Proc. Natl. Acad. Sci. USA
Vol. 74, No. 7, pp. 2923–2927, July 1977
Cell Biology

Hemoglobin F synthesis *in vitro*: Evidence for control at the level of primitive erythroid stem cells

(Hb F regulation/erythroid stem cell differentiation/erythropoietin/hemoglobinopathies)

TH. PAPAYANNOPOULOU, M. BRICE, AND G. STAMATOYANNOPOULOS

Divisions of Hematology and Medical Genetics, Department of Medicine, University of Washington School of Medicine, Seattle, Washington 98195

Communicated by Clement A. Finch, May 2, 1977

ABSTRACT **The *in vitro* regulation of fetal hemoglobin (Hb F) was investigated in clones of cultured adult human erythroid cells by *in situ* immunofluorescent identification of the hemoglobins synthesized. Formation of Hb F-containing clones was enhanced by erythropoietin and by culture conditions favoring the proliferation of less-differentiated stem cells of the burst-forming-unit type. Burst-forming units differed in their capacity to direct Hb F synthesis in their terminally differentiated progeny. A class of early precursors that can produce descendent stem cells with or without commitment to Hb F production was identified. The findings suggest that the capability for expression of Hb F in terminally differentiated cells of the adult is determined at the level of less-differentiated erythroid stem cells with characteristics of burst-forming units. It is proposed that the regulation of Hb F synthesis *in vivo* is also linked to the process of differentiation of the erythroid stem cells and that the patterns of Hb F synthesis during ontogeny reflect the attainment of progressively higher levels of differentiation of erythroid stem cells as development proceeds.**

Hb F is the major Hb component during intrauterine life, but it is almost completely replaced by adult Hb soon after birth, so that only trace amounts, restricted to a few erythrocytes, are synthesized in the normal adult (1, 2). The mechanism of regulation of Hb F remains unclear.

In a previous study (3), we showed that, when hemopoietic cells from adults are cultured under conditions permitting proliferation of erythroid cells in a clonal fashion, increased amounts of Hb F are synthesized. In addition, the *in vitro* erythroid clones differ in their ability to express Hb F synthesis and segregate into clones with absence of Hb F production and others with homogeneous presence of Hb F in their cells. Because each clone derives from a single stem cell progenitor, these observations revealed a heterogeneity among erythroid stem cells regarding their ability to direct Hb F synthesis in their terminally differentiated progeny (3). In the present study we examined whether the heterogeneity of expression of Hb F in erythroid clones is related to the degree of differentiation of the progenitor cells from which the clones were derived.

Current models of erythropoiesis indicate that hemopoietic stem cells, once committed to the erythroid cell pathway, undergo an amplification process accompanied by the appearance of differentiated properties such as the ability to respond to erythropoietin (4). This process is partly uncovered *in vitro* under selective culture conditions favoring proliferation of less-differentiated erythroid stem cells—defined as burst-forming units, erythroid (BFUe)—or more differentiated precursors—colony-forming units, erythroid (CFUe). The CFUe are sensitive to erythropoietin and give rise to early-appearing erythroid colonies (5–7). The BFUe require higher erythropoietin levels for growth, have higher proliferative capacity than the CFUe, and have an ability to give rise to clusters of erythroid colonies (erythroid bursts) that appear later than the colonies originating from CFUe (5–7). These differences in the *in vitro* behavior of CFUe and BFUe were utilized in the present study in order to examine the relationship between erythroid stem cell differentiation and Hb F synthesis. The results indicate that the phenotypic expression of Hb F in erythroid clones is largely dependent on the level of differentiation of the parental stem cells from which the clones derive and that regulatory decisions about γ chain synthesis in erythroblasts are taken in primitive cells with the *in vitro* proliferative characteristics of BFUe.

