A Specific Dimerization of Rabbit β -Globin Messenger Ribonucleic Acid

By VASEK A. MEZL and JOHN A. HUNT

Department of Genetics, John A. Burns School of Medicine, University of Hawaii,
Honolulu, HI 96822, U.S.A.

(Received 13 February 1978)

Rabbit globin mRNA, when layered in low salt on 0.1M-NaCl/sucrose gradients, separates into two peaks of material. Translation of these two RNA fractions in the wheat-germ cell-free system, hybridization against globin complementary DNA (cDNA) and cross-hybridization against cDNA species prepared from each fraction show that the first peak sedimenting at 10S is a α -globin mRNA and the second peak, sedimenting at approx. 15S, is β -globin mRNA. The sedimentation rate of the β -globin mRNA is concentration-dependent. By changing concentration and pH, it is indicated that in low-salt β -globin mRNA adopts a conformation that leads to specific, but weak, self-dimerization during centrifugation in 0.1M-NaCl. This property permits rapid preparation of intact and relatively pure α - and β -globin mRNA species.

Globin mRNA can be prepared in large amounts from the reticulocytes of anaemic rabbits (Housman *et al.*, 1971); however, further separation into α - and β -globin mRNA remains a problem. Originally α -globin mRNA-enriched preparations were obtained from the reticulocyte postribosomal supernatant (Gianni *et al.*, 1972; Jacobs-Lorena & Baglioni, 1972) and β -globin mRNA-enriched samples were prepared by fractionation of the polyribosomes of rabbit reticulocytes treated with L-O-methylthreonine (Temple & Housman, 1972). When the separation of globin mRNA species by polyacrylamide-gel electrophoresis in formamide was reported (Gould & Hamlyn, 1973) it became, and has remained, the method of choice, since it gives separation of rabbit (Hamlyn & Gould, 1975), mouse (Morrison *et al.*, 1974) and human (Nudel *et al.*, 1977) globin mRNA species. These gels give low resolution because of the heterogeneous length of the poly(A) terminus (Maniatis *et al.*, 1976; Vournakis *et al.*, 1975). The method is very sensitive to experimental parameters (Kazazian *et al.*, 1974), and the small amounts of globin mRNA that can be applied to the gels are recovered in poor yield (Maniatis *et al.*, 1976; Nudel *et al.*, 1977).

The present paper reports a specific aggregation of rabbit β -globin mRNA that can be conveniently used to obtain larger amounts of intact α - and β -globin mRNA. Since non-specific aggregation of rabbit globin mRNA to other RNA species under non-denaturing conditions has been reported (Kabat,

1975) the sequence specificity of this separation is confirmed by RNA excess hybridization against cDNA prepared from each peak. Analysis of the change in sedimentation rate of the aggregated β -globin mRNA indicates that this change is consistent with an equilibrium between a dimer and a monomer formed by interaction of a few base pairs between the mRNA molecules.

Experimental

Isolation of reticulocytes and preparation of polyribosomes

Adult New Zealand White rabbits were injected intraperitoneally with 0.3 ml of 2.5% (w/v) phenylhydrazine/kg for 6 days. On day 7 the blood was collected by cardiac puncture into heparinized tubes (haematocrit approx. 15%). Cells were packed by centrifugation at 1600g at 4°C for 10 min and the buffy coat was removed by three washings with NKM buffer (0.14M-NaCl/5 mM-KCl/5 mM-MgCl₂). The cells (greater than 90% reticulocytes) were then lysed with an equal volume of cold water. Cell debris was removed by centrifugation at 15000g for 10 min and the polyribosomes were isolated by a further centrifugation at 165000g and 2°C for 1 h.

Isolation of poly(A)-containing RNA

Poly(A)-containing RNA was isolated by a modification of the method of Krystosek *et al.* (1975). The polyribosomal pellet was washed with a small volume of binding buffer (0.12M-NaCl/0.01M-Tris/HCl/1 mM-EDTA, pH 7.5) and dissolved at room temperature (23°C) in 20 ml of the same

Abbreviations used: cDNA, complementary DNA; R_{ot} , product of concentration of nucleotides (M) $\times$ time (s); SDS, sodium dodecyl sulphate; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; Pipes, 1,4-piperazinediethanesulphonic acid.

buffer containing 0.5% SDS and 0.1 mg of proteinase K/ml (EM Biochemicals, Elmsford, NY, U.S.A.). This solution was diluted to an absorbance of no more than 50 A_{260} units with binding buffer and applied to a column (60 mm $\times$ 9 mm) containing 1 g of oligo(dT) (type T-2; Collaborative Research, Waltham, MA, U.S.A.). After washing with binding buffer until the eluate had an absorbance of less than 0.05 A_{260} unit, the retained RNA was eluted with low-salt buffer (0.01 M-Tris/HCl/1 mM-EDTA, pH 7.4). After adding NaCl to 0.1 M, the RNA was precipitated with 2 vol. of ethanol; this procedure gave a yield of approx. 1 mg of poly(A)-containing RNA from three rabbits (1.5–2% of the A_{260} unit applied to the column). The concentration of RNA is determined in 0.1 M-sodium phosphate buffer, pH 7.0, at A_{260} by using an $A_{1\text{cm}}^{1\%}$ of 250.

