Analysis of the Ratio of α - to β -Globin and Globin Messenger Ribonucleic Acid Content of Fractionated Rabbit Erythroid Bone-Marrow Cells

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The ratio of α - to β -globin mRNA was measured by hybridization of a constant amount of highly purified α - or β -globin cDNA (complementary DNA) with increasing amounts of RNA in the range up to 20% cDNA hybridization, where an essentially linear reaction is obtained. Statistical analysis indicates that the ratio of α - to β -globin can be measured within a maximal error of ± 0.3 and in most cases is better than ± 0.15 . Under these conditions there is no significant deviation from the ratio of 1.3 in the α - to β -globin mRNA ratio of RNA isolated from erythroid cells rich in pronormoblasts through to reticulocytes. If the ratio of α - to β -globin mRNA exceeded 1.7 or was less than 0.9 in pronormoblasts, it would be detected in these experiments. The overall globin mRNA content increases to a maximal value in the fractions rich in basophilic normoblasts of 30000–50000 molecules/cell. However, the accuracy of these determinations is not as great as for the ratio determinations, and no significant deviations were seen except in the cells rich in pronormoblasts, which contained less globin mRNA than the later stages.

The mature mammalian erythrocyte contains equimolar amounts of α - and β -globin chains associated as the $\alpha_2\beta_2$ -tetramer, and in the reticulocyte and bone marrow synthesis of the globin chains is equal. However, it appears that in many species there are duplicate α -chain genes but only single β -chain genes active in adult tissues (see review by Hunt, 1976), and, although the overall rate of globin synthesis is related to the total concentration of globin mRNA in the cell (Ramirez *et al.*, 1975; Stewart *et al.*, 1976), there appears to be an imbalance in the relative amount of α - to β -globin mRNA (Lodish, 1971). In the reticulocyte, Lodish & Jacobsen (1972) have shown that the balanced α - to β -globin chain synthesis is maintained, even though there is a 40% higher rate of initiation of β -globin-chain synthesis and an equal elongation rate of the two polypeptide chains, predicting a 40% excess of α -globin mRNA in polyribosomes (Lodish, 1971). This α -globin mRNA excess has been confirmed in mouse, rabbit and sheep reticulocytes (Phillips *et al.*, 1977).

There are conflicting reports from studies of two of the erythropoiesis model systems which indicate that the final α - to β -globin mRNA ratio observed in reticulocytes is only attained at the end of erythro-

poiesis. Orkin *et al.* (1975) observed a 4-fold excess of α -globin mRNA over β -globin mRNA in mouse erythroleukaemic cells shortly after induction by dimethyl sulphoxide. This ratio changed to close to unity as erythropoiesis proceeded. The opposite trend was reported by Cheng & Kazazian (1976) in the anaemic mouse, where an excess of β -globin mRNA was observed in spleen cells collected at the earliest times after phenylhydrazine treatment, and the β - to α -globin mRNA ratio increased to a value of 2 when the erythropoietic activity was maximal. In the spleen study, the mRNA species were analysed by fractionation on oligo(dT)-cellulose followed by electrophoresis on polyacrylamide gels in formamide buffers, a system that gives insufficient resolution or identification for accurate work. Phillips *et al.* (1977) have reported that preliminary results obtained by hybridization with purified cDNA species indicate a constant α - to β -globin mRNA ratio of 1.25 during splenic erythropoiesis.

Other workers have shown that the ratio of α - to β -globin mRNA in the induced mouse erythroleukaemic cells depends on the nature of the inducer, and in most cases that the ratio changes during induction (Nudel *et al.*, 1977). However, they find an excess of β -globin mRNA at early times after dimethyl sulphoxide induction, in contrast with the α -globin mRNA excess reported by Orkin *et al.* (1975). It is therefore still unclear whether differential expression of globin genes occurs *in vivo*.

We have addressed this question by determining the α - to β -globin mRNA ratios in different erythroid-cell fractions obtained directly from the rabbit. A

Abbreviations used: cDNA, complementary DNA; SDS, sodium dodecyl sulphate; Pipes, 1,4-piperazine-diethanesulphonic acid.

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highly purified β -globin mRNA and its cDNA (Mezl & Hunt, 1978) were used to prepare an α -globin cDNA probe having a similar degree of purity. Bone-marrow cells obtained from anaemic rabbits were rapidly fractionated on bovine serum albumin gradients, and α - to β -globin mRNA ratios were determined in each fraction by hybridization with the respective cDNA. Our results show that, although the relative amount of each globin mRNA is increased in the more differentiated cell types, a constant α - to β -globin mRNA ratio of 1.3 is maintained throughout differentiation in all fractions. It is estimated that the method used can detect a 30% variation in the ratio in the earliest cell fractions.

