

# Translation and Stability of Human Globin mRNA in *Xenopus* Oocytes

GEORGE M. MANIATIS, FRANCESCO RAMIREZ, ALMIRA CANN, PAUL A. MARKS,  
and ARTHUR BANK

*From the Departments of Human Genetics and Development, and Medicine, College of Physicians and Surgeons, Columbia University, New York 10032*

**ABSTRACT** Human globin messenger RNA (mRNA) prepared from erythroid cells of patients with sickle cell anemia has been translated in *Xenopus laevis* oocytes. Addition of hemin to the injected mRNA causes total globin synthesis to increase and the ratio of  $\alpha$ - to  $\beta^s$ -globin synthesis ( $\alpha/\beta^s$  ratio) to approach unity. To determine the effect of the length of the poly-(A) segment on human globin mRNA stability, 10 S globin mRNA was fractionated into poly-(A)-rich and poly-(A)-poor fractions by oligo (dT)-cellulose column chromatography. When oocytes are injected with each of these fractions, translation of the poly-(A)-rich globin mRNA is sustained for a longer period than that of the poly-(A)-poor mRNA. Regardless of the mRNA fraction injected, the  $\alpha/\beta^s$  ratio of the synthesized globin decreases as the injected oocytes are incubated for longer periods. The results indicate that in frog oocytes poly-(A)-rich mRNA has greater translational stability than poly-(A)-poor mRNA, and  $\beta$ -mRNA has greater stability than  $\alpha$ -mRNA with comparable poly-(A) content.

## INTRODUCTION

Human globin messenger RNA (mRNA)<sup>1</sup> has been translated in a variety of cell-free systems (e.g., 1, 2). In all these systems, globin synthesis is linear for only a few hours, at most. It is not, therefore, possible

<sup>1</sup> *Abbreviation used in this paper:* mRNA, globin messenger RNA.

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to study the translational stability of exogenous mRNA over a period of days using cell-free systems. The oocytes of the African frog *Xenopus laevis* are capable of translating exogenous, injected mRNAs with high efficiency and for several days (3). A variety of heterologous mRNAs have been translated in the frog oocytes including rabbit (4, 5) and mouse globin mRNA (6-8), human proinsulin mRNA (9), immunoglobulin mRNA (10), and ovalbumin mRNA (11).

Human globin mRNA molecules contain varying lengths of poly-(A)-rich sequences at the 3'-OH terminus and can be fractionated on the basis of their poly-(A) content (12). The function of the poly-(A) sequences in mRNA is not known. There is evidence that the poly-(A) segment undergoes progressive shortening with time and the hypothesis has been advanced that the poly-(A) is involved in determining the stability of the mRNA in the cell (13-15). Huez et al. (16) and Nudel et al. (17) have prepared deadenylated rabbit globin mRNA by limited phosphorolysis using *Escherichia coli* polynucleotide phosphorylase. When this enzymatically deadenylated mRNA was injected into *X. laevis* oocytes, it was found that the stability of the mRNA was dependent upon the length of the poly-(A) segment remaining after enzymatic treatment. In this paper we have used the frog oocyte to study the functional stability of human globin mRNA by measuring the products of translation of the injected mRNA at various times after injection. We have demonstrated that human  $\alpha$ - and  $\beta^s$ -globin mRNA can be translated faithfully in the frog oocyte, and that the addition of hemin greatly increases the rate of globin mRNA translation. The translational stability of globin mRNA containing the longest poly-(A) sequences is greater than that of globin mRNA containing shorter poly-(A) sequences. In addition, our results indicate that  $\beta^s$ -globin mRNA is more stable than  $\alpha$ -globin mRNA.

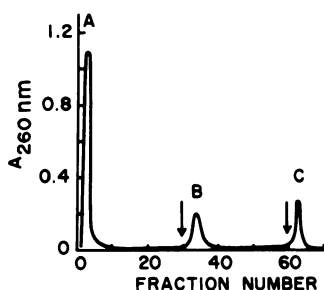


FIGURE 1 Elution profile of human 10S RNA from an oligo (dT)-cellulose column. Fraction A is the material not retained by the column in 0.01 M Tris (pH 7.5)–0.5 M KCl. Fraction B was eluted with 0.01 M Tris (pH 7.5)–0.1 M KCl and Fraction C was eluted with 0.01 M Tris (pH 7.5). The above three fractions were used in experiment III (Table III).

## METHODS

**Preparation of human globin mRNA.** Human reticulocyte RNA was prepared separately from blood obtained from three patients with sickle cell anemia collected in acid-citrate-dextrose buffer. The peripheral blood of these patients contained more than 90% hemoglobin S. The cells were washed with 0.9% NaCl and the buffy coat was removed. The procedures for RNA extraction and fractionation by centrifugation through two successive sucrose density gradients have been previously described (12). The resulting 6 to 16 S region (designated 10S) was pooled and precipitated at  $-20^{\circ}\text{C}$  overnight after adding NaCl to a final concentration of 0.3 M and 2 vol of ethanol. The precipitate was washed successively with 95% ethanol, 70% ethanol containing 2% potassium acetate, and 95% ethanol, dissolved in water, and lyophilized. The lyophilizate was either dissolved in injection buffer when injected into oocytes as unfractionated mRNA or in 0.01 M Tris-HCl (pH 7.5) containing 0.5 M KCl in preparation for oligo (dT)-cellulose chromatography.

