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Biochemistry

Unequal accumulation of α- and β-globin mRNA in erythropoietic mouse spleen

[total cellular RNA/oligo(dT)-cellulose chromatography]

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ABSTRACT **Relative amounts and rates of synthesis of α- and β-globin mRNAs were determined during splenic erythropoiesis in mice. At times after injection of mice with phenylhydrazine, α- and β-globin mRNAs were separated by gel electrophoresis and quantitated by densitometric scanning of stained gels. At 66 hr after injection, the ratio of β to α mRNA is about 1.2. By 138 hr, total globin mRNA is 5-fold greater in spleen cells, and the β to α mRNA ratio approaches 2. This ratio remains around 1 in reticulocytes throughout this period. Analyses of globin products directed by these mRNAs from spleen cells and reticulocytes in the ascites cell-free system reflect the β to α mRNA ratio observed by electrophoresis. Relative rates of synthesis of globin mRNAs were estimated after incubation of spleen cells with either [^{3}H]uridine or [^{3}H]adenosine. Although synthesis of both mRNAs is maximal at 114 hr and then declines sharply, β mRNA is synthesized at a greater rate than α mRNA at every developmental stage. In contrast to the excess accumulation of β mRNA in spleen cells, synthesis of α- and β-globin chains remains balanced throughout erythroid development. These data suggest that during erythropoiesis in this system, equal synthesis of α and β globin involves regulation at both transcriptional and post-transcriptional levels.**

The erythropoietic spleen can be used as a model system for the study of erythroid development (1–6). In a nonanemic mouse, the spleen is a small lymphoid organ which contains few erythroid precursor cells. As the mouse is made anemic, the spleen increases greatly in size and its cells follow an orderly sequence of cellular and biochemical events in developing towards late-stage erythroblasts. At 0, 66, and 138 hr after phenylhydrazine injection of mice, hemoglobin synthesis accounts for 5, 40, and 85%, respectively, of total protein synthesized by spleen cells* (5). At 66–90 hr after injection, proerythroblasts and basophilic erythroblasts predominate in the spleen. Activities of nuclear RNA polymerases I and II peak at 66 hr (7), whereas ribosomal RNA synthesis reaches a maximum at 90 hr (3). Globin mRNA template activity accumulates in later stages of splenic erythropoiesis to a maximum at 114–138 hr after injection (4, 5).

The formamide gel technique originated by Staynov *et al.* (8) has permitted physical separation of 10S RNA from rabbit reticulocytes into two RNA species (9). Cell-free translation of these separated RNAs from different species (10–12), along with other data (11, 13–15), indicates that after electrophoresis the faster migrating 10S RNA is α and the slower, β mRNA. We have used this technique to study relative amounts and rates of synthesis of α- and β-globin mRNA during erythroid cell development in mouse spleen.

METHODS

Preparation and Fractionation of Total Cellular RNA from Spleen Cells. Male C57 Bl/6J mice (Jackson Lab) were injected three times with phenylhydrazine (4). At time intervals after the first administration of phenylhydrazine, spleens were removed and flushed with medium A [minimal essential Eagle's medium (GIBCO) containing 12% fetal calf serum and 0.3 mg/ml of glutamine]. Splenic capsules were disrupted and cells were dispersed with medium A. The cells, of which 50% were monodisperse and the remainder in clumps containing up to 25 cells, were then filtered through 10XX silk mesh, washed once in medium A, and resuspended in 0.9% NaCl to give a final cell concentration of approximately 1.25×10^8 cells per ml. The cells were then dripped into an equal volume of phenol:chloroform:isoamyl alcohol (50:50:1) (16) saturated with 50 mM Tris·HCl (pH 9.0)–0.1 mM EDTA, and 0.25% sodium dodecyl sulfate. After the mixture was stirred for 20 min at 25°, the aqueous phase was recovered by centrifugation at 18,000 × *g* for 10 min. The phenol phase was then re-extracted with 50 mM Tris·HCl–0.1 mM EDTA in 0.9% NaCl. After the combined aqueous phases were re-extracted twice with 1 volume of phenol:chloroform: isoamyl alcohol, 1/10 volume of 20% potassium acetate (pH 5.5) and 2 volumes of ethanol were added and the RNA was precipitated at −20°.

