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Erythropoietin changes the globin program of an interleukin 3-dependent multipotential cell line

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ABSTRACT **B6SUtA is a factor-dependent murine cell line of adult origin displaying the functional properties of a multipotent hematopoietic stem cell. We analyzed the globin programs of B6SUtA cells undergoing erythroid differentiation in both suspension and clonal cultures. In the absence of added erythropoietin, a small number of hemoglobinized cells were present, and these expressed predominantly embryonic globin. Addition of erythropoietin increased the number and maturation of hemoglobinized cells and led to a preferential augmentation of adult globin. Analysis of individual B6SUtA erythroid bursts showed that embryonic and adult globin can be expressed in cells derived from a single progenitor. Furthermore, by studying globin expression in cultured cells from mouse embryos, we found that the globin programs of B6SUtA cells are similar to those of erythroid progenitors at the period of transition from yolk sac to fetal liver erythropoiesis. Since B6SUtA cells are derived from adult bone marrow and they have the capacity to express embryonic globin, we speculate that the globin locus is not irreversibly modified during development and that adult cells at early stages of erythroid differentiation can transiently express ontogenetically primitive globin programs.**

The globin gene family has long served as a model genetic locus for the study of developmental stage- and tissue-specific gene control. Globin gene expression is restricted to terminally differentiated erythroid cells, which express different globins at different developmental stages—a phenomenon termed hemoglobin switching. These terminally differentiated erythroid cells are constantly being turned over and replenished through the erythroid commitment and subsequent differentiation of a self-renewing population of stem cells, which also sustain the other hematopoietic lineages (1). Some insights have been gained into the molecular events associated with late stages of globin gene activation, but little is known as to how the erythroid phenotype is selected, what the early stages of globin gene activation are, or how the developmental decision as to which globin to produce is taken. To begin to approach these questions one needs access to early erythroid progenitor cells. However, these cells are rare *in vivo* and difficult to isolate (2). Leukemic cells and cell lines have provided a valuable window on the biology of hemopoietic stem cells (3). Unfortunately these cells are, by and large, unresponsive to normal regulatory control mechanisms (e.g., response to hematopoietic growth factors) and fail to terminally differentiate. However, in recent years nonmalignant murine multipotent hematopoietic cell lines have been isolated that seem to respond to several hematopoietic growth factors [e.g., interleukin 3 (IL-3), granulocyte/macrophage-colony-stimulating factor (GM-CSF), granulocyte-colony-stimulating factor (G-CSF), and erythropoietin (Epo)] either separately or in combination (4–7). One of these cell lines, B6SUtA (4), was derived through continuous culture of adult mouse bone marrow. Based on several functional, biochemical, and cytogenetic criteria, these cells do not appear to be transformed. B6SUtA cells form erythroid, nonerythroid (neutrophil, macrophage, basophil), and mixed colonies in clonal cultures containing IL-3 and Epo. As stable, nonmalignant, multipotent hematopoietic cells, they provide a convenient model for study of the early events of hematopoiesis, particularly at the molecular level.

METHODS

Cell Culture. B6SUtA cells were maintained in suspension culture in Kincaide's medium with 15% fetal bovine serum and 10% WEHI-3 conditioned medium (4). Cells were incubated at 37°C in a highly humidified 5% CO_2 incubator and fed twice weekly. They reach saturation density at $\approx 2 \times 10^6$ cells per ml, at which point growth is slowed. Clonal growth and differentiation were carried out in semisolid culture medium consisting of 0.8% methylcellulose (Fisher), 30% fetal bovine serum (GIBCO), 10% bovine serum albumin (Armour Pharmaceutical), 10% human plasma, 10% WEHI-3 conditioned medium, and 2.5 units of recombinant human Epo (Genetics Institute) per ml in Iscove's modified Dulbecco's medium (GIBCO).

