IN SITU LOCALIZATION OF GLOBIN MESSENGER RNA FORMATION

I. During Mouse Fetal Liver Development

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ABSTRACT

Globin mRNA levels in 11–15-day mouse fetal liver cells have been estimated by *in situ* hybridization of a highly labeled DNA copy (cDNA) of adult globin messenger RNAs (mRNAs) (globin cDNA) to fixed preparations of cells. Under the conditions employed, no significant *in situ* hybridization occurred to lymphoma cells (L 51787), mouse L cells, or hepatocytes; whereas reticulocytes from phenyl hydrazine-treated mice showed extensive *in situ* hybridization. The proportion of fetal liver cells showing predominantly cytoplasmic *in situ* hybridization increased from about 30% at the 11th day of development to 80–85% by days 13–15. Unlike more mature cells, proerythroblasts did not show *in situ* hybridization, except to a slight extent at later stages of development. These studies therefore indicate that globin mRNAs begin to accumulate during or shortly after the proerythroblast-basophilic erythroblast transition.

The fact that certain immature erythroid cells from 14-day fetal liver contain substantial amounts of globin mRNAs has been confirmed by comparing the hybridization in solution of globin cDNA to cytoplasmic RNA extracted from total fetal liver cells or from immature erythroid cells obtained by treatment of fetal liver cells with an antiserum raised against erythrocytes.

During the 11th–16th day of fetal mouse development, the liver becomes the main erythropoietic organ. At this time, the fetal liver consists of up to 70% erythroid cells, comprising both multipotential and intermediate "stem cells" and all the recognizable erythroid cell types. Although erythroid cell development represents a continuous process, certain erythroid cell types have been classified, as summarized in the scheme below [see (1, 2) for review]:

proerythroblast → basophilic erythroblast → polychromatic erythroblast → orthochromatic erythroblast → reticulocyte → erythrocyte

The most immature recognizable erythroid cells (proerythroblasts and basophilic erythroblasts) do not contain detectable amounts of hemoglobin as determined by histochemical staining or synthesis of globin chains (3); hemoglobin is first detected in a more mature cell type, the polychromatic erythroblast stage. An important question is, therefore, whether globin messenger RNA (mRNA) can be detected at an earlier developmental stage than hemoglobin, in the proerythroblast or basophilic erythroblast. An answer to this question would enable certain conclusions to be drawn concerning the mechanism of control of hemoglobin formation during erythroid cell maturation.

In a previous report (4) we described a method whereby cellular globin mRNA can be detected autoradiographically in fixed preparations of specific cell types by *in situ* hybridization to a very highly labeled DNA copy (cDNA) of purified globin mRNA obtained by transcribing mouse reticulocyte 9S RNA with viral reverse transcriptase. This work also demonstrated the presence of globin mRNA in basophilic erythroblasts from 13-day fetal livers; whereas little globin mRNA could be detected in the more immature proerythroblasts. However, Terada et al. (3) have not detected significant amounts of translatable globin mRNA in immature erythroid cells (a mixture of proerythroblasts and basophilic erythroblasts obtained by treatment of total fetal liver cells with an antiserum raised against mature red blood cells), as judged by the ability to direct globin synthesis in the Krebs ascites lysate system. These reports therefore suggest the interesting possibility that during erythroid cell differentiation globin mRNA may be synthesized before the immature erythroid cell has the capacity to translate it into globin chains.

A related question concerns the stabilization of the hemoglobin synthetic capacity at about the 12th–13th day of fetal liver development. Before this time, hemoglobin synthesis in fetal livers is dependent on continued RNA synthesis; subsequently, inhibition of RNA synthesis does not affect the synthesis of hemoglobin. This change is not due to accumulation of larger numbers of more mature erythroblasts during development (5). It is, therefore, of considerable interest to ascertain whether this change is reflected in the types of erythroid precursor cells in which globin mRNA can be detected.

The present paper therefore attempts to analyze these two questions in more detail. First, globin mRNA levels in specific erythroid cell types from fetal livers of different ages are determined by *in situ* hybridization to globin cDNA. These results are then confirmed by estimating globin mRNA levels in total or immature erythroid cells (obtained by treatment of total fetal liver cells with antiserum, as described above) by titrating extracted cytoplasmic RNA with globin cDNA. Although the latter approach does not yield such detailed information with respect to the distribution of globin mRNA in specific erythroid cell types at different stages of fetal liver development, it does facilitate an analysis as to whether the globin mRNAs synthesized in total or immature fetal liver cells are the same as those in adult reticulocytes, namely α- and β-globins.

MATERIALS AND METHODS

Preparation of Globin cDNA

[^{3}H]DNA complementary to globin mRNA (globin cDNA) was synthesized by copying mouse reticulocyte 9S RNA with reverse transcriptase prepared from avian myeloblastosis virus as described previously (6–9). cDNA prepared in this way represents a faithful but partial, transcript of α and β globin mRNAs (8, 9).

In Situ Hybridization

Procedures for obtaining fetal livers, preparation of slides, and *in situ* hybridization of cDNA have been described in detail (4). After treatment for 25 min in 0.2 N HCl at 20°C and dehydration in ethanol, slides bearing about 10^5 fixed cells were incubated at 43°C for 18 h with about 4 μl cDNA (25–50 $\times$ 10^6 dpm/μg; 4 $\times$ 10^7 dpm/ml) dissolved in 0.45 M NaCl, 0.045 M citrate (3$\times$ SSC) containing 40% formamide; washed in 2$\times$ SSC at 20°C; incubated for 1–2 h at 55°C in 2$\times$ SSC; and finally washed in 2$\times$ SSC. Autoradiographs (AR10) were prepared and exposed for 6–7 wk. Cells were then stained with May-Grünwald and Giemsa stains.

