Switch from Fetal to Adult Hemoglobin Is Associated with a Change in Progenitor Cell Population

RONA S. WEINBERG, JUDITH D. GOLDBERG, J. MATTHEW SCHOFIELD, ALEXANDRA L. LENES, ROGER STYCZYNSKI, and BLANCHE P. ALTER, *The Polly Annenberg Levee Hematology Center, Departments of Medicine, Pediatrics, and Biomathematical Sciences, Mount Sinai School of Medicine, New York 10029*

ABSTRACT To examine the switch from fetal to adult hemoglobin at the cellular level, erythroid progenitor cells from newborn infants and adults were cultured in methyl cellulose with erythropoietin. Individual erythroid colonies were labeled with [^{3}H]-leucine at various times, and globin synthesis patterns examined by gel electrophoresis and fluorography. The percent γ- or β-globin synthesis was determined from the total of $\gamma + \beta$, and the percent Gγ from the total of Gγ + Aγ. The nonparametric correlation coefficients of percent Gγ with percent γ or β were obtained. Each group of colonies at each time point was examined separately. In colonies from adult blood, the proportion of Gγ-synthesis did not correlate with the proportion of γ-synthesis. Colonies from newborn blood fell into two groups. Those that developed from relatively mature progenitor cells, and were seen on day 14, showed a strong negative correlation of Gγ with β-globin synthesis. However, those newborn colonies that developed from immature progenitors, and were seen later in culture (days 17 and 21), showed no correlation of Gγ with β-synthesis. These findings are compatible with a clonal model for hemoglobin switching. Fetal progenitors, in which Gγ- and β-syntheses are negatively correlated, are gradually replaced during ontogeny by adult progenitors. The adult progenitors produce more β (less γ), and the proportions of Gγ- and γ- or β-synthesis are not correlated.

INTRODUCTION

During normal human ontogeny there is a switch from fetal to adult hemoglobin (Hb)[1] (Hb F, $\alpha_2\gamma_2$ to Hb A, $\alpha_2\beta_2$), due to a change in non-α-globin synthesis (1). Patients with hemoglobinopathies, such as sickle-cell anemia or thalassemia, might be improved clinically if Hb F synthesis persisted. A second switch that occurs at approximately the same time during development involves the two γ-globin genes, Gγ and Aγ, which code for glycine or alanine at amino acid position 136. The proportion of Gγ declines from ~70% in fetuses and newborn infants to ≤50% in the small amount of fetal Hb found in adults (2, 3).

Investigation of Hb regulation at the cellular level provides one approach to understanding of the switch. The pattern of Hb synthesis in colonies that develop in vitro from erythroid progenitor cells is assumed to reflect the Hb potential of that progenitor cell class. Hb F synthesis is increased in cultures derived from adult erythroid progenitors (4); several theoretical models have been proposed to explain this finding (5–8). In cultures of erythroid progenitors from fetuses or newborns, the reverse is seen. Adult Hb is synthesized in proportions equal to or greater than seen in vivo (9). Similar results obtained from studies of erythropoiesis during ontogeny in the rhesus monkey led us to suggest that ontogeny of erythropoiesis could be associated with the gradual appearance of new classes of erythroid progenitors, with different Hb programs (10).

Further study of this clonal model requires analysis of the Hb program of single progenitor cells. These progenitors produce in culture colonies of hemoglobinized erythroblasts. The presence of two cell populations during ontogeny might be postulated from skewed or bimodal distributions of Gγ- or β- (or γ-) globin synthesis. Alternatively, the relationship between Gγ- and β- or γ-proportions might be different in the two populations. The resulting observed distribution may be a mixture of the two individual distributions. There are several reports of single colony studies involving newborn or adult blood (11–15). In all but one (15), the proportion of Hb F synthesis was distributed normally, interpreted as suggesting a single

This work was presented, in part, at the American Society of Hematology, 7 December 1981, and appeared in abstract form. 1981 *Blood.* 58(Suppl. 1): 68*a*. (Abstr.)

Received for publication 9 July 1982 and in revised form 30 November 1982.

[1] *Abbreviations used in this paper*: BFU-E, erythroid burst-forming unit; Ep, erythropoietin; Hb, hemoglobin; KRP, Krebs-Ringer phosphate buffer.

population. In addition, the proportion of Gγ-synthesis was reported to correlate with the level of Hb F synthesis.

Our studies, although similar in design to those mentioned above, have provided different results. We measured globin synthesis in individual erythroid colonies cultured from newborn and adult blood. We examined large numbers of colonies and studied various time points in the newborn experiments. Data were analyzed by several statistical methods. Although Gγ- and γ-synthesis proportions were correlated in some of the early newborn studies, this correlation was no longer present in later newborn cultures, or in those obtained from adults. We have thus characterized the "fetal" erythroid progenitor as one which gives rise to colonies in which the levels of Gγ- and γ- or β-synthesis are correlated. The colonies from the "adult" progenitor, however, can be distinguished by the lack of correlation of these two parameters. Hb switching during ontogeny may thus be explained by the gradual replacement of the fetal by the adult progenitor.

METHODS

Blood was obtained from the umbilical cords of term newborn infants, and the antecubital veins of normal adults. All procedures were approved by the Research Advisory Committee of the Mount Sinai School of Medicine. Samples were collected in heparin (Elkins-Sinn, Inc., Cherry Hill, NJ), 50 U/ml of blood. The blood was diluted with an equal volume of alpha medium lacking nucleosides (Gibco Laboratories, Grand Island Biological Co., Grand Island, NY), and 25 ml of the diluted blood was layered onto 20 ml of Ficoll-Paque (Pharmacia Fine Chemicals, Piscataway, NJ). The blood was then centrifuged at 450 *g* for 30 min at 18°C in a Sorvall RC3B centrifuge (Du Pont de Nemours, E. I. & Co., Inc., Sorvall Instruments Div., Newtown, CT). The mononuclear cell layer was removed and washed three times with alpha medium. In one experiment, adherent cells were removed by incubation of 1.2×10^7 cells in 3 ml of RPMI medium (Gibco Laboratories) containing 30% fetal calf serum (Armour Pharmaceutical Co., Tarrytown, NY) in a 60-mm culture dish (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA). The adherence took place for 1 h in a 37°C incubator with 5% CO_2. The supernatant was then transferred to another culture dish for a 2nd h of adherence (16). The nonadherent cells were washed once in alpha medium. All cell counts were obtained with a Coulter ZBI counter (Coulter Electronics Inc., Hialeah, FL). Cells were then suspended in alpha medium at the appropriate concentrations for cultures.