**Oligo (dT)-cellulose chromatography.** The conditions of oligo (dT)-cellulose chromatography have been previously described (12). The material not retained by the column when the sample was applied in 0.01 M Tris-HCl, pH 7.5, 0.5 M KCl is designated as Fraction A. The material eluted in 0.01 M Tris-HCl, pH 7.5, 0.1 M KCl is called Fraction B, and that eluted in 0.01 M Tris-HCl, pH 7.5, is called Fraction C. Each of the A, B, and C fractions of RNA was precipitated by addition of NaCl to a final concentration of 0.3 M followed by 2 vol of ethanol. After standing overnight at  $-20^{\circ}\text{C}$ , the precipitate was pelleted, washed, and lyophilized. The lyophilizate was dissolved in injection buffer consisting of 88 mM NaCl, 15 mM Tris-HCl (pH 7.6) before injection into oocytes. Hemin was prepared by first dissolving it in 1 M KOH followed by the addition of an equal volume of 1 M Tris buffer (pH 7.8), as described by Zucker and Schulman (18) to a 20 mM solution and added to the RNA preparation in 1:20 vol/vol ratio. The final concentration of hemin in the injection mixture was 1 mM unless indicated otherwise.

**Oocyte injection.** *X. laevis* frogs were obtained from Charles W. Fletcher, Hampstead, Md. Oocytes were injected with 50 nl each of injection mixture using a glass needle made from a 10- $\mu\text{l}$  Drummond capillary pipette (Drummond Scientific Co., Broomall, Pa.). For each experimental point 10 full-sized oocytes were incubated in 100  $\mu\text{l}$  of Barth's medium (3) at room temperature, except in

experiment III, Table III, and experiment II, Table IV where 15 oocytes were used per point. For labeling, the oocytes were incubated in 100  $\mu\text{l}$  of Barth's medium containing 1–2 mCi/ml of [ $^3\text{H}$ ]L-leucine (70 Ci/mmol, New England Nuclear, Boston, Mass.). The time and duration of labeling is indicated in the tables. At the end of the incubation period, the oocytes were washed with Barth's medium and stored at  $-70^{\circ}\text{C}$  until analyzed.

**Homogenization of the oocytes.** The oocytes were homogenized in 52.2 mM glycine–52.2 mM Tris containing 0.1% unlabeled leucine and 5 mg/ml of a mixture of human hemoglobins A and S. The homogenate was then either applied on a Sephadex G-100 column (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) as described by Lane et al. (5) or mixed with more carrier hemoglobin to a final concentration of 25 mg/ml and used to prepare globin by the acid-acetone method (19).

**Carboxymethyl cellulose chromatography.** The oocyte product was analyzed for the presence of human  $\alpha$ - and  $\beta$ -globin chains by urea-carboxymethyl cellulose (CM-23, Whatman Inc., Clifton, N. J.) column chromatography using a modification of the method of Clegg et al. (20). An eight-chamber gradient of  $\text{Na}_2\text{HPO}_4$  was employed, as previously described (2). The amount of globin synthesized was calculated from the radioactivity which co-chromatographed with the  $A_{280}$   $\beta^{\text{S}}$ - and  $\alpha$ -peaks of the carrier hemoglobin eluted from the carboxymethyl cellulose column. Since human globin  $\alpha$ - and  $\beta$ -chains contain the same number of leucine residues, no correction was necessary when calculating the  $\alpha/\beta^{\text{S}}$  ratio. Recovery from the carboxymethyl cellulose chromatography was 85–90%.

## RESULTS

**Fractionation of human globin mRNA by oligo (dT) column chromatography.** Purified 6–16S reticulocyte RNA was fractionated by affinity chromatography on oligo (dT)-cellulose (Fig. 1). The three fractions obtained, A, B, and C, were used for injection into the oocytes. The poly-(A) content of the preparations used in experiment I (Table III) has been previously shown by hybridization to [ $^3\text{H}$ ]polyuridylylate to be as follows: Fraction A contains an average of 1–2 adenylate residues per mRNA molecule, fraction B contains an average of 20–60, and fraction C contains an average of 60–120 adenylate residues per mRNA molecule (12).