The mRNA was fractionated from total cellular RNA by oligo(dT)-cellulose chromatography (16, 17). Poly(adenylic acid) [poly(A)]-rich RNA, which eluted in 10 mM Tris·HCl (pH 7.4), was rechromatographed on oligo(dT)-cellulose and the resulting poly(A)-rich RNA was precipitated in ethanol. Total RNA was extracted from peripheral reticulocytes in the manner described for spleen cells.

Hemoglobin Synthesis by Spleen Cells. Spleen cells (approximately 1.25×10^8 cells per ml) were incubated in medium A containing 70 μg/ml of human transferrin, 0.02 M ferrous ammonium sulfate, 50 units/ml of penicillin, 50 μg/ml of streptomycin, and 10 μCi/ml of [^{3}H]alanine (38 Ci/mmol) for 2 hr at 37°. After incubation, cells were washed in medium A and lysed twice in 5 mM $MgCl_2$; protein was precipitated by acidic acetone (18). After globin labeled with [^{14}C]alanine prepared by incubation of mouse reticulocytes for 4 hr at 37° (19) was added, α and β peptide chains were analyzed by cellulose acetate gel electrophoresis (20).

Incorporation of [^{3}H]Uridine or [^{3}H]Adenosine by Spleen Cells. Washed spleen cells (about 1.25×10^8 cells per ml) were suspended in medium A supplemented by 50 μg/ml of streptomycin, 50 units/ml of penicillin, 3 μCi/ml of [^{3}H]uridine (45 Ci/mmol) or [^{3}H]adenosine (30 Ci/mmol), and 1×10^{-5} M each of the other nonradioactive ribonucleosides. After incubation at 37° for 2 hr, cells were washed and total cellular RNA was extracted.

Cell-Free Assay and Polyacrylamide Gel Electrophoresis. The Krebs II ascites cell-free system was used with [^{3}H]leucine added (21, 22). Specific α- and β-chain tryptic peptides were analyzed (23). Formamide electrophoresis in 7.5% polyacryl-

Abbreviation: Medium A, minimal essential Eagle's medium containing 12% fetal calf serum and 0.3 mg/ml of glutamine.

* G. D. Ginder, unpublished observation.

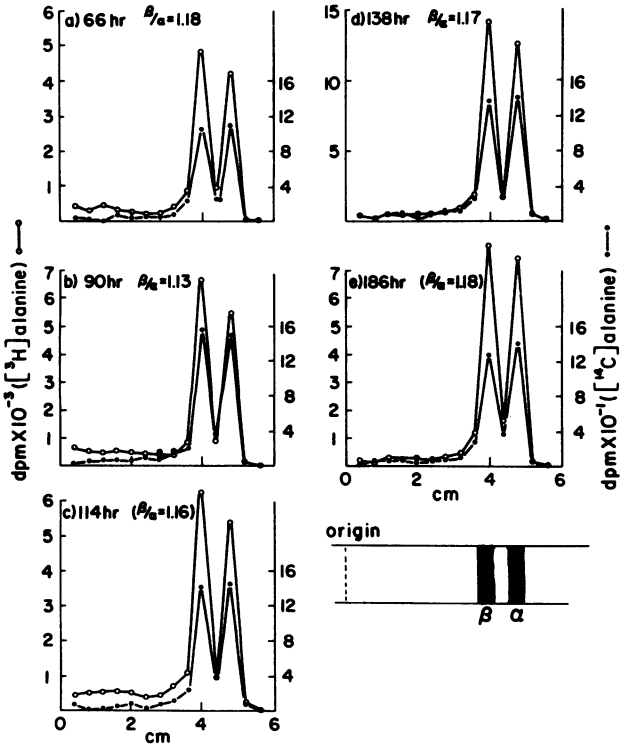

FIG. 1. Synthesis of α- and β-globin chains during erythroid development. [^{14}C]Alanine-labeled mouse globin (9000 cpm in 2 mg) was mixed with [^{3}H]alanine-labeled proteins derived from incubation of whole cells. α and β chains were separated on cellulose acetate gel electrophoresis. The strip on the right depicts stained α and β chains. After the [^{3}H]alanine content of slices containing the α- and β-chain peaks in each sample was standardized for the [^{14}C]alanine content in each chain, the ^{3}H dpm of the β chain divided by the ^{3}H dpm of the α chain gave the β/α results shown.

amide gels was done (10), and RNA was stained with "Stains-all" (24). 10S RNAs were separately eluted by electrophoresis from slices of unstained gels (10).