Sucrose-gradient centrifugation

RNA dissolved in 0.5 ml of low-salt buffer was heated at 60–65°C for 10 min, quickly cooled in ice/water and layered on to 17 ml 5–20% (w/w) linear sucrose gradients made up either in low-salt buffer (0.01 M-Tris/HCl/1 mM-EDTA, pH 7.4) or in high-salt buffer (0.1 M-NaCl/0.01 M-Tris/HCl/1 mM-EDTA, pH 7.4). The gradients were centrifuged for 24 h at 4°C and 27000 rev./min in a Beckman SW 27.1 rotor. Fractions (0.52 ml) were collected by upward displacement with 30% (w/w) sucrose through a 2 mm cuvette (ISCO model UA u.v. analyser). The appropriate fractions were pooled and precipitated with ethanol as described above. The 10S RNA obtained from low-salt sucrose gradients was used as the unfractionated globin mRNA standard.

Sedimentation values were initially determined with 4S tRNA, 10S globin mRNA and 18S rRNA as markers. For the α - and β -globin mRNA separations the α -globin mRNA was used as a 9.5S internal marker for each gradient. The assumption of linearity of s value in the 10–15S region appeared valid, since the 18S rRNA peak was always within 0.6S (s.d.) of its calculated position. Molecular weights of 202000 and 227000 were used for α -globin mRNA and β -globin mRNA (Gould & Hamlyn, 1973) and these were related to s value by the empirical relationship: $S = 0.0177 \times \text{mol.wt.}^{0.514}$ (obtained by using molecular weights of the 18S and 9.5S RNA as 700000 and 202000 respectively). Analysis of the relative amounts of the two peaks of material was done by assuming Gaussian distributions, and computer fitting the total area and the mean, s.d. and fraction of each peak to the data given by the absorbance profile by using the non-linear least-squares method of Wolberg (1967).

Assay of globin mRNA by the wheat-germ cell-free system

Wheat germ (General Mills, Vallejo, CA, U.S.A.) was prepared as described by Marcu & Dudock (1974) with the difference that 1500 A_{260} units of wheat-germ extract were layered on the Sephadex column and the centrifugation after elution from this column was avoided. Instead, the eluate was either used directly or quickly frozen in solid CO_2 /acetone and stored at -70°C .

The complete system contained, in a final volume of 50 μl : 25 μl of wheat-germ extract (120–160 A_{260} units), 20 mM-Hepes, pH 7.7, 100 mM-KCl, 3 mM-magnesium acetate, 2 mM-dithiothreitol, 1 mM-ATP, 0.1 mM-GTP, 8 mM-phosphocreatine, 100 μg of creatine kinase/ml (rabbit muscle, type 1; Sigma, St. Louis, MO, U.S.A.), each of the 19 unlabelled amino acids at 39 μM and the labelled amino acid at 2–4 μM ($[\text{U-}^{14}\text{C}]$ leucine, 330 mCi/mmol, or $[\text{4,5-}^3\text{H}]$ leucine, 55 Ci/mmol; Amersham-Searle, Arlington Heights, IL, U.S.A.). For preparative assays (samples for gels) 3 μCi of ^3H -labelled or 0.3 μCi of ^{14}C -labelled amino acid was used per assay; for analytical purposes, 0.5 μCi of ^3H -labelled or 0.1 μCi of ^{14}C -labelled amino acid was used. After incubation at 22°C for 1 h, 10 μl samples were analysed by precipitation on strips of Whatman 3MM paper (Loening, 1967) and the remainder was frozen. The radioactivity on the filter papers was determined in a toluene scintillator [0.5% 2,5-diphenyloxazole, 0.05% 1,4-bis-(5-phenyloxazol-2-yl)benzene in toluene].

With the unfractionated standard globin mRNA prepared on low-salt gradients, the assay was linear up to 2 μg . This range was used to prepare samples for the assay. When saturating amounts of globin mRNA were used, a preferential translation of the β -globin mRNA was found as has been reported for the reticulocyte cell-free system (Krstosek *et al.*, 1975). Variability between preparations resulted in an incorporation of 20–90 pmol of the labelled amino acid with 1 μg of unfractionated standard globin mRNA; controls without added mRNA incorporated less than 1 pmol per assay.