Abbreviations: BFUe, burst-forming units, erythroid; CFUe, colony forming units, erythroid; anti-Hb F-FITC, anti-Hb F antibody conjugated with fluorescein isothiocyanate; anti-Hb S-FITC, anti-Hb S conjugated with FITC; anti-Hb C-FITC, anti-Hb C conjugated with FITC; F-colony, Hb F-containing colony; S-colony, Hb S-containing colony; C-colony, Hb C-containing colony.

METHODS

Bone Marrow Cultures from Normal Adults. The plasma clot culture system was used as described (3) with the exception that fetal calf serum was replaced by adult human serum. Effects of erythropoietin on Hb F formation were tested in cultures maintained for 8–9 days in the presence of 0.25, 2.0, or 8.0 international units (IU) of erythropoietin (anemic sheep plasma erythropoietin step III preparation, Connaught Research Laboratories, Toronto, Canada) per ml of medium. For temporal observations of Hb F formation in culture, erythroid colonies were evaluated at various intervals and mostly at the peak formation of early-appearing (8–9 days) and late-appearing (14–16 days) colonies; erythropoietin concentrations (per ml of medium) were 2.0 IU (14 experiments) and 0.25 or 0.5 IU (8 experiments). Cell inocula contained 5×10^4 nucleated cells per ml.

Peripheral Blood Cultures. Samples of blood collected in NCTC-109 (Microbiological Associates, Bethesda, MD) containing 2% (vol/vol) fetal calf serum, penicillin (50 IU/ml), streptomycin (50 μg/ml), and heparin (10 IU/ml) were layered over Ficoll-Hypaque (Pharmacia Fine Chemicals, Piscataway, NJ) in 50-ml centrifuge tubes. After 60 min of centrifugation at $400 \times g$ at room temperature, interphase mononuclear cells were removed, washed twice in fresh medium, adjusted to approximately 1×10^7 cells per ml, and incubated for 2 hr at 37° in tissue culture flasks. The recovered nonadhering cells were cultured in plasma clots in the presence of adult human serum and 4.0 IU of erythropoietin. Cultures were evaluated with immunochemical and cytochemical staining at days 11 and 13.

Antibodies and Immunofluorescent Labeling. Antibodies against Hb F (anti-Hb F), hemoglobin S (anti-Hb S), and hemoglobin C (anti-Hb C) were raised in horses and purified by affinity chromatography as described (2, 9). Anti-Hb F sera were absorbed against Sepharose–Hb S and Sepharose–Hb C, anti-Hb S sera against Sepharose–Hb C, and anti–Hb C sera against Sepahrose–Hb S. Conditions of *in situ* labeling with

Table 1. Effect of three erythropoietin levels on F-colony formation*

	Erythroid colonies								
	At 0.25 IU/ml			At 2.0 IU/ml			At 8.0 IU/ml		
Exp.	Total scored	Per 10^5 cells†	F-colonies, %	Total scored	Per 10^5 cells†	F-colonies, %	Total scored	Per 10^5 cells†	F-colonies, %
75				908	364	9.1	924	370	11.6
78				868	248	13.8	1160	232	14.2
79				1198	300	17.4	914	368	20.7
83	439	146	6.2	548	182	12.8			
88	197	66	7.6	267	90	21.7			
90	332	110	3.6	250	166	5.2			
92	166	110	7.2	888	178	24.6	546	156	37.5
94a	499	166	5.2	671	268	8.8	427	284	22.2
94b	569	228	6.5	612	244	8.8			
98	863	214	2.5	366	183	15.3			
103	70	20	1.4	286	144	16.4	429	172	10.7
104	513	172	7.0	796	199	16.3	1079	270	24.1
114	244	98	5.7	450	226	8.6	239	240	15.5
Mean		132.6	5.3		214.8	13.8		261.5	19.6
SEM		20.3	0.7		19.8	1.6		28.1	2.9

* All marrow cells were from adults without hemoglobinopathy.
† Colonies recovered per 10^5 inoculated nucleated cells.

fluorescein isothiocyanate- or rhodamine-conjugated antibodies were as described (3). Hb F-containing colonies (F-colonies), Hb S-containing colonies (S-colonies), or Hb C-containing colonies (C-colonies) were defined as those binding the corresponding fluorescent antibodies and emitting strong fluorescence. For double-immunofluorescent labeling, plasma clots already treated with anti-Hb S-FITC or anti-Hb C-FITC were counterlabeled with anti-Hb F antibodies conjugated to rhodamine (anti-Hb F-rhodamine).