Measurement of Radioactivity in the Formamide Gels. After the stained gels were scanned at 600 nm, they were sliced into equal pieces approximately 1.1 mm in length. Each gel slice

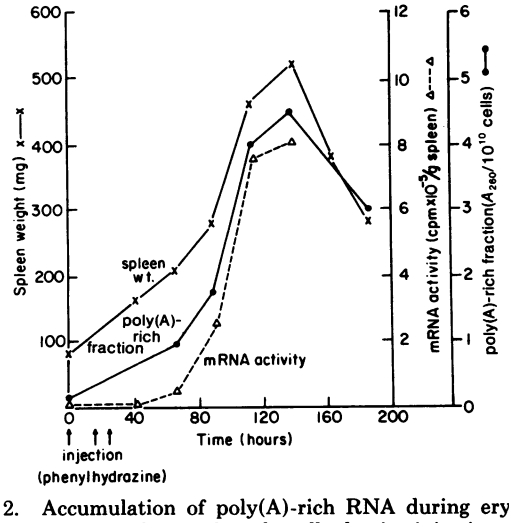

FIG. 2. Accumulation of poly(A)-rich RNA during erythroid development. Time in hours after phenylhydrazine injection of mice is plotted against spleen weight/animal, mRNA activity isolated from free polyribosomes/g of spleen, and poly(A)-rich RNA/spleen cell. The mRNA activity of polyribosomal RNA shown here was reported earlier (4).

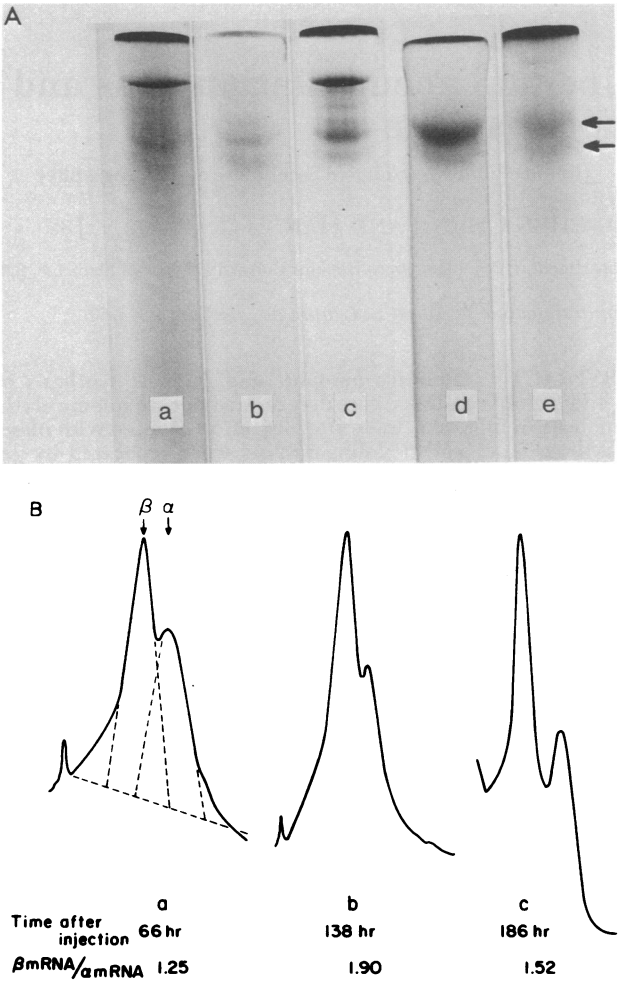

FIG. 3. (A) 7.5% polyacrylamide gel electrophoresis in formamide of poly(A)-rich RNA from spleen cells at various times after phenylhydrazine injection: a and b, 66 hr; c, 114 hr; d, 138 hr; and e, 186 hr. Gels were stained with "Stains-all" and scanned at 600 nm. RNA bands seen at and near the top of the gel are ribosomal RNAs, products of ribosomal RNA breakdown, and artifacts of the electrophoresis procedure. (B) Gel scans of globin mRNA from spleen cells at a, 66 hr; b, 138 hr; and c, 186 hr. Scans are from different mRNA preparations from those shown in part A. Relative amounts of the α and β mRNAs were estimated from the area under the curve for each after subtraction of background (see a). Since the background stain was often greater proximal to β mRNA than distal to α mRNA, background was estimated by a line connecting the portion of the scan proximal to β mRNA with that distal to α mRNA. The area of overlap between the two RNAs was determined, and half of this area was assigned to each.