Induction of Erythroid Differentiation. Erythroid differentiation was induced by the addition of recombinant human Epo to suspension cultures or its incorporation in methylcellulose medium. The effects of different doses of Epo (0.5–5 units/ml) as well as different sources of fetal bovine serum, bovine serum albumin, or human plasma on the differentiation/maturation ability of B6SUtA cells were tested. Cell proliferation was evaluated by frequent cell counts. The effect of Epo on erythroid differentiation was evaluated by scoring benzidine-positive cells or positive colonies, in suspension or clonal culture, respectively (8).

Globin Chain Synthesis. Cells grown in suspension were labeled with [^{3}H]leucine overnight, washed, and lysed. Murine globin chains were purified from these lysates by haptoglobin binding in the manner described for human cells (9) and were identified by isoelectric focusing (10). Individual hemoglobinized bursts plucked from culture plates were lysed and applied directly for isoelectric focusing.

RNA Preparation and Analysis. Total cellular RNA was prepared by the guanidinium/lithium chloride method (11). The concentration and integrity of the RNA were assessed by UV spectroscopy and agarose gel electrophoresis. Unless otherwise specified, 2 μg of RNA was used in each RNase protection analysis, which was performed as described (12). The following globin-specific probes were used: mouse ε, pSP65Mε (13); mouse ζ, pSP64Mζ (13); mouse α, pSP64Mα;

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Abbreviations: Epo, erythropoietin; IL-3, interleukin 3.

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mouse β^{maj}, pSP64Mβ134 (13) and pSP65β^{maj} (14); mouse βh1, pSP65βh1 (15). In addition, a human γ-actin probe (13) that cross-hybridizes to mouse actin mRNA served as a control for a non-Epo-inducible message.

RESULTS

Epo-Induced Differentiation of B6SUtA Cells. In Epo-containing B6SUtA clonal cultures, only about 5% of the inoculated cells give rise to colonies and only a minority of these (6–12%) are erythroid (ref. 4 and data not shown). Molecular analysis of this line would be greatly facilitated if a high proportion of cells could be induced to undergo erythroid differentiation in suspension as opposed to clonal culture. The proportion of benzidine-positive B6SUtA cells in suspension cultures is very low (0–0.01%). However, while the cells were kept in continuous culture, selective passages were noted to have higher constitutive levels of benzidine-positive cells. Based on the assumption that these populations had an increased propensity to become erythroid, they were selectively expanded and used for the studies reported here. The percentage of (wet) benzidine-positive cells present in these cultures (which did not contain added Epo) varied according to the proliferative status of the cells, from about 1–3% to a plateau of 8–12% during logarithmic-phase growth. Basophils make up the bulk of the nonerythroid cells present in these cultures. Addition of Epo increased the percentage of benzidine-positive cells to up to 75% (Fig. 1*A*). The occurrence of hemoglobinized cells in B6SUtA populations cultured without Epo (Fig. 1 *A* and *C*) suggests that added Epo is not necessary for terminal erythroid differentiation by this hematopoietic progenitor cell line. To test whether traces of Epo, either present in the fetal bovine serum or constitutively produced during culture, were responsible for this seemingly Epo-independent effect, we made use of an Epo-neutralizing polyclonal antibody. Doses capable of neutralizing up to 1400 milliunits of Epo per ml did not abolish the generation of benzidine-positive cells (peak levels of 5–9%) in these experiments (data not shown). Culture components other than IL-3 and Epo (e.g., fetal bovine serum) were also found to influence (up to 2-fold) the proportion of benzidine-positive cells seen in Epo-treated suspension cultures (Fig. 1*B*).

Epo-Dependent and -Independent Globin Programs. To determine the globin gene programs associated with the Epo-dependent and -independent erythroid differentiation of B6SUtA progenitors, we analyzed globin gene expression at the transcriptional as well as the protein level in both Epo-treated and control suspension cultures. Total cytoplasmic RNA was analyzed for the presence of α-like (α, ζ) and β-like (βh1, ε^{y3}, β^{maj}, β^{min}) globin mRNAs. In the absence of Epo, embryonic β-like (βh1, ε^{y3}) messengers were detected (Fig. 2). Using a probe specific for the 3′ end of the adult β^{maj} transcript, we detected no β^{maj} mRNA (Fig. 3*A*). However, using a probe derived from the 5′ end of the β^{maj} gene, which cross-hybridizes to the β^{min} message, we detected adult globin mRNA. The identity of this message as β^{min} mRNA in uninduced cells was confirmed by using the two probes simultaneously (Fig. 3*B*). In contrast to β-like embryonic (βh1, ε^{y3}) gene expression in these cells, no α-like embryonic globin mRNA (ζ) was detected (Fig. 4*A*), but adult α-like message was present (Fig. 4*B*).