RESULTS

Association of F-Colonies and Less Differentiated Precursors. This relationship was examined with two approaches. In the first, precursor cells of high or low erythropoietin sensitivity were stimulated for growth by varying the erythropoietin concentrations in the culture medium (5, 7, 10). In the second, colony populations were distinguished on the basis of differences in time of appearance of CFUe- and BFUe-origin colonies in culture (6, 7).

F-colony frequencies in adult human marrow cultures maintained for 8–9 days in the presence of three erythropoietin concentrations are shown in Table 1. Despite the variation between marrows, a positive correlation between erythropoietin level and proportion of F-colonies was observed. At the erythropoietin levels used, 7, 29.6, and 51.2 F-colony forming cells, respectively, were recovered per 10^5 inoculated nucleated cells. The data suggest that F-colony forming cells have high erythropoietin requirements for *in vitro* growth.

F-colony formation in two periods of colony growth in the presence of a high erythropoietin stimulus (2.0 IU/ml of medium) is depicted in Table 2. In human marrow cultures, early erythroid colonies of CFUe origin show a peak growth at 7–9 days of culture and subsequently disintegrate; with application of high erythropoietin stimuli, a wave of immature colonies of BFUe origin appears at 8–11 days of culture and they achieve maximal mature growth at days 14–16. Cultures observed at 8–9 days produced 221.4 ± 21.5 (mean ±SEM) colonies per 10^5 inoculated cells, 13.1% ± 1.5 of which were F-colonies. In the 14- to 16-day cultures, there was an average of 151.9 ± 23.2 erythroid colonies per 10^5 inoculated cells (the decrease reflecting the disappearance of colonies of CFUe origin) with a frequency of F-colonies of 34.1% ± 3.9. The absolute number of F-colonies among late colonies was increased by a factor of 1.76 over that in the 8- to 9-day cultures (from 28.9 ± 4.8 to 50.8 ± 8.4 per 10^5 inoculated nucleated cells), indicating that the increased frequency of F-colonies among the late colony population was due to new F-colony formation. Similar information was obtained in five experiments (among eight attempts) in which growth of late colonies was achieved at low erythropoietin levels (0.25 or 0.5 IU/ml of medium).

Expression of Hb F in Erythroid Bursts. Bone marrow and blood cultures (both initiated with low cell inocula) containing *discrete* erythroid bursts were used to examine the expression of Hb F in the subcolonies composing each burst. Labeling with anti-Hb F-FITC revealed three patterns of Hb F formation in well-hemoglobinized bursts: (*i*) F-subcolonies only (Fig. 1); (*ii*) bursts in which subcolonies had no detectable levels of Hb F (Fig. 1); and (*iii*) bursts with a significant variation in intensity of fluorescence among subcolonies as well as subcolonies showing sectorial appearance of Hb F (Fig. 2). In peripheral blood cultures from two normal persons, the frequencies of the three types of bursts were 5–10%, 30–40%, and 50–60% for burst types 1, 2, and 3, respectively.