Analysis of α - and β -globins

The globins were separated by electrophoresis in acid urea/Triton X-100: 100 mm $\times$ 4.5 mm 7.5% polyacrylamide disc gels (0.1% bisacrylamide, 0.12% ammonium persulphate and 0.5% $\text{NNN}'\text{N}'$ -tetramethylenediamine), polymerized in 5% acetic acid/3.75 M-urea/0.5% Triton X-100, were pre-electrophoresed at 2.5 mA per gel for 4 h with 5% acetic acid in the buffer compartments. Cathodic electrophoresis of 100 μl samples containing 10 μl of preparative wheat-germ assay and 30 μg of marker

globin in 5% acetic acid/10% sucrose was carried out for 2h at 2mA per gel in fresh 5% acetic acid. Gels were stained for 10min with 0.1% Amido Black in 5% acetic acid/25% methanol and destained by washing in 7.5% acetic acid/5% methanol. The band positions were noted and the gels cut into 5mm slices. The slices were dissolved by heating at 60°C with 0.3ml of 30% H_2O_2 in capped scintillation vials. After cooling, 4.5ml of a mixture of toluene scintillator and Triton X-100 (6:7, v/v) was added for radioactivity counting with an efficiency of 78% for ^{14}C and 14% for 3H . Comparison with α - and β -globin separated by CM-cellulose column chromatography shows that the faster moving band is α -globin and the slower band is β -globin.

Synthesis of globin cDNA species

This was based on the procedure of Efstratiadis *et al.* (1976). A 50 μ l reaction mixture consisted of 50mM-Tris/HCl, pH8.3, 60mM-NaCl, 6mM-magnesium acetate, 10mM-dithiothreitol, 100 μ g of bovine serum albumin/ml, 50 μ g of actinomycin D (Sigma)/ml, 0.4mM-dATP, 0.4mM-dGTP and 0.4mM-dTTP, 0.1mM-[5- 3H]dCTP (20.9Ci/mmol; ICN, Irvine, CA, U.S.A.), 20 μ g of oligo(dT₁₂₋₁₈)/ml (P-L Biochemicals Milwaukee, WI, U.S.A.), 120 μ g of template mRNA/ml and 150 units of AMV RNA-dependent DNA polymerase/ml (Office of Program Resources and Logistics, Viral Cancer Program, Viral Oncology Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, MD, U.S.A.; 1 unit adds 1nmol of dTMP to a poly(rA·dT₁₂₋₁₈) template in 10min at 37°C). After incubation for 90min at 37°C, the template was hydrolysed with 0.1M-NaOH/8mM-EDTA at 65°C for 20min (total vol. 0.5ml). At this stage about 30% of the initial radioactivity was acid-precipitable. The sample was neutralized and extracted with an equal volume of water-saturated phenol. The interphase was re-extracted with an equal volume of water and the pooled aqueous layers were applied to a column (240mm $\times$ 10mm) of Sephadex G-100. A sharp peak of material was eluted with water at the void volume, which contained about a half of the initial acid-precipitable material. Fractions containing radioactivity of 1000c.p.m./ μ l or greater were pooled, stored at -60°C and used for hybridization. The specific radioactivity of the cDNA was estimated to be 37×10^6 d.p.m./ μ g.

Hybridization of cDNA with an excess of RNA

Hybridizations were performed in 100 μ l siliconized glass capillary tubes at 68°C in 0.3M-NaCl/10mM-Pipes/0.1% SDS/2mM-EDTA, pH6.8. Each tube contained at least a 10-fold molar excess of RNA and 2000c.p.m. of cDNA. Samples were

denatured at 100°C for 10min and, after the appropriate incubation time at 68°C (5min to 24h), the tubes were rapidly cooled in ice/water. The contents were then added to 4ml of 100mM-NaCl/30mM-sodium acetate (pH4.5)/1mM-zinc sulphate/10 μ g of denatured calf thymus DNA/ml. The solution was divided into two 1.9ml portions, to one of which was added 4 μ g of *Aspergillus oryzae* S1 nuclease (Miles Laboratories, Elkhart, IN, U.S.A.) and the other was used as a control. Samples were incubated at 45°C for 45min, then 150 μ g of yeast RNA was added and the hybrids were collected on Whatman GF/C filters by precipitation with cold 10% (w/v) trichloroacetic acid and washed with 5% trichloroacetic acid. The filters were dried with ethanol, incubated for 30min at 70°C in 200 μ l of 0.5M-HCl, then neutralized and counted for radioactivity in a mixture of toluene scintillator and Beckman Bio-Solv BBS-3 (5:1, v/v). Under these conditions about 95% of the cDNA in the controls lacking RNA was degraded. The experimental data were fitted to eqn. (1) (Bishop *et al.*, 1974):

$$D_H = B + \sum F_i(1 - e^{-K_i R_0 t}) \quad (1)$$

where D_H is the fraction of cDNA in hybrid, B is the fraction of cDNA hybrid that is present as a background, F_i is the fraction of cDNA hybridized in the i th transition and K_i is the apparent rate constant for this transition, by using the non-linear least-squares program described by Wolberg (1967).