**Translation of total globin mRNA (10S).** When *X. laevis* oocytes are injected with total human reticulocyte 10S RNA, the oocyte product, analyzed by gel filtration on Sephadex G-100, contains radioactive molecules which co-chromatograph with carrier human hemoglobin (Fig. 2). Oocytes injected with only the buffer and hemin do not show any radioactive peak in the carrier hemoglobin region. Fractions corresponding to the carrier hemoglobin peak as well as those eluting between the hemoglobin and the free leucine peaks were pooled. If globin chains, not equilibrated with the carrier hemoglobin, were present, they would be eluted in the latter pool. The pooled fractions were analyzed by carboxymethyl cellulose chromatography to identify and quantitate

globin chains (Fig. 3). Two radioactive peaks co-chromatograph with the absorbance peaks of the  $\alpha$ - and the  $\beta^s$ -chains of the carrier hemoglobin. The amount of globin chains synthesized in the oocyte

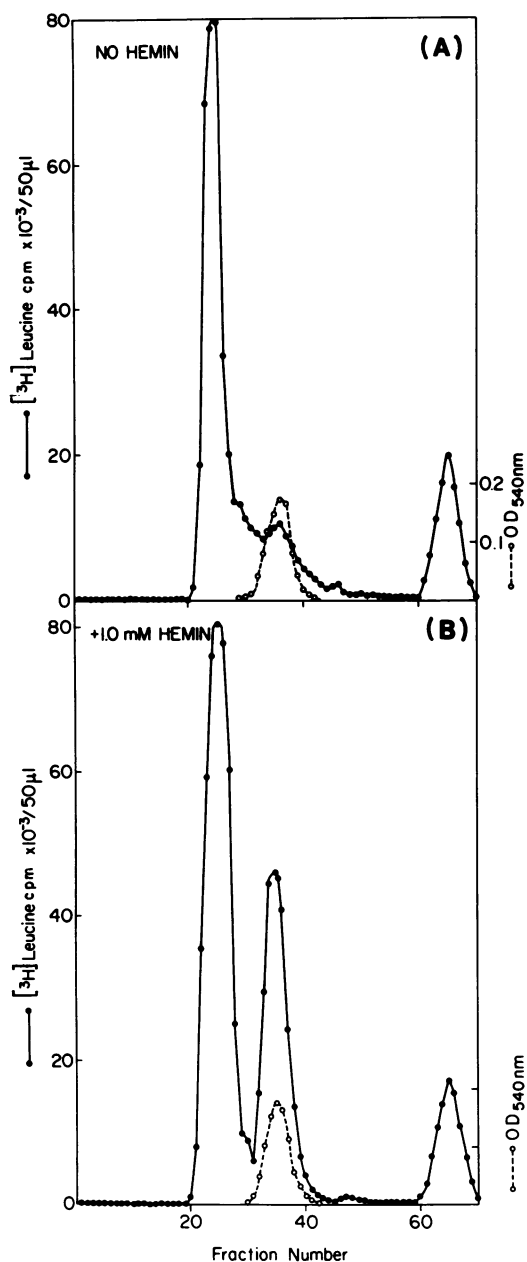


FIGURE 2 Sephadex G-100 fractionation of proteins extracted from oocytes injected with human globin mRNA and incubated in the presence of  $[^3\text{H}]$ leucine for 20 h. The first radioactive peak represents endogenous oocyte proteins, the second peak corresponds to the carrier hemoglobin, and the third peak corresponds to free leucine. Fractions of 1.5 ml each were collected. (---) OD at 540 nm; (---) radioactivity. (A) The injection mixture contained no hemin. (B) The injection mixture contained 1 mM hemin.

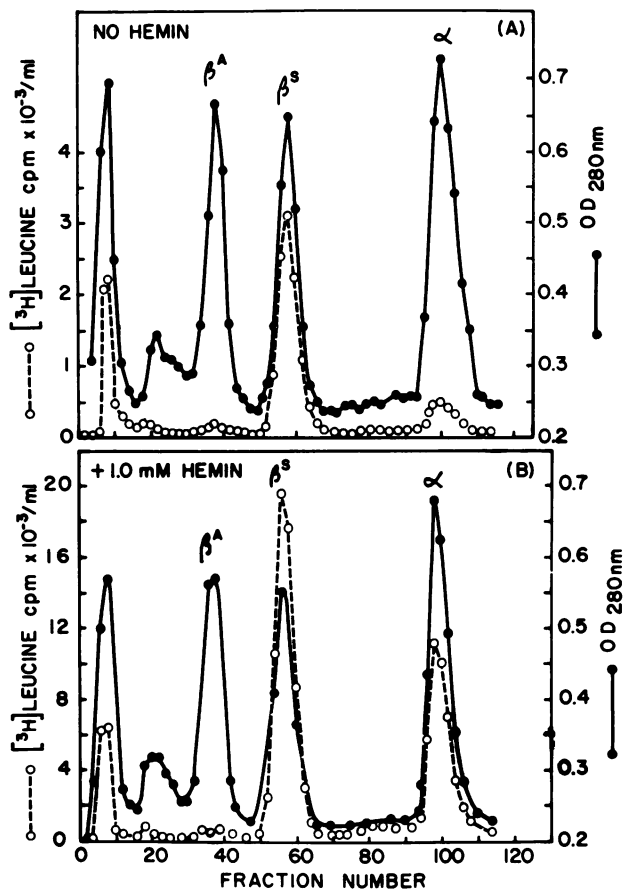


FIGURE 3 Carboxymethyl cellulose chromatography of globin synthesized in oocytes injected with globin mRNA. Globin was prepared from the Sephadex hemoglobin peak (Fig. 2) after addition of carrier hemoglobin consisting of a mixture of Hb A and Hb S. 5.5-ml fractions of eluate were collected. (---) OD<sub>260</sub> of carrier Hb; (---) radioactivity. (A) No hemin in injection mixture. (B) Injection mixture contained 1 mM hemin.

relative to the total protein synthesis was calculated as follows: Total protein synthesis was measured as the radioactivity of all the Sephadex fractions excluding the free leucine peak. The amounts of globin chains were measured as the radioactivity under the  $\alpha$ - and  $\beta^s$ -peaks in the eluate of the carboxymethyl cellulose column corrected for 90% recovery. The ratios of globin chain synthesis to total protein synthesis are presented in Tables I, III, and IV.