Addition of Epo increased the steady-state level of all globin mRNAs present in the uninduced cells. This effect seemed to be globin-specific, as Epo did not augment the steady-state level of mouse actin mRNA in these cells (Fig. 4*C*). Both βh1 and ε^{y3} messages were induced early after Epo addition, as was β^{min}, whereas induction of the β^{maj} transcript was not apparent until later. This was best seen when RNA from cells induced with Epo was probed simultaneously for

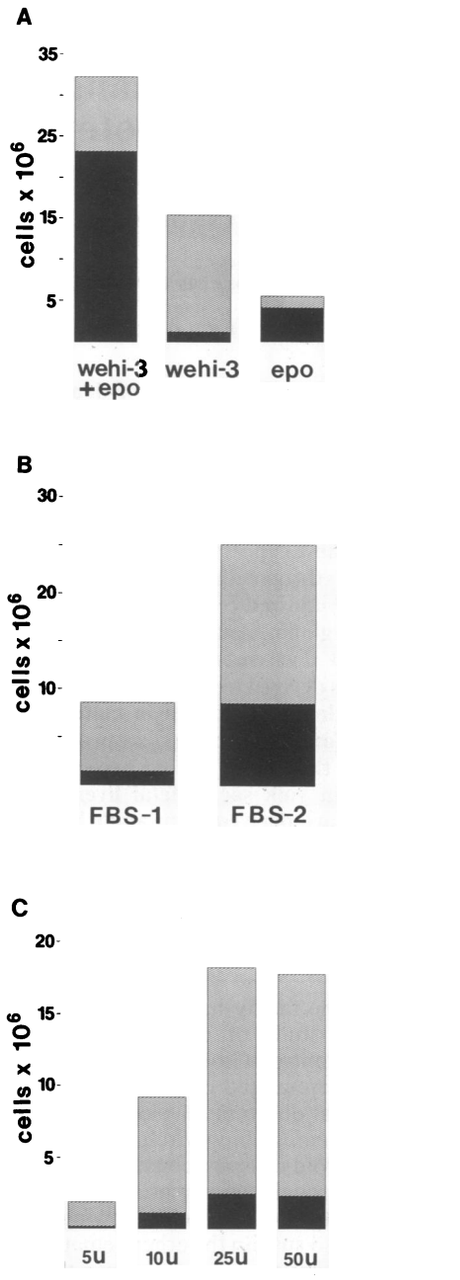

FIG. 1. Effects of IL-3, Epo, and medium components on the proliferation and erythroid differentiation of B6SUtA cells in suspension culture. Light and dark regions of the bars correspond to nonerythroid and erythroid cells respectively, as judged by benzidine positivity. All cultures were initiated with the same inoculum of B6SUtA cells, and total cell counts were done 6 days after the addition of Epo and/or IL-3. (*A*) Cells cultured in the presence or absence of 10% WEHI-3 conditioned medium (as a source of IL-3) and 2.5 units of recombinant human Epo per ml. (*B*) Cells cultured with WEHI-3 conditioned media and recombinant human Epo, in the presence of two different lots of fetal bovine serum (FBS). (*C*) Cells cultured in the presence of recombinant murine IL-3 at 5, 10, 25, or 50 units/ml, in the absence of WEHI-3 conditioned medium and the absence of Epo.