To determine whether, in the third type of bursts, a single BFUe was responsible for formation of subcolonies with Hb F as well as of subcolonies without Hb F, the single-cell origin of erythroid bursts was examined. An artificial mosaic was established by coculturing stem cells from two persons, one homozygous for Hb C and the other homozygous for Hb S. The genetic identity of the colonies composing each erythroid burst was then determined by sequential immunofluorescent labeling with anti-Hb S-FITC and anti-Hb C-FITC (Fig. 3). In cultures initiated with low cell inocula, bursts containing only Hb S subcolonies or only Hb C subcolonies were observed, and no discrete erythroid bursts containing both types of subcolonies were noted, providing direct evidence for single BFUe origin of the colonies comprising each erythroid burst.

Cultures of this artificial mosaic, first labeled with anti-Hb

Table 2. F-colonies in early and late bone marrow cultures*

Exp.	Days in culture	Erythroid colonies/ 10^5 cells	F-colonies %	F-colonies Per 10^5 cells
75	8	282	11.3	31.9
	16	272	26.9	73.2
78†	8	248	13.8	34.2
	16	114	67.5	76.9
79	8	357	18.9	67.5
	16	281	36.5	102.6
83†	8	182	12.8	23.3
	16	82	26.2	21.5
86	9	236	3.8	8.9
	15	71	13.1	9.3
	16	54	16.7	9.1
87	8	197	4.6	9.1
	14	103	16.0	16.5
88†	9	90	21.7	19.5
	14	42	52.4	22.0
90†	8	166	5.2	8.6
	14	141	23.4	33.0
92	8	168	18.5	31.1
	14	118	31.6	37.3
94b†	8	244	8.8	21.5
	15	150	29.3	43.9
98†	8	183	15.3	28.0
	14	158	33.2	52.5
104	8	202	18.1	36.6
	15	147	49.6	72.9
105	9	143	14.3	20.5
	14	105	40.5	42.3
131	8	389	15.9	61.8
	14	340	35.9	122.1
	15	342	31.2	106.7
	18	314	54.2	170.2
	20	144	59.3	71.0

* All marrow cells were from adult persons without a hemoglobinopathy.

† Values at culture day 8 or 9 are those of the "2.0 erythropoietin units" column of Table 1.

S-FITC or anti-Hb C-FITC to identify each burst according to genetic origin, were counterlabeled with anti-Hb F-rhodamine to evaluate Hb F synthesis in the subcolonies of the genetically defined bursts. The clear-cut differences in Hb F formation among well-developed colonies of such bursts (Fig. 4) show that a single BFUe can provide stem cell descendents that form subcolonies with Hb F synthesis as well as stem cell descendents that form subcolonies without Hb F production. Fifty to 60% of the BFUe of the Hb S homozygote and the Hb C homozygote were of this type.

DISCUSSION

The finding, in this study, of Hb F-synthesizing clones in cultures of adult human hemopoietic cells suggests that there exists a relationship between fetal Hb synthesis and the level of differentiation of the erythroid stem cells from which the *in vitro* erythroid clones derive. Supporting evidence is provided by (*i*) the erythropoietin dose-response experiments, (*ii*) the determination of F-colony frequency in late colonies of BFUe origin, and (*iii*) the direct immunofluorescent studies of erythroid bursts.

The synthesis of Hb F in adult erythroid cultures was found

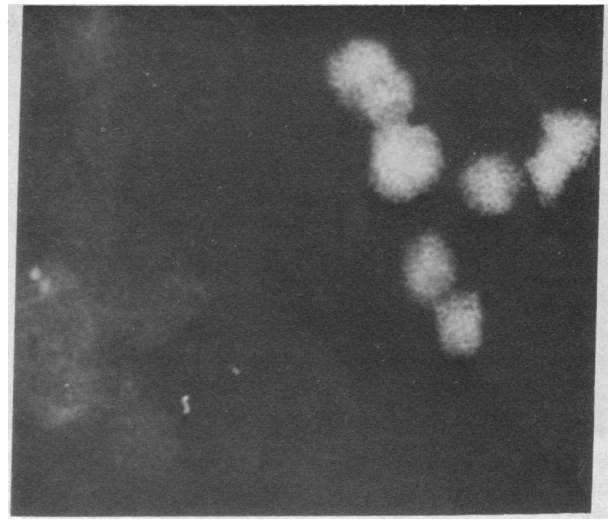

FIG. 1. Erythroid bursts labeled with anti-Hb F-FITC. At lower left, burst comprised of six subcolonies all of which fail to bind anti-Hb F-FITC. At right, burst comprised of seven subcolonies homogeneously labeled with the fluorescent antibody. (×75.)