*Effect of hemin on the translation of globin mRNA.* When purified 10S RNA is injected into oocytes without hemin, the ratio of globin to total protein synthesis is 0.02 and the  $\alpha/\beta^s$  ratio is 0.15–0.17 (Table I). This is observed after both 2.5 h and 20 h of incubation. Addition of hemin to the injection mixture has two effects: First, there is more globin synthesis relative to total protein synthesis; the ratio of globin/total protein increases from 0.02, in the absence of hemin,

TABLE I  
Effect of Hemin on the Synthesis of  $\alpha$ - and  $\beta^s$ -Globin Chains by  
Frog Oocytes Injected with Human Globin mRNA\*

Experiment	Incubation period	Hemin in injection mixture	Total protein synthesis	Globin synthesis			Globin/total protein synthesis
				$\alpha$	$\beta^s$	$\alpha/\beta^s$	
	<i>h</i>	<i>mM</i>	<i>cpm</i> $\times 10^{-3}$	<i>cpm</i> $\times 10^{-3}$	<i>cpm</i> $\times 10^{-3}$		
I	0-2.5	—	8,000	22.3	151.0	0.15	0.02
		1	6,100	463.3	653.2	0.71	0.20
		2	6,570	396.0	545.4	0.73	0.16
II	0-20	—	13,780	45.0	260.0	0.17	0.02
		0.5	15,990	505.6	1,284.4	0.39	0.11
		1	18,040	1,480.0	2,277.6	0.65	0.23

\* Human reticulocyte mRNA, prepared as described in METHODS was injected into frog oocytes. The injection mixture contained RNA at 5.0  $A_{260}$  U/ml and hemin at the concentration indicated in Table. At the end of the incubation period the oocytes were homogenized and the homogenate was applied to a Sephadex G-100 column. Total protein synthesis was calculated from the Sephadex fractions (Fig. 2) and included the radioactivity of fractions eluting before free leucine. The fractions between the first peak (oocyte proteins) and the last peak (free leucine) were pooled, mixed with carrier hemoglobin, and the globin was analyzed by chromatography on a urea-carboxymethyl cellulose column (Fig. 3).  $\alpha$ - and  $\beta^s$ -globin chain synthesis was calculated from the radioactivity of the fractions corresponding to the  $\alpha$ - and  $\beta^s$ -peaks of the carrier globin. Columns 5 and 6 show the actual counts per minute under the globin peaks. In the last column globin counts per minute were corrected for 90% recovery.

up to 0.20 in the presence of 1 mM hemin in the injection mixture, and to 0.16 in the presence of 2 mM hemin at 2.5 h (Experiment I, Table I). After 20 h of incubation, the globin/total protein ratio increases from 0.02 to 0.11 in the presence of 0.5 mM hemin and to 0.23 at 1 mM hemin (Experiment II, Table I). Second, on addition of hemin, the relative amount of  $\alpha$ -globin

to that of  $\beta^s$ - is closer to 1. Thus, the  $\alpha/\beta^s$  ratio increases in experiment I from 0.15 in the absence of hemin to 0.73 in the presence of 2 mM hemin; in experiment II, it increases from 0.17 without hemin to 0.39 in the presence of 0.5 mM hemin, and to 0.65 in the presence of 1 mM. From these experiments, the optimum concentration of hemin in the injection mixture is about 1 mM.

TABLE II  
Effect of the Method of Preparation on the  
Recovery of  $\alpha$ - and  $\beta^s$ -Globin Chains

Hemin in injection mixture	Method of preparation	Globin synthesis		
		$\alpha$	$\beta^s$	$\alpha/\beta^s$
		<i>cpm</i> $\times 10^{-3}$	<i>cpm</i> $\times 10^{-3}$	
—	Sephadex G-100	22.3	151.0	0.15
—	Directly in acid acetone	309.3	661.4	0.47
1 mM	Sephadex G-100	463.3	653.2	0.71
1 mM	Directly in acid-acetone	799.8	972.2	0.82

\* Batches of 10 oocytes each were injected with human globin mRNA (5.0  $A_{260}$  U/ml) in the presence or absence of hemin and incubated in the presence of [ $^3H$ ]leucine for 150 min. The oocytes were then homogenized and the homogenate was either applied on a Sephadex G-100 column (as described in Table I) or dripped directly in acid-acetone to prepare globin.  $\alpha$ - and  $\beta^s$ -globin chains were isolated by carboxymethyl cellulose chromatography.