ε^{y3} and β^{maj} message (Fig. 5*A*). This differential accumulation of embryonic and adult globins during Epo-dependent erythroid differentiation resulted in a shift from a predominantly embryonic globin program in the uninduced cells to a program with significant adult (β^{maj}) globin production in the induced cells (Fig. 5*B*). This shift in programs was also seen at the protein level (Fig. 6*A*). Uninduced cells expressed a

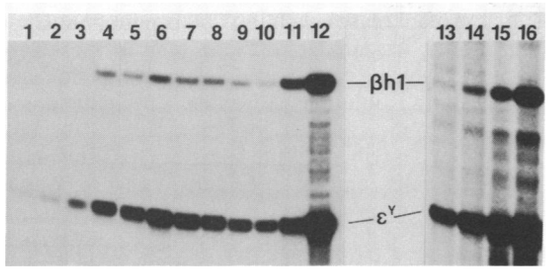

FIG. 2. Expression of β-like embryonic globin mRNA during B6SUtA differentiation. B6SUtA cells were analyzed for the presence of βh1 (182-base-pair RNase protection product) and ε^{y3} (145-base-pair protection product) mRNAs before (day 0, d0) and at various days after addition of Epo in suspension culture. Lanes: 1, d0; 2, d2; 3, d4; 4, d6; 5, d10; 6, d11; 7, d13; 8, d15; 9, d17; 10, d20; 11 and 12, controls (10 and 100 ng of 11.5-day mouse embryo blood RNA); 13–16, longer exposures of lanes 1–4.

predominantly embryonic ($\varepsilon^{y3} > \beta^{maj}$) globin program, whereas Epo-mediated induction enhanced the production of adult globin. However, the switch from embryonic to adult globin production appeared more pronounced at the protein level than at the RNA level, suggesting a possible involvement of posttranscriptional mechanisms in this process.

Cellular and Developmental Basis of the B6SUtA Globin Program. What is the cellular basis of the different globin programs observed in B6SUtA suspension cultures and their modulation by Epo? We approached this question by analyzing the globin program of individual erythroid colonies from clonal cultures of B6SUtA cells containing IL-3 and Epo. Hemoglobinized colonies appearing at different times during culture were morphologically distinct. Those that appeared early were small, rather uniformly benzidine-positive, and probably derived from cells with an activated erythroid program; their number was higher when Epo stimulation preceded the clonal culture or when the number

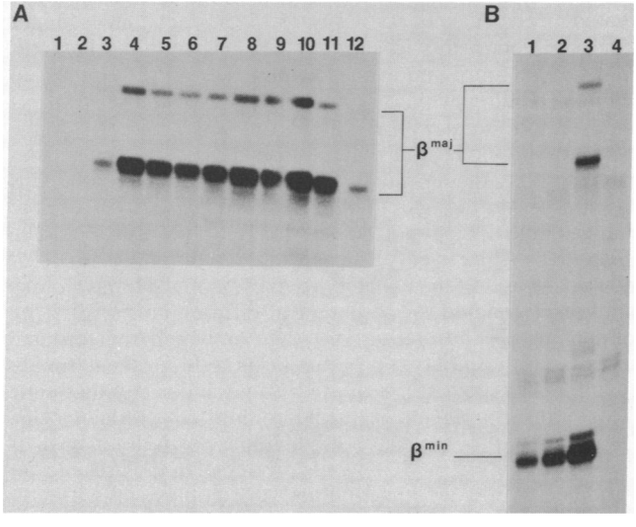

FIG. 3. Expression of adult β-globin mRNA during B6SUtA differentiation. Cells induced to differentiate in suspension culture by Epo were analyzed for β^{maj} and β^{min} mRNAs. A probe derived from the 3′ end of the β^{maj} gene is specific for β^{maj} mRNA (RNase protection products of 298 and 250 base pairs), whereas the probe derived from the 3′ end of the β^{maj} gene hybridizes to both the β^{maj} and β^{min} messengers (134-base-pair protection product). (*A*) Analysis using only the 3′ probe. Lanes: 1, day 0 (d0, before addition of Epo); 2, d2; 3, d4; 4, d6; 5, d8; 6, d10; 7, d11; 8, d13; 9, d15; 10, d17; 11, d20; 12, 11.5-day mouse embryo blood RNA (100 ng). (*B*) Analysis using 5′ and 3′ probes simultaneously. Lanes: 1, d0; 2, d2; 3, d4; 4, negative control (10 μg of yeast tRNA).