to be erythropoietin-dependent. Direct induction of γ chain synthesis by erythropoietin is an unlikely explanation of the finding because the appearance of Hb F in the erythroid colonies was clonal. The data are most compatible with enhanced recruitment, at the high erythropoietin levels, of less erythropoietin-sensitive (and hence less differentiated) erythroid stem cells that are also capable of directing Hb F synthesis in their terminally differentiated progeny. In support are the experiments on temporal variation of F-colony formation in culture, showing highest frequencies (and absolute numbers) of Hb F-synthesizing clones among the late-appearing erythroid colony populations. Because the early erythroid colonies (derived mostly from the more differentiated CFUe) had disintegrated by the time the observations in late cultures were made, the high frequencies of F-colonies among the late colony populations provide an accurate reflection of the increased potentials for Hb F synthesis in the colonies of BFUe origin. Additional evidence for the relationship between Hb F synthesis and the less-differentiated erythroid stem cells was obtained

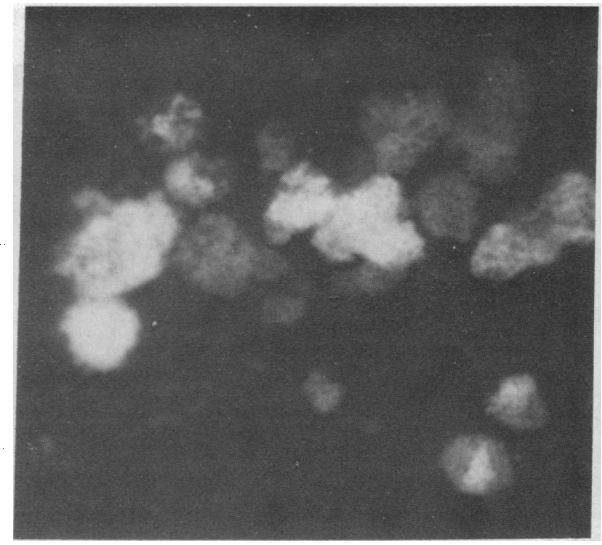

FIG. 2. Erythroid burst showing variation of Hb F synthesis among subcolonies, some of which display sectorial appearance of Hb F. (×75.)