Experimental

Cell fractionation

New Zealand White rabbits were made anaemic by five daily subcutaneous injections of 2.5% neutralized phenylhydrazine (0.3 ml/kg), and the animals were killed on the seventh day (haematocrit 18–25%; reticulocytosis 85–90%). Each animal's bone marrow was then separately disaggregated by a single passage through a nylon mesh sieve (approx. 200 μ m) and layered on a linear bovine serum albumin (fraction V; Armour Pharmaceutical, Phoenix, AZ, U.S.A.) density gradient (ρ 1.06–1.09 g/ml) at 20°C (Borsook *et al.*, 1969). After 30 min of centrifugation at 1500g, the most prominent band (fraction-3 cells), the large but more diffuse band below it (fraction-4 cells) and the pellets were collected from each gradient and pooled for further analysis. The two small closely spaced bands (fractions 1 and 2) seen just above the main band were pooled from three gradients and re-run on a second identical gradient for 45 min to obtain a better separation from the fraction-3 cells. This method yields approx. 2×10^8 fraction-1 cells, 9×10^8 fraction-2 cells, 30×10^8 fraction-3 cells, 20×10^8 fraction-4 cells, and 35×10^8 pellet cells from three animals. At least 1000 cells, stained with MacNeal's tetrachrome (Harleco, Gibbstown, NJ, U.S.A.), were counted in each fraction. Blood cells were stained with 1% Brilliant Cresyl Blue in 0.14 M-citrate/0.14 M-NaCl for reticulocyte determination.

RNA extraction

Osmotic lysis with rat liver supernatant. The lysis was carried out with rat liver supernatant containing ribonuclease inhibitor as described by Grau & Favelukes (1968), except that the supernatant was desalted by passing through a Sephadex G-25 column equilibrated with 0.01 M-Tris/HCl/0.015 M-KCl/0.0015 M-MgCl₂ (pH 7.6).

Detergent lysis. To obtain total cytoplasmic RNA, cells were disrupted with Triton X-100 lysis me-

dium [0.25 M-sucrose/0.01 M-Tris/HCl/0.015 M-KCl/0.0015 M-MgCl₂/0.4% Triton X-100 (pH 7.6)], and the nuclei were removed by centrifugation at 10000g for 10 min. One ml of this medium was used to lyse 10^8 cells. The nuclei were washed with an equal volume of lysis medium. The combined supernatants were extracted with phenol to obtain total cytoplasmic RNA.

Phenol extraction. Lysates were made to 4% with sodium *p*-aminosalicylate and extracted with an equal volume of water-saturated phenol/*m*-cresol/hydroxyquinoline (100g of redistilled phenol, 14 ml of redistilled *m*-cresol, 0.1g of hydroxyquinoline, 300 ml of water). The supernatant was saved and the phenol and interphase were re-extracted with an equal volume of 0.1 M-sodium acetate, pH 5.0. The combined supernatants were adjusted to 0.3 M-NaCl and extracted with 0.5 vol. of the phenol solution. The RNA was precipitated from the final supernatant with 2 vol. of 95% ethanol.

Cytoplasmic RNA determination

The combined supernatants obtained by detergent lysis of a known number of cells were adjusted to 0.3 M-NaCl, and the nucleic acids were precipitated with 2 vol. of ethanol. After being washed with ethanol, the dried precipitates were hydrolysed for 20 min in 0.3 M-NaOH at 70°C. The solutions were chilled, adjusted to 1 M in HClO₄ and centrifuged to remove DNA and proteins. The absorbance of the supernatant at 260 nm was used to determine the RNA content (1 mg = 30.3 absorbance units). DNA in nuclei or whole cells after ethanol precipitation was determined in the HClO₄-washed pellet after hydrolysis in 1 M-HClO₄ at 70°C for 20 min (1 mg = 30.3 absorbance units).

Preparation of standard globin mRNA

Purified β -globin mRNA, containing 3–5% of α -globin mRNA, and partially purified α -globin mRNA, containing 7–17% of β -globin mRNA, were prepared from RNA isolated from the polyribosomes of anaemic rabbit reticulocytes by β -globin mRNA dimerization on sucrose gradients in 0.1 M-NaCl (Mezl & Hunt, 1978). Cellulose containing covalently bound β -globin cDNA sequences was synthesized with reverse transcriptase by the method of Wood & Lingrel (1977) by using oligo(dT)-cellulose (900 mg; type T₂; Collaborative Research, Waltham, MA, U.S.A.) as a primer and 600 μ g of purified β -globin mRNA as a template. The amount of β -globin cDNA synthesized was estimated as 40 μ g by hybridization with excess globin mRNA. A column containing this cellulose was then used to remove β -globin mRNA sequences from the partially purified α -globin mRNA (see, e.g., Table 1).