Purification of Immature Erythroid Cells by Treatment with Antiserum

Rabbit antiserum against adult mouse red blood cells [obtained as described by Cantor and co-workers (10)] had a titer of 1:2,000 for lysis of mouse red blood cells in the presence of guinea pig complement. 14-day fetal liver cells were treated with antiserum exactly as described by Terada et al. (3), except that antiserum was used at a dilution of 1:200. About 1–2% of the total fetal liver cells were recovered by this procedure and these comprised 86% immature erythroid cells (i.e. proerythroblasts and basophilic erythroblasts) and 7% hemoglobinized cells. Accurate cell counts were obtained by adding a known number of red blood cells to immature cell samples before preparing slides and then by obtaining a differential cell count.

Extraction of Cytoplasmic RNA

Erythroid cells were suspended (2×10^7 cells/ml) by gentle homogenization in 0.14 M NaCl, 0.01 M Tris-HCl, 1.5 mM magnesium acetate and 0.5% Nonidet NP-40, allowed to stand at 0°C for 2 min, and then centrifuged for 10 min at 1,000 *g*. Microscope examination of the pellet fraction showed that most of the cells had been lysed by this procedure and that most erythroid cell nuclei remained intact. Sodium dodecyl sulphate

(0.5%) was added to the supernatant fraction and the mixture extracted repeatedly with an equal volume of phenol/chloroform (1:1 vol/vol) containing 1% amyl alcohol until no protein at the interface was visible. RNA was obtained by precipitation with 2 vol of ethanol after adjusting the salt concentration to 0.2 M, and purified by dissolving in 0.2 M NaCl and reprecipitating again with ethanol.

Hybridization of RNA to cDNA

cDNA (sp act 45 × 10^6 dpm/μg) was dissolved in hybridization buffer (0.5 M NaCl, 25 mM HEPES, 0.5 mM EDTA, containing 50% formamide, pH 6.8) at about 0.2 μg/ml. Serial dilutions of RNA from erythroid cells (0–15 mg/ml) were prepared in hybridization buffer also containing 4 mg/ml *E. coli* RNA as carrier. 5-μl portions each of cDNA and each dilution of erythroid RNA were mixed, sealed in a capillary, and incubated at 43°C for 7 days. We have shown previously (8, 9) that under these conditions, hybridization to cDNA of any complementary RNA sequences present in the RNA samples will be complete. Each sample was then assayed for hybrid formation by treatment with single-strand specific nuclease or by banding in CsCl after ribonuclease treatment exactly as described previously (8, 9).

Determination of Melting Temperatures

Hybrids were formed as described above at an RNA/cDNA ratio slightly in excess of that required to give the maximum hybridization of cDNA. Hybrids were diluted in 0.5 M NaCl, 25 mM HEPES, 0.5 mM EDTA, pH 6.8, resealed in capillaries, and then heated for 5 min at various temperatures in the range 43–90°C. Capillaries were then immersed in an ice/salt mixture, expelled with S1 nuclease buffer and assayed for resistance to S1 nuclease exactly as described previously (8).

Determination of Cellular RNA Content.

Known numbers of cells were treated at 0°C with 0.2 N $HClO_4$ and the precipitate was washed extensively with 0.2 N $HClO_4$. After digesting the precipitate in 1 N NaOH for 4 h at 37°C, 1/4 vol of 6 N $HClO_4$ was added and the precipitate centrifuged down after allowing the acidified mixture to stand for 30 min in ice. RNA determinations were calculated from the E_{260} of the supernatant fraction.