Results

Discrete aggregation of rabbit globin mRNA on 0.1M-NaCl/sucrose gradients

RNA prepared from reticulocyte polyribosomes by the proteinase K/SDS method and oligo(dT) chromatography migrates as the expected 10S peak on low-salt gradients with an occasional trace of contaminating 18S RNA (Fig. 1a). However, when layered in low salt on a 0.1M-NaCl gradient, the same material, on the same gradient run, gives an additional somewhat broader peak at approx. 15S (Fig. 1b). This additional peak of material is still obtained when the RNA is extracted with phenol before centrifugation, or when proteinase K is not used, or when the sample is not heat-treated, or when the sample is heated in SDS before layering. If the polyribosomes are extracted by phenol/0.5% SDS, pH9 (Brawerman *et al.*, 1972), before oligo(dT) chromatography, there is an increase (2.5-5% of the A_{260} units applied to the column) in RNA retention on the column, even if the sample is heat-treated before chromatography. The increased retention appears to be due to the presence of a very large peak of 18S rRNA in these phenol-extracted samples; however, a discrete peak of 15S RNA is still present.

Fig. 1. Fractionation of globin mRNA by sucrose-gradient centrifugation

Heat-treated reticulocyte polyribosomal poly(A)-containing RNA was centrifuged on 5–20% (w/w) sucrose gradients at 27000 rev./min for 24 h at 4°C in different buffers (see the Experimental section). (a) RNA layered in low salt on a low-salt gradient. (b) The same RNA (330 μ g in 0.5 ml) layered in low salt on a high-salt gradient.

The appearance of the 15S peak appears therefore to be dependent on the salt content of the gradient. A peak of 15S RNA was collected from a gradient (Fig. 1b) and run on a low-salt gradient, where it

gave only a peak of 10S RNA (Fig. 1a); this peak of 10S RNA was collected and re-run on a high-salt gradient, where it again migrated as a discrete peak of 15S RNA (gradients not shown). To obtain a discrete peak of 15S RNA the sample must be applied to the gradient in water or in low-salt buffer; if it is applied in high-salt buffer, the amount of RNA in the 10S peak decreases and a broad peak of higher S-value RNA with a variable shape appears.

10S and 15S RNA code for different globins

The RNA species in the 10S and in the 15S peaks are as active as standard globin mRNA in the wheat-germ cell-free-system assay. In the sample shown in Fig. 2(a), the RNA collected from the peak of 10S RNA (fraction A) was 95% as active as standard globin mRNA and the RNA collected from the 15S RNA (fraction B) had a translational efficiency of 117%. However, analysis of the cell-free system products on acid urea/Triton X-100 gels shows that each peak of material is enriched in a different globin translational activity. The translation of the 10S RNA (fraction A, Fig. 2a) gives 2.2 times more α -globin than β -globin, whereas the translation of the 15S RNA (fraction B, Fig. 2a) gives 2.4 times more β -globin than α -globin. It should be mentioned that with this assay, standard globin mRNA gives an α - β -globin ratio of 1–1.5 to 1, whereas mRNA species prepared from the reticulocyte polyribosomal supernatant, which are enriched in α -globin mRNA (Gianni *et al.*, 1972), give an α - β -globin ratio of 3–5 to 1.

Pooled 10S and 15S RNA fractions from several gradients, such as Fig. 2(a), when re-run on high-salt gradients give predominant 10S (Fig. 2b) and 15S (Fig. 2c) RNA with only small amounts of the second component. RNA obtained from these peaks shows high purity in the wheat-germ cell-free-system assay: Fig. 3(a) shows that translation of the re-run 10S RNA (Fig. 2b, fraction C) gives an α - β -globin ratio of 6 and Fig. 3(b) shows that translation of the re-run 15S RNA (Fig. 2c, fraction D) gives a greater than 8-fold β - α -globin ratio.

These results (summarized in Table 1) show that, in terms of translation, the 10S RNA contains α -globin mRNA activity and the 15S RNA contains β -globin mRNA activity. To differentiate these from the 10S RNA observed for total globin mRNA on low-salt gradients (Fig. 1a), the high-salt gradient 10S RNA (Figs. 2a, 1b) will be referred to as α -10S.

α -10S and 15S RNA are intact α -globin mRNA and intact β -globin mRNA respectively

To obtain a higher degree of purification the trailing half of a peak of α -10S RNA and the leading half of a peak of 15S RNA were collected

Fig. 2. Separation of α -10S and 15S globin mRNA

Heated RNA was layered on high-salt gradients as described in Fig. 1. (a) First fractionation of 340 μ g of reticulocyte poly(A)-containing RNA. (b) Second fractionation of 288 μ g of pooled RNA samples from region A in (a). Region C is designated 10S α -globin mRNA. (c) Second fractionation of 400 μ g of pooled RNA samples from region B in (a). Region D is designated 15S globin mRNA.