Table 1. Purification of α -globin mRNA by chromatography on β -globin-cDNA-cellulose

Partially purified α -globin mRNA was hybridized to the column for 5 h at 68°C in 0.1 M-NaCl/0.01 M-Tris/HCl/1 mM-EDTA/0.2% SDS (pH 7.4). The column was washed with 10 ml of this buffer and then eluted with 6 ml of water at the same temperature. Fractions of volume 0.5 ml were collected. Total RNA per fraction was determined from the A_{260} (1 mg = 25 absorbance units) and the β -globin mRNA content of each sample was determined by hybridization with β -globin cDNA (see Fig. 2 and the text).

	Amount (μ g)	β -Globin mRNA (%)	β -Globin mRNA (ng)
Sample applied to column	180	8.1	14.6
Retained on column	12	83	Approx. 10
Recovered in eluate	171	1.1	1.9

Preparation of α - and β -globin cDNA

[5- 3 H]dCTP-labelled cDNA (37×10^6 d.p.m./ μ g) was synthesized with reverse transcriptase by using 0.4 mM-unlabelled triphosphates, 0.1 mM-[5- 3 H]dCTP (20.9 Ci/mmol; ICN, Irvine, CA, U.S.A.) and 100 μ g of actinomycin D/ml as previously reported (Mezl & Hunt, 1978). This isolation procedure, using base hydrolysis followed by phenol extraction and chromatography on Sephadex G-100, gave cDNA preparations with weight-average molecular weights of 150 000–190 000 and number-average molecular weights of 90 000–120 000, determined on alkaline sucrose gradients.

In Expt. 1 the β -globin cDNA used was fractionated on an alkaline sucrose gradient, only those fractions with a mol.wt. between 100 000 and 255 000 being used. This preparation had a calculated weight-average mol.wt. of 167 000 and a number-average mol.wt. of 153 000. The weight-average mol.wt. of the total sample was 149 000 and number-average mol.wt. 110 000.

In the case of the α -globin cDNA a combination of fractions from three preparations was used. The original preparations had weight-average mol.wts. of 104 000, 100 000 and 125 000, and the number-average mol.wts. of 76 000, 66 000 and 88 000 respectively. The fractions combined in approximately equal amounts had weight-average mol.wts. of 141 000, 135 000 and 157 000 with number-average mol.wts. of 130 000, 125 000 and 144 000 respectively. In addition, a second preparation of α -globin cDNA was used that was of higher purity. The fraction used had a weight-average mol.wt. of 170 000 and a number-average mol.wt. of 153 000, and the original preparation had a weight-average mol.wt. of 140 000 and number-average mol.wt. of 58 000. The sucrose gradients used in these preparations are shown in Fig. 1. Similar fractionation of α - and β -globin cDNA was used for the other experiments.

The hybridization curve in Fig. 2(a) shows that the purified α -globin mRNA is faithfully transcribed into a cDNA of similar purity, thereby permitting the preparation of α -globin cDNA, containing 1–3% of β -globin cDNA, that is even less contaminated than the β -globin cDNA prepared by the β -globin-

mRNA-dimerization method (Mezl & Hunt, 1978).

Hybridization

Hybridizations were performed in silicone-treated capillary tubes containing 50 μ l of 0.3 M-NaCl/10 mM-Pipes (pH 6.8)/0.1% SDS/2 mM-EDTA, 10 μ g of *Escherichia coli* tRNA/ml, 750–1300 c.p.m. of [3 H]-cDNA (0.06–0.11 ng) and various amounts of RNA per sample. Reaction mixtures were denatured at 100°C for 10 min, then hybridized for 5 h at 68°C. The reaction mixtures were washed into 1 ml of 30 mM-sodium acetate (pH 4.5)/1 mM-ZnSO₄/10 μ g of calf thymus DNA/ml, and digested at 45°C with 1 μ g of *Aspergillus oryzae* S1 nuclease (Miles Laboratories, Elkhart, IN, U.S.A.) for 45 min. After the addition of 150 μ g of carrier yeast RNA, the hybrids were collected on Whatman GF/C filters by precipitation with 10% trichloroacetic acid and dried with ethanol. The dried filters were incubated for 30 min at 70°C in 200 μ l of 0.5 M-HCl, neutralized and 3 H was assayed in a mixture of toluene scintillator [0.5% 2,5-diphenyloxazole/0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene] and Beckman Bio-Solv BBS-3 (5:1, v/v) with an efficiency of 33%. Total input cDNA was determined by omitting S1 nuclease from several samples.