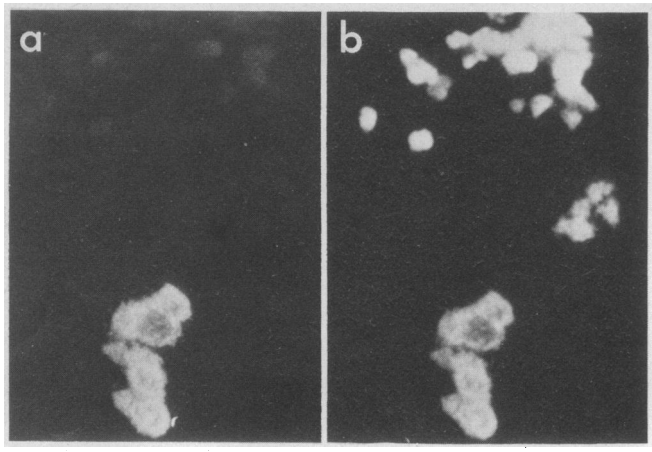

FIG. 3. Demonstration of the single-cell origin of subcolonies comprising erythroid bursts. The preparation, from an artificial mosaic of cultured homozygous S and homozygous C cells, was labeled first with anti-Hb S-FITC, photographed, and then counterlabeled with anti-Hb C-FITC. (*a*) Anti-Hb S-FITC labeling: an erythroid burst appears at the lower part of figure, with unlabeled colonies seen near the top. (*b*) After labeling with anti-Hb C-FITC, an erythroid burst appears at the top of the same field with all its subcolonies labeled with the fluorescent anti-Hb C antibody. No *discrete* bursts containing both S-subcolonies and C-subcolonies were noted with the sequential labeling of the preparations of the artifical Hb S/Hb C mosaic. (×30.)

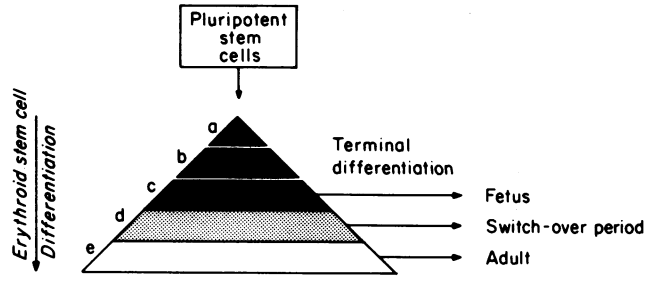

FIG. 5. Model for stem cell differentiation-dependent regulation of Hb F synthesis. The triangle corresponds to the erythroid stem cell compartment and the postulated stages of differentiation, *a* to *e*. Commitment to Hb F synthesis is considered to be inversely related to degree of differentiation of erythroid stem cells. Differentiation stages *a*, *b*, and *c* (black area) are associated with the ability to produce large amounts of Hb F in terminally differentiated cells derived from these stages. Commitment to Hb F is decided in a stochastic fashion in the progeny of burst-forming units of the differentiation stage *d* (stippled area). Stage *e* (white area) is associated with only Hb A production in the terminally differentiated progeny. The perinatal switch from fetal to adult Hb formation is attributed to the addition, around the perinatal period, of the differentiation stages *d* and *e*.

by the direct study of Hb F production in the subcolonies of erythroid bursts; it revealed existence of differentiative heterogeneity among BFUe regarding their ability to direct Hb F synthesis in their progeny-subcolonies. This heterogeneity of Hb F synthesis among erythroid bursts (Figs. 1 and 2) and the existence of precursors that produce erythroid stem cell descendents with different commitments to Hb F synthesis (Figs. 2 and 4) indicate that regulatory decisions about γ-gene activity in erythroblasts are made during the process of differentiation of those earlier erythroid stem cells that behave in culture as burst forming units.

Several observations *in vivo* are compatible with regulation of Hb F synthesis at the level of stem cells. The cellular restriction of Hb F in the erythrocytes of the normal adult (1, 2), the clonal increase of Hb F in acquired Hb F elevations (2, 11), the reversion to a fetal pattern of Hb formation in certain hematologic malignancies (12, 13), and the reappearance of Hb F during the regeneration phase after bone marrow transplantation (14) all are in accordance with proliferation, in the adult hemopoietic tissue, of stem cells with ability to direct Hb F formation. The suggested derivation of Hb F-containing cells in the adult from a separate stem cell line (13) of (by definition) pluripotent stem cells is not supported by the present findings in culture. Because, as shown here, early erythroid precursors can provide progeny that differ in commitment to Hb F synthesis, erythrocytes *in vivo* that contain Hb F as well as those that do not must share common erythroid stem cell progenitors. If the commitment to Hb F were made at the pluripotent stem cell level, only two classes of erythroid bursts would have been observed (with either presence or absence of Hb F synthesis but not both). It thus appears more likely that mechanisms of Hb F regulation synchronized with other processes of differentiation of erythroid stem cells operate *in vivo*.