The effect of hemin on the  $\alpha/\beta^s$  ratio could be due either to a regulatory effect at the translational level which preferentially favors  $\alpha$ -mRNA translation or, alternatively, could be due to a post-translational stabilizing effect on the newly synthesized chains, i.e., it is possible that in the absence of hemin a large portion of  $\alpha$ -chains precipitate and are lost during the preparation of the homogenate and gel filtration. The purpose of the experiment presented in Table II was to distinguish between these possibilities. The oocytes were injected with mRNA but without hemin and incubated for a relatively short period (150 min). They were then homogenized in the presence of carrier hemoglobin, but instead of going through centrifugation and gel filtration, the homogenate was added directly to cold acid-acetone for globin preparation. Such a treatment resulted in a large increase in the recovery of  $\alpha$ -chains relative to  $\beta^s$ -chains: the  $\alpha/\beta^s$  ratio increased more than threefold from 0.15 when gel filtration preceded globin preparation, to 0.47 when globin was prepared directly from the homogenate. Table II shows also the effect on  $\alpha/\beta^s$  ratio of preparing globin directly, without gel filtra-

**TABLE III**  
 *$\alpha$ - and  $\beta^s$ -Globin Synthesis in Frog Oocytes Injected with Human Globin and mRNA*  
*Fractionated by Oligo (dT)-Cellulose Chromatography\**

Experiment	mRNA injected	Labeling period	Total protein synthesis	Globin Synthesis			$\alpha$ /Total protein synthesis†	$\alpha$ -synthesis compared to 0h‡	$\beta^s$ Total protein synthesis‡	$\beta^s$ -synthesis compared to 0h‡
				$\alpha$	$\beta^s$	$\alpha/\beta^s$				
		h	cpm $\times 10^{-3}$	cpm $\times 10^{-3}$	cpm $\times 10^{-3}$		%		%	
I	A fraction 4.5 A <sub>280</sub> U/ml	0-3.5	3,555	56.7	118.8	0.48	0.0177	100	0.0371	100
		24-27.5	2,480	14.3	57.9	0.25	0.0064	36	0.0259	70
		48-51.5	467	2.1	12.7	0.17	0.0047	27	0.0302	81
	B fraction 0.9 A <sub>280</sub> U/ml	0-3.5	3,099	38.3	75.8	0.51	0.0137	100	0.0272	100
		24-27.5	2,391	23.3	53.8	0.43	0.0108	79	0.0250	92
		48-51.5	687	5.5	12.2	0.45	0.0089	65	0.0197	72
	C fraction 1.1 A <sub>280</sub> U/ml	0-3.5	3,208	90.8	139.0	0.65	0.0314	100	0.0481	100
		24-27.5	2,352	71.0	132.9	0.53	0.0335	107	0.0628	131
		48-51.5	929	25.2	58.8	0.43	0.0301	96	0.0703	146
II	A fraction 7.5 A <sub>280</sub> U/ml	0-3	2,385	69.3	126.0	0.55	0.0323	100	0.0587	100
		30-33	2,080	14.6	65.3	0.22	0.0078	24	0.0349	59
		54-57	1,990	2.9	26.5	0.11	0.0016	5	0.0148	25
	B fraction 1.7 A <sub>280</sub> U/ml	0-3	2,640	74.0	125.0	0.59	0.0311	100	0.0526	100
		30-33	2,590	30.1	73.3	0.41	0.0129	41	0.0314	60
		54-57	1,150	8.9	25.5	0.35	0.0086	28	0.0246	47
	C fraction 2.3 A <sub>280</sub> U/ml	0-3	2,848	114.3	139.4	0.82	0.0446	100	0.0544	100
		30-33	2,504	84.0	171.5	0.49	0.0373	84	0.0761	140
		54-57	2,132	56.0	118.0	0.47	0.0292	65	0.0615	113
III	A fraction 4.8 A <sub>280</sub> U/ml	0-4	7,023	127.5	223.7	0.57	0.0202	100	0.0354	100
		24-28	4,942	29.5	120.1	0.25	0.0066	33	0.0270	76
		47-51	5,786	6.2	51.6	0.12	0.0012	6	0.0099	28
	C fraction 2.0 A <sub>280</sub> U/ml	0-4	7,365	389.8	464.8	0.84	0.0588	100	0.0701	100
		24-28	5,971	241.0	365.1	0.66	0.0448	76	0.0679	97
		47-51	5,392	158.9	273.3	0.58	0.0327	56	0.0563	80
	Mixture of A fraction (4.8 A <sub>280</sub> U/ml) and C fraction (2.0 A <sub>280</sub> U/ml)	0-4	7,785	411.2	552.3	0.74	0.0587	100	0.0788	100
		47-51	6,272	166.8	338.4	0.49	0.0295	50	0.0599	76

\* All injection mixtures contained 1 mM hemin. [<sup>3</sup>H]Leucine was added at the time indicated and the incubation continued for 3.5 h (Expt. I), 3 h (Expt. II), or for 4 h (Expt. III). Total protein and globin synthesis were calculated or explained in Table I.