Methyl cellulose cultures were established according to a modification of Iscove's method (17). Each milliliter contained $1–5 \times 10^5$ newborn or $2–20 \times 10^5$ adult mononuclear cells in 0.8% methyl cellulose (Fisher Scientific Co., Pittsburgh, PA), 30% fetal calf serum (Armour Pharmaceutical Co.), 1% bovine serum albumin (Cohn fraction IV, Sigma Chemical Co., St. Louis, MO) deionized according to Worton et al. (18), 10^{-4} M 2-mercaptoethanol (Sigma Chemical Co.), 0.1 U penicillin and 0.1 μg streptomycin/ml (Gibco Laboratories). The erythropoietin (Ep) was from sheep plasma (step III, lot 3038, 2.7 U/mg, Connaught Laboratories, Toronto, Canada) or human urine (National Institutes of Health lot PS 831, 391 U/mg, kindly provided by the Division of Blood Diseases and Resources of the National Heart, Lung, and Blood Institute). The Ep was diluted in alpha medium and used at various concentrations up to 2 U/ml. Flat bottom 96-well tissue culture plates (Linbro Chemical Co., Hamden, CT) were cut into sections of 6 wells each, placed in 100-mm dishes (Falcon Labware), and sterilized by UV irradiation in a Biogard laminar flow hood for 30 min. Three wells were then plated with 0.3 ml of culture mix, and the remaining three wells with sterile deionized water to maintain humidity. The cultures were incubated for up to 27 d in a National water-jacketed incubator at 37°C with 5% CO_2 and high humidity. Each 0.3-ml culture is referred to as a "whole plate", to distinguish it from studies of individual colonies. Three whole plates were usually studied at each point, and the data reported as the mean±1 SD. Colonies were counted using a Bausch & Lomb stereozoom dissecting microscope (Bausch & Lomb Inc., Rochester, NY). Identification of erythroid colonies was confirmed by photography of the unstained plates as well as by removal of single colonies and staining with benzidine-Wright's-Giemsa.

For studies of globin synthesis in whole plates, 100 μCi of previously lyophilized [^{3}H]leucine (New England Nuclear, Boston, MA, >100 Ci/mmol) was dissolved in 30–50 μl of alpha medium and added dropwise to each 0.3-ml plate. After incubation at 37°C for 16–24 h, each whole plate was harvested by dilution of the methyl cellulose with cold Kreb's-Ringer phosphate buffer at pH 7.4 (KRP), transferred to a tube containing 4×10^5 nonradioactive newborn erythrocytes, and washed three times with cold KRP at 4°C. The cell pellet was stored at −80°C until further analysis.

Individual colonies were removed under the dissecting microscope using 4-μl microcaps (Drummond Scientific Co., Broomall, PA), and placed in 1.5-ml Eppendorf tubes (Brinkmann Instruments, Inc., Westbury, NY) containing 50 μCi of lyophilized [^{3}H]leucine plus 10 μl of leucine-free incubation medium (19). After incubation at 37°C for 16–24 h, the cells were recovered by washing twice in cold KRP for 2 min in an Eppendorf centrifuge (Brinkmann Instruments) at 4°C. Carrier was added as above. The pellets were stored at −80°C.

Globin chain synthesis was evaluated by electrophoresis on slab gels of polyacrylamide, acid, urea, and Triton X-100, modified from the previously described method (20). The gel was 0.8-mm thick, 11-cm long, and 15-cm wide. No more than 10 μg of total protein was used per lane. Electrophoresis was at 16 mA for 4.5 h. The gels were stained with 0.5% Coomassie blue, diffusion destained in 7% acetic acid-30% methanol, and impregnated with 2,5-diphenyloxazole (PPO) in dimethylsulfoxide (DMSO) for fluorography (21, 22). We used preflashed x-ray film, XR5 and XAR5 (Eastman Kodak Co., Rochester, NY). The x-ray films were developed in a Kodak X-Omat, and scanned at 615 or 550 nm (for XR and XAR film, respectively) in a Gilford model 240 spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH) equipped with a linear transporter. The areas under the peaks were determined by connection of the lowest points surrounding each peak, and measurement with a Numonics 1250 planimeter (Numonics Corp., Lansdale, PA). The percent Gγ-synthesis was calculated from $G\gamma/(G\gamma + A\gamma) \times 100$, and percent β- or γ-synthesis from β or $\gamma/(\beta + \gamma) \times 100$.

The observed distributions of Gγ and γ or β (as well as transformations of each) in the groups of single colonies were plotted and examined for each experiment. That the observed data are samples from normal distributions was tested using the W statistic (23). Nonparametric (Spearman's) correlation coefficients were obtained for Gγ and β or Gγ and γ to measure the association of these quantities (24). $P \leq 0.05$ is considered statistically significant and to suggest the associations described here.

The mean levels of Gγ- and β-syntheses were compared jointly in red, well hemoglobinized colonies and white, poorly hemoglobinized colonies using linear discriminant methods (24). We do recognize that the lack of normality in the distributions of Gγ and γ or β may violate the assumptions of this approach. However, the results support univariate analyses using nonparametric methods (Mann Whitney tests) as well.

RESULTS

Studies of newborn blood

Colony growth. The number of colonies derived from the blood mononuclear cells of newborn infants increased with time in culture (Fig. 1). The peak was 100–120 colonies/10^5 cells plated, and was reached on day 21, 16, and 13, respectively, in the experiments shown in Fig. 1. Colony number and size also increased with increasing concentration of Ep, up to 1.5 U/ml.

Globin chain synthesis in whole plates. The time courses of synthesis of Gγ- (of Gγ + Aγ) and β- (of β + γ) globin are shown in Fig. 2. On day 10, the earliest time studied, Gγ- and β-syntheses resembled the values seen in reticulocytes. The proportion of β-synthesis then increased with time in culture, from 45 to 50% on day 10, to 70–80% on day 21. Gγ-synthesis remained at ~60% in two experiments, and decreased slightly but not signficantly from 56 to 43% in one. Thus Gγ- and β-syntheses were not correlated over time in culture. Although the number of colonies was higher at 1.5 than at 0.5 U of Ep/ml, the relative synthesis of β and Gγ was the same at both Ep concentrations.