Previous workers have suggested that the regulation of the synthesis of Hb F is accomplished through the action of regulatory genes on the closely linked γ and β hemoglobin loci (15, 16), the sequential excisions of globin genes during development

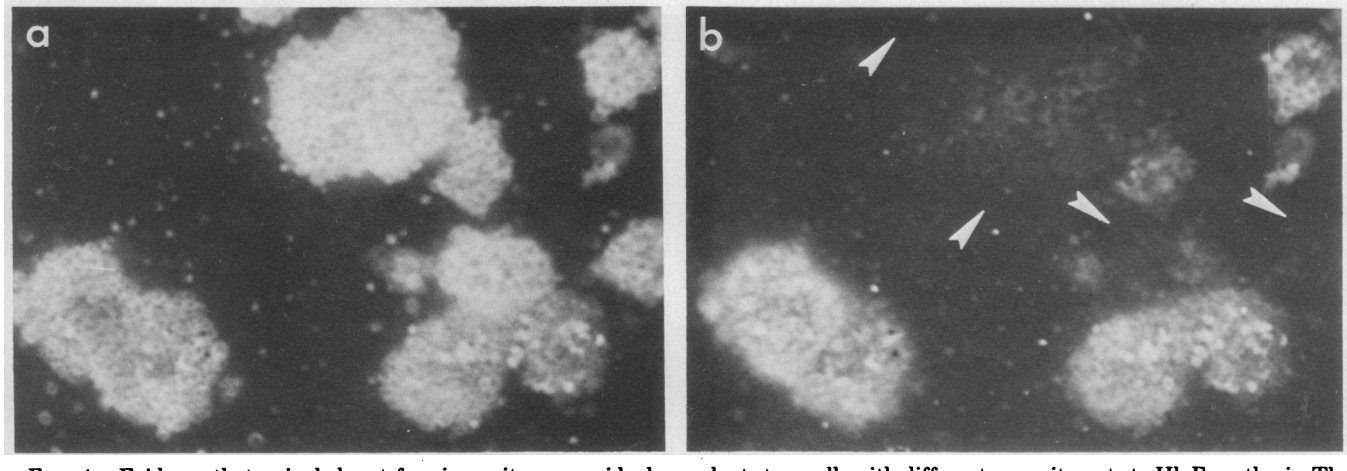

FIG. 4. Evidence that a single burst-forming unit can provide descendent stem cells with different commitments to Hb F synthesis. The preparation is from the artificial Hb S/Hb C mosaic. (*a*) Labeling with anti-Hb S-FITC shows an erythroid burst composed of 10 Hb S subcolonies. (*b*) The same erythroid burst labeled with anti-Hb F-rhodamine. Note the differences in Hb F content between subcolonies and the presence of well-hemoglobinized subcolonies (arrows) that fail to synthesize Hb F. (×95.)

(17), the selective proliferation of precursors programmed for Hb F formation (18), or a translational control operating in less-differentiated cells (19). Other studies have failed to reveal translational (20) or globin gene excision mechanisms (21, 22) in Hb F regulation. We would like to indicate that a stem cell differentiation-dependent model of regulation of Hb F synthesis could account for the observations *in vivo* as well as the findings in culture.

We propose that the *in vivo* expression of Hb F is inversely related to the level of differentiation of erythroid stem cells, so that erythroblasts derived from earliest precursors synthesize mostly Hb F while those derived from fully differentiated stem cells produce only adult Hb (Fig. 5). The patterns of Hb F production during normal ontogeny can then be attributed to the proliferation of erythroid stem cells of successively higher levels of differentiation as development proceeds. We suggest that the drastic change in erythropoiesis around the perinatal period induces stem cell differentiation to progress first to a level at which commitment to Hb F is decided in a stochastic fashion (Figs. 2 and 4) and finally to a level at which commitment to Hb F is lost; the switch from fetal to adult Hb formation is thus viewed as a reflection of changes in cell kinetics rather than direct action of inducers or repressors (15, 16) on the non-α-globin genes. In the normal adult, the erythroid precursors complete their differentiation process, so that production of Hb F occurs only in an occasional erythroblast that derives directly from less-differentiated precursors. Under the proposed mechanism, the reactivation of Hb F synthesis in certain diseases of adults represents secondary alteration of the kinetics of differentiation or proliferation of erythroid stem cells, leading to terminal differentiation of earlier erythroid precursors or precursors at different stages of responsiveness to factors affecting erythroid stem cell proliferation.