was then placed in a screw-capped scintillation vial with 0.5 ml of NCS:H_2O (9:1) (25). After the tightly capped vials remained overnight at 37°, they were cooled, and 10 ml of scintillation fluid (6 g of 2,5-diphenyloxazole, 75 mg of 1,4-bis[2(5-phenyloxazolyl)]benzene, in 1 liter of toluene) along with 0.02 ml of 4% ascorbic acid was added to each before counting.

RESULTS

Synthesis of α- and β-Globin Chains During Erythroid Development. Spleen cells were incubated with [^{3}H]alanine, cells were lysed, and α and β chains were separated and analyzed for radioactivity. β-Globin synthesis is 1.1–1.2 times that of α globin throughout erythroid development (Fig. 1). These ratios are similar to those obtained from peripheral reticulocytes

Table 1. Cell-free synthesis directed by separated 10S RNAs of spleen cells

Gel fraction	Relative synthesis		
	β Chain	α Chain	β/α
Slower 10S RNA	2.7	0.06	45.0
Both 10S RNA (midregion)	0.8	0.3	2.7
Faster 10S RNA	0.75	1.7	0.44

The separated 10S RNAs (<1 μg each) of spleen cells were added to the cell-free system with [^{3}H]alanine. To each assay tube, after incubation, 3000 cpm of [^{14}C]alanine-labeled globin was added (4). Chain synthesis was calculated from the average syntheses of four α peptides (αT3, αT5, αT6, and αT9) and six β peptides (βT1, βT2, βT7, βT9, βT13, and βT14) (10).

of mice, indicating that approximately equal amounts of α and β globin are made in both erythroid precursors and reticulocytes.

Poly(A)-Rich RNA During Splenic Erythropoiesis. When the poly(A)-rich RNA content per spleen cell was determined at different stages of development, it was barely detectable in spleens of uninjected mice, reached a maximum at 138 hr, and then declined by 186 hr (Fig. 2). Furthermore, the yield of poly(A)-rich RNA at a late stage of development (138 hr) was almost 5-fold greater than that at an early stage (66 hr). Similarly, globin mRNA activity isolated previously from spleen polyribosomes was first detected 66 hr after phenylhydrazine injection of mice, and reached a maximum at 138 hr (4, 5).

The poly(A)-rich fraction isolated from spleen cells 66–186 hr after injection consistently had globin mRNA activity by cell-free assay and contained a single diffuse band on aqueous polyacrylamide electrophoresis with the same mobility as globin mRNA prepared from mouse reticulocytes. When this poly(A)-rich RNA was subjected to polyacrylamide electrophoresis in formamide, two major RNA species were consistently observed (Fig. 3). Experiments in which reticulocyte and spleen poly(A)-rich RNA were mixed showed that these two 10S RNAs of spleen origin have electrophoretic mobilities indistinguishable from those of the known α and β mRNAs of mouse reticulocytes. Indeed, studies of translational specificity of spleen RNAs isolated separately show that the slower RNA contains β mRNA activity and the faster RNA is rich in α mRNA activity (Table 1). That these globin mRNAs are not significantly contaminated with other nonglobin mRNAs is indicated by the following data: (*i*) poly(A)-rich RNA from spleens of uninjected mice lacks these RNAs, (*ii*) changes in the ratio of the quantity of faster migrating RNA to that of slower RNA are reflected in cell-free mRNA assays presented below, and (*iii*) when these RNAs are isolated from spleen cells, synthesis of globin chains accounts for 40–85% of total protein synthesized by these cells.