Fig. 3. Separation of α - and β -globin chains synthesized in the wheat-germ cell-free system

The products from the cell-free system were separated on acid urea/Triton X-100 polyacrylamide gels as described in the Experimental section. The positions of α - and β -globin markers are indicated. Slices of 6mm were assayed, except that the top slice was only 1mm and was removed before staining the gel. (a) Products of translation of 1 μ g of 10S α -globin mRNA (see Fig. 2b) with 3.7 μ M-[4,5- 3 H]leucine; 10 μ l of the 50 μ l assay was run on the gel. (b) Products of translation of 0.95 μ g of 15S globin mRNA (see Fig. 2c) with 3.7 μ M-[4,5- 3 H]leucine; 2 μ l of the 50 μ l assay was run on the gel.

from a gradient such as shown in Fig. 2(a). These samples were analysed by transcribing limiting amounts of these RNA species with RNA-dependent

DNA polymerase. On a weight basis, both of these RNA species are as active as standard globin mRNA in this assay, thereby indicating that each species has

Table 1. Analysis of α -10S and 15S globin mRNA by translational analysis

The globin mRNA fractions after one or two rounds of sucrose-gradient centrifugation in high salt were translated in the wheat-germ cell-free system and the products fractionated by polyacrylamide-gel electrophoresis in Triton X-100/acetic acid/urea (for details see the Experimental section) and the ratio of [^{14}C]leucine in the α -globin and β -globin bands was measured.

RNA fractionation	Ratio of α -globin/ β -globin synthesis		
	α -10S RNA	15S RNA	Unfractionated RNA
First gradient	2.2	0.42	1–1.5
Second gradient	5.7	0.12	—

a 3'-poly(A) terminus comparable with globin mRNA. Since the α -10S RNA and 15S RNA were respectively 95 and 108% as active as unfractionated globin mRNA in the wheat-germ assay, these experiments indicate that these mRNA species have intact 5'- and 3'-ends.

Analysis of the fractionated and unfractionated globin mRNA species, by hybridization in an excess of RNA with cDNA to unfractionated globin mRNA, is shown in Fig. 4. Both the 15S RNA (Fig. 4a) and the 10S RNA (Fig. 4c) hybridize to the same total extent as the unfractionated RNA (Fig. 4b). The low total hybridization of the cDNA is due to the use of [^3H]dTTP label in the cDNA (Ross *et al.*, 1973), since use of [^3H]dCTP label in subsequent experiments results in a high total percentage hybridization. Both the 10S and 15S RNA hybridizations are bimodal and can be fitted to theoretical curves by using eqn. (1) and two K values. This indicates the presence of two RNA components, which are both present in the unfractionated globin mRNA, but in different concentrations.

A more complete analysis of the cross-contamination of the fractionated mRNA was obtained by making cDNA to these two fractions and hybridizing these with α -10S and 15S globin mRNA in an excess (Fig. 5). The $R_{0.4}$ values obtained by the curve-fitting procedures are shown in Table 2. From these data the purity of the α -10S mRNA fraction is 93% α -globin mRNA and of the 15S mRNA fraction 96% β -globin mRNA, assuming that the single components found in high yield in these fractions are α - and β -globin mRNA. This is shown by the wheat-germ cell-free-system assay, where the α -10S mRNA fraction synthesizes approx. 90% α -globin chains and the 15S mRNA 95% β -globin chains.

β -Globin mRNA migrates as a dimer-monomer equilibrium on high-salt gradients

Although the peak of β -globin mRNA has been referred to as 15S, close examination shows that its position on the gradient is dependent on the amount of β -globin mRNA applied to the gradient. Fig. 6 shows the effect of layering different amounts of β -globin-mRNA-enriched samples to high-salt gradients. In Fig. 6(a), where the β -globin mRNA concentration is high (218 μg of total RNA, 196 μg of calculated β -globin mRNA), the β -globin mRNA is at 14.2S, essentially the value expected for a dimer of β -globin mRNA (14.4S). However, in Fig. 6(b), where less β -globin mRNA was applied to the gradient (40 μg of total RNA, 28 μg of calculated β -globin mRNA), the β -globin mRNA sedimentation rate is now 12.4S. This trend is observed over the entire β -globin mRNA concentration range studied (Fig. 7). The same relationship was observed for 30h centrifugation runs. The β -globin mRNA position is independent of the amount of α -globin mRNA or 18S rRNA in the sample.

