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Globin Messenger RNA Activity in Erythroid Precursor Cells and the Effect of Erythropoietin

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ABSTRACT **Cell populations enriched for erythroid precursor cells were fractionated from 13-day fetal-mouse livers by a method of immune hemolysis. These preparations of precursors, contaminated by less than 7% hemoglobinized erythroblasts, synthesize globin at a rate less than 6% that of the unfractionated liver erythroid cell population. RNA was isolated from these precursor cells and assayed for globin mRNA activity in a cell-free system from Krebs ascites tumor. The 6–16S RNA fraction from precursor cells has less than 5% of the globin mRNA activity of RNA isolated from unfractionated populations. Precursor cells incubated with erythropoietin show an increment in the rate of synthesis of globin only after 5–10 hr of incubation. After 10 hr of culture with this hormone, precursor cells show a 6- to 10-fold increase in globin mRNA activity. These results suggest that the precursor cells of hepatic erythropoiesis, responsive to erythropoietin, do not contain globin mRNA in a biologically active form. Erythropoietin-induced differentiation of these cells to erythroblasts is associated with an increase in globin mRNA.**

Fetal-mouse liver erythropoiesis provides a model for study of mammalian cell differentiation (1). The erythroid cell population obtained from 13-day fetal livers contains cells at all stages of differentiation, from the immature, erythropoietin-responsive precursor to the fully hemoglobinized erythrocyte. Erythroid precursor cells can be concentrated from the livers of fetal mice of 12- and 13-days gestation (2). Incubation of these precursor cells with erythropoietin stimulates both proliferation and differentiation to cells that actively synthesize hemoglobin. The questions posed in the present study are: (*i*) Does the precursor cell population contain biologically active mRNA for globin and synthesize globin, and (*ii*) Does erythropoietin-induced stimulation of erythroid cell differentiation involve an increase in the amount of biologically active globin mRNA? The evidence indicates that precursor cell populations (contaminated with 2–7% hemoglobinized erythroblasts) contain less than 5% of the globin mRNA activity recovered from an unfractionated erythroid cell population from fetal liver, and that they synthesize globin at a rate less than 5% that of the unfractionated cells. Erythropoietin-induced differentiation of precursor cells is characterized by a marked increase in globin mRNA activity and in the rate of globin synthesis.

MATERIALS AND METHODS

C57BL/6J mice were obtained from the Jackson Laboratory. [*4,5*-^{3}H]Leucine (39 Ci/mmol) and [*U*-^{14}C]leucine (286 mCi/mmol) were obtained from New England Nuclear. The [^{14}C]-leucine was lyophilized to remove HCl before use. Leucine-free Waymouth's MB752/1 medium was purchased from GIBCO (Grand Island, N.Y.). Fetal-calf serum (Microbiological Assoc.) was dialyzed against 0.15 M NaCl overnight with three changes of NaCl solution. Human urinary erythropoietin was provided by the NIH.*

Hormonal priming of mice and procedures to obtain disaggregated fetal-liver erythroid cells were described (3). Immune cytolysis of mature erythroblasts was accomplished with rabbit antiserum prepared against mouse erythrocytes and complement. We have demonstrated (4) that antibodies directed against mature mouse erythrocytes attach to hemoglobinized erythroblasts, reticulocytes, and erythrocytes. These cells are selectively sensitized for hemolysis by complement (4). The unlysed cells were demonstrated to be precursor erythroid cells (2). These precursor cells were cultured, with or without erythropoietin, according to methods detailed elsewhere (2). In order to determine the rate of synthesis of total proteins and of globin, cells were suspended in leucine-free Waymouth's medium with [^{14}C]leucine, supplemented with dialyzed 10% fetal-calf serum, four ribonucleosides (U,C,G, and A) at 1 μg/ml, four deoxyribonucleosides (dT, dC, dG, and dA) at 10 μg/ml, and 3.6 μg/ml $FeCl_3$. All concentrations are final concentrations in the incubation mixture. Incubation was for 60 min at 37° in a Dubnoff metabolic shaker with 5% CO_2 in air. In preliminary experiments with precursor cells and unfractionated cells, we found that the rate of incorporation of [^{14}C]leucine into total cytoplasmic protein and into purified globin is linear for at least 60 min. The cells were lysed by one or the other of the following methods: (*a*) Detergent lysis: Washed cells (not over 5×10^7 cells) were suspended in 1 ml of 0.1% NP 40 (Shell Chemical Co.) in 10 mM Tris·HCl–5 mM $MgCl_2$–10 mM NaCl (pH 7.4) and centrifuged at $750 \times g$ for 3 min at 4°. The supernatant was removed, the nuclear fraction was washed once with 0.5 ml of this buffer, and the wash was combined with the first supernatant solution. (*b*) Mechanical lysis: the cells were suspended in 2 ml of 30 mM Tris·HCl–5 mM Mg acetate–10 mM KCl (pH 7.5) and lysed in a Dounce homogenizer with five strokes of the lightly-fitted pestle (5). Nuclei and unlysed cells were removed by centrifugation at