Changes in Relative Amounts of α- and β-Globin mRNA During Erythroid Development. When poly(A)-rich RNA was analyzed at different stages of splenic erythropoiesis by polyacrylamide electrophoresis in formamide and densitometric scanning of stained gels, the ratio of β to α mRNA isolated at 66 hr after injection was about 1.2, but increased to around 2 by 138 hr† (Figs. 3 and 4). By 186 hr, the β to α mRNA ratio had declined to 1.7. In Fig. 4, each plotted value represents the mean of four to six different preparations of spleen cells, with a range for the estimated β/α mRNA ratio at various times as follows:

† Ratios obtained by repeated runs of the same sample varied by less than 15% from the mean.

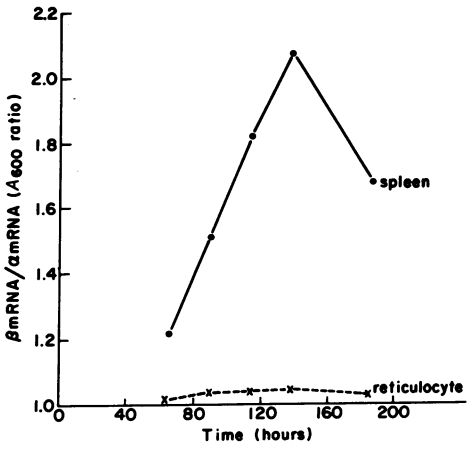

FIG. 4. Relative amounts of α and β mRNAs in spleen erythroid precursors and peripheral reticulocytes.

66 hr, 1.06–1.35; 90 hr, 1.37–1.6; 114 hr, 1.6–1.9; 138 hr, 1.8–2.2; and 186 hr, 1.5–1.8. On the other hand, the β to α mRNA ratio of mouse reticulocytes remained around 1 at all times. To test for preferential loss of α mRNA during extraction of splenic cells, we mixed mouse reticulocytes and spleen cells obtained 138 hr after injection in volume ratios of 3:1 and 1.5:1 (spleen:reticulocyte). After RNA extraction and formamide electrophoresis of the poly(A)-rich RNA of these mixtures, β to α mRNA ratios were 1.9 and 1.6, respectively, similar to those expected (1.94 and 1.65) for simple mixing of reticulocyte and spleen mRNAs.

Additionally, poly(A)-rich RNA at different stages of erythroid development was added in identical limiting amounts to the Krebs II ascites cell-free system (Fig. 5). The changes in β to α mRNA ratios observed by electrophoresis (Fig. 4) were reflected in these mRNA assays (Fig. 5). Cell-free synthesis of β chains relative to that of α chains was 2.5 times greater in the later erythropoietic cells (138 hr) than in the early phase (66 hr). In contrast, mRNA of mouse reticulocytes isolated throughout this period directed the cell-free synthesis of β and α chains in a 2:1 ratio, similar to the ratio obtained in our laboratory when

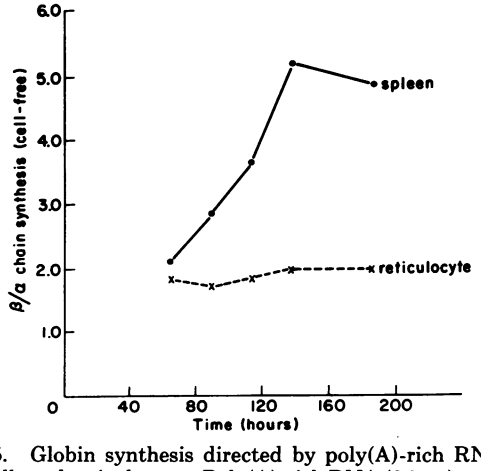

FIG. 5. Globin synthesis directed by poly(A)-rich RNA from spleen cells and reticulocytes. Poly(A)-rich RNA (2.5 μg) was added to 60 μl of cell-free reaction mixture containing [^{3}H]leucine (10 μCi, specific activity 40–45 Ci/mmol) (21). After 1 hr of incubation, [^{14}C]leucine-labeled mouse globin (approximately 4000 cpm) was added, globin was precipitated, and radioactivity was analyzed after cellulose acetate gel electrophoresis as in Fig. 1. Globin synthesis is linear through a poly(A)-rich RNA concentration of 8 μg per reaction mixture.