Only the linear portion of the standard curve (up to 20% hybridization) was used for quantification, since this area of the curve offers the highest sensitivity, does not require any subjective curve-drawing and permits an estimate of the analytical variation. In each assay appropriate amounts of purified standard mRNA and sample RNA were hybridized with each cDNA (see Figs. 2b and 2c). The values for each mRNA calculated from the standard regression line were transformed into percentage mRNA, and the mean and standard error of the mean determined. The ratios were calculated and the standard error (s.e.) of the ratio was calculated by the formula:

$$\frac{\bar{x}}{\bar{y}} \sqrt{\frac{\text{S.E.}_x^2}{\bar{x}^2} + \frac{\text{S.E.}_y^2}{\bar{y}^2}}$$

The degrees of freedom for the ratios were assumed to be $N-1$, when N is the number of determinations for either α - or β -globin mRNA. The t statistic used

was the method of Welch (1947), and for the null hypothesis:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\text{S.E.}_1^2 + \text{S.E.}_2^2}$$

with degrees of freedom (f^1) defined by:

$$\frac{1}{f^1} = \frac{c^2}{f_1} + \frac{(1-c)^2}{f_2}$$

Fig. 1. Sedimentation of globin [³H]CTP-labelled cDNA preparations in alkaline sucrose gradients

The samples were sedimented on a 5–20% (w/w) sucrose gradient in 0.2M-NaOH/0.8M-NaCl/0.002M-EDTA at 5°C for 17h at 40000rev./min in the Spinco 50.1 rotor. Direction of sedimentation is from left to right. The arrow marks the position of the 360-nucleotide marker, and the bar the fractions that were combined for use in hybridization. (a)–(c) α -Globin cDNA samples which were bulked and used in Expt. 1; (d) β -globin cDNA used in Expt. 1.

when

$$c = \frac{S.E._1^2}{S.E._2^2 + S.E._1^2}$$

At least eight samples of RNA were hybridized at different RNA/cDNA ratios for each determination and the mean and standard deviation of each determination were calculated.

Fig. 2. Hybridization analysis of α - and β -globin mRNA

(a) Hybridization of purified α - and β -globin mRNA to α -globin cDNA. [3 H]CTP-labelled cDNA was made from the α -globin mRNA purified as shown in Table 4. Samples containing 1300 c.p.m. were hybridized with the original α -globin template (○) or a purified β -globin mRNA (●). The point of intersection of the linear portion of the β -globin mRNA curve (12–20%) with the ordinate indicates that there is less than 2% β -globin cDNA present in the α -globin cDNA. The difference in slopes between the α - and β -globin-mRNA-driven curves indicate that the β -globin mRNA contains 3–4% α -globin mRNA. (b) Standard curves for α - and β -globin mRNA analysis. α -Globin cDNA (800 c.p.m.) was hybridized with α -globin mRNA (○), and β -globin cDNA (750 c.p.m.) was hybridized with β -globin mRNA (●). The lines shown were calculated by linear regression. For the α -globin cDNA the regression line had a slope of 106.6%/ng of RNA, intercept 3.48%, R^2 0.978 and s.e. of estimate of 1.23%; for the β -globin the corresponding parameters were 100.6%/ng of RNA, 3.08%, 0.973 and 1.45%. (c) Analysis of total RNA isolated from two cell fractions. The α - and β -globin cDNA from species (b) were hybridized with total cytoplasmic RNA isolated from total reticulocytes (△, α), (▲, β) and fraction-3 cells (○, α), (●, β). The lines drawn through these points are for illustration. The globin mRNA amounts were calculated from the regression lines determined for the standard curves as described in the text.

In general, the degrees of freedom in our experiments are close to 7 for two ratios and may be as high as 13. Since the maximum s.e. found in our ratios is 0.18, it would be expected that a difference between ratios of 0.6 would be significant at the 5% level.