RESULTS

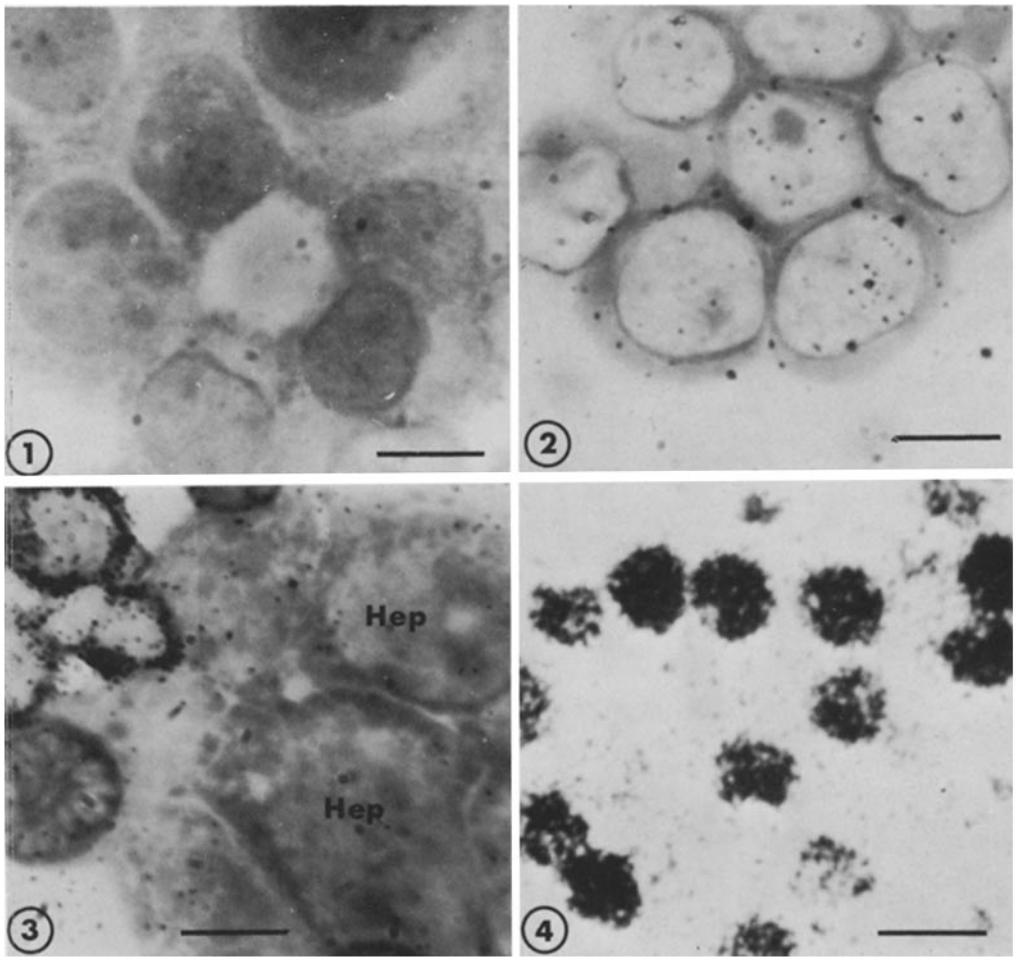
In Situ Hybridization

NONERYTHROID CELLS: The conditions of *in situ* hybridization and subsequent washing of cells at elevated temperature were designed to eliminate nonspecific adsorption of cDNA to fixed preparations of cells (4). Under these conditions, only slight *in situ* hybridization of cDNA to mouse LS cells, lymphoma cells (L 5178Y) or hepatocytes as observed (Figs. 1–3).

RETICULOCYTES: Since cDNA is obtained by transcribing 9S RNA from mouse reticulocytes, one would expect cDNA to show good *in situ* hybridization to reticulocytes. In the past, this has been difficult to achieve, and various explanations have been suggested (4). However, recently we have repeatedly obtained extensive *in situ* hybridization of cDNA to fixed preparations of reticulocytes (Fig. 4). In these successful experiments, the cell concentration on the slides was reduced considerably compared to the previous unsuccesful experiments, so that the reticulocytes were far from confluent (Fig. 4). Assuming that the globin mRNA content of a single reticulocyte is 3 × 10^{-2} pg [i.e. 1.5% of the total RNA (11)], calculations show that the ratio of cDNA to reticulocyte globin mRNA during *in situ* hybridization was about 1:3 in the previous experiments and about 3:1 in the present experiments. This may mean that it is important to maintain an adequate cDNA to cell ratio during *in situ* hybridization.

13–15-DAY FETAL LIVER CELLS: As is evident from Fig. 5 and Table I, most cells from 13–15-day fetal livers undergo extensive *in situ* hybridization with globin cDNA. However, the very largest cells (diameter greater than 15 μm), with characteristics of proerythroblasts, form *in situ* hybrids much less frequently than the smaller cells including those which exhibit characteristics of basophilic erythroblasts (Fig. 5). In fact, only about 15% of the proerythroblast compartment shows diffuse labeling over the whole cell (Table I). Owing to the high grain counts on these slides, it is difficult to be more precise in the identification of more mature erythroid cell types, except on the basis of size. Nevertheless, it is clear that during the13th–15th day of development erythroid cell types of size 8–12 μm show extensive *in situ* hybridization to globin cDNA.

11–12-DAY FETAL LIVERS: During growth from the 11th–12th day of fetal liver development, the proportion of proerythroblasts decreases markedly from about 80% to about 20%, and mature erythroid cells (polychromatic and orthochromatic erythroblasts), which are totally absent at the 11th day, begin to accumulate. The results collected in Table I show that the proportion of cells showing *in situ* hybridization to globin cDNA in-



FIGURES 1–3 *In situ* hybridization of globin cDNA to nonerythroid cells.

FIGURE 1 Amouse L cell line (LS).

FIGURE 2 Lymphoma cells (L 5178Y).

FIGURE 3 A group of hepatocytes (*Hep*), adjacent to erythroid cells which are labeled. Autoradiographic exposure: 6 wk. The magnification in Figs. 1–9 is × 1,400, and the bar represents 10 μm.

FIGURE 4 *In situ* hybridization of globin cDNA to reticulocytes. Autoradiographic exposure: 6 wk.

creases progressively as the livers develop during the 11th–13th days. As is evident from Figs. 6 *a–c*, this occurs because proerythroblasts from 11–12-day fetal livers do not form *in situ* hybrids with globin cDNA to any great extent, as is also the case for proerythroblasts from 13–15-day fetal livers (Fig. 5 *a*). Moreover, any *in situ* hybridization to proerythroblasts is relatively diffuse over the whole cell rather than localized over the cytoplasm (Fig. 6 *c*). However, proerythroblasts showing such diffuse labeling represent only about 2% of the proerythroblast compartment; this figure is much lower than that for 13–15-day fetal livers (Table I). The remarkable impression obtained from thorough scanning of these *in situ* hybridization slides is that there appears to be a sudden, rather than a gradual, increase in globin mRNA content in cells of decreasing size: larger cells appear to be labeled slightly in a diffuse manner, if at all; smaller cells are very heavily labeled exclu-

