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Genetics

Three mouse models of human thalassemia

(DNA·RNA hybridization/restriction endonuclease mapping/globin synthesis)

J. MARTINELL*, J. B. WHITNEY III*†, R. A. POPP‡, L. B. RUSSELL‡, AND W. F. ANDERSON*

*Laboratory of Molecular Hematology, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, Maryland 20205; and ‡Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830

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ABSTRACT **Three types of mice with globin gene mutations, called 352HB, 27HB, and *Hba*$^{th-J}$, appear to be true animal models of human thalassemia. Expression of the α-globin genes in three stocks of mice, each one heterozygous for one of the α-globin mutations, was examined at the polypeptide, RNA, and DNA levels. α-Globin polypeptide chains, relative to β-globin chains in heterozygous thalassemic mice, are present at approximately 80% of normal. The ratios of α-globin to β-globin RNA sequences are also 75–80% of normal, exactly reflecting the α-globin to β-globin chain ratios. In the case of mutant 352HB, at least one α-globin gene is deleted. Thalassemic mouse erythroid cells appear to compensate partially for the loss of half of their α-globin genes.**

Two radiation-induced mutations (namely 352HB and 27HB) at the α-hemoglobin locus in mice (1, 2) and a third mutation, which was chemically induced (*Hba*$^{th-J}$) (3, 4), have characteristics phenotypically similar to α-thalassemia in humans. Some of these characteristics are: (*i*) lethality in the homozygous state (5), (*ii*) neither of the two α-globin chains of the irradiated strain SEC parent is expressed in the viable heterozygotes, (*iii*) microcytic anemia (2), (*iv*) shortened erythrocyte lifespan (6), and (*v*) the formation of homotetramers of embryonic (4, 7) or adult hemoglobins (8).

To determine if these three mouse stocks contain an α-thalassemia mutation analogous to the human genetic anemias, levels of α- and β-globin chain synthesis in reticulocytes were measured, relative amounts of α- and β-globin RNA sequences in reticulocytes were determined, and an *Eco*RI restriction endonuclease digest of DNA of one thalassemic type (352HB) was compared to digests from normal mice. The data indicate that these mouse mutants are true models of human α-thalassemia. Our results are consistent with the defect being a deletion of at least part of the α-globin gene complex.

MATERIALS AND METHODS

Materials. *Mice.* Heterozygous thalassemic mice from stocks 352HB and 27HB (1) were from the Oak Ridge National Laboratory; *Hba*$^{th-J}$ heterozygous thalassemics (3, 4) and normal inbred SEC, C57BL, and CE mice were descendants of mice from the Jackson Laboratory. Thalassemic heterozygotes denoted 352HB and 27HB used in this study were progeny of four backcrosses to C57BL/6. *Hba*$^{th-J}$ mice were also bred onto a C57BL/6 genetic background. *Hba*$^{th-J}$ thalassemic heterozygotes were identified by increased resistance to erythrocyte lysis by hypotonic saline. The 352HB and 27HB heterozygous thalassemic individuals were identified by clinical hematological methods, using reticulocytosis and microcytosis as the principal criteria.

Reagents. The restriction enzymes *Hha* I, *Bgl* II, and *Eco*RI were purchased from New England BioLabs; *Kpn* I, *Hin*dIII, and formamide were obtained from Bethesda Research Laboratories (Rockville, MD). All references to restriction endonuclease units are consistent with the supplier's definitions. Agarose was supplied by SeaKem (Rockland, ME). Nonradioactive deoxyribonucleoside triphosphates, *Escherichia coli* RNA and DNA, DNase I, and phenylhydrazine·HCl were all from Sigma. Tritiated deoxyribonucleoside triphosphates were purchased from New England Nuclear, and Amersham supplied the ^{32}P-labeled dCTP. Boehringer Mannheim was the source of DNA polymerase I. Miles Laboratories was the source of S1 nuclease (*Aspergillus oryzae*). Bacteriophage stocks of λ Charon (Ch) 3A Hb117 and Charon (Ch) 3A Hb4 and a stock of Mα1 (a mouse α-globin genomic clone) were generously given to us by O. Smithies and P. Leder, respectively.