Globin chain synthesis in single colonies. Globin chain synthesis was examined in individual colonies at several times. Table I shows that the values for Gγ- and β-syntheses in each group of individual colonies were similar to those obtained in whole plates examined at the same time. Thus, the colonies examined were representative of the overall growth in each case.

Representative spectrophotometric scans and fluorograms are shown in Fig. 3. In these two individual colonies from 14-d cultures, the Gγ-synthesis was ~40% in both, while β-synthesis was 85% in one and 65% in the other. Nonglobin protein synthesis was minimal. The levels of Gγ- and β-synthesis were uncorrelated in these examples.

The results of Gγ- and β-synthesis are compared for the three newborn studies in Figs. 4–6 and the data are summarized in Table I. In the example in Fig. 4 (study A), on day 14, Gγ-synthesis decreased as β-synthesis increased; this negative correlation was highly significant. On day 17, however, Gγ-synthesis did not decrease as β-synthesis rose and the degree of negative correlation between these two parameters was reduced.

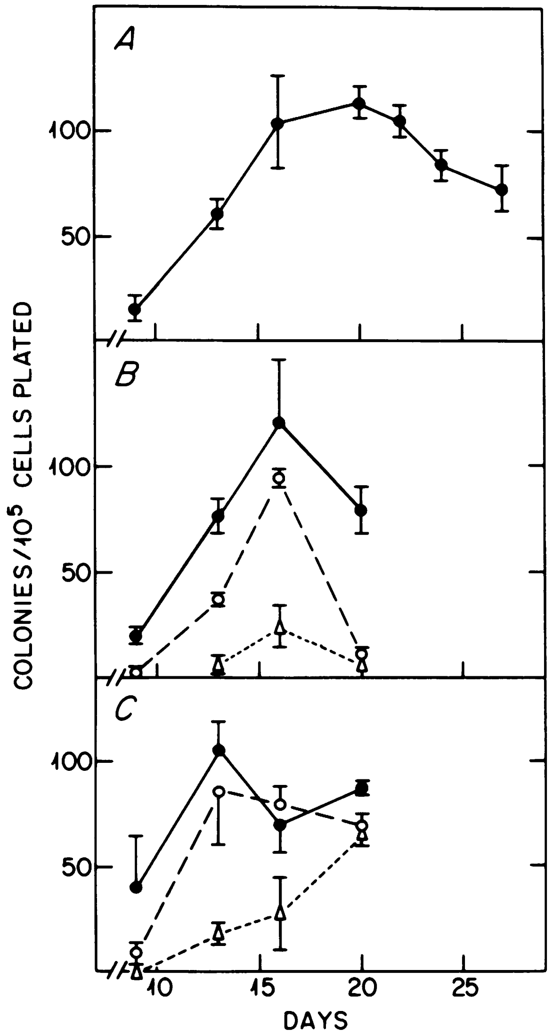

FIGURE 1 Growth curves of newborn blood. Colonies per 10^5 nucleated cells in three separate experiments (A–C) with 0.1 (Δ), 0.5 (O), and 1.5 (●) U Ep/ml.

In the study shown in Fig. 5, globin synthesis was analyzed in red, well hemoglobinized, as well as white, poorly hemoglobinized colonies (Table I, study B). Although there was a trend toward less Gγ- and more β-synthesis in the red colonies, the differences were not significant ($F_{1,42} = 0.62$, $P > 0.25$). We recognize the lack of normality in the distributions of the Gγ, but the results do not suggest that there are any differences in these groups of colonies. The values were not significantly different by the Mann Whitney test either (U for Gγ = 174, $P = 0.48$, U for β = 233, $P = 0.42$). In the total group as well as the red and

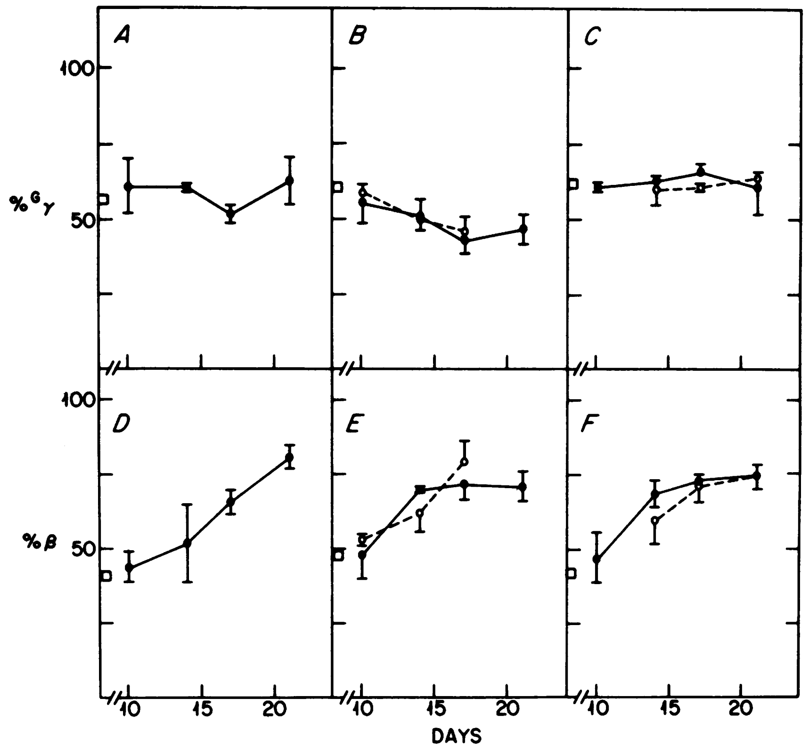

FIGURE 2 Time course of percent Gγ- and percent β-syntheses by newborn blood reticulocytes (□) and whole plate cultures in three separate experiments (A and D, B and E, C and F, respectively). Cultures were grown with 0.5 (O) and 1.5 (●) U Ep/ml.

white colonies separately, Gγ- and β-syntheses were strongly negatively correlated. Only five of each type of colony were examined on day 17. As before, the globin synthesis patterns were the same in the red and the white colonies, and Gγ- and β-syntheses were correlated.

Fig. 6 shows the third study, in which the removal of adherent cells before plating was investigated. Gγ- and β-syntheses were the same (Table I, study C) in both groups on days 14 and 17, and were not correlated in either group. Only the standard culture was used on day 21. As on the earlier days, Gγ and β were not correlated.