* The erythropoietin was collected and concentrated by the Department of Physiology, University of the Northeast, Corrientes, Argentina and further processed and assayed by the Hematology Research Laboratories, Childrens Hospital of Los Angeles [under Research Grant HE 10880 (National Heart and Lung Institute)].

TABLE 1. *Protein synthesis in unfractionated and precursor-cell preparations*

Exp.	Cells	Hemoglobin (μg/μg of ribosomes)	[^{14}C]Leucine incorporation into total cytoplasmic protein (cpm/total culture $\times 10^{-4}$)	[^{14}C]Leucine incorporation into globin (cpm/total culture $\times 10^{-4}$)	Total cytoplasmic protein synthesis (cpm/μg of ribosomes)	Globin synthesis (cpm/μg of ribosomes)
*1**	Precursor cells	<0.21	5.0	0.29	540	32
	Unfractionated cells	2.1	8.5	5.6	1210	800
2†	Precursor cells	<0.06	34	3.7	500	53
	Unfractionated cells	1.95	69	45	1500	920

* The precursor cells and unfractionated cells were incubated with 15 μCi of [^{14}C]leucine and lysed mechanically; the incorporation of [^{14}C]leucine into ribosome-free supernatant and other fractions was analyzed.

† Fetal-liver cells were incubated with 40 μCi of [^{14}C]leucine, then 75% of the cells were treated with antibody to obtain precursor cells. 25% of the cells comprised the unfractionated cell population for analysis.

750 $\times$ *g* for 3 min at 4°. The crude cytoplasmic fraction was centrifuged at 27,000 $\times$ *g* for 10 min at 4°. The supernatant was recovered and recentrifuged in a Spinco 65 rotor for 3 hr at 55,000 rpm at 4°. The ribosome pellets recovered were rinsed two to five times with 10 mM Tris·HCl–5 mM $MgCl_2$–10 mM NaCl (pH 7.4) and dissolved in the same buffer; the ribosome content was determined by A_{260nm}. The hemoglobin content in the supernatant was determined by the method of Drabkin (6). These two methods of lysis proved comparable with respect to rates of globin synthesis.

For Na dodecyl SO_4–gel electrophoretic analysis of protein, the ribosome-free supernatant was incubated with 20 μg of bovine-pancreatic RNase A (boiled 10 min before use) at 37° for 30 min. Samples were concentrated by precipitation by 20 volumes of cold acetone. The precipitates were washed three times with acetone, and were dissolved in 0.1 M Na phosphate buffer (pH 7.0)–8 M urea–1% Na dodecyl SO_4–1% 2-mercaptoethanol. Samples were boiled for 5 min and electrophoresed in 10% acrylamide gels (7). Analysis of globin by chromatography (5) was performed on CM52 carboxymethyl cellulose instead of CM 23. Ribosome-free supernatant from adult-mouse reticulocytes, labeled with [^{14}C]- or [^{3}H]leucine, was used both as a marker to identify the position of globin chains in the elution pattern upon chromatography and to calculate recovery of globin from the columns. Fractions corresponding to α- and β-globin chains were pooled and precipitated with 10% Cl_3CCOOH. The precipitates were collected, washed twice with 5% Cl_3CCOOH, once with acetone, dissolved in 1 ml of NCS (Nuclear Chicago Co.) and counted in Aquasol after the addition of 0.2 ml of acetic acid.

RNA was extracted from cytoplasmic fractions and from total cells with Na dodecyl SO_4–phenol at 4° as described (8), except that 10 ml of 0.05 M Na acetate buffer (pH 5.1, containing 1% Na dodecyl SO_4 and 0.01 M EDTA) was used for each extraction.