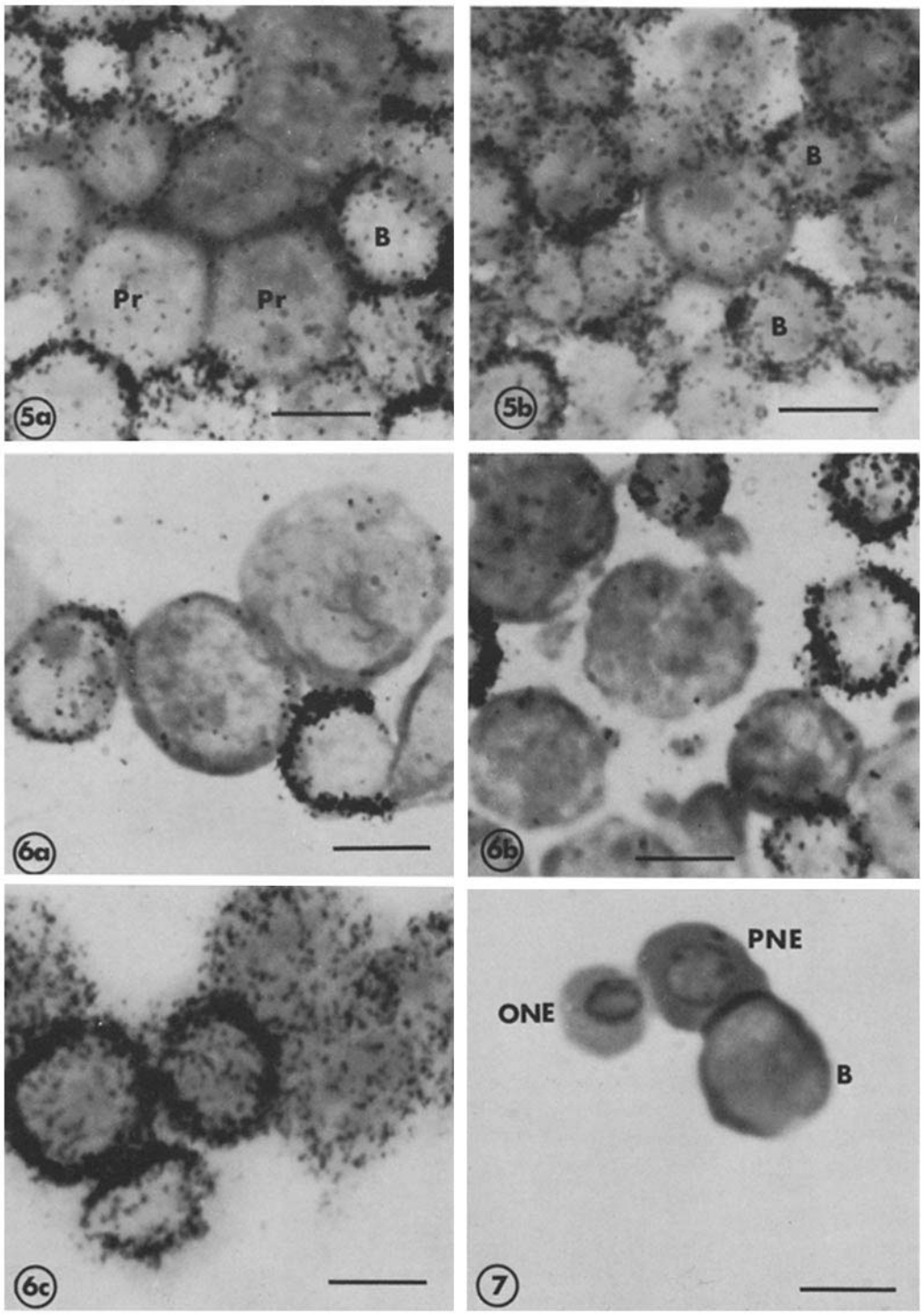


FIGURE 5*a–b* *In situ* hybridization of globin cDNA to 13-day fetal liver cells. *Pr*, proerythroblast; *B*, basophilic erythroblast. Autoradiographic exposure: 6 wk.

FIGURE 6*a–c* *In situ* hybridization of globin cDNA to 11–12-day fetal liver cells. Autoradiographic exposure: (*a*) and (*b*), 6 wk; (*c*) 10 wk.

FIGURE 7 Basophilic erythroblast (*B*), polychromatic nucleated erythrocyte (*PNE*), and orthochromatic nucleated erythrocyte (*ONE*) from 11-day fetal liver.

TABLE I

Percentage of Fetal Liver Cells Showing In Situ Hybridization to Globin cDNA

Age	Positive cells	Proerythroblast compartment showing diffuse labeling
days	%	%
11	33	~2
12	55	
13	78	~15
14	85	
15	84	

Each value is the average of two measurements on slides exposed for 6–7 wk or 10 wk. Cells showing predominantly cytoplasmic labeling with more than 15 grains were scored as positive.

sively in the rim of cytoplasm. Some of the smaller, labeled cells appear to be typical basophilic erythroblasts, but the majority are somewhat smaller basophilic cells. The smaller cells which form *in situ* hybrids are too numerous to be accounted for by the very small proportion of mature hepatic erythroid cells in livers of this age. They are also too numerous to be polychromatic or orthrochromatic nucleated erythrocytes derived from the yolk-sac; moreover, their appearance is characteristically different from that of the nucleated erythrocytes (Fig. 7).

IMMATURE CELLS FROM 14-DAY FETAL LIVERS: Since the proportion of immature cells in fetal liver declines rapidly after the 11th–12th day, it is not possible to determine directly whether all proerythroblasts remain free of hybrid after *in situ* hybridization and whether the characterstically small immature erythroblasts forming extensive *in situ* hybrids with cDNA persist during fetal liver development. However, it is possible to approach this question indirectly by removing mature erythroblasts from preparations of 14-day livers by the use of a specific antiserum. After such treatment, the proportions of the various erythroid cell types are altered as shown in Fig. 8 *a–b* and quantified in Tabe II. Immature cells obtained by this procedure were then allowed to hybridize with globin cDNA *in situ* in the usual way. It is clear from the results (Fig. 9 *a–b*) that the cells which form extensive *in situ* hybrids with globin cDNA have characteristics very similar to those of the immature cells from 11–12-day fetal liver. Any large proerythroblasts which show *in situ* hydridization are much less heavily labeled and tend to show hybridization generally distributed over both nucleus and cytoplasm.