Collection of Reticulocytes. Each mouse received three or four injections of 0.4–0.5 ml of phenylhydrazine (2 mg/ml, pH 6.4–7.0) over a period of 5 days. When reticulocyte levels of about 60% were obtained, approximately 0.5 ml of blood was collected from each aminal by retro-orbital bleeding (9), and the cells were washed by standard methods (10). After labeling of globin chains or prior to extraction of RNA, lysates were made (10) and stored in liquid nitrogen.

Synthesis and Analysis of Radioactive Globin Chains. Fresh reticulocytes (0.4 ml of packed cells) were incubated in 0.2 ml of improved minimal essential medium (minus leucine, minus tryptophan) plus 2% fetal calf serum. The incubation medium also contained 0.2 ml of Dulbecco's phosphate-buffered saline (minus Ca^{2+}, minus Mg^{2+}) and 100 μCi of [4,5-^{3}H]leucine (specific activity, 60 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels). After incubation for 5.5 hr at 37°C, lysates were prepared and stored. Acid/acetone extraction and globin chain analysis by carboxymethyl-cellulose chromatography were as described (11).

Preparation of RNA. RNAs were extracted from reticulocyte lysates by the pH 9 method of Brawerman (12) and were stored in aqueous solution in liquid nitrogen until assayed for globin sequences.

Preparation of Single-Stranded Globin cDNA Probes. Both α- and β-globin cDNA probes were prepared from DNA fragments purified from mouse globin cDNA-derived bacteriophage clones (i.e., Ch 3A Hb117 and Ch 3A Hb4).

α-Globin cDNA. DNA from Ch 3A Hb117 (500 μg) was incubated with restriction enzymes under the following conditions: 250 units of *Bgl* II, 1000 units of *Kpn* I, and 100 units of *Hin*dIII in 6 mM Tris·HCl, pH 7.4/50 mM NaCl/6 mM $MgCl_2$ in a total volume of 6 ml. Incubation was for 2 hr at 37°C, after which the enzymes were inactivated by heating for 10 min at 68°C. The resulting 2.9-kilobase (kb) fragment containing the α-globin gene (E. F. Vanin, personal communication) was purified by electrophoresis through a 2% agarose gel (14 cm × 10

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Abbreviation: kb, kilobase.

† Department of Cell and Molecular Biology, Medical College of Georgia, Augusta, Georgia 30912.

cm × 6 mm) in 40 mM Tris·HCl, pH 7.8/20 mM NaOAc/2 mM EDTA at 25 V for 16 hr at room temperature.

β-Globin cDNA. DNA from Ch 3A Hb4 (1 mg) was incubated in the presence of 200 units of *Hha* I in 6 mM Tris·HCl, pH 7.4/50 mM NaCl/6 mM $MgCl_2$ in a total volume of 1.1 ml for 1 hr at 37°C. The enzyme was heat inactivated and the 1.8-kb fragment containing the β-globin gene (13) was purified in a manner similar to that described for the α-globin DNA.

After electrophoresis through agarose gels, DNA bands containing restriction fragments with either α- or β-globin cDNA sequences were recovered from the agarose and were extracted with phenol as described (14). After precipitation with ethanol, the DNA was resuspended in water and desalted by passage over Sephadex G-25 prior to nick-translation.