Since the S value of the β -globin mRNA peak decreases with β -globin mRNA concentration and has a maximum value corresponding to a dimer, this indicates that a dimer-monomer equilibrium is responsible for the change in S value. For such an equilibrium if only monomer (M) and dimer (D) are present, the observed S value (S_0) will represent a weight-average according to the equation (Kirshner & Tanford, 1964, eqn. 4):

$$S_0 = (1 - p)S_D + pS_M \quad (2)$$

where p is the weight fraction of monomer, S_M and S_D the S values of monomer and dimer respectively. The dimer $\rightleftharpoons$ monomer dissociation can be described by the relation (Kirshner & Tanford, 1964, eqn. 5):

$$K = (M)^2/D = \frac{4p^2 C_0}{F(1 - p)} \quad (3)$$

where K is the equilibrium constant (molar) under the given conditions, F the molecular weight of the dimer and C_0 the concentration in g/l. By combining eqns. (2) and (3), we obtain the expected relationship between the observed S value and the dimerizing macromolecule concentration:

$$S_0 = \left(\frac{S_M - S_D}{8C_0} \right) [-KF + (K^2 F^2 + 16KFC_0)^{1/2}] + S_D \quad (4)$$

S_M and F were set at 10.09 and 454000 (see the Experimental section) and the data of Fig. 7 were fitted to eqn. (4) by using a non-linear least-squares program (Wolberg, 1967). The best-fit curve (root mean square $\pm 0.31\text{S}$; Fig. 7) has an equilibrium

Fig. 4. Hybridization analysis of fractionated globin mRNA with cDNA to unfractionated globin mRNA. The cDNA was prepared with [methyl- ^3H]dTTP and had a calculated specific radioactivity of 14.7×10^6 d.p.m./ μg . The arrows mark the $R_0 t_{1/2}$ transitions as determined by curve-fitting procedures. (a) Hybridization with excess of 15S globin mRNA (root mean square of curve fit is 3.3% with $R_0 t_{1/2}$ of 3.8×10^{-4} and 1.37×10^{-2}). (b) Hybridization with an excess of homologous unfractionated globin mRNA (root mean square of curve fit is 3.7% with $R_0 t_{1/2}$ of 5.6×10^{-4}). (c) Hybridization with an excess of α -10S globin mRNA (root mean square of curve fit is 2.4% with $R_0 t_{1/2}$ of 4.5×10^{-4} and 9.7×10^{-3}).

Fig. 5. Hybridization of fractionated globin mRNA species with their corresponding cDNA species. The arrows mark the $R_0t_{1/2}$ transitions as defined in Table 2. (a) cDNA to 10S α -globin mRNA used in Fig. 4(c) hybridized with an excess of 15S globin mRNA ($\circ$) and of 10S α -globin mRNA ($\bullet$). (b) cDNA to 15S globin mRNA used in Fig. 4(a) hybridized with an excess of 15S globin mRNA ($\bullet$) and 10S α -globin mRNA ($\circ$).

constant of 54 ± 18 nm and a dimer sedimentation value of 14.6 ± 0.3 S (expected 14.4 S). It should be noted that when the expected relationship between S_M and S_D is included in eqn. (4) [$S_M = S_D \times (0.5)^{0.514}$, see the Experimental section] the curve fit is similar (root mean square = 0.33 S) and the calculated K and S_D show only small differences ($K = 0.1 \pm 0.03 \mu\text{M}$, $S_D = 15.1 \pm 0.05$).

From the K value obtained, one can calculate a free energy (ΔG) of -36 kJ for the monomer-dimer reaction under these conditions ($\Delta G = RT \cdot \ln K$). Since free energies of RNA base-pairing are available (-5.1 kJ/A·U base-pair, -10.0 kJ/G·C base-pair; Tinoco *et al.*, 1961), we can estimate that the sequence involved in β -globin mRNA dimerization is, as expected, small, involving from 5 to 9 base-pairs. It should be noted that since these data were obtained

with sucrose gradients, these calculations give minimum values because the RNA concentration (C_0) used in eqn. (4) and in Fig. 7 is that observed at the β -globin mRNA peak at the end of the run, and therefore is the lowest value obtained during the centrifuge run. When the initial β -globin mRNA concentration layered on the gradients is used in the calculations, only small differences are obtained, K becomes $0.24 \pm 0.07 \mu\text{M}$ and the sequence involved in dimerization is 5 to 8 base-pairs.

β -Globin mRNA adopts a dimerizable conformation in the layering solution

To obtain well-resolved β -globin mRNA dimerization the conditions under which the sample is layered on the gradient are crucial. It has been stated

Table 2. Analysis of the RNA-hybridization curves of an excess of 10S and 15S mRNA with cDNA to the 10S and 15S mRNA

10S and 15S mRNA fractions were purified by two rounds of sucrose-gradient sedimentation and cDNA was prepared to the fractionated mRNA. The hybridization curves are shown in Fig. 5. The percentage of α - and β -globin mRNA is obtained by comparison of the $R_{0t_{\frac{1}{2}}}$ values of the 15S globin cDNA with those of α -10S globin mRNA and β -globin mRNA to determine the contamination of β -globin mRNA in the α -10S globin mRNA. The comparison of the $R_{0t_{\frac{1}{2}}}$ values of the α -10S globin cDNA with those of α -10S globin mRNA and 15S-globin mRNA gives the contamination of α -globin mRNA in the 15S globin mRNA.

