10th day. The rise corresponds to an increase in colony number and the fall to a decrease in the colony number. Fig. 3*B* illustrates that, conversely, the $\beta/(\beta+\gamma)$ ratio rises on about the 10th day and reaches a maximum about the same time as erythroid burst numbers. The Pearson product moment correlation of this latter ratio with the number of days cultured beginning at day 4/5 is 0.913 ($P < 0.001$), indicating a highly significant linear relationship. Fig. 3*C* demonstrates that the maximal synthesis of nonglobin protein measured is on day 4/5, whereas the maximal synthesis of globin measured is on day 9/10. Fig. 3*D* shows that the $(\beta+\gamma)/\alpha$ globin synthetic ratio is relatively constant throughout the period of culture despite the changes in the synthesis of globins vs. nonglobin protein and of β vs. γ chains.

Table 3. Effect of duration of culture on globin synthesis in cultured fetal hepatic erythroid cells

Estimated fetal age, wk	Day of culture*	Cells recovered, %	Cells used for synthesis, no. $\times 10^{-6}$ †	[^{3}H]Leucine incorporation into globin, cpm			$\frac{\beta+\gamma}{\alpha}$	$\frac{\gamma}{\beta+\gamma}$	$\frac{\beta}{\beta+\gamma}$
				γ	β	α			
14.5	1/2	—	3.0	1,870	623	1,970	1.27	0.750	0.250
	3/4	66	1.6	2,040	654	1,880	1.43	0.757	0.243
	5/6	50	1.3	24,100	2,330	26,000	1.02	0.912	0.088
	7/8	66	2.0	378,000	53,100	433,000	1.00	0.877	0.123
	11/12	150	4.5	68,700	14,700	87,500	0.95	0.824	0.176
	13/14	140	3.5	7,170	1,390	7,300	1.17	0.837	0.163
18.0	0/1	100	5.0	8,050	1,550	10,500	0.92	0.838	0.162
	1/2	50	1.5	7,700	1,210	9,200	0.97	0.864	0.136
	3/4	—	—	2,320	440	2,390	1.15	0.841	0.159
	4/5	50	1.8	19,900	2,010	19,900	1.10	0.908	0.092
	5/6	86	2.6	36,200	4,830	39,800	1.03	0.882	0.118
	6/7	100	2.5	21,600	2,310	24,900	0.96	0.903	0.097
	7/8	100	2.5	75,910	8,530	78,100	1.08	0.899	0.101
	9/10	47	1.4	52,400	6,360	54,000	1.09	0.892	0.108
	11/12	216	4.5	15,100	2,720	18,500	0.96	0.847	0.153
	13/14	263	6.6	23,500	4,700	30,000	0.94	0.833	0.167
	15/16	160	4.0	2,530	500	3,000	1.02	0.834	0.166
	19/20	220	11.0	1,590	333	1,890	1.02	0.827	0.173

* Twenty-four-hour period.

† Globin synthesis was measured at a cell concentration of 5×10^5 nucleated cells per ml.

DISCUSSION

The results reported here demonstrate that, for studies of hemoglobin synthesis in the human midtrimester fetus, the liver of the dilatation and evacuation abortus provides a suitable source of erythroid progenitor cells. Although the liver is often delivered in fragments, it can be identified unless fragmentation is unusually extensive. Ample material is available from a single fetus for multiple comparisons. Globin synthesis in cultured nucleated cells appears to be more active than in adult marrow (1). Calculation of the $(\beta + \gamma)/\alpha$ globin synthetic ratio gives a value near unity, as is found in fetal reticulocytes of approximately the same age (13), confirming the physiological nature of hemoglobin synthesis in these cultured cells.

The principal interest attaches to the changes over time in the relative synthesis of β and γ chains. On days 5–7, when colonies predominate, $\beta/(\beta + \gamma)$ was 0.09–0.11, a value characteristic of fetal reticulocytes (13, 14). By day 11/12, when bursts predominate, this ratio had risen to 0.15–0.17. Rosenblum *et al.* (15) have reported in abstract form a similar value for the burst phase of cultures of liver from younger (first trimester) fetuses.

The changes we observed in the relative synthesis of β and γ chains in cultures of fetal liver are not predictable from the formulation of Papayannopoulou *et al.* (3–5) based on the studies of adult marrow cited above. They found that the earlier progenitor, the burst-forming units, gave rise to a larger proportion of erythroid colonies making fetal hemoglobin than did the later progenitor, the colony-forming unit. In contrast, we have found that the earlier progenitor synthesizes a larger proportion of β chains, characteristic of adult hemoglobin, than does the later progenitor. Thus it appears that their generalization does not apply to the second trimester fetal liver and perhaps not until after the perinatal γ-to-β switch has occurred.

Three questions are relevant to the interpretation of this change in the relative synthesis of β and γ chains. First, are the same cells active in globin synthesis throughout the period of change? It is possible that they are and that this phenomenon does represent intracellular "switching." However we think it is more likely that different cell populations are active at different times and specifically that the cells synthetically active late in culture have arisen from earlier progenitors than those active early in culture. In mouse marrow, the conclusions that the first appearing erythroid colonies arise from the later progenitors and that the subsequently appearing colonies (small and large bursts), resulting from a greater number of cell divisions prior to hemoglobin formation, arise from earlier progenitors are supported by evidence from colony size, erythropoietin requirement, cycling characteristics, effect of leukocyte conditioned medium, response to transfusion of the donor, and spleen colony assay of the progenitors (2, 16–19). In human marrow the same conclusion is supported by more limited studies (20–22).

Second, is the pattern of globin synthesis observed by the methods used masking cellular heterogeneity in synthetic activity at any given time? This question is difficult to answer with present methods. The immunologic methods used to identify fetal hemoglobin-containing cells in adult marrow (3, 5, 23) indicate presence or absence but not relative amounts of this hemoglobin. It seems likely that all the cells in the fetal liver have both fetal and adult hemoglobins, though possibly in differing amounts.

Third, does this change in the relative synthesis of β and γ chains represent physiological differentiation or an artifact of culture? Since the cultures were not fed, the rapid decline in globin synthesis per cell after the 10th day could be due to a deficiency of erythropoietin, to a nutritional deficiency, or to another unphysiological aspect of culture. In any case, based on globin synthetic rates per cell, there is no specific indication that the properties described do not reflect physiological differentiation.

Whereas other investigators have found unexpectedly high γ chain synthesis in cultures of adult marrow (3–5), we have found unexpectedly high β chain synthesis in cultures of fetal liver. In each case, the colony expressed the gene characteristic of the age of the organism to a greater degree, whereas the burst expressed β and γ genes more equally. Thus we might say that the burst-forming unit, the earlier progenitor, is less selective and the colony-forming unit, the later progenitor, is more selective. That is, the capacity for highly selective gene expression

characteristic of differentiating cells appears to be less well developed in the burst-forming unit than in the colony-forming unit.

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