Nick-Translation. The α- and β-globin cDNAs were made radioactive by nick-translation (15, 16). Conditions were as described (14) except the reaction for the α-globin cDNA probe contained 40 μM each of nonradioactive dATP, dGTP, and dTTP along with 1 mCi of [α-^{32}P]dCTP (specific activity, 400 Ci/mmol). Reaction mixtures for the β-globin cDNA probes contained 40 μM each dATP and dTTP; 75 μCi of [^{3}H]dCTP (specific activity, 24.9 Ci/mmol) and 30 μCi of [^{3}H]dGTP (specific activity, 10 Ci/mmol) were also included. After incubation, the reaction mixture was extracted with phenol and passed over Sephadex G-50, and DNA was precipitated with ethanol as described (14), except that the Sephadex G-50 equilibration buffer was 10 mM Tris·HCl, pH 7.5/10 mM NaCl/2 mM EDTA. DNA was collected by centrifugation and was desalted as described. Specific activities of 1–2 × 10^8 cpm per μg for ^{32}P were obtained.

Single-stranded DNAs were prepared from radioactive double-stranded DNA nick-translated products by hybridization in solution to excess α- and β-globin RNA sequences under conditions that favor RNA·DNA heteroduplexes over DNA·DNA duplexes (17). The hybridization reaction mixture contained a ratio of total mouse reticulocyte RNA to DNA (A_{260}:A_{260}) of 4600 and had a total volume of 250 μl as described (14). After digestion with 2000 units of S1 nuclease (see below), RNA in the mixture was hydrolyzed by incubation in 0.3 M NaOH at 65°C for 20 min. The solution was then neutralized with HCl and extracted with phenol, and the DNA was precipitated and desalted as described.

Probes prepared in this manner were 1–2% resistant to digestion by S1 nuclease when "hybridized" to zero input eukaryotic RNA under conditions described below. Various preparations of probes were protected to maxima of 35–65% by excess mouse reticulocyte total RNA. Probes prepared by this method had the properties of single-stranded DNA when eluted from a column of hydroxyapatite (18) (data not shown).

Liquid Hybridization. Saturation hybridizations of α- and β-globin cDNA probes were performed in a total reaction volume of 10 μl in 50% (vol/vol) formamide and 0.5 M NaCl (17). In addition, each reaction mixture contained a variable quantity between 0 and 280 ng of mouse reticulocyte RNA (1 A_{260} unit was assumed to be equivalent to 35 μg of RNA) plus the ^{32}P-labeled α-globin probe and ^{3}H-labeled β-globin probe in approximately equal amounts (ranging between 0.2 and 0.4 ng). Hybridizations were carried out at 67°C for 48 hr (17) and S1 nuclease resistance was determined as described (19).

Southern Blot Analysis. Genomic mouse DNA prepared by the method of Blin and Stafford (20) (35 μg per sample) was digested by *Eco*RI and analyzed by the method of Southern (21). The double-stranded probe was a nick-translated ^{32}P-labeled, 3-kb *Sac* I DNA fragment derived from DNA of the mouse genomic bacteriophage clone Mα1 (22). This DNA fragment contains an entire α-globin gene with considerable 5′ and 3′ flanking regions.

RESULTS

α/β-Globin Polypeptide Chains. Reticulocytes from phenylhydrazine-treated normal or mutant mice were incubated in the presence of [^{3}H]leucine for 5.5 hr, after which the newly synthesized α- and β-globin chains were separated by carboxymethyl-cellulose chromatography. No correction was made in converting the dpm eluted in each peak to a molar α/β-globin ratio because α- and β-globin chains of mice each contain 17 leucine residues (23, 24). Results in Fig. 1 and Table 1 show that the α-globin chain synthesis in 352HB and 27HB mice is depressed by approximately 20%. The α-globin chain deficiency is not 50%, as might be expected (see *Discussion*).