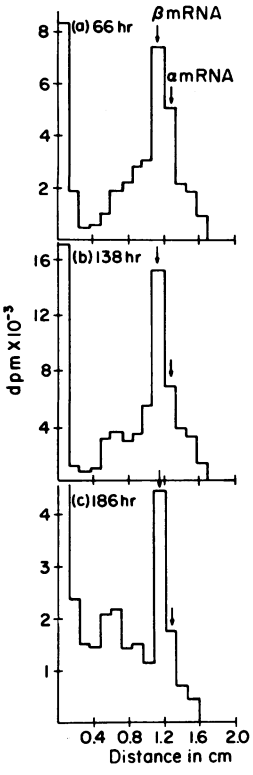

FIG. 6. Radioactivity profile of poly(A)-rich RNA labeled with [^{3}H]uridine after formamide gel electrophoresis.

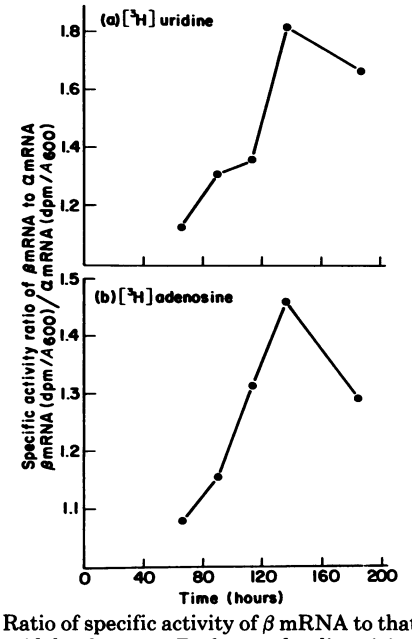

FIG. 7. Ratio of specific activity of β mRNA to that of α mRNA during erythroid development. Background radioactivity for β mRNA was estimated from the average dpm of two gel slices proximal to β mRNA, whereas that for α mRNA was derived from the values of two slices distal to α mRNA. For example, the dpm attributed to β mRNA and α mRNA in Fig. 6a were calculated as follows: β mRNA, 7600 − 3330 = 4270; α mRNA, 5100 − 2150 = 2950. Specific activity ratios shown here were obtained by averaging results of two to three comparable experiments.

roughly equal amounts of β and α mRNA from different sources are assayed in this cell-free system.

RNA of reticulocytes and spleen cells that did not adhere to oligo(dT)-cellulose in high salt contained negligible amounts of the globin mRNA bands on electrophoresis and insignificant cell-free activity, which was insufficient for analysis of globin chain synthesis.

Synthesis of α- and β-Globin mRNA by Erythroid Precursor Cells. Synthesis of globin mRNAs was estimated after incubation of spleen cells with either [^{3}H]uridine or [^{3}H]adenosine. After electrophoresis of the poly(A)-rich RNA, stained gels were sliced manually into equal pieces so that by eye each specific mRNA band was free of the other. The radioactivity patterns at 66, 138, and 186 hr in one experiment in which [^{3}H]uridine was incorporated are shown in Fig. 6. The radioactivity associated with α mRNA was slightly less than that of β mRNA at 66 hr, but much less than that of β mRNA at 138 and 186 hr.

Specific activities of β- and α- globin mRNAs were estimated from the radioactivity of each mRNA fraction and the amount of each mRNA observed by densitometric scanning of the same gel. When spleen cells were labeled with [^{3}H]uridine (Fig. 7a), the ratio of the specific activity of β to α mRNA increased from 1.1 to 1.8 between 66 and 138 hr, then decreased to 1.7 by 186 hr. When [^{3}H]adenosine (Fig. 7b) was the labeled nucleoside, this specific activity ratio showed a similar increase of lesser degree from early erythroid phase (1.1) to late phase (1.5), followed by a decline (1.3). These experiments suggest that the synthesis of β mRNA is greater than that of α mRNA throughout splenic erythropoiesis, accounting at least in part for the excess accumulation of β mRNA demonstrated by both gel analysis and cell-free mRNA assay. Furthermore, the results suggest that the different rates of α and β mRNA synthesis are not attributable to different rates of post-transcriptional addition of poly(A).