† Ratios in these columns were calculated according to the formula:  $\alpha$  or  $\beta^s$ /total protein synthesis = cpm under  $\alpha$ - or  $\beta^s$ -peak/0.9/total protein synthesis, where 0.9 is the approximate recovery after carboxymethyl cellulose chromatography.

‡ Percent of globin synthesis compared to 0 h; the globin counts per minute have been corrected for changes in total protein synthesis.

tion, from oocytes injected with hemin. The  $\alpha/\beta^s$  ratio shows only a 1.15-fold increase, from 0.71 to 0.82. Thus, it appears that in the absence of hemin,  $\alpha$ -globin recovery from Sephadex column is disproportionately low. Because of the hemin effect on total globin synthesis and on  $\alpha$ -globin recovery, hemin was used in all further experiments.

*Translational stability of human globin mRNA.* The three fractions of mRNA obtained by chromatography on oligo (dT)-cellulose (Fig. 1) were tested for translational stability in the oocyte (Table III). The RNA used in each of the experiments shown was prepared from a different patient. The synthesis of  $\alpha$ - and  $\beta^s$ -globin by the injected oocytes were followed for 51.5 h in the first experiment, for 57 h in the second

experiment, and for 51 h in the third one. Fraction A was injected into the oocytes at a higher concentration than fractions B and C because, as shown previously for preparations used in experiment I (12), fraction A contained only about 20% as much globin mRNA compared to fraction C, as assayed by hybridization to complementary DNA. The results of these experiments indicate that fraction C, which contains the greatest amount of polyadenylate segments, is the most stable in the oocyte. The amount of  $\alpha$ -globin synthesis for fraction A RNA declined to 5-27% of control levels in the three experiments, while  $\beta$ -globin synthesis declined to 25-81% of control (Table III). By contrast,  $\alpha$ -globin synthesis by fraction C RNA declined to only 56-96% of control

TABLE IV  
Effect of Reinjection of Hemin or Globin mRNA on the Synthesis of  $\alpha$ - and  $\beta^s$ -Globin Chains\*

Experiment	Reinjection	Labeling period	Total protein synthesis	Globin synthesis			Globin/total protein synthesis
				$\alpha$	$\beta^s$	$\alpha/\beta^s$	
				$cpm \times 10^{-3}$	$cpm \times 10^{-3}$		
I	—	0–3	1,395	22.6	46.2	0.49	0.05
	—	20–23	1,266	4.5	19.4	0.23	0.02
	1 mM hemin	21–24	1,090	3.9	19.9	0.20	0.02
	A-fraction + 1 mM hemin	21–24	1,246	18.2	54.0	0.34	0.06
II	A-fraction + 1 mM hemin	47–51	6,762	114.2	223.9	0.51	0.06

\* In expt. I oocytes were injected with fraction A (nonadenylated). The injection mixture contained 5.0 A<sub>260</sub> U/ml RNA and 1 mM hemin. After 21 h of incubation one batch was reinjected with the same preparation of RNA and hemin while another batch was injected with hemin alone. Total protein synthesis and globin synthesis were calculated as explained in Table I. In expt. II oocytes were injected with buffer at the beginning of the experiment. After 47 h they were reinjected with fraction A and labeled for an additional 4 h.

values in 2 days, while  $\beta$ -globin synthesis declined to 80% in one experiment and was 146% and 113% in two other experiments (Table III).

**Relative functional stability of  $\alpha$ - and  $\beta^s$ -globin mRNA.** In all experiments, regardless of the mRNA fraction used, the  $\alpha/\beta^s$  ratio is always less than 1 and there is a decrease of the  $\alpha/\beta^s$  ratio with increasing time of incubation (Table III). This suggests that the oocytes translate the  $\alpha$ -mRNA less efficiently than the  $\beta^s$ -mRNA, or that the functional stability of the  $\alpha$ -mRNA in the oocyte is less than that of the  $\beta^s$ -mRNA. The change in the  $\alpha/\beta^s$  ratio with time is smaller for the C fraction than for the A fraction. After 2 days of incubation, the  $\alpha/\beta^s$  ratio for the C fraction decreases by a factor of 1.51, 1.74, and 1.45 in experiments I, II, and III, respectively. In oocytes injected with A fraction, the  $\alpha/\beta^s$  ratio decreases during the same period by a factor of 2.72, 5.0, and 4.75 in three experiments (Table III). The greater decline in the  $\alpha/\beta^s$  in oocytes injected with fraction A as compared with those injected with fraction C RNA could be due to either the inherent properties of the globin mRNAs or, alternatively, to some interference by nonglobin RNAs present in fraction A on  $\alpha$ -globin mRNA translation. To distinguish between these possibilities, a mixture of fractions A and C was injected at the same concentration as when each was injected alone (Experiment III, Table III). The  $\alpha/\beta^s$  ratios obtained and the globin/total protein synthesis ratio are those expected for the mixture of fraction A and C RNA and do not indicate any specific effect by nonglobin RNAs in fraction A on  $\alpha$ -globin mRNA translation.

