Instability of β^E-messenger RNA during Erythroid Cell Maturation in Hemoglobin E Homozygotes

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ABSTRACT Hemoglobin E interacts with β-thalassemia to produce a disorder of variable severity that is the most common form of symptomatic thalassemia in Southeast Asia. The β^E-globin gene acts as a mild thalassemia gene; there are low levels of β^E-messenger RNA (mRNA) in reticulocytes, and preliminary evidence had suggested that this might be due to instability of the β^E-mRNA. Analysis of β^E-mRNA levels in the nuclei and cytoplasm of bone marrow erythroblasts compared with reticulocytes has shown higher levels of β^E-mRNA in the former, providing direct evidence that this is the case.

INTRODUCTION

Hemoglobin E (Hb E) ($\alpha_2\beta_2^{26\ glu \rightarrow lys}$) is one of the most common hemoglobin structural variants, reaching very high frequencies throughout much of Southeast Asia (1, 2). On interaction with a β-thalassemia gene, the β^E-gene produces a disorder that may be as severe as β-thalassemia major, yet in the homozygous state produces only a mild anemia. The severity of the interaction with β-thalassemia is explained by a reduced rate of β^E-chain synthesis (3–5); in other words, the β^E-gene acts as a mild β-thalassemia determinant. The reason for the low level of β^E-chain synthesis is a deficiency in β^E-mRNA, an unexpected finding in a disorder caused by a single base substitution in the coding part of the β-globin gene (3, 5).

Previously, we reported that in Hb E heterozygotes the specific activity of β^E-chains synthesized in reticulocytes was lower than that of the β^A-chains; moreover the ratio of β^A/β^E-chain synthesis was lower in bone marrow than in reticulocyte incubations (3). These observations suggested asynchrony in β^A- and β^E-synthesis during erythroid maturation (the latter declining more rapidly), raising the possibility that the low level of β^E-mRNA in reticulocytes might be due to its instability. We have now examined this question directly by measuring the mRNA levels in bone marrow erythroblast nuclei and cytoplasm and comparing it with that in reticulocytes. The results support the hypothesis that β^E-mRNA is less stable than β^A-mRNA.

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METHODS

Peripheral blood and bone marrow samples were collected in Bangkok from five Thai individuals homozygous for Hb E. All samples were washed in reticulocyte saline (130 mM NaCl, 5 mM KCl, and 7.4 mM $MgCl_2 \cdot 6$ mM H_2O) and immediately frozen on dry ice for transportation. In addition, peripheral blood and bone marrow samples were obtained from five controls (AA), who had various disorders (Table I) but with normal bone marrow morphology at the time of sampling. These samples were also washed and frozen at −90°C until required. Hematologic details and hemoglobin analysis of all cases are summarized in Table I.

RNA extraction. Total RNA was extracted from peripheral blood samples by phenol-chloroform extraction as described (6). The frozen bone marrow samples (~0.2 ml packed cells) were thawed in the presence of 10 vol 1 mM $MgCl_2$/0.1% diethyl pyrocarbonate. The thawed cells were homogenized in a Teflon glass homogenizer, diluted with an equal volume of 0.1 M citric acid/1 mM $MgCl_2$, and homogenized again. The lysate was spun at 2,500 *g*, after which the pelleted nuclei were resuspended in 2 ml of 0.1 M citric acid/1 mM $MgCl_2$, homogenized, and spun again. The supernatant from both spins was retained for extraction of the cytoplasmic RNA with phenol-chloroform (7). The nuclei were further purified by centrifugation through sucrose cushions (7). Light microscopy revealed minimal contamination of these nuclei by cytoplasmic fragments. The nuclei were resuspended in 10 ml of 0.1 M NaCl/10 mM NaOAc, pH 5.0, per 1 mM EDTA/0.5% sodium dodecyl sulfate and the RNA extracted as for the cytoplasmic fraction.