Results

Isolation of cells at early stages of erythropoiesis

Bovine serum albumin density gradients were used to obtain large numbers of fractionated bone-marrow cells at different stages of development as described in the Experimental section (Borsook *et al.*, 1969). The procedure is relatively fast and all cell fractions are ready for extraction after 2.5 h. After incubation in tissue culture medium, more than 80% of the fraction-3 cells were viable after 8 h, as judged by Trypan Blue exclusion. The cell composition of each fraction is shown in Table 2. Fractions 1 and 2 are enriched in very immature cells (pronormoblasts, large basophilic erythroblasts), and fractions 3 and 4 contain a higher proportion of more mature erythroid cells (small basophilic and polychromatophilic erythroblasts). No orthochromatic erythroblasts were found in these marrow samples, which is expected for animals with severe anaemia and indicates that the cell population is homogeneous (Borsook, 1964).

Extraction and analysis of RNA

Two methods of cell lysis were used to obtain the cytoplasmic RNA, which accounts for 85% of the RNA in these cells. The detergent (Triton X-100) method has the advantage that it lyses all of the cells and gives close to 70% yields of RNA after phenol extraction (Table 3). When RNA extracted from each cell fraction was analysed for α - and β -globin mRNA by hybridization with purified cDNA, the proportion of globin mRNA is seen to increase from fractions 1 to 3 (Table 4). It is apparent that there is a low yield of globin mRNA in cell fraction 4 and the pellet. This low yield is most likely due to ribonuclease activity during detergent lysis, since the pellet does contain a large amount of cell debris and damaged cells. In two other experiments when fraction 4 was extracted by the osmotic-lysis method in the presence of rat liver supernatant the proportion of globin mRNA was the same as in cell fraction 3, which is consistent with the cell composition (Table 2). Although it is clear that the use of osmotic lysis in the presence of rat liver supernatant gives superior preparations of undegraded RNA, it was unclear whether the early erythroblasts would be lysed by this method. This is in part borne out by the fact that only 50% of the cytoplasmic RNA of fraction-1

Table 2. Cell composition of bone-marrow cells fractionated on bovine serum albumin gradients
Anaemic bone-marrow cells were fractionated on bovine serum albumin gradients as described in the text. Slices were prepared and 1000 cells were counted for each fraction. The results of two different experiments are shown. The non-erythroid cells category consists of 2-5% white cells (8% in fractions 1 and 2, 15% in pellet) up to 1.5% free nuclei (3% in fraction 4, 6% in pellet) and up to 5% reticular cells (10% in unfractionated bone marrow, 9% in fraction 1, 23% in the pellet). The underlined values represent major components (averaging more than 20%) in the cell fractions.

Cell fraction	Percentage of cell type													
	Basophilic erythroblast							Polychromatophilic erythroblast						
	Pronormoblast		Large		Small		Reticulocyte	Non-erythroid		Total erythroid				
	Expt. 1	Expt. 2	Expt. 1	Expt. 2	Expt. 1	Expt. 2		Expt. 1	Expt. 2		Expt. 1	Expt. 2	Expt. 1	Expt. 2
1	28	27	24	27	11	10	11	18	21	82	79			
2	13	20	29	13	20	23	14	13	17	87	83			
3	0.7	2.4	11	14	27	23	18	6	7	94	93			
4	0.2	2.0	6.4	15	24	26	14	11	8	89	92			
Pellet	0.8	0.2	0.8	1.8	7	10	23	44	25	56	75			
Unfractionated	6.0	4.2	15	10	19	30	17	15	12	85	88			

Table 3. *Nucleic acid content of cell fractions*

The measurement of RNA/cell was by three methods. In column 1, the RNA in the Triton X-100 lysates was estimated by alkaline hydrolysis after precipitation with ethanol as described in the Experimental section. In the second column the amount of RNA and the percentage yield extracted after Triton X-100 lysis is given and in the third column the amount of RNA and percentage yield extracted after osmotic lysis is given (values for column 4 are means $\pm$ S.E.M. for three determinations). The ratios of A_{280}/A_{260} and A_{230}/A_{260} in all of the samples in columns 2 and 3 were between 0.44 and 0.51. The mean $\pm$ S.E. for the A_{280}/A_{260} ratio was 0.47 ± 0.004 (10) and that for the A_{230}/A_{260} ratio 0.46 ± 0.008 (9). Abbreviation: N.D., not determined.