α/β-Globin RNA Sequences. In order to compare α/β-globin RNA ratios of thalassemic mice to one another and to those of nonthalassemic animals, RNA saturation hybridization experiments were performed with a common nonthalassemic mouse (namely, C57BL/6J) as a control, rather than normal littermates of each mutant. The use of C57BL RNA in each experiment thus allows comparison of hybridization data for several types of mice. The relative amounts of α- and β-globin RNA sequences were measured by hybridization of various amounts of total mouse reticulocyte RNA to single-stranded globin cDNA probes. Figs. 2 and 3 illustrate such saturation hybridization curves. A comparison of the hybridization curves in Figs. 2 and 3 shows that the slopes of the α-globin RNA curves for the 352HB, 27HB, and *Hba*$^{th\text{-}J}$ mice are lower than the α-globin slopes for C57BL mice. This difference is clearer when the α-globin curves are compared to the β-globin curves. Data from two (or three) separate experiments were used to calculate average α/β-globin RNA ratios (see Table 2). A least-squares straight line was calculated for the linear portion of each hybridization curve and the amount of RNA necessary to protect 50% of each probe available for hybridization from S1 nuclease degradation was then found by referring to the straight line. These values were used to calculate α/β-globin RNA sequence ratios (25).

$$\alpha/\beta \text{ RNA sequences} = \frac{\text{Ratio RNA/DNA}_\beta \text{ required for 50\% hybrid DNA}_\beta}{\text{Ratio RNA/DNA}_\alpha \text{ required for 50\% hybrid DNA}_\alpha}$$

The α/β RNA sequence ratios were then normalized for each experiment (see Table 2 legend). These data indicate that the amount of α-globin RNA relative to β-globin RNA is decreased by 20–25% in thalassemic mice. This difference is the same as the decrease of α-globin polypeptide relative to β-globin polypeptide in 352HB and 27HB mice as shown in Table 1.

α-Globin DNA Structure. The results of a Southern blot analysis of DNAs of thalassemic and normal mice are shown in Fig. 4. Lane A contained an *Eco*RI digest of DNA of strain SEC (the same as the irradiated father of the original mutant 352HB thalassemic mouse) and shows two bands of α-globin DNA at

Table 1. α/β ratios of globin chains labeled in intact reticulocytes

Mouse	Total dpm α	Total dpm β	α/β	Normalized α/β
352HB NS	196,000	178,000	1.10	1.00
352HB TH	221,000	263,000	0.84	0.76
27HB NS	210,000	208,000	1.01	1.00
27HB TH	350,000	395,000	0.89	0.88
101	539,000	486,000	1.11	—

The total amount of radioactivity contained in each α- and β-globin region of each column (bracketed regions in Fig. 1) was determined and the α/β-globin ratios were calculated. Normalized ratios are shown to compare thalassemics (TH) to their normal siblings (NS).