The studies of single colonies derived from newborn blood thus showed varying patterns of globin synthesis. In study B, Gγ- and β-syntheses were correlated on days 14 and 17. In study A, correlation was seen on day 14, but to a much lesser extent on day 17. In the third study (C), Gγ- and β-syntheses showed very low order correlation at any time point (days 14, 17, or 21).

Studies of adult blood

Colony growth. Variables which influence colony growth include cell and Ep concentrations, time in culture, and donor. The number and size of adult erythroid burst-forming unit (BFU-E)-derived colonies plateaued at 2 U of Ep/ml. Four studies from adult donors are summarized in Table II. The peak colony number was achieved on day 14 in three and day 15 in one study. The number of colonies per 10^5 cells plated ranged from 5 to 40.

Globin synthesis in whole plates and single colonies. The results of the studies of single colonies and whole plates are compared in Table III. As in the newborn experiments, the values for single colonies and whole plates were similar, and thus the colonies were representative of the cultures. The distribution of Gγ-synthesis appeared normal in all but one study, while the distribution of γ-synthesis appeared normal in only one study (both were study B, Table III).

The comparisons of Gγ with γ-synthesis are shown in Fig. 7 and summarized in Table III. Only red, mature, well hemoglobinized colonies were examined in studies A–C. The mean Gγ-synthesis was similar (53, 47, and 57%) in these experiments, while mean γ-synthesis varied (41, 16, and 25%, respectively). In each case, Gγ- and γ-syntheses were not significantly correlated. In the fourth experiment, shown in Fig. 7D, globin synthesis was examined separately in 20 red and

TABLE I
Gγ- and β-Synthesis by Newborn Blood Colonies

				Percent Gγ					Percent β					Correlation of Gγ and β‡	
							Distribution*					Distribution*			
Study	Day	Sample	*n*	Mean ±1 SD	Median	Range	W	*P*	Mean ±1 SD	Median	Range	W	*P*	r_s	*P*
A	0	Reticulocytes	1	57					41						
	14	Whole plates	3	54±5		49–59			58±1		56–59				
		Singles	30	52±12	53	28–71	0.95	0.27	60±18	61	29–86	0.92	0.04	−0.75	0.0001
	17	Whole plates	2	44		43–45			79		76–82				
		Singles	21	44±7	47	34–56	0.92	0.09	79±11	84	53–90	0.77	<0.01	−0.23	0.31
B	0	Reticulocytes	1	61					48						
	14	Whole plates	2	51		47–55			70		70–70				
		Red singles	31	50±11	47	34–73	0.92	0.03	69±14	68	43–91	0.96	0.34	−0.67	0.0001
		White singles	13	52±11	49	37–72	0.94	0.48	63±17	71	31–81	0.87	0.06	−0.40	0.07
		Total singles	44	51±11	48	34–73	0.92	<0.01	67±15	70	31–91	0.96	0.25	−0.63	0.0001
	17	Whole plates	3	43±4		39–46			72±5		67–77				
		Singles	10	43±7	41	37–56	0.76	<0.01	74±15	77	37–92	0.85	0.06	−0.66	0.04
C	0	Reticulocytes	1	62					42						
	14	Whole plates	3	63±2		62–65			68±5		66–74				
		Singles	24	59±6	58	46–70	0.97	0.56	57±13	56	23–83	0.97	0.70	0.06	0.79
		NA§ whole plates	3	62±2		60–64			65±3		63–69				
		NA singles	12	58±8	61	41–67	0.87	0.07	59±10	60	42–78	0.99	0.99	0.21	0.50
	17	Whole plates	3	66±3		63–69			72±1		71–73				
		Singles	24	56±9	57	33–72	0.96	0.48	60±16	63	27–82	0.95	0.29	0.12	0.58
		NA whole plates	3	61±1		60–61			66±7		60–74				
		NA singles	23	59±7	60	36–73	0.89	0.02	65±11	66	40–82	0.95	0.35	−0.14	0.51
	21	Whole plates	3	61±8		53–69			75±3		72–78				
		Singles	12	64±7	62	54–76	0.95	0.60	75±15	79	44–91	0.91	0.27	−0.29	0.35

* W, Wilk's w statistic, $P < 0.05$ indicates data are not normally distributed.
‡ r_s, Spearman's rank correlation coefficient, $P < 0.05$ indicates correlation of Gγ and β.
§ NA, nonadherent.

14 white colonies. Mean Gγ-synthesis was 60 and 47% in these colonies. Mean γ-synthesis was 10 and 18%, respectively. Thus, Gγ-synthesis was higher and γ-synthesis lower in the red, well hemoglobinized colonies than in the white colonies, ($F_{1,31} = 8.73$, $P < 0.005$), again recognizing that the distribution of γ was not normal. (The Mann Whitney test U statistic is 60 for Gγ, $P = 0.5$, and U for $\gamma = 70$, $P = 0.01$). Thus, the γ-values were significantly lower in the red colonies. As before, Gγ- and γ-syntheses were unassociated in each group. However, analysis of the combined red and white colonies showed the same level of correlation of Gγ with γ as the red colonies alone.

The studies shown in Figs. 7 C and D were from the same donor on two different occasions. Mean γ-synthesis by the red colonies in these experiments was 25 and 10%, significantly different ($U = 130$, $P < 0.001$), while mean Gγ-synthesis was similar in both (57 and 60%) ($U = 519$, $P = 0.19$). This emphasizes the lack of association between Gγ- and γ-syntheses in colonies derived from the peripheral blood BFU-E of adults.