RNA was fractionated by gradient centrifugation in 5–20% sucrose in 5 mM Tris·HCl (pH 7.4) with a SW40 rotor at 36,000 rpm for 8 hr at 4°. After centrifugation, the gradient was collected in five fractions; RNA was recovered from each fraction by precipitation with 0.3 M NaCl and 2 volumes of 95% ethanol, washed, dissolved in 50 μl of water, lyophilized, and redissolved in water (8). Half of the sample was assayed for globin mRNA activity in a cell-free system from Krebs ascites tumor (5) with an incubation time of 60 min and a total reaction mixture of 100 μl.

RESULTS

Globin and total protein synthesis in precursor cells

Four paired preparations of precursors and unfractionated cells were examined, and detailed data from two of these are presented in Table 1. Precursor cell populations, fractionated by immune cytolysis, were examined morphologically; they contained proerythroblasts and basophilic erythroblasts by the criteria reported previously (2, 3). These preparations were contaminated with small numbers (2–7%) of benzidine-positive (hemoglobinized) erythroblasts. The hemoglobin content of the precursor cell population is less than 10% than that of the unfractionated erythroid cell population (Table 1), and may be ascribed to contamination by hemoglobinized erythroblasts. Preparations of precursor cells synthesized globin at a rate less than 4% that of the unfractionated erythroid cell population, and no preparation formed globin at a rate higher than 6% of that of the unfractionated population (Table 1). The rate of synthesis of total cytoplasmic proteins, normalized to ribosome content, was 2- to 3-fold greater in the unfractionated cell populations than in precursor cells (Table 1).

In unfractionated populations of erythroid cells, globin synthesis comprises 60–70% of the total cytoplasmic protein formed (Table 1). In precursor cell populations, globin comprises 5–10% of the total cytoplasmic protein formed (Table 1) or 2–5% of the total cellular (nuclear plus cytoplasmic) protein formed. The proteins synthesized by precursor and by unfractionated cells were compared by column chromatography (Fig. 1) and by Na dodecyl SO_4–gel electrophoresis (Fig. 2). By these criteria, no differences in the types of proteins produced by the two populations of cells could be detected other than the synthesis of globin by unfractionated cells, but not by precursor cells. To determine whether exposure to cytolytic antibodies alters the types of proteins synthesized in precursor cells, the rates of total protein and globin synthesis were determined (Table 1, compare Exp. 1 and Exp. 2) and gel electrophoresis was performed (Fig. 2) on proteins of cells labeled with [^{14}C]leucine either before or after immune cytolysis. These studies indicate that exposure

to antibody does not demonstrably affect the pattern of proteins synthesized.

Globin mRNA activity

Total cellular RNA was extracted from precursor and from unfractionated erythroid-cell populations of liver. Total RNA was separated on sucrose density gradients into five fractions (Fig. 3). Each RNA fraction was assayed for activity in directing protein and globin synthesis in a cell-free system from Krebs ascites tumor. Each of these fractions of RNA, from both unfractionated erythroid cells and from precursor cells, stimulate protein synthesis. The highest specific activity is associated with the 6–16S RNA fraction from either cell population. However, only the 6–16S RNA component derived from unfractionated erythroid cells directs significant amounts of globin synthesis (Fig. 3). 60–70% of the protein synthesized in the cell-free system in the presence of this RNA fraction is globin, whereas less than 2% of the protein synthesis directed by the 6–16S RNA fraction derived from precursor cells is globin. The specific stimulatory activity for globin (globin synthesis per μg of RNA) of the RNA from precursor cells is less than 5% that of the same RNA component from the total, unfractionated erythroid-cell population.

The following control experiment was performed to examine the possibility that the procedures used to prepare precursor cells might involve agents that selectively destroy biological activity of globin mRNA. Precursor cell preparations were mixed with unfractionated cells in about equal numbers and the total RNA was extracted, fractionated, and assayed for globin mRNA as in the experiments illustrated in Fig. 3. The globin mRNA activity recovered from these mixed cell populations was similar to that recovered from the same amount of unfractionated cells alone.

Globin synthesis in precursor cells incubated with erythropoietin

There is no detectable increase in the total number of cells per culture during 10 hr of incubation with erythropoietin. Nevertheless, early signs of morphological differentiation—namely, faintly positive benzidene staining for hemoglobin and nuclear maturation (chromatin condensation)—are detected within 10 hr of culture with the hormone.