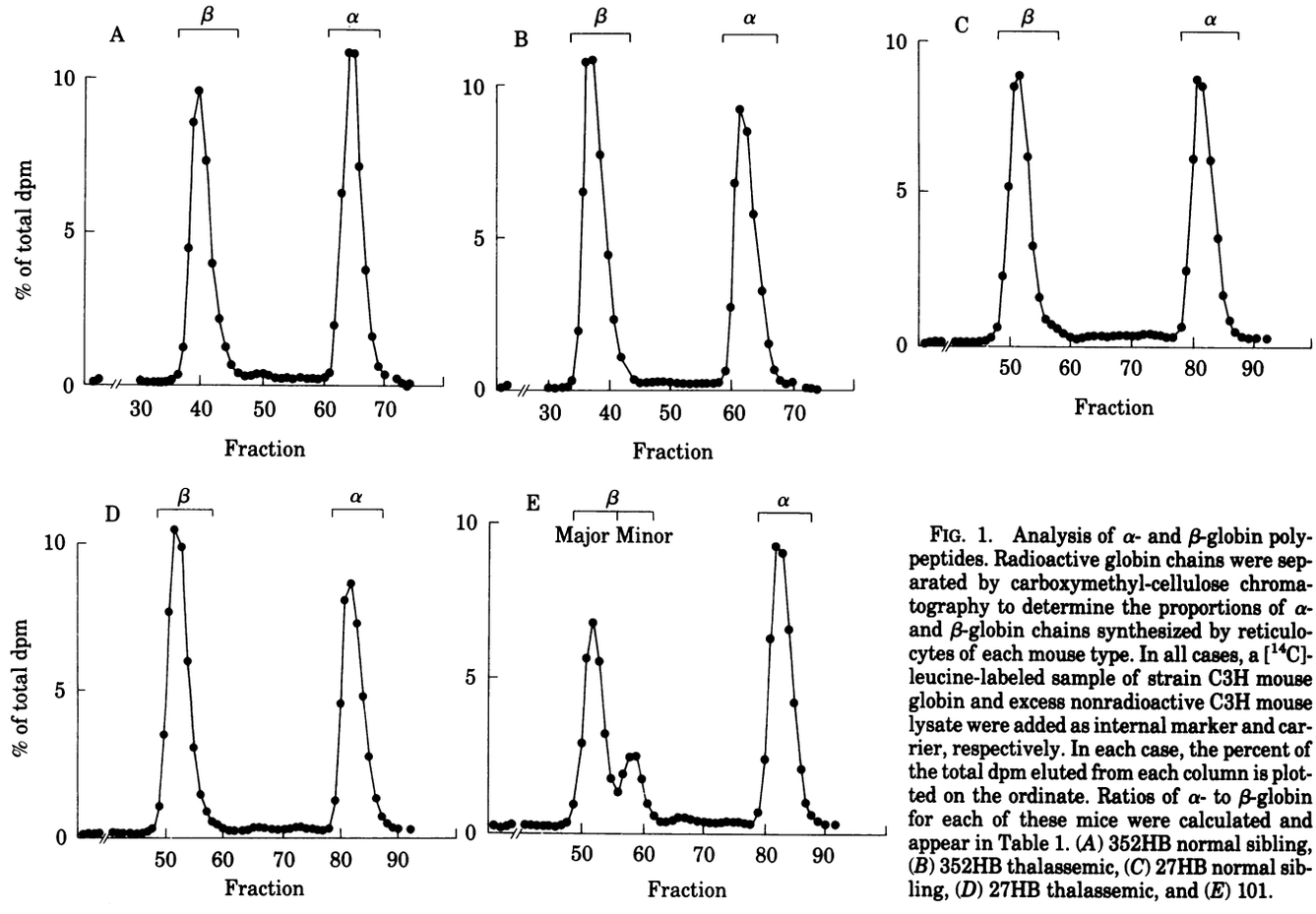

FIG. 1. Analysis of α- and β-globin polypeptides. Radioactive globin chains were separated by carboxymethyl-cellulose chromatography to determine the proportions of α- and β-globin chains synthesized by reticulocytes of each mouse type. In all cases, a [^{14}C]-leucine-labeled sample of strain C3H mouse globin and excess nonradioactive C3H mouse lysate were added as internal marker and carrier, respectively. In each case, the percent of the total dpm eluted from each column is plotted on the ordinate. Ratios of α- to β-globin for each of these mice were calculated and appear in Table 1. (*A*) 352HB normal sibling, (*B*) 352HB thalassemic, (*C*) 27HB normal sibling, (*D*) 27HB thalassemic, and (*E*) 101.

10.5 and 12.0 kb. Lane C contained DNA of normal strain CE and shows a strong band at 10.5 kb, but no band at 12.0 kb. A normal hybrid bearing one CE chromosome and one SEC chromosome would be expected to show a 10.5-kb band of full intensity but to have the 12.0 band at only 50% of the normal SEC intensity. However, a mouse bearing one normal CE chromosome and the mutated 352HB chromosome (lane B) shows no 12.0-kb band whatsoever, even at long exposures of autoradiography, indicating that this region is deleted from the mutant chromosome. While the absence of the 12.0-kb band is invariant in samples from mice with only the CE (or similar) chromosomes 11, higher molecular weight bands of variable intensity are sometimes observed in DNA samples of thalassemic and normal mice with this chromosome. The weakness of the 10.5-kb band in lane B may indicate that the second α-globin gene is also deleted from the 352HB mutant chromosome.