Conventional Hybridization Studies

It is obviously desirable to confirm by an independent method the conclusions derived from studies using *in situ* hybridization. Immature cells from 14-day fetal livers were first obtained by treatment with antiserum raised against mature red blood cells as described above. Cytoplasmic RNA was then extracted from either total or immature erythroid cells and the globin mRNA content in these RNA preparations was determined by titration with globin cDNA. The theoretical and experimental basis of this titration method has been validated rigorously in previous papers (8, 9). A constant amount of highly labeled globin cDNA was annealed under stringent conditions with increasing amounts of RNA of interest, under conditions in which hybridization of any complementary sequences present is known to be complete (9). In this way, a titration curve is obtained showing how the proportion of cDNA hybridized varies with RNA/cDNA ratio.

It is clear from Fig. 10 that negligible amounts of cDNA are converted into hybrid by annealing with 8,000 times excess cytoplasmic RNA from lymphoma cells. Thus cytoplasmic RNA sequences present in a nonerythroid mouse cell line (but of hematopoietic origin) do not hybridize at all with globin cDNA under the conditions used as assayed by resistance to single strand-specific (S1) nuclease. However, about 85% of cDNA sequences are hybridized after annealing under the same conditions with about 2 times excess globin mRNA from adult mouse reticulocytes or about 1,000 times excess cytoplasmic RNA from 14-day fetal liver erythroid cells, or about 8,000 times excess cytoplasmic RNA from immature erythroid cells (Fig. 10).

It is obviously of interest to determine precisely the extent of sequence complementarity between globin cDNA and the RNA sequences from fetal liver cells to which it hybridizes. Therefore, hybrids formed between globin cDNA and RNA from total or immature erythroid cells were treated with ribonuclease and banded in CsCl. After annealing of globin cDNA with either total or immature erythroid cell cytoplasmic RNA under conditions which converted 60–70% of globin cDNA to S1 nuclease-resistant hybrid, a similar proportion of the cDNA was converted to

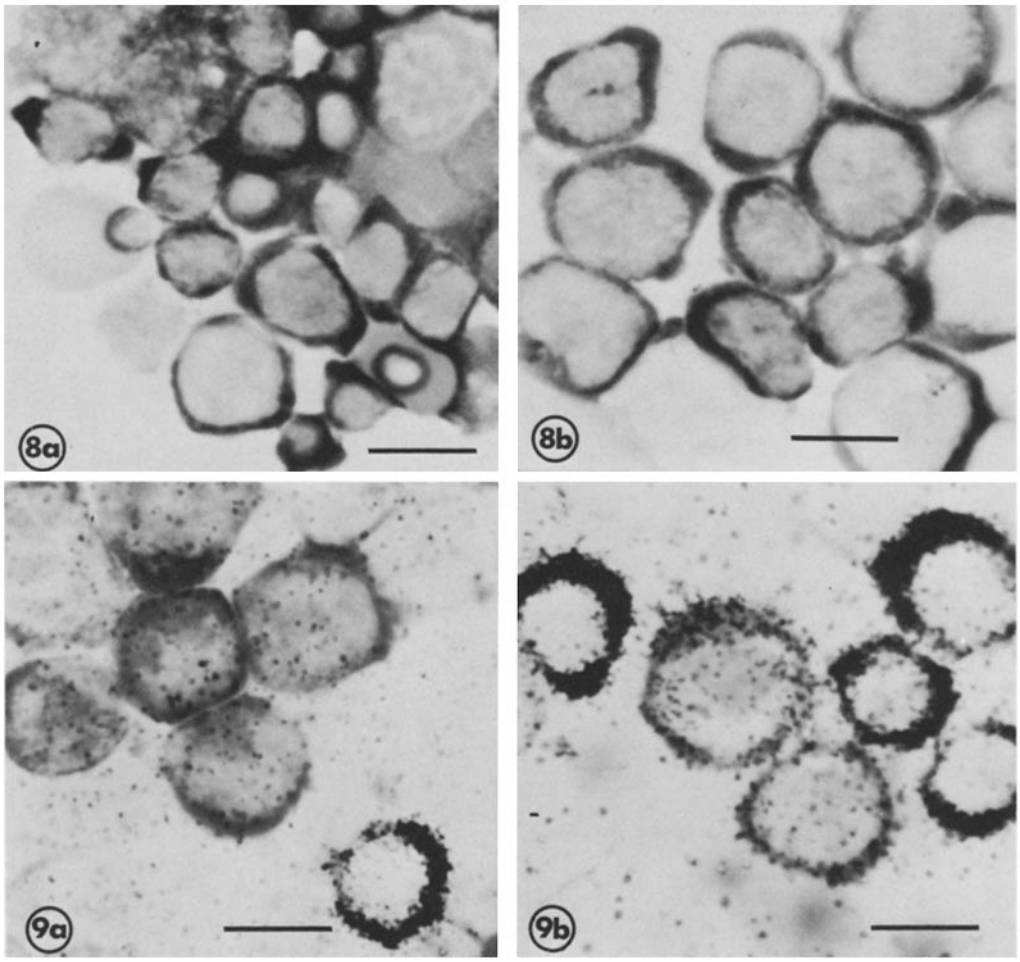


FIGURE 8 14-day fetal liver cells (*a*) before or (*b*) after treatment with antiserum raised against erythrocytes.