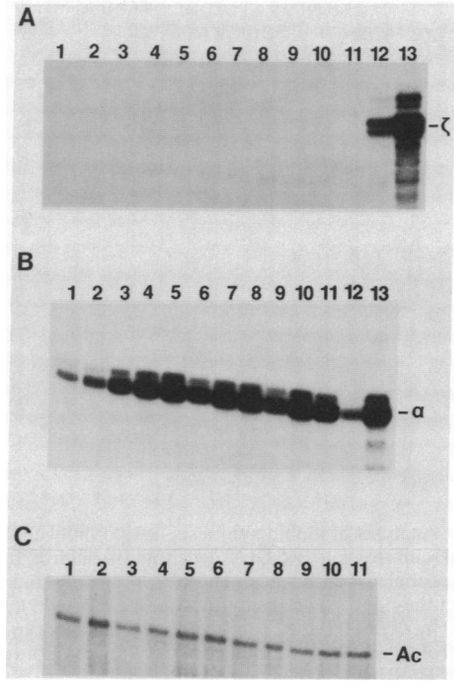

FIG. 4. Expression of α-like globin mRNA during B6SUtA differentiation. Globin mRNA expression was monitored by RNase protection analysis before and at various days (d) following the addition of Epo to B6SUtA cells in suspension culture. The ζ- and α-globin probes produce protection products of 151 (*A*) and 128 (*B*) base pairs, respectively. The same B6SUtA samples were analyzed with a probe that cross-hybridizes to mouse actin mRNA (Ac), producing a protected product of ≈70 base pairs (*C*). Lanes: 1, d0; 2, d2; 3, d4; 4, d6; 5, d8; 6, d10; 7, d11; 8, d13; 9, d15; 10, d17; 11, d20; 12 and 13, 11.5-day mouse embryo blood RNA (10 and 100 ng).

of benzidine-positive cells was higher. By contrast, colonies that appeared later were larger and were more heterogeneous with respect to their benzidine positivity, maturity, and cell-type composition (data not shown). Although the relative synthesis of embryonic and adult chains varied greatly from colony to colony, all (41/41) individual, immature colonies present late in clonal culture (day 10) clearly coexpressed adult and embryonic globins (Fig. 6*C*). In contrast, a signif-

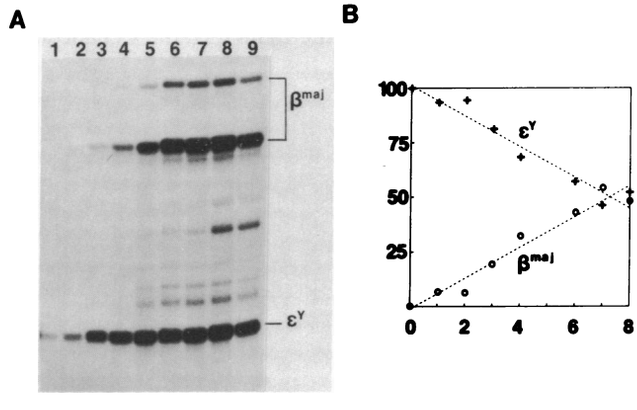

FIG. 5. Differential accumulation of embryonic and adult globin mRNAs during Epo-induced B6SUtA differentiation. (*A*) RNA was prepared at various days (d) after addition of Epo and analyzed for the presence of adult (β^{maj}) and embryonic (ε^{y3}) mRNAs. Lanes: 1, d0; 2, d1; 3, d2; 4, d3; 5, d4; 6, d6; 7, d7; 8, d8; 9, d8 from a different induction series. (*B*) Percentage (*y* axis) of globin constituted by each mRNA on the days indicated (*x* axis) was determined by densitometric analysis of the data in *A*. $\varepsilon^{y} = \varepsilon^{y3}/(\varepsilon^{y3} + \beta^{maj})$; $\beta^{maj} = \beta^{maj}/(\beta^{maj} + \varepsilon^{y3})$.