The decrease of the  $\alpha/\beta^s$  ratio with incubation time noted in all experiments could be due either to the preferential inactivation of  $\alpha$ -mRNA, exhaustion of an oocyte factor required for the efficient translation of  $\alpha$ -mRNA, or preferential degradation of newly

synthesized  $\alpha$ -chains. Evidence for the existence of such factors for the translation of mouse and rabbit globin mRNA has been presented by Lane et al. (8). If there were exhaustion of an oocyte factor required for  $\alpha$ -mRNA translation, reinjection of mRNA into the oocytes, at a time when a decrease in the  $\alpha/\beta^s$  ratio is evident, should not restore the  $\alpha/\beta^s$  ratio to the initial level. If, however, the decrease in  $\alpha$ -globin synthesis is due to loss or inactivation of  $\alpha$ -globin mRNA, reinjection of mRNA should restore the  $\alpha/\beta^s$  ratio. Table IV presents the results of such reinjection experiments. Fraction A was used because it shows the greatest decrease in the  $\alpha/\beta^s$  ratio with time. Reinjection of mRNA at 21 h causes an increase of the  $\alpha/\beta^s$  ratio from 0.23 to 0.34, while reinjection of hemin alone does not increase the ratio (Experiment I, Table IV). In another experiment (Experiment II, Table IV), oocytes were injected at zero time with injection buffer and after 47 h with fraction A. The  $\alpha/\beta^s$  ratio obtained (0.51) is similar to those obtained initially from oocytes injected with fraction A (0.48, 0.55, 0.57, Table III and 0.49, Table IV). These results lend support to the preferential inactivation of  $\alpha$ -mRNA as the reason for the change in the  $\alpha/\beta^s$  ratio with time.

## DISCUSSION

Our results show that the *X. laevis* oocyte system can faithfully translate injected human 10S globin mRNA and constitutes an efficient system for testing small amounts of human mRNA: in our experience a total of 100 ng of 10S RNA, injected into 10 oocytes, leads to the incorporation of more than  $1.5 \times 10^6$  cpm in globin after 2.5 h of incubation. Chromatography of the oocyte products on a carboxymethyl cellulose column, after gel filtration of the oocyte homogenate,

gives a uniform low background and allows an accurate estimation of the amount of human globin synthesized in the oocyte.

Gigliani et al. (21) have reported that the efficiency of translation of rabbit globin mRNA can be greatly increased by addition of hemin into the injection mixture and Lane et al. (8) found a similar effect on the translation of mouse  $\alpha$ -globin mRNA. In our experiments, translation of human globin mRNA is imbalanced, with synthesis of less  $\alpha$ - than  $\beta$ -globin chain ( $\alpha/\beta^s$  between 0.15 and 0.17). Inclusion of hemin into the injection mixture increases the ratio to 0.39–0.84, depending on the concentration of hemin used. Part of the hemin effect appears to be due to an increased recovery of  $\alpha$ -chains during the preparation, since the  $\alpha/\beta^s$  ratio of the globin synthesized in the absence of hemin increases from 0.15 to 0.47 (Tables I and II) when storage of the oocytes after incubation, centrifugation, and gel filtration is avoided. This is contrary to the findings of Gurdon et al. (6) who reported that, for mouse and rabbit globin mRNA, direct preparation of globin from the oocyte homogenate does not result in an increase of the low  $\alpha/\beta$  ratio. Improved recovery of  $\alpha$ -chains cannot account, however, for the total hemin effect since the  $\alpha/\beta^s$  ratio is not increased to greater than 0.47 and does not approach the ratios obtained in the presence of hemin. In addition, a preferential loss of  $\alpha$ -chains in the absence of hemin cannot explain the ninefold increase in total globin synthesized in the presence of hemin (Table I). Hemin has no appreciable effect on the synthesis of endogenous oocyte proteins. Our results are at variance with the results obtained with rabbit globin mRNA, where the experimental evidence suggests that the balancing effect of hemin is achieved by stimulating  $\alpha$ -mRNA translation, without affecting  $\beta$ -globin synthesis (21). The mechanism of the hemin effect is not clear and may, indeed, be multifaceted. There is evidence that hemin plays a role in the regulation of globin chain synthesis in reticulocytes (22). In reticulocyte cell-free systems, hemin controls the rate of globin synthesis, in part, by preventing the accumulation of an inhibitor of chain initiation (23–25). In these reticulocyte-lysate systems hemin stimulates the synthesis not only of globin but of other proteins as well (26, 27).