FIGURE 9*a–b* *In situ* hybridization of globin cDNA to immature erythroid cells from 14-day fetal liver cells treated with antiserum. Autoradiographic exposure: 6 wk.

ribonuclease-resistant hybrid banding in CsCl at a density of 1.78 (Fig. 11). Melting temperatures (Tm) of usch hybrids were also determined in 0.5 M NaCl, 50% formamide in the presence of excess *E. coli* RNA (Fig. 12). Under these conditions the Tm of globin cDNA/mouse reticulocyte 9S RNA was 68°C; the same Tm was obtained if the hybridization and melting were performed in the presence of 40,000 times excess lymphoma cytoplasmic RNA. However, the Tm of hybrids between globin cDNA and immature erythroid cell cytoplasmic RNA was found to be 5–6°C below that of hybrid between globin cDNA and total erythroid cell cytoplasmic RNA, which was, in turn, 4°C below that of hybrid between globin cDNA and mouse reticulocyte 9S RNA. Evidence that these reductions in Tm are not entirely due to nucleases contaminating the fetal liver cell RNA preparations or to other trivial reasons was obtained by measuring the Tm of hybrid formed between cDNA and 5 times excess 9S RNA in the presence of excess fetal liver RNA. These RNA ratios were arranged so that the amount of "endogenous" globin mRNA in the fetal liver RNA was only one-fifth of the amount of 9S RNA added. Thus the sequences which hybridize to

TABLE II

Effect of Treatment with Antiserum on the Erythroid Cell Types in 14-Day Fetal Liver

	Proerythro-blasts	Erythroblasts			Reticulo-cytes	Nucleated erythrocytes	Hepato-cytes	Other types
		Basophilic	Poly-chromatic	Ortho-chromatic				
Untreated	13	32	23	13	7	3	6	2
After treatment with immune antiserum	51	35	3	2	1	1	4	3

Results from the three experiments described in the text. The proportion of orthochromatic erythroblasts in the preparations of total fetal liver cells is somewhat lower than that reported previously using the trypsinization procedure to obtain single cell suspensions (18).

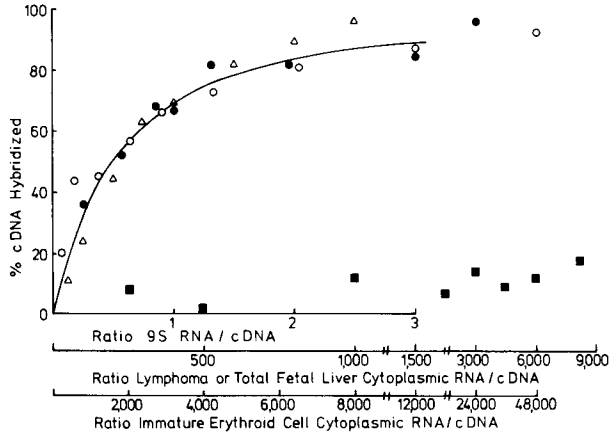


FIGURE 10 Titration of globin cDNA with mouse reticulocyte 9S RNA (Δ), lymphoma cell cytoplasmic RNA (■), 14-day fetal cell cytoplasmic RNA (●), or immature erythroid cell cytoplasmic RNA (○). The values for the proportion of globin cDNA hybridized have been normalized to correct for the percentage of the cDNA which failed to form S1 nuclease-resistant hybrid even after annealing at the highest RNA/cDNA ratios (23%, ●; 27%, ○).

cDNA are mainly derived from the added 9S RNA; yet the hybridization mixture contains a very large excess of fetal liver RNA. The results shown in Fig. 12 A indicate that the Tm of the 9S RNA/cDNA hybrid was not reduced by the presence of excess cytoplasmic RNA from total fetal liver cells; whereas the Tm of 9S RNA/cDNA hybrid was reduced by about 3–4°C by the presence of excess cytoplasmic RNA from immature fetal liver cells. These control experiments thus show that the Tm's of hybrids between cDNA and cytoplasmic RNA from both total and immature fetal liver cells are both about 5–6°C lower than expected by comparison with that of 9S RNA/cDNA hybrid. This means that on average the cytoplasmic RNA sequences from both total and immature erythroid cells which hybridize to globin cDNA contain about 4% base sequence differences relative to α- or β-globin mRNA (12, 13). It is therefore possible that both total and immature erythroid cells from 14-day fetal liver contain globin mRNAs other than those specifying α- and β-globins, for example those specifying z-globin chains, which have 14% amino acid differences to β-chains (14).

The precise concentration of these globin mRNAs in a given cytoplasmic RNA preparation can be estimated by comparing its titration curve with globin cDNA with the corresponding titration curve for globin mRNA. The titration curves only approximate to linear forms due to the size heterogeneity of cDNA (9). The results shown in

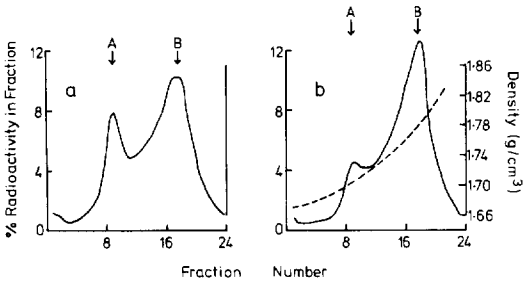


FIGURE 11 Banding of RNA/cDNA hybrids in CsCl. Globin cDNA was annealed with cytoplasmic RNA from (*a*) 14-day fetal liver cells or (*b*) from immature erythroid cells at RNA/cDNA ratios of 1,000 or 40,000, respectively. The annealed mixture (containing about 5,000 cpm. of labeled cDNA) was then treated with ribonuclease and then banded in CsCl. A and B mark the buoyant densities of cDNA and 40% G + C synthetic hybrid respectively. –, density of CsCl.