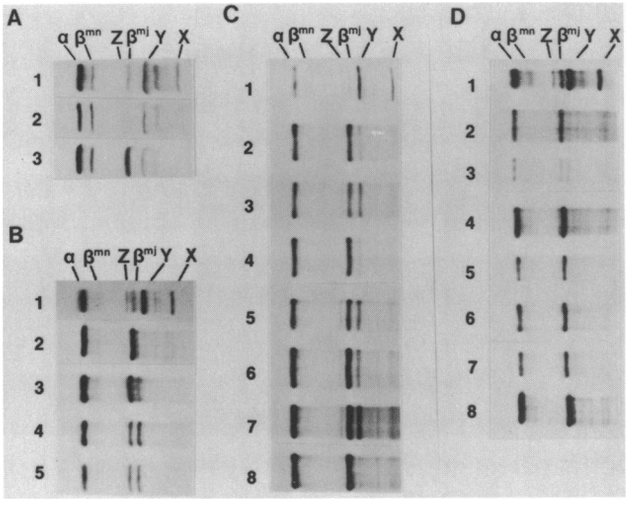

FIG. 6. Analysis of globin synthesis in Epo-induced suspension and clonal cultures of B6SUtA cells by isoelectric focusing of [^{3}H]leucine-labeled cell lysates. The globin chains (and the genes from which they derive) are as follows: α (α), β^{mn} (β^{min}), β^{mj} (β^{maj}), X (ζ), Y (ε^{y3}), Z (βh1). Lanes 1: a 13.5-day mouse embryo peripheral blood control to indicate the mobility of the various globin chains. (*A*) Samples of B6SUtA suspension cultures before (lane 2) and 8 days after (lane 3) the addition of Epo. (*B*) Pools of erythroid bursts plucked from Epo-containing clonal cultures of B6SUtA cells at days 7, 8, 11, and 13 (lanes 2–5, respectively). (*C*) Individual erythroid bursts plucked at day 10 from Epo-containing B6SUtA clonal cultures (lanes 2–8). (*D*) Individual (lanes 2–7) and pooled (lane 8) erythroid bursts lifted from day 7 methylcellulose cultures of B6SUtA cells containing IL-3 and Epo.

icant number of the more mature single colonies present early in culture (day 7), expressed only adult globin (Fig. 6*D*). These findings with individual colonies were confirmed by analyzing large pools of colonies (>150) from days 7–13 of clonal culture (Fig. 6*B*).

Is the globin program of B6SUtA cells aberrant or can a similar globin program be seen during normal mouse ontogeny? We carried out clonal erythroid cultures of mouse embryo and yolk sac cells and analyzed the globin program of colonies present for the next 3–6 days in culture (Fig. 7). Initially, both β-like embryonic chains (z,y) were expressed, while β^{maj} accounted for only a small fraction of the total globin (Fig. 7*A*). As developmental time advanced, a decrease in the synthesis of first the z and then the y chain was

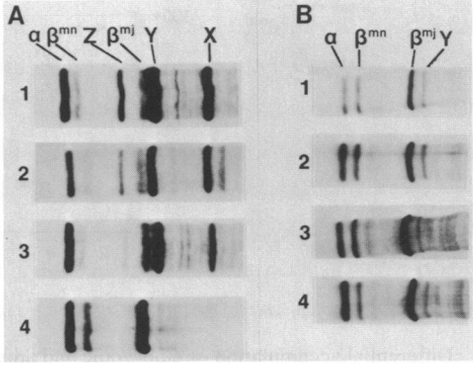

FIG. 7. Globin chain expression during normal mouse development. Globin synthesis in colonies derived from clonal cultures of day 8 mouse embryo and yolk sac erythroid progenitors was analyzed by isoelectric focusing of [^{3}H]leucine-labeled globin chains. (*A*) Pools of colonies from plasma clot cultures on days 3, 4, 5, and 6 (lanes 1–4). (*B*) Individual erythroid bursts plucked from day 6 methylcellulose clonal cultures.

paralleled by an increase in adult (β^{maj}) production. Changes in globin production similar to those seen in these *in vitro* cultures were also observed *in vivo*, in blood collected at days 10–13 of gestation (unpublished data). This developmental sequence of globin changes in the mouse is essentially identical to that described for the hamster (16). Furthermore, as in B6SUtA cells, and in agreement with previous work (17–20), individual colonies derived from murine progenitors after 6–7 days in culture (corresponding to days 11–14 of normal development) can coexpress embryonic (mainly y) and adult globin, i.e., a pattern similar to one seen in B6SUtA cells.