In all of our experiments there is a progressive decrease in the  $\alpha/\beta^s$  ratio with time regardless of mRNA preparation used. This indicates that the functional stability of human  $\alpha$ -mRNA in the frog oocyte is less than that of  $\beta$ -mRNA. This decrease with time in relative  $\alpha$ -globin synthesis may be due either to an actual decrease in the amount of functional  $\alpha$ -mRNA or to a progressive loss of an oocyte factor specific for the translation of  $\alpha$ -mRNA. Injection of RNA may have caused some damage to the oocytes resulting in the

release of ribonucleases which preferentially attack  $\alpha$ -mRNA. In some of our experiments (e.g. IA, Table III) the rate of total protein synthesis declines markedly during the incubation period, presumably because of damage to the oocytes during the injection. In other experiments, e.g., experiment III, Table III, there is no significant decline in total protein synthesis. It is, therefore, unlikely that damage of the oocytes is the explanation for the decline in the  $\alpha/\beta^s$  ratio with time since this decline is similar, regardless of the decline in the total protein synthesis (e.g. compare experiment IA with IIA and IIIA). It should be noted that the factors in the oocyte which affect human globin mRNA stability are unknown, and may well be different from those which ordinarily degrade human globin mRNA in human erythroid cells. It is still possible that the change in  $\alpha/\beta^s$  ratio is, at least partially, due to the effect of nucleases released as a result of damage in the oocytes. However, regardless of the mechanisms, the preferential susceptibility of  $\alpha$ -mRNA relative to  $\beta$ -mRNA in oocytes is still indicative of inherent differences of  $\alpha$ - and  $\beta$ -mRNAs.

The experiments in Table IV also provide evidence that the exhaustion of a specific factor required for the translation of  $\alpha$ -mRNA is not responsible for the decline in  $\alpha/\beta^s$  ratio with time. Oocytes reinjected with mRNA 21 and 51 h after a first injection showed  $\alpha/\beta^s$  ratios similar to those obtained during the first few hours after injection with the same mRNA fraction (Table IV).

In all three experiments presented in Table III, the decline with time of the  $\alpha/\beta^s$  ratio is more pronounced in oocytes injected with the A fraction than in those injected with the C fraction. One explanation for this difference could be that the A fraction contains RNA species other than globin mRNA which interfere preferentially with the translation of  $\alpha$ -mRNA. However, when a mixture of A and C fractions were injected together, the results are consistent with an additive effect of mixing the A and C fractions and do not support any such preferential interference with the translation of the  $\alpha$ -mRNA (Table IV).

In our experiments the initial  $\alpha/\beta^s$  ratio was never one. This may be due to a greater affinity of  $\beta$ -mRNA than  $\alpha$ -mRNA for the initiation complex, as the hemin effect suggests and as Lodish (28) has shown for rabbit globin mRNA; as a result more  $\alpha$ -mRNA would remain free rather than associated with ribosomes. The stability of mRNAs injected into oocytes seems to depend on their interaction with ribosomes (29). Our finding of progressive decrease in the  $\alpha/\beta^s$  ratio with time is different from that of Gurdon et al. (16) who found that for mouse mRNA the  $\alpha/\beta$  ratio remained more or less constant for up to 320 h of incubation despite the imbalanced initial synthesis ( $\alpha/\beta = 0.11$ ) indicating equal stability of  $\alpha$ - and  $\beta$ -mRNAs.

It is possible that some of the differences between our findings with human mRNA and those obtained with animal globin mRNA are due to the fact that our human mRNA is prepared from reticulocytes of patients whose erythropoiesis is at steady state, while animal globin mRNA is prepared from animals made acutely anemic so their reticulocytes are younger and more synchronously produced.

Our results indicate that the translational stability in the oocyte of the poly-(A)-rich human globin mRNA is greater than that of the poly-(A)-poor mRNA. The exact role of the poly-(A) sequences in the translation of eukaryotic mRNAs is not known. Its presence is not a requirement for translation since histone mRNA is known to lack poly-(A), and mRNA from which poly-(A) has been removed enzymatically can be translated in cell-free systems (30–33). Recently, the addition of poly-(A) to RNA was found to inhibit human spleen ribonuclease (34); whether poly-(A) serves a similar function in vivo remains unknown.

The data (Table III) indicate that in the absence of poly-(A) sequences,  $\alpha$ -mRNA activity declines much more rapidly (to 27, 5, and 6% of initial value after 2 days) than the activity of  $\beta$ -mRNA (which decreases to 81, 25, and 28% of control levels). Using poly-(A)-rich RNA, the  $\alpha$ -mRNA activity declines to a much lesser extent (to 96, 65, and 56% of control after 2 days). The activity of poly-(A)-rich  $\beta$ -mRNA, on the other hand, is either increased or slightly decreased. These results show that the functional stability of  $\alpha$ -mRNA is less than that of  $\beta$ -mRNA regardless of poly-(A) content. In addition, the translation of both  $\alpha$ - and  $\beta$ -mRNA containing significant lengths of poly-(A) is more prolonged than that of poly-(A)-poor globin mRNA. We conclude that the stability of human globin mRNA, at least, in the frog oocyte, is correlated with its poly-(A) content. If such a correlation exists in the native erythroid cell and if the differential stability between  $\alpha$ - and  $\beta$ -mRNA observed in the oocyte is an inherent property of the mRNAs, then our findings would suggest that post-transcriptional regulatory mechanisms may have a role in the balanced synthesis of  $\alpha$ - and  $\beta$ -globin chains. Some of the thalassemia syndromes in which there is a decrease in the amount of mRNA for one of the globin chains (35, 36) may involve disturbances of such a mechanism.

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