Complementary DNA (cDNA)-RNA hybridization assays. Full details of the hybridization assays to measure ratios of α/β-mRNA using cDNA probes have been described (6). The α/β-mRNA ratios obtained do not represent absolute values owing to variation in length and purity of the

TABLE I
Haematologic Data and mRNA Analysis on the Patients Studied

	Case No.	Diagnosis	Hb	MCV*	MCH‡	MCHC§	α/β-mRNA ratio Marrow nuclei	Marrow cytoplasm	Retics
			g/dl	fl	pg	%			
AA	1	Treated iron deficiency	13.9	87	28	33	1.59	1.52	1.42
	2	AML in remission	13.9	101	32	32	—‖	2.30	2.07
	3	Chronic idiopathic thrombocytopenia	12.6	85	28	33	1.07	1.73	1.64
	4	ALL in remission	15.6	98	33	34	1.45	2.24	2.18
	5	Treated pernicious anemia	14.9	92	31	33	1.04	1.41	1.62
						mean±SD	1.29±0.27	1.84±0.41	1.79±0.32
EE	1	—	11.5	68	23	34	1.35	2.97	3.66
	2	—	12.8	68	22	32	1.75	2.30	5.18
	3	—	12.5	65	21	33	1.59	2.50	3.29
	4	—	9.7	64	22	35	1.65	2.60	5.30
						mean±SD	1.59±0.17	2.59±0.28	4.36±1.03
	5	α-thal-2.	14.2	72	23	32	1.22	—‖	1.97

*Mean cell volume.
‡ Mean cell hemoglobin.
§ Mean cell hemoglobin concentration.
‖ The hybridization curves in these samples did not meet the criteria for calculating ratios and insufficient RNA was available to repeat these assays.

α- and β-cDNA probes, but are valid for comparative purposes (6). For each individual, the same probes were used for nuclear, cytoplasmic, and reticulocyte RNA samples. The majority of hybridizations were carried out in duplicate with close agreement (±10%).

Globin gene mapping. Because of the high incidence of α-thalassemia in Thailand, all of the EE samples were subjected to α-globin gene analysis (8, 9) to detect the presence of any deletion forms of α-thalassemia.

RESULTS

Hybridization curves obtained from bone marrow nuclei, bone marrow cytoplasm, and reticulocytes from one AA and one EE patient are shown in Fig. 1. Data on individual cases is presented in Table I and summarized in Fig. 2.

Among the five AA controls there is no significant difference in the mean α/β-mRNA levels between the three RNA fractions (range, 1.0–2.3), although the ratio in the nuclear samples was slightly lower than in the others.

Among the EE cases the ratios of α/β-mRNA in the reticulocytes (3.3–5.3) were similar to those reported (3) and much higher than in the AA controls (1.4–2.2, $P < 0.002$) with the exception of one EE sample with a ratio of 2.0. This individual was shown to be an α-thalassemia 2 heterozygote (with one α-globin gene deleted) by gene mapping, thus explaining this low ratio. In the EE cases, marrow cytoplasmic ratios ranged from 2.3 to 3.0, significantly lower than in the EE reticulocytes ($P < 0.02$) but still significantly higher than the equivalent sample from the AA controls (1.4–2.3, $P < 0.02$), while the ratios in the nuclear RNA from the EE samples showed complete overlap with the AA controls ($P > 0.1$). The lowest ratio among the EE cases was obtained in the case with α-thalassemia, although this was still within the range of the AA controls. Thus in contrast to the relatively constant ratio observed in the AA cases, the Hb E homozygotes showed a marked decrease in the amount of β^E-mRNA in going from marrow nuclei, to marrow cytoplasm, and to reticulocytes.

DISCUSSION

The results presented in Fig. 2, clearly demonstrate that in Hb E homozygotes there is a progressive decrease in the proportion of β-mRNA (relative to α-mRNA) from bone marrow nuclei to bone marrow