	Cytoplasmic RNA			DNA
	1 (pg/cell)	2 [pg/cell (and % yield) extracted after Triton X-100 lysis]	3 [pg/cell (and % yield) extracted after osmotic lysis]	4 (pg/cell)
Erythroid cell fraction				
1	13.1	9.5 (73)	6.6 (50)	8.0 ± 0.2
2	13.4	9.0 (67)	5.9 (44)	9.5 ± 0.7
3	3.6	2.1 (58)	1.6 (44)	4.6 ± 0.1
4	2.3	1.6 (70)	1.2 (52)	5.3 ± 0.3
Pellet	1.1	0.5 (45)	0.4 (36)	7.7 ± 0.6
Reticulocyte	0.95	N.D.	N.D.	—

and -2 cells is extractable by osmotic lysis as compared with 70% by detergent lysis, but essentially the same fraction of α - and β -globin mRNA was found in the RNA extracted by osmotic lysis from fractions 1 and 2, as is shown in Table 3. Moreover, the 28S rRNA isolated from cells lysed by the detergent method was degraded when heat-denatured and sedimented on low-salt/sucrose gradients, whereas the 28S rRNA from osmotic lysates remained intact under the same conditions.

It is likely that the difference in yields of RNA is due to preferential lysis of the more mature erythroblasts by osmotic lysis and that the Triton X-100 lysis allows extraction of RNA from all cells. However, it should not be overlooked that the Triton X-100-lysis technique will also extract polyribosomes from endoplasmic reticulum, which may be present in the erythroblasts. However, since little difference is found in analysis of the α - and β -globin mRNA especially in fraction-1 and -2 cells, the method of osmotic lysis in the presence of rat liver supernatant appears to be preferable because of its superior protection from ribonuclease degradation.

The accuracy of the determination of α - and β -globin mRNA was checked in two of the experiments by assaying samples of each RNA for total globin mRNA with an equimolar mixture of α - and β -globin cDNA. In the experiments shown in Table 4 this assay shows that the sum of the α - and β -globin mRNA determinations (Table 4, column 3) is equal to the total globin mRNA in the sample (Table 4, column 4). The reticulocyte polyribosomal RNA contains about 1.5% of α - and β -globin mRNA in a similar ratio to that found by others, whereas the supernatant fraction obtained after centrifugation from the polyribosomal fraction is significantly

enriched in α -globin mRNA (Jacobs-Lorena & Baglioni, 1972).

The determinations show a 30-fold difference in relative mRNA content between the immature cell fraction and the circulating reticulocytes. However, since there is a significant decrease in cell size and RNA content per-cell during differentiation (Table 3), the calculated number of globin mRNA molecules per cell is very similar in each cell fraction (Table 4), especially when the proportion of erythroid cells in each fraction is taken into account.

Globin mRNA ratios in different cell fractions

Since a ratio requires the combination of two different determinations, it is by necessity subject to more variation than are individual determinations from the same sample. The advantage of the computation procedure used in these assays (see the Experimental section) is that it permits calculation of the errors of each determination and therefore the error of the ratios. Table 5 shows the results obtained in these different experiments. In each experiment the α - to β -globin mRNA ratio is similar in all cell fractions and comparable with the values reported for blood reticulocytes. By applying Student's *t* test for small samples the small differences in ratios observed between different cell fractions in each experiment are not significant at the 5% confidence level, the exception being the ratios for the RNA species from subcellular fractions of the reticulocyte in Expt. 1, which are significantly different from the other samples assayed in that experiment. Although there is an apparent systematic difference between experiments, most notably between Expts. 1 and 3, statistical analysis shows that, except for the averages of all cell types, this difference is not significant at the 5%

Table 5. Ratios of α - to β -globin mRNA in fractionated bone-marrow cells

Fractionated bone-marrow cells were pooled from three anaemic rabbits for each experiment. The cell compositions for Expts. 1 and 2 are shown in Table 2. RNA was extracted from the fractionated cells by the detergent-lysis procedure in Expt. 1 and by the osmotic-lysis procedure in Expts. 2 and 3. Analyses were carried out as described in Table 2, and the means $\pm$ S.E. and estimated degrees of freedom (DF) obtained for each ratio are shown.