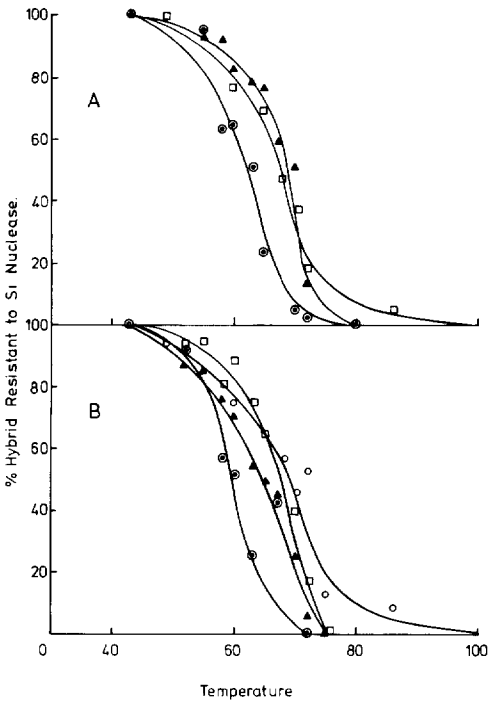


FIGURE 12 Tms of RNA/cDNA hybrids. A. □ Mouse reticulocyte 9S RNA/cDNA hybrid; ▲ hybrid formed between cDNA and a mixture of 5 times excess reticulocyte 9S RNA and 2,000 times excess cytoplasmic RNA from total fetal liver cells; ⊙ hybrid formed between cDNA and 8,000 times excess cytoplasmic RNA from total fetal liver cells. B. □ Mouse reticulocyte 9S RNA/cDNA hybrid; ○ hybrid formed between cDNA and a mixture of 5 times excess reticulocyte 9S RNA and 40,000 times excess cytoplasmic RNA from lymphoma cells; ▲ hybrid formed between cDNA and a mixture of 5 times excess reticulocyte 9S RNA and 10,000 times excess cytoplasmic RNA from immature fetal liver cells; ⊙ hybrid formed between cDNA and 10,000 times excess cytoplasmic RNA from immature fetal liver cells.

Fig. 10 show that the three titration curves for 9S RNA, total and immature erythroid cell cytoplasmic RNA are nearly superimposable if the scale of the abscissae for the three RNA fractions are in the ratios 1:400:3,200. This means that globin mRNAs comprise 0.25% and 0.03%, respectively, of total and immature 14-day fetal liver cell cytoplasmic RNA. The results of three such experiments indicate that the concentration of globin mRNA in immature erythroid cell cytoplasmic RNA is 15–40% (actual values: 24, 13, and 41% of the concentration of globin mRNA in total erythroid cell cytoplasmic RNA). Since the average amounts of cytoplasmic RNA in total and immature erythroid cells are about 6 and 14 pg/cell, respectively, this means that the average globin mRNA content of immature erythroid cells is about 60% of total 14-day liver cells.

It might be argued that the lower content of globin mRNA in immature cells could be due to degradation of globin mRNA during treatment of cells with antiserum and complement. Since our antiserum acts against mature erythroid cells mainly by agglutination, it is possible to recover total erythroid cells treated with complement and antiserum simply by omitting the usual filtration steps. Cells obtained by such a modified procedure have virtually the same distribution of erythroid cell types as the total, untreated, fetal liver cells. In two experiments, the proportion of globin mRNA in cytoplasmic RNA obtained from antiserum- and complement-treated total fetal liver cells, as determined by titration with globin cDNA. Thus, globin mRNA is not specifically degraded during treatment of fetal liver cells with antiserum and complement.

Alternatively, it might be argued that increased degradation of globin mRNA occurs during extraction of cytoplasmic RNA from immature cells, for example due to increased nuclease levels in immature erythroid cells. In order to examine this possibility the globin mRNA content was determined for approximately equal numbers of total or immature cells and for a mixture of the same numbers of total and immature cells (Table III). The globin mRNA content of the mixture of total

TABLE III

Globin mRNA Content of Total and/or Immature Cell Cytoplasmic RNA

Source of RNA	Amount RNA extracted	RNA as globin mRNA
	μg	%
Total cells	156	0.135
Immature cells	322	0.055
Mixed total and immature cells	426	0.079 (Predicted*: 0.081)

* Predicted average for mixture from the individual results for total and immature cells.

and immature cells is exactly the value expected from the individual values (Table III). This indicates that degradation of globin mRNA during extraction of immature cells is not the explanation as to why the globin mRNA content of cytoplasmic RNA from these cells is lower than that for total fetal liver cells.

DISCUSSION

Validity of In Situ Hybridization of Globin cDNA to Measure Cystoplasmic Globin mRNA

The present results show that globin cDNA forms *in situ* hybrids with fixed slide preparations of reticulocytes under conditions in which no such hybridization to nonerythroid mouse cells (LS or lymphoma cells, hepatocytes) occurs. Since reticulocytes hybridized under these conditions develop at least 200 grains in 7 wk, this implies, assuming an autoradiographic efficiency of 10%, that at least 10^{-3} pg of globin cDNA is hybridized per reticulocyte, i.e. about 2% of the input level of cDNA. Since globin cDNA probably comprises transcripts of rather more than half the sequences present in both α- and β-globin mRNAs (8, 9), this means that the *in situ* hybridization detects about 2×10^{-3} pg of α- and β-globin mRNAs per reticulocyte, i.e. about 0.1% of the total cytoplasmic RNA. This is about one-twentieth of the proportion of globin mRNAs in reticulocyte polysomal RNA as estimated in this laboratory by conventional RNA-cDNA hybridization (11). This failure to form *in situ* hybrids with the bulk of the mRNA in fixed preparations of reticulocytes illustrates the difficulties in obtaining quantitative estimates of globin mRNAs by this method.