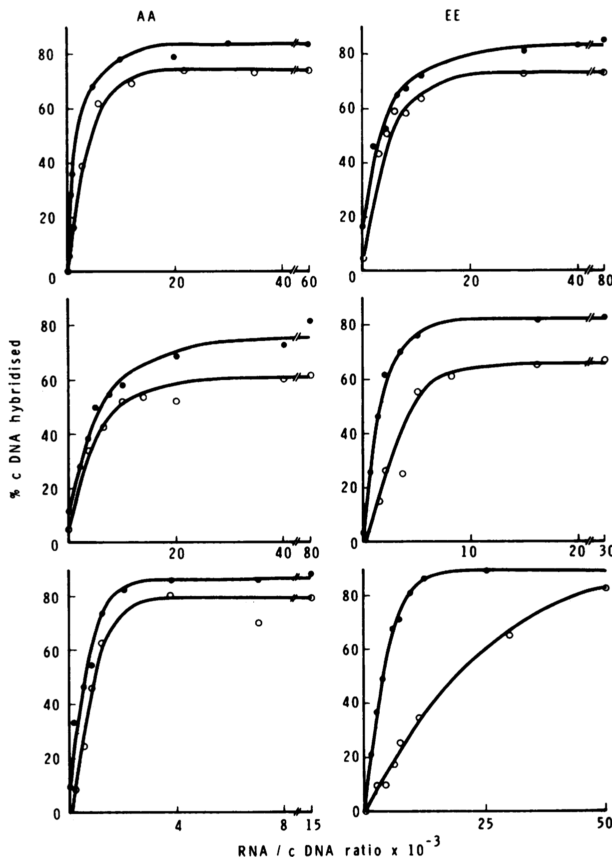

FIGURE 1 The hybridization of cDNA α (●) and cDNA β (O) to total RNA prepared from bone marrow nuclei (top), bone marrow cytoplasm (middle), and peripheral blood (bottom) of AA case 1 (left), and EE case 1 (right).

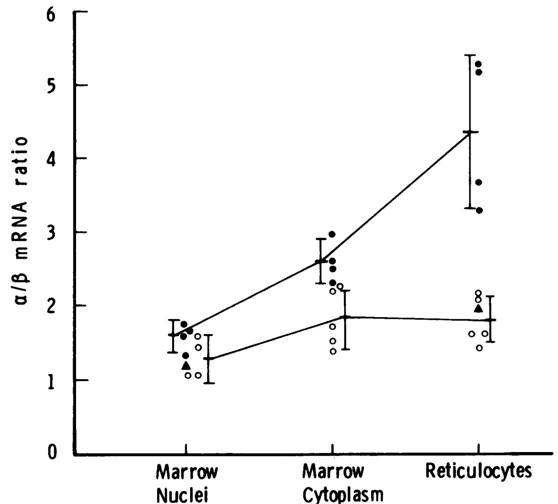

FIGURE 2 α/β-mRNA ratios, determined by cDNA solution hybridization in bone marrow nuclei, bone marrow cytoplasm, and peripheral blood of AA controls and Hb E homozygotes. ●, EE; ▲, EE/α_2-thal, O, AA.

cytoplasm to reticulocytes in contrast to the relatively constant ratio observed in the controls. Thus this direct analysis of mRNA ratios strongly supports the previous evidence from protein synthesis studies (3) that β^E-mRNA is unstable.

The similar ratios obtained in marrow nuclei from both normal and EE cases indicates that there was little or no contamination of the nuclei by cytoplasm in the EE cases. Any contamination of the marrow cytoplasm samples by bone marrow reticulocytes will only tend to diminish the difference between these two fractions but would exaggerate any difference between the nuclear and cytoplasmic fractions of the nucleated cells. This suggests that much, if not all of the loss of β^E-mRNA occurs after transportation of the mRNA molecules from the nucleus to the cytoplasm.

Instability of β-mRNA has been suggested as the mechanism for decreased levels of β-mRNA in some of the β^+-thalassemias (7, 10). In those cases, however, the demonstration of a lower α/β-mRNA level in reticulocytes compared with marrow nuclei and cytoplasm has to be interpreted with caution because they were measured in β-thalassemia homozygotes in which there is considerable ineffective erythropoiesis and selection for cells producing Hb F. In the case of Hb E homozygotes this problem is avoided because there is little ineffective erythropoiesis and Hb F levels in all of the cases were <5%.

It remains to be seen whether the β^E-mutation, presumably GAG to AAG at codon 26, is responsible for both the mRNA instability and the amino acid substitution, or whether there are two separate defects in this gene. Measurements of globin synthesis ratios and mRNA levels in other variants around position 26 might be helpful in distinguishing between these possibilities [eg., Hb Henri Mondor, $\alpha_2\beta_2{}^{26\,glu\rightarrow val}$, probable substitution GAG → GTG (11)]. However, to exclude the possibility of a second defect in the β^E gene, it may be necessary to clone and sequence it.

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