RNA analysed	Expt. 1			Expt. 2			Expt. 3			Mean of three experiments $\pm$ S.E.M.
	Mean	S.E.	DF	Mean	S.E.	DF	Mean	S.E.	DF	
Bone marrow										
Fraction 1	1.47 $\pm$ 0.19		(8)	1.30 $\pm$ 0.09		(8)	1.20 $\pm$ 0.14		(4)*	1.32 $\pm$ 0.08 (2)
Fraction 2	1.34 $\pm$ 0.19		(7)	1.22 $\pm$ 0.07		(7)	1.24 $\pm$ 0.15		(4)*	1.27 $\pm$ 0.04 (2)
Fraction 3	1.40 $\pm$ 0.10		(16)	1.31 $\pm$ 0.16		(5)	1.16 $\pm$ 0.11		(4)	1.29 $\pm$ 0.07 (2)
Fraction 4	1.41 $\pm$ 0.11		(15)	1.15 $\pm$ 0.13		(7)	1.08 $\pm$ 0.14		(1)	1.21 $\pm$ 0.10 (2)
Pellet	1.25 $\pm$ 0.10		(16)	1.27 $\pm$ 0.16		(5)	—			[1.26 $\pm$ 0.01 (1)]
Blood										
Total reticulocyte RNA	1.31 $\pm$ 0.09		(7)	1.58 $\pm$ 0.10		(4)	—			[1.44 $\pm$ 0.14 (1)]
Reticulocyte polyribosomal RNA	0.94 $\pm$ 0.10		(13)	1.15 $\pm$ 0.07		(10)	0.98 $\pm$ 0.04		(1)	1.02 $\pm$ 0.06 (2)
Reticulocyte post-polyribosomal RNA	3.46 $\pm$ 0.46		(9)	—			—			—
Average of all cell types	1.36 $\pm$ 0.03		(5)	1.30 $\pm$ 0.06		(5)	1.17 $\pm$ 0.03		(3)	

* In Expt. 3 the early fractions were not re-run on a second gradient and therefore contained a smaller percentage of pronormoblasts and large basophilic erythroblasts (fraction 1, 27%; fraction 2, 22%).

level. It is therefore concluded that there is no detectable difference in α - to β -globin mRNA ratio between the cell fractions, and that a ratio of about 1.3 is maintained in rabbit bone-marrow cells during erythropoiesis.

Discussion

Specific mRNA analysis

We justify the use of a linear approximation of the cDNA hybridization curve up to 20% hybrid using a fixed cDNA concentration and differing RNA concentration for a set time on two grounds. First, the equation:

$$\frac{H}{D_0} = \frac{R_0[1 - e^{-(R_0 - D_0)kt}]}{D_0 - R_0 [e^{-(R_0 - D_0)kt}]}$$

(Bishop, 1972), which describes the reaction we have used, when simulated by using 8–10 points up to fraction of cDNA in hybrid $H/D_0 = 0.2$ with a cDNA concentration (D_0) of 0.06 ng/50 μ l, $k = 1.98 \times 10^3 \text{ M}^{-1} \cdot \text{s}^{-1}$ and $t = 18000 \text{ s}$ (5 h), with a varying concentration of RNA (R_0), these being essentially the conditions we use, has a slope of 172%/ng of RNA, with an intercept of 1% and correlation coefficient (R^2) of 0.998. This is indistinguishable from a straight line. Interestingly, if the mRNA and cDNA have two components, the slope of the line and, incidentally, the half-reaction point, varies by approx. 10% for each 5% of impurity. We consider that the use of a 50% hybridization point (see Phillips *et al.*, 1977) and the same titration method used

here is less accurate, since we can use all of our data in the linear regression, whereas the assessment of the 50% hybridization point is not adaptable to the same type of statistical analysis, or at least has not been analysed in such a manner.

Second, since in each experiment the cDNA is standardized with purified full-size globin mRNA and the slope of the regression line is determined, no correction is necessary for changes in kinetics due to the size of the cDNA, since this is taken into account with the calibration curve. However, we have taken care to use cDNA that was no less than half-length by fractionation of the cDNA on alkaline sucrose gradients (Fig. 1).

One factor that cannot be monitored by this technique (or any other) is the possible presence of a new β -globin mRNA with a 10% sequence difference from normal β -globin mRNA (Clissold *et al.*, 1977). We have checked the 'melting' curves of the β -globin cDNA hybrids with RNA from fractions of cells from anaemic and non-anaemic rabbits by thermal chromatography on hydroxyapatite, but can find no significant difference in 'melting' temperatures. We have therefore not been able to confirm the presence of a new β -globin mRNA in our cells.

Ratio of α - to β -globin mRNA

The results reported here clearly show that, contrary to the situation in the mouse spleen system or the Friend erythroleukaemic cell line, the globin mRNA ratio does not vary severalfold during erythropoiesis in rabbit bone marrow. However, the resolution of the cell types and the precision of the

analytical procedures would not permit the detection of small changes. The statistically significant difference found between the reticulocyte polyribosomal RNA and the cell fractions in Expt. 3 indicates that a difference of at least 0.3 in ratio must exist to be detectable by this method. However, the use of the worst-case standard errors (see the Experimental section and Table 4) indicates that the maximum difference in ratios detectable is 0.6. It may be estimated that in some fractions (see Tables 2 and 4) up to 20% of the assayable globin mRNA is due to reticulocyte contamination of the fraction. Combining these two limitations it is estimated that a difference in ratio would only be detectable in the early erythroid cells in fraction 1 if these cells had an α - to β -mRNA ratio greater than 1.7 or less than 0.9.