DISCUSSION

The characteristics of our adult cultures were similar to those reported by others, except that we did not remove adherent cells. The peak time and plating efficiency for the adult studies were day 14 and 5–40

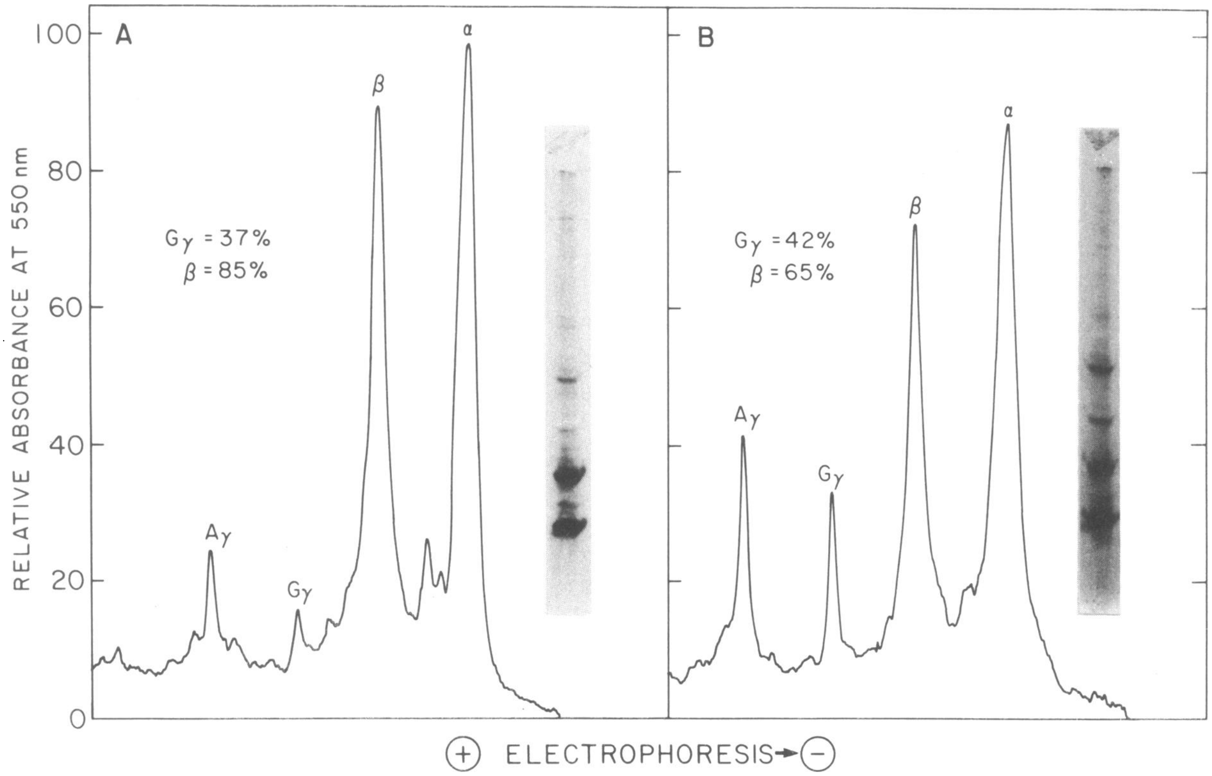

FIGURE 3 Representative spectrophotometric scans and fluorograms of two individual colonies (A and B) from a day 14 newborn culture.

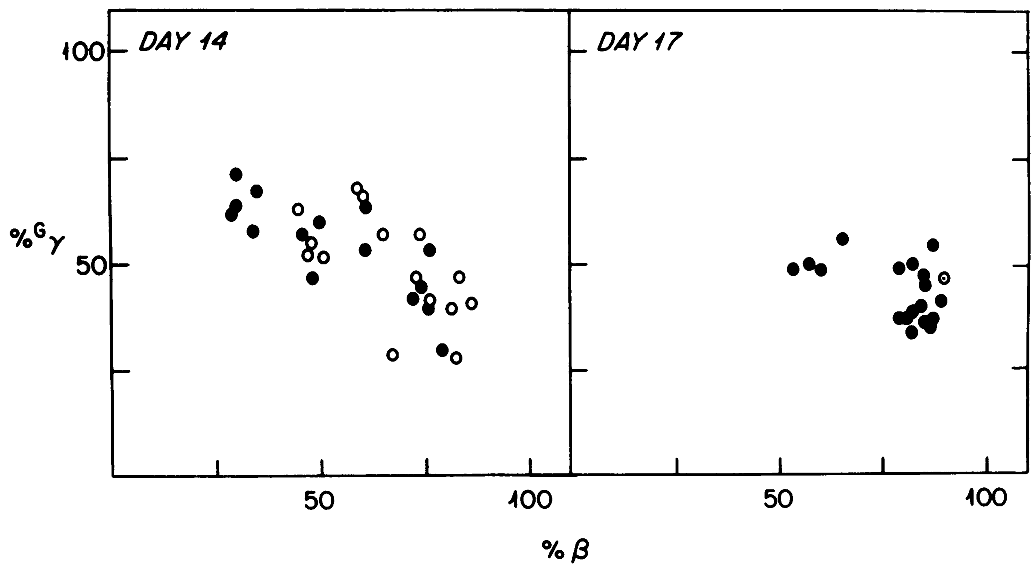

FIGURE 4 Percent Gγ- and percent β-syntheses by single newborn blood colonies from study A. Cultures were plated at 1×10^5 (●) or 5×10^5 (○) cells/ml. Overlapping filled and empty circles indicates two identical data points.

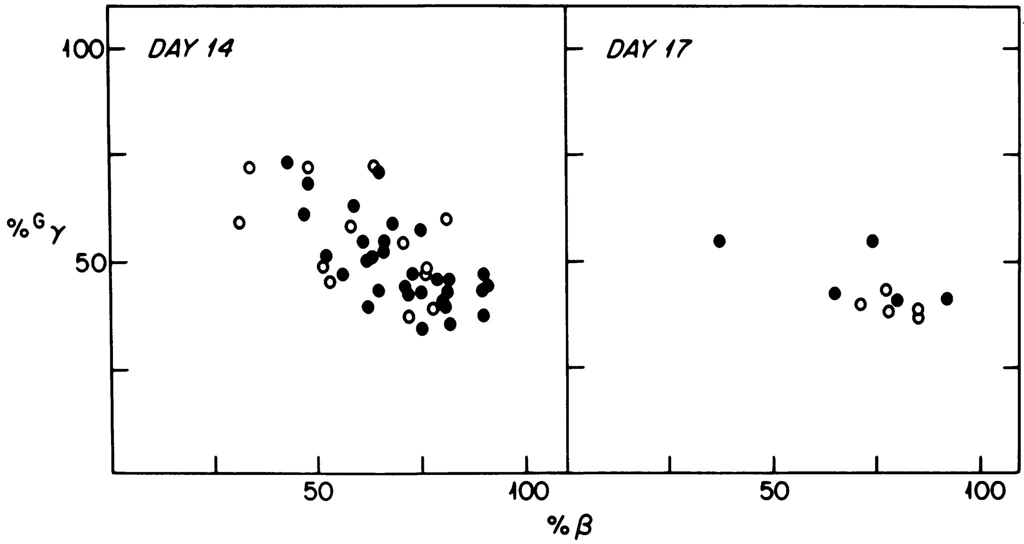

FIGURE 5 Percent Gγ- and percent β-syntheses by single newborn blood colonies from study B. Each symbol represents one red (●) or white (○) colony.

colonies/10^5 cells plated at 2 U of Ep/ml. In the newborn experiments, the plating efficiency was higher, at 100–120 colonies/10^5 cells. The peak colony number was usually at days 16–21, later than was apparently examined in most of the published reports of newborn studies.