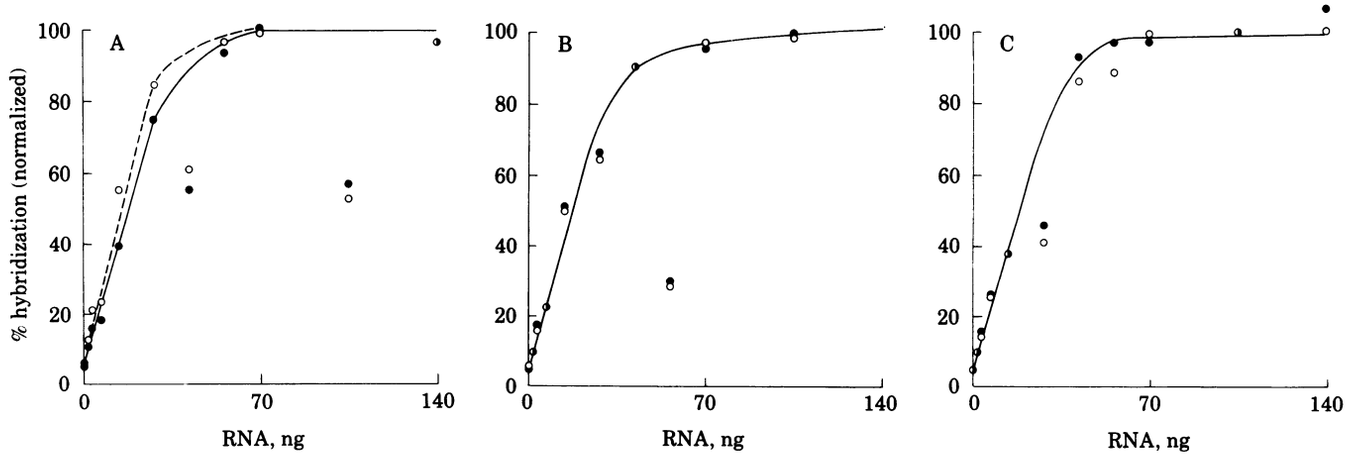

FIG. 2. Measurement of α- and β-globin RNA sequences in reticulocytes of normal and thalassemic mice. Single-stranded DNA probes derived from Ch 3A Hb117 and Ch 3A Hb4 were hybridized to increasing amounts of total reticulocyte RNA from various mice. The degree of hybridization in each sample was determined by probe resistance to digestion by S1 nuclease. All hybridization values were normalized, the saturation plateau of each curve being defined as 100%. Actual hybridization values for each saturation plateau are as follows: C57BL α, 33%; C57BL β, 45%; 352HB α, 35%; 352HB β, 46%; 27HB α, 36%; 27HB β, 47%. Probes for α-globin RNA sequences were labeled with ^{32}P and probes for β-globin RNA sequences with ^{3}H. Therefore, at any given RNA concentration, α-globin and β-globin sequences were measured within the same hybridization reaction vessel. Globin RNA sequences were measured in C57BL (i.e., nonthalassemic) mice (*A*), 352HB thalassemic mice (*B*), and 27HB thalassemic mice (*C*). ●, β-Globin probe; ○, α-globin probe.