The rate of globin synthesis in precursor cells was measured after 0, 1.5, 5, and 10 hr of incubation, with and without erythropoietin (Fig. 4). The rate of globin synthesis in precursor cells incubated without erythropoietin remains

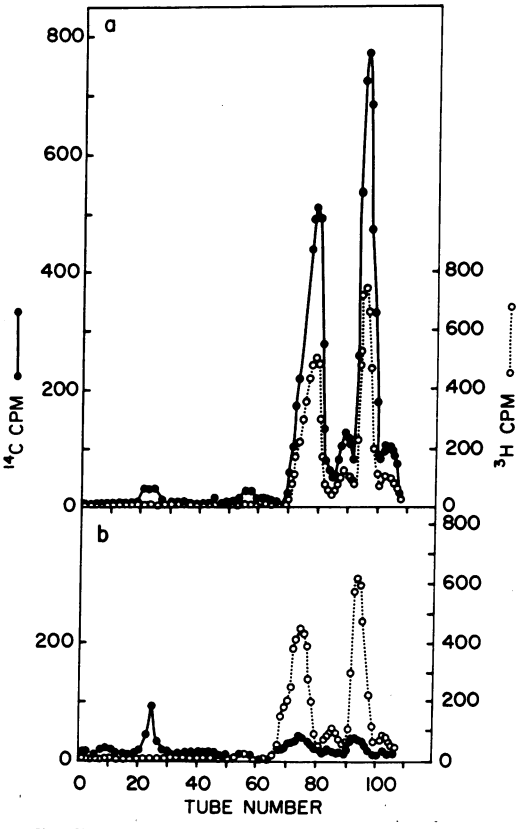

FIG. 1. CMC-cellulose chromatography of globin synthesized by unfractionated cells and precursor cells. 1.3 × 10^7 unfractionated cells (panel *a*) and 1.1 × 10^7 precursor cells (panel *b*) labeled with [^{14}C]leucine (●——●) were used to prepare ribosome-free supernatants for these experiments. The ribosome-free supernatant was chromatographed. To each preparation of ribosome-free supernatant, we added 6 × 10^4 cpm of [^{3}H]leucine (O- - -O)-labeled ribosome-free supernatant prepared from adult-mouse reticulocytes to provide labeled globin as a marker. These experiments were performed on the same cells that were used in Exp. *2* of Table 1.

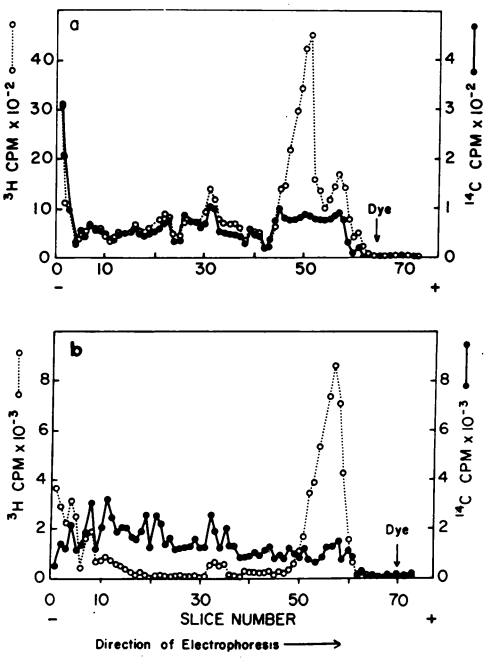

FIG. 2. Acrylamide-gel electrophoresis of proteins synthesized by unfractionated cells and precursor cells. (*a*) 3.5 × 10^6 precursor cells labeled with [^{14}C]leucine (●——●) and 3.5 × 10^6 unfractionated cells labeled with [^{3}H]leucine (O- - -O) were mixed and used to prepare ribosome-free supernatant for this experiment. This ribosome-free supernatant was analyzed by electrophoresis in Na dodecyl SO_4–acrylamide gels. The precursor cells in this experiment were labeled before immune cytolysis. (*b*) 5 × 10^6 precursor cells labeled with [^{14}C]leucine (●——●) were used to prepare the ribosome-free supernatant, which was mixed with [^{3}H]leucine-labeled (O- - -O) ribosome-free supernatant from adult-mouse reticulocytes to provide labeled globin as a marker for the electrophoresis. The precursor cells in this experiment were labeled after immune cytolysis.