In the newborn cultures, the proportion of β-globin synthesis increased with time in culture, as has been observed by others (13, 25, 26). The proportion of Gγ-synthesis was essentially unchanged, as has also been noted previously (13, 25). The rise in β-synthesis has been ascribed to a relative loss of γ-gene expression as erythroblasts progress from immature to mature (26–29). In most of our studies we therefore deliberately examined only well-hemoglobinized colonies at the various time points.

Previously published studies of single colonies from newborn infants were only from day 14 cultures, or often included only small numbers of colonies at the later time points (13). Data from more than one cord or adult study were sometimes pooled, thus obscuring any individual lack of correlation. We did not pool data, and we examined newborn cultures on several days (days 14, 17, and 21). We found that the com-

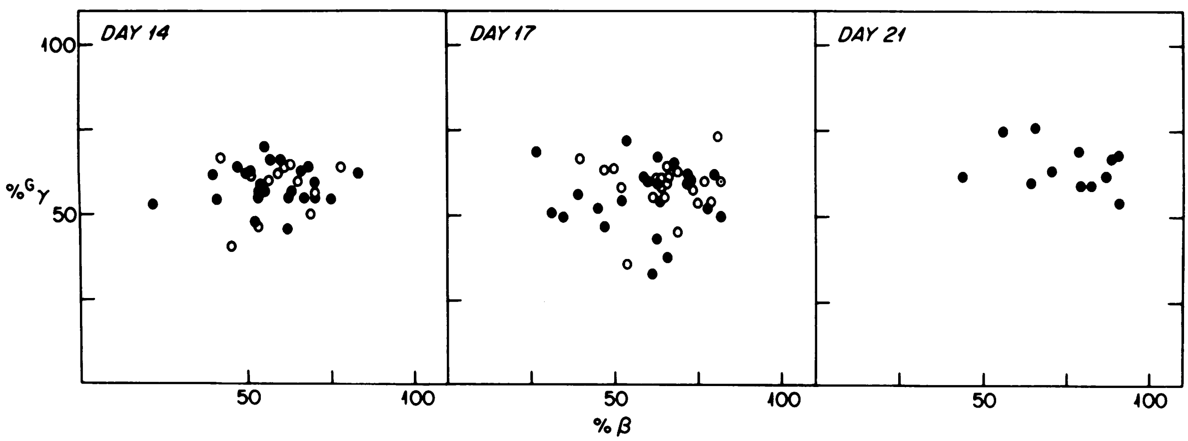

FIGURE 6 Percent Gγ- and percent β-syntheses by single newborn blood colonies derived from cultures of mononuclear (●) or nonadherent cells (○) in study C. Overlapping filled and empty circles indicates one colony from each type of culture, with identical data.

TABLE II
*Growth Characteristics of Adult Blood Colonies**

Study	Plating concentration	Day of maximum colonies	Colonies per 10^5 cells
	cells/ml		
A	2×10^6	14	5±1
B	2.5×10^5	15	40±23
C	2×10^6	14	17±10
D	5×10^5	14	18±11

* All experiments were done at 2U Ep/ml.

bination of Gγ- and γ- or β-proportions served to identify two classes of erythroid colonies. The fetal class was characterized by correlation of these parameters, and the adult class by a lack of this correlation.

Our data can, thus, be explained by a clonal model for Hb switching (Table IV). In this model, there are two types of erythroid progenitors that emerge during ontogeny. The truly fetal progenitor is unique to the fetus. It is characterized by a high proportion of γ-synthesis (low β), which correlates with the proportion of Gγ-synthesis on a clonal basis. The other progenitor, which is "adult", or "fetal-like", results in some Hb F (i.e., γ) synthesis in vitro, which exceeds that seen in vivo, but is less than is seen in truly "fetal" colonies. In this adult population, Gγ- and γ-syntheses are not correlated.

Both types of progenitors may be detected at birth. In our studies, the fetal progenitor was identified by the production of colonies on day 14 alone, or on days 14 and 17. The adult, fetal-like progenitor produced colonies only on day 17, or on both days 14 and 17. The exact timing of the growth of colonies from each type of progenitor may reflect the individual variation in the development of these "term" infants.

Our data show a temporal separation of the two types of colonies in newborn infants. "Fetal" colonies were produced from fetal erythroid progenitors, which presumably arose from the fetal pluripotent stem cell during in utero-ontogenic development. Those fetal erythroid progenitors replicated and amplified to fill the erythroid compartment with mature erythroid progenitors. These in turn could develop colonies rapidly in vitro, i.e., on days 14 and perhaps 17. The colonies assayed at later culture times, such as days 17 and 21, presumably derived from less mature erythroid progenitors, committed to erythropoiesis from pluripotent stem cells later in ontogeny. These stem cells were of the "adult" class. These erythroid progenitors, which arose later in ontogeny (near term), had not yet had time to amplify and fill the mature erythroid progenitor compartments. Thus, their colonies took longer to emerge in culture.