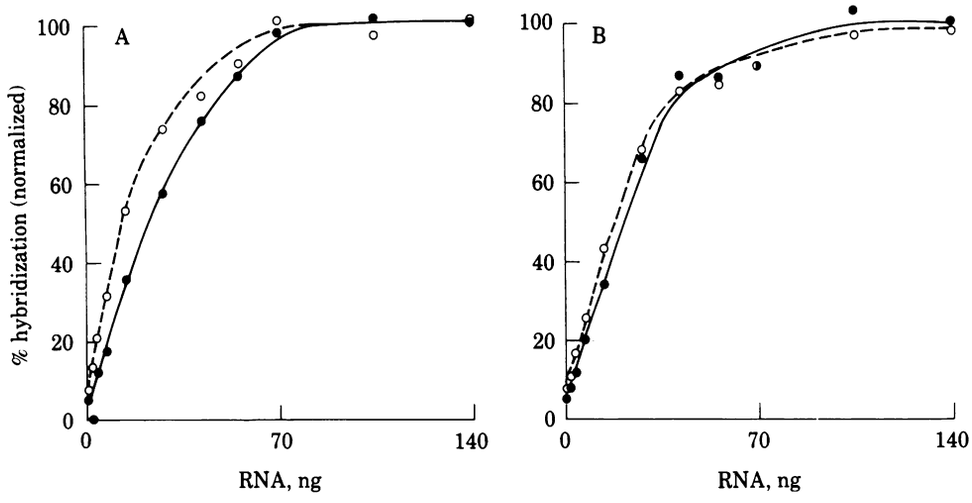

FIG. 3. Measurement of α- and β-globin RNA sequences of reticulocytes of normal and $Hba^{th\text{-}J}$ mice. Radioactive single-stranded DNA probes were hybridized to various amounts of total reticulocyte RNA from normal and $Hba^{th\text{-}J}$ mice (see legend to Fig. 2). Values were normalized as in Fig. 2. Actual saturation plateau values were: C57BL α, 42%; C57BL β, 53%; $Hba^{th\text{-}J}$ α, 38%; $Hba^{th\text{-}J}$ β, 48%. (*A*) C57BL mouse, (*B*) $Hba^{th\text{-}J}$ mouse. ●, β-Globin probe; ○, α-globin probe.

DISCUSSION

We show that the three mouse α-globin gene mutations, namely, 352HB, 27HB, and $Hba^{th\text{-}J}$, are true thalassemia mutations, and are thus far the only known experimental animal models of thalassemia. We have measured the relative amounts of α- and β-globin polypeptide for two of the mutations and RNA sequences in the reticulocytes of heterozygous mice for each of the three independent mutations. We have also analyzed the α-globin gene structure of one of the thalassemic mice by restriction endonuclease digestion followed by the Southern blotting technique.

The ratio of α- to β-globin chain synthesis was measured for the 352HB and 27HB mutations and was found to be depressed by about 20% compared to the same ratio for normal mice. The α/β-globin RNA ratio of all three mutants is likewise lower in comparison to normal mice, the thalassemics having an α/β-globin RNA ratio of 75–80%. These data are consistent with the observed presence of 6–8% β_4 in these mice (unpublished results).

The haploid genome of C57BL mice has been shown to contain two β-globin genes, even though only one type of β-globin chain is expressed (26). Genomic DNAs of C57BL mice show the same restriction patterns (for *Eco*RI and *Hin*dIII; unpublished observations) as SEC; thus, we presume that C57BL has two α-globin genes per chromosome 11, even though only one type of α-globin chain is expressed (23). It is likely then that the α/β-globin gene ratio in these mice is 1. As judged on the basis of the Southern blot analysis of 352HB DNA, at least one and possibly both α-globin genes that could have been inherited from the x-irradiated progenitor of the 352HB stock (1) have been deleted. Because x-irradiation frequently results in the

Table 2. Measurement of α- and β-globin RNA sequences of reticulocytes

Exp.	Mouse	S1 nuclease-resistant probe saturation, ng		RNA necessary for 50% saturation of probe, ng		Normalized α/β
		α-Globin	β-Globin	α-Globin	β-Globin	
1	C57BL	0.085	0.12	16.2	19.2	1.00
	352HB	0.090	0.12	24.9	26.2	0.92
	27HB	0.090	0.12	21.0	20.1	0.83
2	C57BL	0.10	0.23	21.8	22.3	1.00
	352HB	0.11	0.21	30.6	17.8	0.73
	27HB	0.12	0.22	37.1	26.8	0.86
3	C57BL	0.12	0.19	14.2	17.7	1.00
	352HB	0.12	0.14	24.1	17.9	0.77
	27HB	0.12	0.15	22.2	14.4	0.64
4	C57BL	0.17	0.19	14.5	18.4	1.00
	$Hba^{th\text{-}J}$	0.18	0.19	17.3	16.5	0.76
5	C57BL	0.14	0.18	15.7	23.2	1.00
	$Hba^{th\text{-}J}$	0.12	0.16	18.7	20.9	0.74
Mean	352HB	—	—	—	—	0.81 ± 0.10
	27HB	—	—	—	—	0.78 ± 0.12
	$Hba^{th\text{-}J}$	—	—	—	—	0.75