In order to compare the relative synthesis of the α and β mRNAs per cell during erythroid development, synthesis of α mRNA per cell at 66 hr was set at 1 (Fig. 8). From 66 to 114 hr, synthesis of both mRNAs per cell increased 5- to 7-fold, and

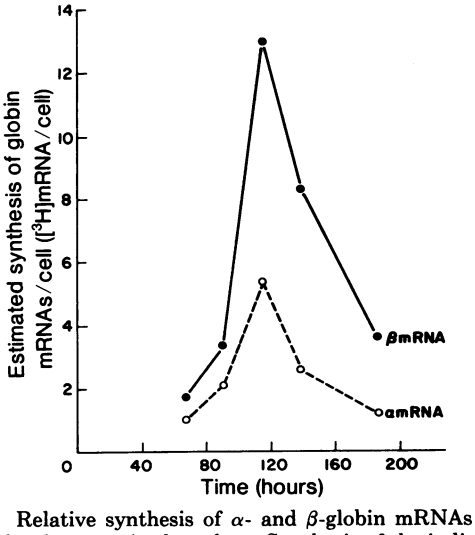

FIG. 8. Relative synthesis of α- and β-globin mRNAs during erythroid development in the spleen. Synthesis of the individual α and β mRNAs was measured from the specific activity of each mRNA ([^{3}H]uridine-labeled mRNA of Fig. 7a in dpm/A_{600}) and the amount of each mRNA per spleen cell. Synthesis of α mRNA per cell at 66 hr was set at 1. For comparisons of mRNA synthesis rates from one time point to another, knowledge of pool size of radioactive precursor is important. Spivak *et al.* (3) have found that the pool of UTP and the cellular uptake of uridine do not change as the spleen differentiates. In addition, the concentration of added radioactive uridine (7×10^{-8} M) is greater than the concentration of UTP within the cell (4×10^{-8} M), so that small changes in the cellular pool of uridine nucleotides will have little effect on the specific radioactivity of the precursor.

incorporation of label into β mRNA was 2.5 times greater than into α mRNA at the latter time. At 186 hr, synthesis of both mRNAs had markedly diminished. These data indicate that maximum synthesis of the globin mRNAs occurs 24 hr prior to their maximum accumulation (4, 5).

DISCUSSION

Following procedures for isolation of total cellular RNA modified from those previously described (26, 27), we, and Markel *et al.* (6), have obtained intact globin mRNA from spleen cells. Our analyses by gel electrophoresis and cell-free mRNA assay indicate that during erythroid development β mRNA accumulates to a greater extent than α mRNA. Although a more rapid turnover of α mRNA than of β mRNA has not been excluded, the excess accumulation of β mRNA in these experiments can be explained by greater synthesis of β mRNA than of α mRNA. However, since the β/α mRNA ratio is nearly 1 in the reticulocyte stage and spleen cells synthesize β and α chains in equal amounts, excess β mRNA in spleen cells is clearly unanticipated. Further evidence of a β mRNA excess may come from hybridization of total spleen RNA to complementary DNA specific for the α or β mRNA.

Beyond these experiments, the data suggest several questions for study. One is, how does the erythroid precursor cell provide for balanced globin synthesis in the face of an excess of β mRNA? Two testable hypotheses follow. (*i*) The rate of initiation or translation of β mRNA is less than that of α mRNA in these cells. (*ii*) A fraction of β mRNA molecules is unavailable for translation by cytoplasmic polyribosomes.

Another important question is, how does one account for a greater ratio of β to α mRNA in spleen erythroblasts at 138 hr (2:1) than in reticulocytes (1:1)? In acutely anemic animals, polychromatophilic erythroblasts skip maturation steps to become peripheral reticulocytes (28). Thus, the β to α mRNA ratio in stress reticulocytes studied here might approximate that of the spleen cells at 90 hr (1.5), accounting in part for the different ratios of β to α mRNA observed in reticulocytes and late erythroid precursors. Alternatively, the excess of β mRNA may be lost along with the nucleus during the transition from erythroblast to reticulocyte.

In murine erythroleukemic cells in culture, Orkin *et al.* have shown a deficiency of β mRNA in early erythropoiesis and essentially equal numbers of β and α mRNAs in late normoblasts (29). The difference between their results and ours, though not understood, may be attributed to the difference in cells used.

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