Globin mRNA	$10^4 R_{0t_{\frac{1}{2}}}$ cDNA		α -Globin mRNA in mRNA (%)	β -globin mRNA in mRNA (%)
	α -10S	15S		
α -10S	2.1 ± 0.3	57 ± 6	92.7 ± 1.0	7.3 ± 1.0
15S	45 ± 5	4.1 ± 0.4	4.8 ± 0.8	95.2 ± 0.8

that if a polyribosomal mRNA sample is layered in high-salt buffer, specific β -globin mRNA dimerization is lost and generalized aggregation occurs.

The pH of the layering solution affects the resolution of the two peaks of RNA. Freeze-dried mRNA samples were dissolved and heat-treated in solutions of different pH values, then layered on the usual high-salt gradients. Table 3 shows that the S value of the β -globin mRNA peak decreases markedly as the pH of the layering solution decreases, going from a value of 12.6S for the pH8.4 layering solution to a value of 11.2S for the pH5.7 layering solution. The computer resolution of these profiles indicates that the amount of RNA in each peak does not change (Table 3, column 3). This suggests that the observed differences in S value are due to a change in the dissociation constant of the dimer (calculated in column 4 of Table 3). An interpretation of these results is that as the β -globin mRNA regains some of its secondary structure after heat treatment, at lower pH it adopts conformations that are less favourable for dimerization.

Discussion

We have described conditions under which rabbit globin mRNA sediments as two peaks on sucrose gradients. The separated peaks are also shown to be specific for translation into either α - or β -globin chains (Fig. 3). The confirmation that this fractionation was real rather than apparent, such as by interaction of a protein not removed by proteinase K treatment or phenol extraction, is shown by the

Fig. 6. Change in sedimentation of the 15S globin mRNA with concentration

Sedimentation conditions were as for Fig. 1(b). The broken lines show the computer-resolved Gaussian peaks. (a) mRNA (218 μ g) calculated as 90% β -globin mRNA. (b) mRNA (40 μ g) calculated as 70% β -globin mRNA.

hybridization analysis with cDNA made to RNA from the different fractions.

As far as possible mechanisms are concerned, our data show that the association of the β -globin mRNA is consistent with a weak bimolecular association. It

Fig. 7. Plot of the concentration of β -globin mRNA versus its sedimentation coefficient in high-salt sucrose gradients

The gradient profiles using different concentrations of β -globin mRNA were resolved by Gaussian analysis as shown in Fig. 6 and the S value of the major (β -globin mRNA) peak of RNA was determined relative to the α -globin mRNA contaminant, with an S value of 9.5 for this peak. The concentration values are peak concentrations determined from the sucrose-gradient profile. The curve is the best fit to eqn. (4) and has a root-mean-square value of 0.31S.

Table 3. Dissociation constants calculated for β -globin mRNA layered at different pH values

mRNA fractions collected between the α - and β -mRNA peaks from several gradients were pooled and freeze-dried as 174 μ g samples. These were dissolved in 600 μ l of buffer of the indicated pH (for pH 8.4, 7.4, 6.4: 10 mM-Tris/HCl/1 mM-EDTA; for pH 5.7: 1.1 mM-sodium acetate/0.1 mM-EDTA), heat-treated and run on the usual pH 7.4 0.1 M-NaCl gradients. Profiles were resolved by computer as described in the Experimental section and K values were calculated from eqn. (4).

pH of layering solution	Apparent S	β mRNA/ α + β mRNA from curve analysis	K (μ M)
8.4	12.6 $\pm$ 0.03	0.58 $\pm$ 0.004	0.113
7.4	12.4 $\pm$ 0.04	0.60 $\pm$ 0.006	0.145
6.4	11.6 $\pm$ 0.08	0.63 $\pm$ 0.02	0.49
5.7	11.2 $\pm$ 0.14	0.60 $\pm$ 0.04	0.83

is not possible in our kinetic analysis to distinguish a pure bimolecular reaction from a bimolecular reaction in which a small cofactor is involved, such as is found in the dimerization of antibodies. It would appear that the latter case is unlikely, since the β -globin mRNA in the 15S RNA can be re-

centrifuged in low-salt gradients, where it sediments as a monomer at 10S, precipitated from the 10S region and re-centrifuged in high salt, where the 15S peak is re-formed. Considering the weak association found, it is unlikely that a low-molecular-weight cofactor would survive the dissociation sedimentation. It is thus likely that the association is between the β -globin mRNA molecules themselves. The proposed mechanism predicts that it will be difficult to remove small amounts of β -globin mRNA from the α -globin mRNA.

This is generally found; however, it must be emphasized that because of the overlap of the two peaks, the degree of purification will depend on the manner in which the gradients are fractionated (compare Table 1 with Table 2). By running the enriched half of each peak from the first gradient (e.g. Fig. 2a) on a second gradient and collecting the enriched half of the major peak (e.g. Figs. 2b and 2c) one obtains β -globin mRNA samples containing 3–7% α -globin mRNA and, as expected, less-pure α -globin mRNA preparations contaminated with 7–17% β -globin mRNA.