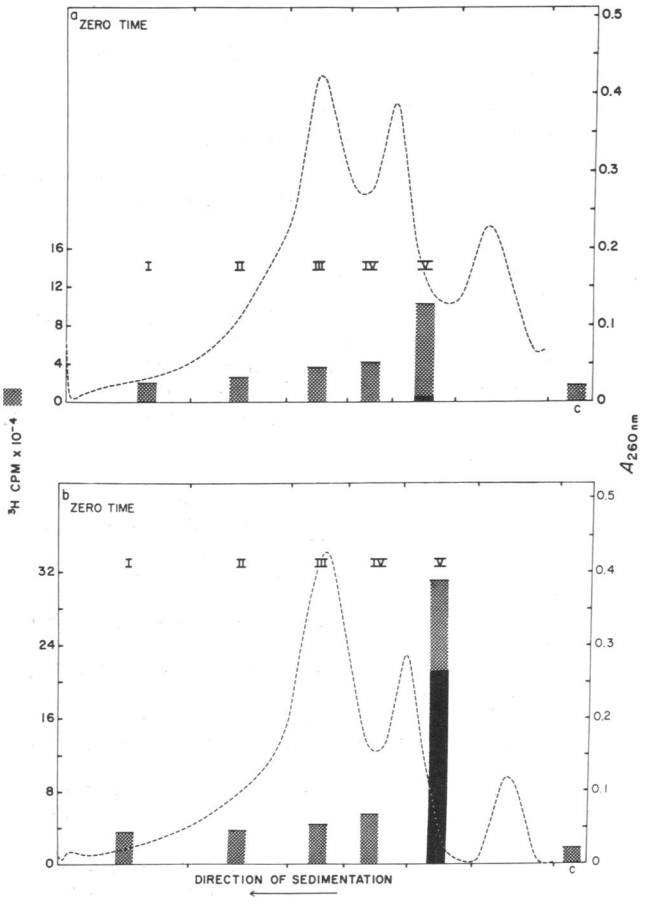

FIG. 3. Assay of total-cell RNA from unfractionated and precursor cells, separated by sucrose gradient centrifugation. 6.6×10^7 precursor cells (*a*) and 6.6×10^7 unfractionated cells (*b*) were used to prepare total-cell RNA. The RNA was fractionated by centrifugation in a 5–20% sucrose gradient in 5 mM Tris·HCl (pH 7.4) at 36,000 rpm in an SW40 rotor for 8 hr at 4°. Fractions *I* through *V* were collected, and the RNA recovered was assayed in the cell-free system from Krebs ascites tumor. The *bar* labeled *C* represents the acid-precipitable radioactivity from the incubation mixture in the absence of added RNA. *Cross-hatched bars* represent total protein synthesis; the *solid bar* represents globin synthesis. Globin synthesis was observed only in the presence of RNA from fraction *V*.

essentially unchanged throughout the incubation, at a rate less than 5% that of the unfractionated erythroid cell population. In the presence of added erythropoietin, the rate of globin synthesis increases relatively little during the initial 5 hr, but by 10 hr the rate of globin synthesis has increased 6- to 7-fold, to a level about half of the rate in the unfractionated erythroid cell population. Total protein synthesis about doubles during this 10-hr incubation with erythropoietin (Fig. 4). At 10 hr, about 30% of the total protein formed is globin.

Globin mRNA activity in precursor cells incubated with erythropoietin

Total cellular RNA was extracted from precursor cells before and after 10 hr of incubation with erythropoietin. The 6–16S RNA fraction was prepared and was assayed for the capacity to direct globin synthesis in the cell-free system. By 10 hr there is a 6- to 10-fold increase in globin mRNA activity (Table 2). The activity of mRNA for globin recovered from total-cell RNA and from the cytoplasmic fraction were comparable.

DISCUSSION

Preparations of precursors of fetal-mouse hepatic erythroid cells were isolated by immune hemolysis (2). They are contaminated by less than 7% hemoglobinized erythroblasts. It was found that in such precursor cell populations, the total-cell RNA contains less than 5% of the globin mRNA activity that can be recovered from unfractionated erythroid cells. In addition, these precursor cell populations synthesize globin at a rate less than 5% that of unfractionated erythroid cells. This amount of globin mRNA activity and this rate of globin synthesis could reflect the observed contamination of precursor cells by differentiated erythroblasts. If translatable globin mRNA is present in precursor cell nuclei, polyribosomes, monoribosomes, ribosomal subunits, or other ribonucleoprotein particles (9, 10), it would have been detected in the assays of total-cell RNA fractions in the cell-free system. On the basis of these findings, we conclude that precursor cells for fetal hepatic erythropoiesis do not contain globin mRNA in a biologically active form.