One alternative model for Hb switching involves a biological time clock, which predicts a continuous evolution of the Hb programs in progenitor cells. This model is not supported by our finding of two temporally separated classes of progenitors. Failure by others to observe a bimodal distribution of Hb F in day 14 colonies is clearly not incompatible with a clonal modal in which the fetal and adult cohorts appear at different

TABLE III
Gγ- and γ-Synthesis by Adult Blood Colonies

Study	Sample	n	Percent Gγ: Mean ±1 SD	Percent Gγ: Median	Percent Gγ: Range	Percent Gγ: Distribution* W	Percent Gγ: Distribution* P	Percent β: Mean ±1 SD	Percent β: Median	Percent β: Range	Percent β: Distribution* W	Percent β: Distribution* P	Correlation of Gγ and β†: r_s	Correlation of Gγ and β†: P
A	Singles	41	53±11	54	30–77	0.99	0.92	41±20	38	11–81	0.95	0.09	0.16	0.31
B	Whole plates	2	56		51–61			14		13–15				
	Singles	22	47±12	49	22–62	0.91	0.04	16±7	15	6–27	0.96	0.54	0.37	0.09
C	Whole plates	2	63		55–71			26		20–31				
	Singles	43	57±9	58	37–77	0.98	0.88	25±15	26	4–71	0.94	0.03	0.18	0.26
D	Whole plates	2	58		55–62			23		15–32				
	Red singles	20	60±10	60	38–73	0.94	0.30	10±5	8	4–22	0.90	0.04	−0.36	0.12
	White singles	14	47±15	48	25–72	0.96	0.73	18±12	15	7–50	0.81	<0.01	−0.05	0.88
	Total singles	34	54±14	56	25–73	0.94	0.08	13±9	11	4–50	0.77	<0.01	−0.36	0.04

* W, Wilk's w statistic, $P < 0.05$ indicates data are not normally distributed.

† r_s, Spearman's rank correlation coefficient, $P < 0.05$ indicates correlation of Gγ and γ.

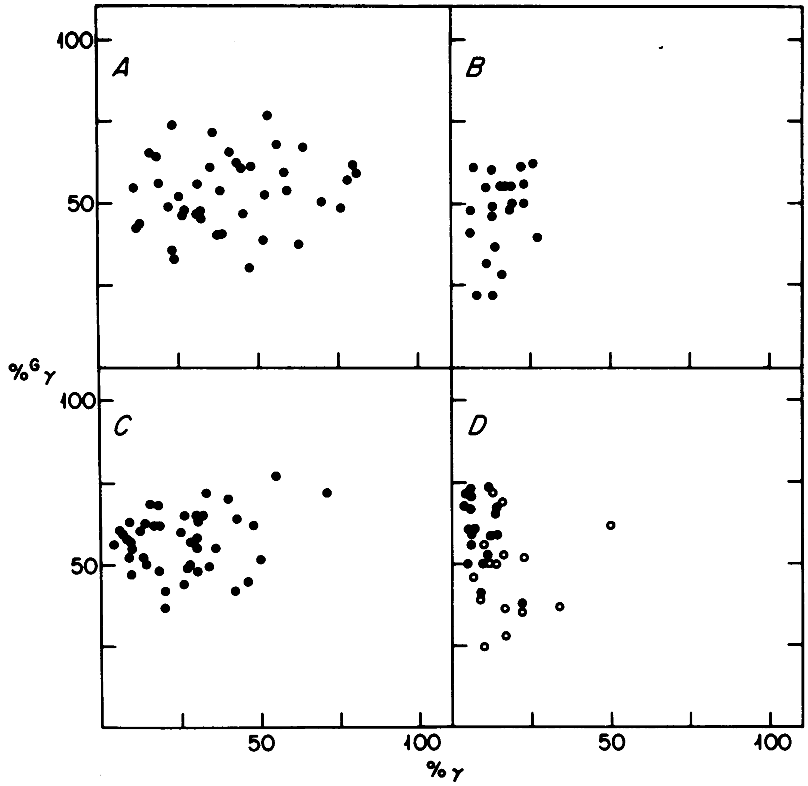

FIGURE 7 Percent Gγ- and γ-syntheses by single adult colonies. A-C show red colonies only (●). D includes white colonies (O).

times in culture. Another model relates to differences in the environments in fetuses compared with adults, in which the environment determines the expression of the globin genes. However, we found both fetal and adult progenitors in the same infants, where there would only be a single environment. Published data in a simian model indicate that Hb F synthesis in fetal colonies is not modulated by exogenous regulators such as Ep concentration (10), while Hb F synthesis in adult colonies is influenced by Ep or other factors (30). This is additional evidence for discrete differences between fetal and adult progenitors. Thus, the clonal model, while not proven by our results, provides the simplest compatible explanation.

TABLE IV
Erythropoiesis during Ontogeny

Progenitor	Level of HbF	Correlation of Gγ and γ	Stage of ontogeny		
			Fetus	Newborn	Adult
Fetal	High	Yes	+	+	−
Fetal-like (adult)	Low	No	−	+	+

+ Indicates presence of this progenitor class.
− Indicates absence of this progenitor class.

ACKNOWLEDGMENTS

We are grateful to David G. Nathan and Paul D. Berk for their support and advice.

This work was supported by National Institutes of Health grant HL 26132, National Foundation March of Dimes grant 1-716, a National Institute of Health New Investigator Award AM 30141 (to Dr. Weinberg) and an Irma T. Hirschl Career Scientist Award (to Dr. Alter).

REFERENCES

1. Huehns, E. R., N. Dance, G. H. Beaven, F. Hecht, and A. G. Motulsky. 1964. Human embryonic hemoglobins. *Cold Spring Harbor Symp. Quant. Biol.* **29:** 327–331.
2. Huisman, T. H. J., W. A. Schroeder, A. Felice, D. Powars, and B. Ringelhann. 1977. Anomaly in the γ chain heterogeneity of the newborn. *Nature (Lond.).* **265:** 63–65.
3. Huisman, T. H. J., H. Harris, and M. Gravely. 1977. The chemical heterogeneity of the fetal hemoglobin in normal newborn infants and in adults. *Mol. Cell Biol.* **17:** 45–55.
4. Papayannopoulou, T., M. Brice, and G. Stamatoyannopoulos. 1976. Stimulation of fetal hemoglobin synthesis

in bone marrow cultures from adult individuals. *Proc. Natl. Acad. Sci. USA.* **73:** 2033–2037.