The estimates of the variation of the ratios of the globin mRNA species are not significantly different between experiments. Since the standard curves for the α - and β -globin mRNA were the same within an experiment, the errors of measurement of mRNA and RNA concentrations are constant, and therefore, in a given experiment, these errors do not affect the detection of variations in the globin mRNA ratios between different cell fractions. However, these errors do become of major importance when absolute amounts of globin mRNA are considered. Hybridizations of cDNA in mRNA excess carried out with purified preparations of globin mRNA, although giving good fit to single-component pseudo-first-order reaction kinetics, display up to a 4-fold difference in the rate of hybridization determined from these fits. The method used in this work, hybridization of increasing RNA/cDNA ratios for a fixed period of time, is more accurate but still shows differences of up to 50% between the slopes of standard curves. However, in order to estimate the error of the RNA content per cell one must consider other errors, in particular, the error of the cell counts obtained by using a haemocytometer. This error can be estimated from the DNA content found in the same experiments as the RNA determinations. The DNA/cell was found to be 7.1 ± 0.74 pg (mean $\pm$ S.E.M. for three determinations on six different cell fractions). Thus the 95% confidence limits for this determination are $\pm 27\%$.

The standard error of the individual triplicate determinations (Table 3) is much lower, ranging from 2 to 8%, which indicates that the error does not come from the method of measurement, but most likely from errors in the cell-counting procedure. Some increase in accuracy may be obtained by using the DNA analysis as a measure of cell content and these results are also shown in Table 4. Bearing these considerations in mind, it is estimated that the measurement of globin mRNA molecules per cell shown in Table 4 cannot be determined more accurately than within a factor of 2, so that the best con-

clusion from these data is to state that the maximal number of globin mRNA molecules per cell is between 30000 and 50000 molecules/cell in the basophilic erythroblasts and polychromatophilic erythroblasts, and it must be somewhat lower in circulating reticulocytes, owing to degradation of mRNA. It appears that the fraction rich in pronormoblasts contains a significantly lower amount of globin mRNA than the more mature cells and this must be due to a significantly lower globin mRNA content in pronormoblasts. These results are consistent with the prediction that globin mRNA synthesis is constant in each nucleated cell stage once it starts and the concentrations of globin mRNA found in the various cell fractions (Table 4) correspond to those calculated from such a model (Table 2 of Hunt, 1974). It should be noted that the numbers of globin mRNA molecules per cell calculated for the earlier fractions are about three times higher than the values reported by Clissold *et al.* (1977) for similar rabbit bone-marrow cell fractions obtained by unit gravity sedimentation. Since in both studies the globin mRNA sequences constitute the same percentages of the total proerythroblast and basophilic erythroblast RNA, the primary cause of the discrepancy in the number of molecules per cell is the higher amount of RNA per cell found by our analysis (Table 3). Our value of 14 pg of RNA/cell for the early erythroid fractions does not appear to be too high, since the same value has been reported for immature mouse erythroid cells (Harrison *et al.*, 1974); in any case the minimum concentration would be 9 pg/cell. It would therefore appear that either the differential treatment of the animals (1 day of rest after phenylhydrazine treatment in our case, compared with 3 days of rest) or the different methods of cell fractionation may account for this difference, but it should not be overlooked that the difference may be caused by a technical one in methods of analysis of cellular RNA content. A further possibility is that there are two cohorts of cells present in marrow used by Clissold *et al.* (1977) represented by the late cells controlled by the high degree of anaemia and the early cell fraction represented by the recovering non-anaemic conditions. This possibility is demonstrated in an earlier publication from the same group (Denton & Arnstein, 1973), where a marked difference in fractionation profiles was shown between highly anaemic marrow and the 3-day-rested marrow.

Under these conditions, if there was a different β -globin mRNA activated by the induction of anaemia in a manner analogous with the sheep β^A - to β^C -globin switch (Benz *et al.*, 1978), it would be predicted that the new β -globin mRNA found by Clissold *et al.* (1977) is, in fact, the normally produced β -globin mRNA and would explain why, under our conditions where a high degree of anaemia is maintained, we can find only one β -globin mRNA.

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