As reported from this laboratory (2), these precursors are specifically responsive to erythropoietin, which induces them to differentiate to erythroblasts capable of globin synthesis. The present studies show that differentiation of these precursors is associated with an increase in the amount of globin mRNA, demonstrable by assay of total-cell RNA or cytoplasmic RNA in the Krebs ascites cell-free system. The increase in active mRNA for globins and in globin synthesis that is detected by 10 hr of incubation of precursor cells with erythropoietin occurs before there is a detectable increase in total cell number in these cultures. However, the evidence does not exclude the possibility that cell division occurs

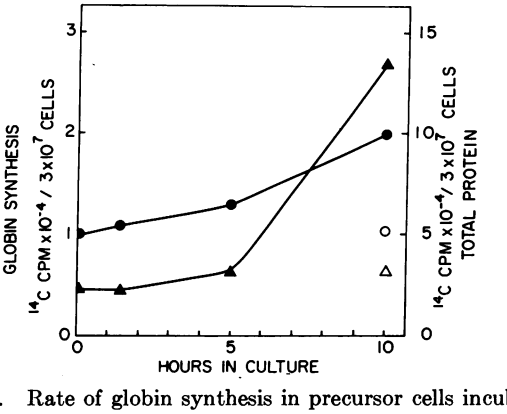

FIG. 4. Rate of globin synthesis in precursor cells incubated with and without erythropoietin. 3×10^7 precursor cells were incubated with and without erythropoietin. At each of the indicated times, samples were washed free of culture medium and suspended in an incubation medium containing 15 μCi of [^{14}C]-leucine for 60 min at 37°. The results are expressed as cpm incorporated into total protein and into purified globin. For cells incubated without erythropoietin, studies were performed at 0 and 10 hr. For cells incubated with the hormone, studies were performed at 0, 1.5, 5, and 10 hr. Total protein: ●——●, with, or ○, without erythropoietin; Globin: ▲——▲, with, or △, without erythropoietin.

TABLE 2. *Activity of mRNA for globin in precursor cells cultured with erythropoietin*

Source of RNA	Hr of culture	Total proteins, (cpm)	Globin, (cpm)
Total cell	0	51,540	980
	10	35,360	6940
Cytoplasmic fraction	0	32,040	500
	10	39,920	7420

RNA was extracted from precursor cells before and after culture with erythropoietin for 10 hr. 40% of the cells were used for extraction of total-cell RNA, and the remainder were used for extraction of RNA from the cytoplasmic fraction. The number of cells used were 7.5×10^7 and 6.2×10^7 for 0-time and 10-hr preparations, respectively. The 6–16S fractions were prepared by sucrose gradient centrifugation and assayed in the cell-free system from Krebs ascites tumor.

before the increase in globin mRNA in any individual cell, since there are insufficient data on the rate of cell proliferation and cell death in these cultures.

Krantz and Goldwasser (11) suggested that in rat-marrow cells erythropoietin selectively stimulates the synthesis of globin mRNA. More recently, however, Gross and Goldwasser (12) reported that erythropoietin causes stimulation of various RNA species. In fetal-mouse erythropoietic cells, the initial effect (in 1–3 hr) of erythropoietin includes the stimulation of synthesis of several classes of RNA, but stimulation of the 10S mRNA fraction was not detected (ref. 13, and unpublished results). In the present experiments, an increase in mRNA activity for globin was detected at 10 hr, a finding that is compatible with these earlier observations.

It has been suggested (1) that the precursor cell for hepatic erythropoiesis is differentiated, by virtue of its selective responsiveness to erythropoietin, but does not itself synthesize globin. The erythroid precursor (or erythropoietin-responsive cell) is distinct from the pluripotential stem cell (14, 15). The present data are consistent with this hypothesis. The erythropoietin-responsive precursor cell contains little or no globin mRNA in biologically active form. The hormone is necessary to induce the transition from precursor stage to erythroblast stages capable of hemoglobin synthesis. This transition involves an increase in the cellular content of biologically active globin mRNA. This increase could reflect either initiation of transcription of globin genes or an increase in the rate of processing of transcribed globin mRNA present in the precursor cells in an inactive form, such as a component of nuclear heterogeneous RNA.

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