5. Papayannopoulou, T., M. Brice, and G. Stamatoyannopoulos. 1977. Hemoglobin F synthesis in vitro: evidence for control at the level of primitive erythroid stem cells. *Proc. Natl. Acad. Sci. USA.* **74:** 2923–2927.
6. Stamatoyannopoulos, G., and T. Papayannopoulou. 1979. Fetal hemoglobin and the erythroid stem cell differentiation process. *In* Cellular and Molecular Regulation of Hemoglobin Switching. G. Stamatoyannopoulos and A. W. Nienhuis, editors. Grune & Stratton, Inc., New York. 323–349.
7. Lipton, J. M., and D. G. Nathan. 1980. Cell-cell interactions in in vitro erythropoiesis. *Blood Cells.* **6:** 645–663.
8. Stamatoyannopoulos, G., D. M. Kurnit, and T. Papayannopoulou. 1981. Stochastic expression of fetal hemoglobin in adult erythroid cells. *Proc. Natl. Acad. Sci. USA.* **78:** 7005–7009.
9. Alter, B. P., B. T. Jackson, J. M. Lipton, G. J. Piasecki, P. L. Jackson, M. Kudisch, and D. G. Nathan. 1981. Three classes of erythroid progenitors that regulate hemoglobin synthesis during ontogeny in the primate. *In* Hemoglobins in Development and Differentiation. G. Stamatoyannopoulos and A. Nienhuis, editors. Alan R. Liss, Inc., New York. 331–340.
10. Alter, B. P., B. T. Jackson, J. M. Lipton, G. J. Piasecki, P. L. Jackson, M. Kudisch, and D. G. Nathan. 1981. Control of the simian fetal hemoglobin switch at the progenitor cell level. *J. Clin. Invest.* **67:** 458–466.
11. Comi, P., B. Giglioni, S. Ottolenghi, A. M. Gianni, E. Polli, P. Barba, A. Covelli, G. Migliaccio, M. Condorelli, and C. Peschle. 1980. Globin chain synthesis in single erythroid burst from cord blood: studies on $\gamma \rightarrow \beta$ and $G\gamma \rightarrow A\gamma$ switches. *Proc. Natl. Acad. Sci. USA.* **77:** 362–365.
12. Terasawa, T., and M. Ogawa. 1980. Hemoglobin biosynthesis in individual bursts from human adult peripheral and umbilical cord blood: analysis of the relative rates of synthesis of $G\gamma$ and $A\gamma$ globin chains. *J. Cell. Physiol.* **105:** 483–488.
13. Papayannopoulou, T., S. Kurachi, M. Brice, B. Nakamoto, and G. Stamatoyannopoulos. 1981. Asynchronous synthesis of HbF and HbA during erythroblast maturation. II. Studies of $G\gamma$, $A\gamma$, and β chain synthesis in individual erythroid clones from neonatal and adult BFU-E cultures. *Blood.* **57:** 531–536.
14. Stamatoyannopoulos, G., T. Papayannopoulou, M. Brice, S. Kurachi, B. Nakamoto, G. Lim, and M. Farquhar. 1981. Cell biology of hemoglobin switching I. The switch from fetal to adult hemoglobin formation during ontogeny. *In* Hemoglobins in Development and Differentiation. G. Stamatoyannopoulos and A. Nienhuis, editors. Alan R. Liss, Inc., New York. 287–305.
15. Dean, A., A. N. Schechter, T. Papayannopoulou, and G. Stamatoyannopoulos. 1981. Heterogeneity of erythroid precursor cells. *J. Biol. Chem.* **256:** 2447–2453.
16. Rinehart, J. J., E. D. Zanjani, B. Nomdedeu, B. J. Gormus, and M. E. Kaplan. 1978. Cell-cell interaction in erythropoiesis. *J. Clin. Invest.* **62:** 979–986.
17. Iscove, N. N., F. Sieber, and K. H. Winterhalter. 1974. Erythroid colony formation in cultures of mouse and human bone marrow: analysis of the requirement for erythropoietin by gel filtration and affinity chromatography on agarose-concanavalin A. *J. Cell. Physiol.* **83:** 309–320.
18. Worton, R. G., E. A. McCulloch, and J. E. Till. 1969. Physical separation of hemopoietic stem cells from cells forming colonies in culture. *J. Cell. Physiol.* **74:** 171–182.
19. Alter, B. P., C. B. Modell, D. Fairweather, J. C. Hobbins, M. J. Mahoney, F. D. Frigoletto, A. S. Sherman, and D. G. Nathan. 1976. Prenatal diagnosis of hemoglobinopathies. *N. Engl. J. Med.* **295:** 1437–1443.
20. Alter, B. P., S. C. Goff, G. D. Efremov, M. E. Gravely, and T. H. J. Huisman. 1980. Globin chain electrophoresis: a new approach to the determination of the $G\gamma/A\gamma$ ratio in fetal hemoglobin and to studies of globin synthesis. *Br. J. Haematol.* **44:** 527–534.
21. Bonner, W. M., and R. A. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46:** 83–88.
22. Laskey, A., and A. D. Mills. 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* **56:** 335–341.
23. Shapiro, S. S., and M. B. Wilk. 1965. An analysis of variance test for normality (complete samples). *Biometrika.* **52:** 591–611.
24. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames, Iowa. 6th edition. **194:** 414–418.
25. Terasawa, T., M. Ogawa, P. N. Porter, and J. D. Karam. 1980. $G\gamma$ and $A\gamma$ globin-chain-biosynthesis by adult and umbilical cord blood erythropoietic bursts and reticulocytes. *Blood.* **56:** 93–97.
26. Darbre, P. D., S. M. Lauckner, J. W. Adamson, W. G. Wood, and D. J. Weatherall. 1981. Haemoglobin synthesis in human erythroid bursts during ontogeny: reproducibility and sensitivity to culture conditions. *Br. J. Haematol.* **48:** 237–250.
27. Papayannopoulou, T., T. Kalmantis, and G. Stamatoyannopoulos. 1979. Cellular regulation of hemoglobin switching: evidence for inverse relationship between fetal hemoglobin synthesis and degree of maturity of human erythroid cells. *Proc. Natl. Acad. Sci. USA.* **76:** 6420–6424.
28. Dover, G. J., and S. H. Boyer. 1980. Quantitation of hemoglobins within individual red cells: asynchronous biosynthesis of fetal and adult hemoglobin during erythroid maturation in normal subjects. *Blood.* **56:** 1082–1091.
29. Papayannopoulou, T., B. Nakamoto, S. Kurachi, and G. Stamatoyannopoulos. 1981. Globin synthesis in erythroid bursts that mature sequentially in culture. I. Studies in cultures of adult peripheral blood BFU-Es. *Blood.* **58:** 969–974.
30. Macklis, R. M., J. Javid, J. M. Lipton, M. Kudisch, P. K. Pettis, and D. G. Nathan. 1982. Synthesis of hemoglobin F in adult simian erythroid progenitor-derived colonies. *J. Clin. Invest.* **70:** 752–761.