DISCUSSION

Certain features of the globin program exhibited by B6SUtA cells during Epo-mediated erythroid differentiation are reminiscent of those of normal murine globin cells during the period of transition from primitive to definitive erythropoiesis. However, B6SUtA cells were not derived from this developmental stage but from adult bone marrow. How then can we best interpret the globin programs of these cells? Perhaps the capacity of this adult-derived cell to express embryonic globin relates to its differentiative multipotency (21). We speculate that multipotent hematopoietic progenitor cells such as B6SUtA are not developmentally restricted in their potential to express globin chains but can express globin programs characteristic of ontogenetically primitive stages. Since terminally differentiated adult erythroid cells express only adult globins, we must further presume that changes in globin programs similar to those seen during ontogeny occur during differentiation down the erythroid pathway. Thus, as the cells progress to more mature stages under the influence of Epo, proportionately more adult globin is produced. The shift in Epo-treated B6SUtA cells from a globin program in which β^{maj} is absent to one in which its production equals or supersedes embryonic globin production could be seen in this way. The persistence of some embryonic globin in Epo-treated B6SUtA cells could relate to the fact that terminal differentiation in this *in vitro* system may not be complete. This may be more important for the experiments conducted in liquid culture, which is thought to favor cell renewal as opposed to differentiation (22).

Several previous studies have shown that Epo, apart from implementing the terminal maturation program, produces differences in the globin species formed (23–29). These changes, like those reported here for B6SUtA cells, could be effected by Epo in any one of several different direct or indirect ways. As the capacity to express embryonic globins is inversely related to the extent of differentiation, Epo, by influencing the differentiation stage at which the terminal erythroid differentiation program is initiated, or the extent to which it is excecuted (maturation), may indirectly modulate the globin program of erythroid-committed B6SUtA cells. Differences in the intensity of benzidine positivity as well as the morphology of B6SUtA cells produced in the presence or absence of Epo in liquid culture are consistent with this scheme. After Epo addition, a more highly hemoglobinized state is achieved, as indicated by benzidine positivity and nuclear maturity. Greater and more synchronized erythroid maturation, however, could be seen in clonal cultures, and a significant proportion of the more mature (day 7) colonies expressed either exclusively or predominantly adult hemoglobin. Our data do not rule out the possibility that these colonies derive from a separate class of exclusively adult programmed progenitors whose elaboration is preferentially favored by Epo. If this were the case, the distinct classes of progenitors would have to be continuously generated from a common stem cell, given that B6SUtA is a clonal population.

Accepting the indirect effect of Epo on the globin program implies that this growth factor merely allows the manifestation of a program already initiated previously in its absence. Although it is widely accepted that the birth of erythroid lineage-committed progenitors is not dependent on Epo (30, 31), the Epo-independent expression of a terminal erythroid differentiation program remains controversial (32–34). Our detection of globin in cells cultured in the presence of an Epo-neutralizing antibody suggests that IL-3 may be adequate to support terminal erythroid differentiation. Whether IL-3 plays an instructive role in this process or simply acts as a mitogen or survival factor for independently programmed cells remains to be determined. Similarly, whether this is physiologically relevant or only an *in vitro* phenomenon is an issue that requires further study. Our data also show that in addition to IL-3 and Epo, other factors such as culture components (e.g., fetal bovine serum) or the proliferative status of the cells can influence the terminal erythroid differentiation of B6SUtA cells. Since similar Epo-independent effects have been observed in normal murine cultures (35, 36), it would seem that the responses of normal murine hematopoietic progenitors to growth factors and culture conditions are shared by B6SUtA cells.

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