α/β-globin RNA sequence ratios of thalassemic mouse reticulocytes were compared to the α/β-globin ratios determined for normal mouse reticulocytes. These ratios were calculated as described by Hunt *et al.* (25) and were normalized within each experiment, defining the normal mouse (i.e., C57BL) α/β-globin ratio as 1.00. The results of three separate determinations are shown, along with the mean values and the standard deviations for the thalassemic mice.

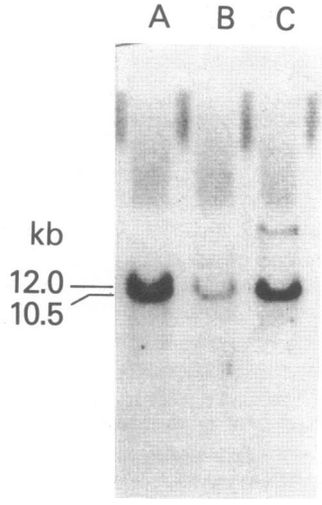

FIG. 4. Restriction endonuclease analysis of 352HB DNA. *Eco*RI digests of DNA from mice of three stocks were electrophoresed through 0.7% agarose gels. The DNA was blotted (21) onto nitrocellulose membranes and was probed with a nick-translated ^{32}P-labeled α-globin gene from clone Mα1. Mouse stocks are: lane A, SEC; lane B, 352HB; and lane C, CE.

loss of relatively large portions of chromosomes spanning several loci (27), and because the α-globin-like embryonic globin is not expressed (4), it would not be surprising for both of the α-globin genes to be deleted from the mutant chromosome in this x-ray induced α-thalassemia.

There are at least two possible explanations for the α/β-globin RNA ratios of approximately 75% that we have observed in the thalassemic mice. If there are three α-globin and four β-globin genes present in diploid cells of thalassemic mice, while there are four globin genes of each type in normal mice, the observed RNA sequence ratios might be expected if transcription, processing, transport, and degradation of α- and β-globin mRNAs were the same for thalassemic and normal mice. However, earlier work (28) has demonstrated the absence of both α-globin products of the x-irradiated SEC parental type. This information, in conjunction with the data relevant to globin synthesis and α/β-globin RNA levels reported here, would indicate that one-third of the α-globin RNA in reticulocytes of the 352HB and 27HB mice is inactive in protein synthesis. Hence, the α-globin mRNA in thalassemic mice should be responsible for twice as much protein product compared to β-globin mRNA if the above hypotheses were correct. An alternative explanation is that two α-globin genes are deleted in thalassemic mice and that the α/β-globin RNA ratios of 0.75–0.80 are the result of differences in the regulation or rates of synthesis, processing, or degradation of RNAs in normal and thalassemic mice. Because the α/β-globin chain ratios in thalassemic mice are reduced by approximately the same amount as the RNA ratios, it is reasonable to suggest that the α- and β-globin RNA sequences measured by hybridization have equal efficiencies of translation. Data shown in Fig. 4 and additional data (unpublished) suggest that two α-globin genes are indeed deleted from each α-thalassemic mutant chromosome and thus offer additional support for the latter hypothesis. It appears likely that these thalassemic mice can partially compensate for the loss of half of their α-globin genes.

The deletion of α-globin sequences from the DNA of the 352HB mutant is analogous to the situation that has been seen in some of the α-thalassemias in man. The normal condition in humans consists of four α-globin loci, two on each chromosome (29). α-Thalassemia variations of this configuration include the deletion of α-globin sequences of one or more of these four genes. Several deletions and partial deletions of α-globin genes occur in various permutations and have been described (30–32).

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