The cooperation of Drs. W. Mills, R. Pinkham, and I. Pierce is acknowledged. This study was supported by National Institutes of Health Grants GM15253 and HL20899. A portion of this work was conducted through the Clinical Research Center Facility of the University of Washington supported by the National Institutes of Health (Grant RR-37).

The costs of publication of this article were defrayed in part by the payment of page charges from funds made available to support the research which is the subject of the article. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

1. Boyer, S. H., Belding, T. K., Margolet, L. & Noyes, A. N. (1974) *Science* **188**, 361–363.
2. Wood, W. G., Stamatoyannopoulos, G., Lim, G. & Nute, P. E. (1975) *Blood* **46**, 671–682.
3. Papayannopoulou, Th., Brice, M. & Stamatoyannopoulos, G. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2033–2037.
4. Lajtha, L. G. & Schofield, R. (1974) *Differentiation* **2**, 313–320.
5. Axelrad, A. A., McLeod, D. L., Shreeve, M. M. & Heath, D. S. (1974) in *Hemopoiesis in Culture*, ed. Robinson, W. A. (U.S. Government Printing Office, Washington, DC), pp. 226–237.
6. Iscove, N. N. & Sieber, F. (1975) *Exp. Hematol.* **3**, 32–43.
7. Gregory, C. J. (1976) *J. Cell. Physiol.* **89**, 289–301.
8. Ogawa, M. & Sexton, J. (1976) *Clin. Res.* **24**, 316A.
9. Papayannopoulou, Th., McGuire, T. C., Lim, G., Garzel, E., Nute, P. E. & Stamatoyannopoulos, G. (1976) *Br. J. Haematol.* **34**, 25–31.
10. Tepperman, A. D., Curtis, J. E. & McCulloch, E. A. (1974) *Blood* **44**, 659–669.
11. Boyer, S. H., Belding, T. K., Margolet, L., Noyes, A. N., Burke, P. J. & Bell, W. R. (1975) *Johns Hopkins Med. J.* **137**, 105–115.
12. Hardisty, R. M., Speed, D. E. & Till, M. (1964) *Br. J. Haematol.* **10**, 551–566.
13. Weatherall, D. J., Clegg, J. B. & Wood, W. G. (1976) *Lancet* **ii**, 660–663.
14. Alter, B. P., Rappeport, J. M., Huismann, T. H. T., Schroeder, W. A. & Nathan, D. G. (1976) *Blood* **48**, 843–853.
15. Neel, J. V. (1961) *Blood* **18**, 769–777.
16. Motulsky, A. G. (1962) *Nature* **194**, 607–609.
17. Kabat, D. (1972) *Science* **175**, 134–140.
18. Baglioni, C. (1973) in *Molecular Genetics, Part 1*, ed. Taylor, J. H. (Academic Press, New York), pp. 405–475.
19. Marks, P. A. & Burka, E. E. (1964) *Science* **144**, 552–553.
20. Lanyon, W. G., Ottolenghi, S. & Williamson, R. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 258–262.
21. Papayannopoulou, Th., Nute, P. E., Stamatoyannopoulos, G. & McGuire, T. C. (1977) *Science* **196**, 1215–1216.
22. Benz, E. J., Jr., Turner, P. H., Barker, J. E. & Nienhuis, A. W. (1977) *Science* **196**, 1213–1214.