With the exception of proerythroblasts, most erythroid cells (including basophilic erythroblasts) from 13–15-day fetal liver show extensive *in situ* hybridization to globin cDNA. In view of the problems of quantitation by this method, it is difficult to deduce the exact relative amounts of globin mRNA in basophilic erythroblasts and more mature erythroid cells including reticulocytes. Therefore, in order to confirm this conclusion concerning the presence of globin mRNA in immature erythroid cells, the content of globin mRNA in immature or total erythroid cells was assayed by hybridization of extracted cytoplasmic RNA to globin cDNA in solution in the conventional manner. These results indicate that immature erythroid cells from 14-day fetal livers contain on average about 60% of the globin mRNA content of total 14-day fetal liver cells. This value is too high to be accounted for solely by the mature erythroid cells surviving the treatment with antiserum, since the proportions of polychromatic erythroblasts, orthochromatic erythroblasts, and reticulocytes are all reduced six- to seven-fold by such treatment (Table II). Therefore, these results obtained independently of *in situ* hybridization confirm that globin mRNAs are present in significant amounts in at least some immature erythroblasts. However, as discussed above (see Results), the globin mRNAs synthesized in immature erythroid cells may not comprise solely the adult α- and β-globin chains.

Relevance to Mechanism of Control of Globin Synthesis

Whereas globin mRNA synthesis appears not to have been initiated or stabilized in the proerythroblast, basophilic erythroblasts and more mature members of the erythroid cell series accumulate globin mRNA. The fact that globin mRNA accumulates in basophilic erythroblasts is an important finding since hemoglobin cannot be detected to any significant extent until a later stage of erythroid cell development, i.e. in the polychromatic erythroblast. Since in fetal liver in vivo the time for transition of a basophilic erythroblast to a polychromatic erythroblast is a few hours (15), this implies that there may be some translational control of globin synthesis at this stage of fetal liver development. Using a sensitive assay, Terada et al. (3) have shown that an immature erythroid

cell preparation (obtained by treatment of 13.5-day fetal liver cells with antiserum against mature red blood cells and containing not more than 7% hemoglobinized erythroblasts) synthesizes α- and β-globin chains at less than 6% of the rate for total fetal liver cells. The same workers showed that the proportion of translatable globin mRNA in the 6–16S RNA fraction from these immature cells (determined by the ability to direct α- and β-globin synthesis in the Krebs ascites cell-free system) was less than 5% of the corresponding value for total fetal liver cells. Taken together with the present evidence, this would appear to indicate that although globin mRNA sequences are synthesized in immature (basophilic) erythroblasts, these sequences do not specify α- and β-globins or cannot be translated until modified in some way. Since globin mRNA from which polyadenylic acid (PolyA) sequences have been removed with polynucleotide phosphorylase can still be translated (16), this modification of globin mRNA in immature erythroblasts which prevents translation cannot be lack of the polyA sequences. Isak and Karsai (17) have presented evidence to suggest that although globin mRNA is present in polysomes of immature (rabbit) erythroblasts, it cannot be translated in the immature cell cytoplasm.

Early Erythroid Cell Development

During the 11th–12th day of fetal liver development, the proportion of proerythroblasts decreases from about 80% to 20%. Most cells in the proerythroblast population do not contain globin mRNA. However, during development from the 11th to 13th days, an increasing proportion of the proerythroblast population contains detectable amounts of globin mRNA, as judged by *in situ* hybridization. This suggests that during maturation of the liver, an increasing proportion of the proerythroblasts begin to transcribe the globin gene. This is analogous to the situation in vitro where treatment of fetal liver cells with erythropoietin increases the proportion of proerythroblasts which contain globin mRNA (D. Conkie, manuscript in preparation). This might explain why, during the 11th–12th day of fetal liver development, characteristically small basophilic cells appear which contain large amounts of globin mRNA. This cell is most probably a small basophilic erythroblast derived directly by division and differentiation of the cells in the proerythroblast population in which expression of the globin gene has been initiated.

The critical transition which results in the globin gene being activated may be connected with the fact that hemoglobin synthesis is no longer dependent on combined RNA synthesis after the 12th–13th day of fetal liver development. This change is probably caused by some change in the microenvironment of the fetal liver (5); Cole and Tarbutt (2) have concluded from cell kinetic data that this change is due to a dramatic reduction at about the 14th day in the number of erythropoietin-sensitive stem cells entering the proerythroblast compartment. For this reason, *in situ* hybridization studies were performed of immature cells from 14-day fetal liver obtained by treatment with antiserum. In fact, the proportion and appearance of cells synthesizing globin mRNA in this immature cell fraction (Fig. 8 *a–b*) are the same as described for cells from 11-day livers (Fig. 6 *a–c*). Thus, the stabilization of hemoglobin synthesis with respect to continued RNA synthesis after the sudden inhibition at the 13th–14th day of stem cell differentiation could be explained simply as follows, assuming that globin mRNA is relatively stable: on the 11th day, most proerythroblasts, which are actively proliferating, have still to undergo the critical transition enabling synthesis of globin mRNA to occur, and therefore the capacity to synthesize hemoglobin subsequently is reduced by inhibition of RNA synthesis by treatment with actinomycin D; by the 14th day, most liver cells have undergone the critical transition and therefore inhibition of RNA synthesis is effective in preventing subsequent globin synthesis.

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