We have examined mouse reticulocyte globin mRNA under the same conditions, but have failed to find a similar fractionation of the mRNA. Other workers have found 10S and 12S RNA in total extracts of mouse and rabbit reticulocytes (Williamson *et al.*, 1971; Loening, 1967), both by centrifugation and electrophoresis. The 12S RNA isolated by Williamson *et al.* (1971) had an anomalous melting profile, which could be due to the melting of a dimer structure; however, the transition in the melting curve was at 45°C, which is well above the temperature and the amount of transition that we would expect for a few base-pairs in the RNA.

Apparently Legon (Legon, 1976; Legon *et al.*, 1976) has observed the same phenomenon that we have described, but did not characterize it as a dimerization of β -globin mRNA. In his experiments he used a '10S' RNA isolated from total reticulocyte RNA fractionated on sucrose gradients in 0.1 M-NaCl. This RNA was characterized by iodination and 'fingerprint' analysis of both the total RNA and the ribosome-binding site and was consistent with the presence of only one type of globin mRNA. He further stated that this problem was not encountered if the poly(A)-containing RNA was fractionated on 10 mM-NaCl gradients after heating the RNA to 70°C before loading. The results are consistent with the results that we have shown in Figs. 1(a) and 1(b).

Although our results describe the molecular basis of this mRNA separation in some detail, we have not attempted to evaluate whether this specific dimerization has any physiological significance. It is a serendipitous association which allows us to isolate large quantities of intact and relatively highly purified α - and β -rabbit globin mRNA with ease.

This work was supported by grants GM 19076 and GM 22312 from the National Institutes of Health and the General Research Support of the John A. Burns School of Medicine.

References

- Bishop, J. O., Morton, J. G., Rosbash, M. & Richardson, M. (1974) *Nature (London)* **250**, 199–204
- Brawerman, G., Mendecki, J. & Lee, S. Y. (1972) *Biochemistry* **11**, 637–641
- Efstratiadis, A., Kafatos, F. C., Maxam, A. M. & Maniatis, T. (1976) *Cell* **7**, 279–288
- Gianni, A. M., Giglioni, B., Ottolenghi, S., Comi, P. & Guidotti, G. G. (1972) *Nature (London) New Biol.* **240**, 183–185
- Gould, H. J. & Hamlyn, P. H. (1973) *FEBS Lett.* **30**, 301–304
- Hamlyn, P. H. & Gould, H. J. (1975) *J. Mol. Biol.* **94**, 101–109
- Housman, D., Pemberton, R. & Taber, R. (1971) *Proc. Natl. Acad. Sci. U.S.A.* **68**, 2716–2719
- Jacobs-Lorena, M. & Baglioni, C. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1425–1428
- Kabat, D. (1975) *J. Biol. Chem.* **250**, 6085–6092
- Kazazian, H. H., Snyder, P. G. & Cheng, T. C. (1974) *Biochem. Biophys. Res. Commun.* **59**, 1053–1061
- Kirshner, A. G. & Tanford, C. (1964) *Biochemistry* **3**, 291–296
- Krystosek, A., Cawthorn, M. L. & Kabat, D. (1975) *J. Biol. Chem.* **250**, 6077–6084
- Legon, S. (1976) *J. Mol. Biol.* **106**, 37–53
- Legon, S., Robertson, H. D. & Prenskey, W. (1976) *J. Mol. Biol.* **106**, 23–36
- Loening, U. E. (1967) *Biochem. J.* **102**, 251–257
- Maniatis, T., Kee, S. G., Efstratiadis, A. & Kafatos, F. C. (1976) *Cell* **8**, 163–182
- Marcu, K. & Dudock, B. (1974) *Nucleic Acids Res.* **1**, 1385–1397
- Morrison, M. R., Brinkley, S. A., Gorski, J. & Lingrel, J. B. (1974) *J. Biol. Chem.* **249**, 5290–5295
- Nudel, U., Ramirez, F., Marks, P. A. & Bank, A. (1977) *J. Biol. Chem.* **252**, 2182–2186
- Ross, J., Leder, P. & Aviv, H. (1973) *Arch. Biochem. Biophys.* **158**, 494–502
- Temple, G. F. & Housman, D. E. (1972) *Proc. Natl. Acad. Sci. U.S.A.* **69**, 1574–1577
- Tinoco, I., Uhlenbeck, O. C. & Levine, M. D. (1961) *Nature (London)* **230**, 362–367
- Vournakis, J. N., Efstratiadis, A. & Kafatos, F. C. (1975) *Proc. Natl. Acad. Sci. U.S.A.* **72**, 2959–2963
- Williamson, R., Morrison, M., Lanyon, G., Eason, R. & Paul, J. (1971) *Biochemistry* **10**, 3014–3021
- Wolberg, J. R. (1967) *Prediction Analysis*, pp. 27–135, Van Nostrand, Princeton