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Genetics

Deletions in the α-globin gene complex in α-thalassemic mice

(Southern blotting technique/mouse genetics/hemoglobin/mutation)

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ABSTRACT **Three induced, heritable mutations in the mouse cause α-thalassemias. The adult α-globin genes on each mutant chromosome are no longer expressed. Embryos heterozygous for one normal and any of the three mutant chromosomes also seem to be deficient in embryonic α-globin-like x-globin, suggesting that the x-globin gene is nearby and also inactivated. A normal genetic polymorphism for a specific *Eco*RI site in or around the mouse α-globin gene complex has been used here to show that each of the three mutated chromosomes has a deletion that includes the segment of a 12-kilobase *Eco*RI band which normally carries one of the two adult α-globin genes. The deletion of the comparable part of the second α-globin gene site is also inferred. Nonetheless, a 4.7-kilobase *Eco*RI segment which carries a characterized α-globin-like pseudogene is still present in each mutant. These mutations were recovered after triethylenemelamine or x-ray treatments.**

Thalassemia in man is a disease in which the synthesis of a globin chain is abnormally low or entirely absent. Mice heterozygous for any one of three induced mutations have hematological abnormalities and decreased α-globin synthesis consistent with their having diseases that can be considered to be models of human thalassemia. Two of these mutations were induced by x-irradiation at The Oak Ridge National Laboratory (1) and the third was induced with triethylenemelamine at The Jackson Laboratory (2). All were discovered because of their effects upon hemoglobin expression—in particular, upon α-globin expression (3, 4).

In man, the two closely linked nonallelic α-globin genes encode identical polypeptide products. Consequently, normal persons have a single type of adult α-globin. Some mice also have a single type of adult α-globin; for example, mice of the inbred strain CE/J have only one adult α-globin, called chain 5 (5, 6). Other mice—for example, those of inbred strain SEC—have two structurally distinct adult α-globins (7), indicating that at least some normal mice also have two nonallelic adult α-globin genes (or more). The nonallelic genes for the two SEC mouse adult α-globins were shown genetically to lie within 0.5 centimorgan of one another (8). The mutations, called 352HB and 27HB, were induced by x-irradiation of SEC mice which normally make adult α-globin chains 2 and 3 in approximately equal amounts. Sequence analyses of the α-globins of heterozygous mutant mice of these types show that neither of the genes for these two chains remains detectably active (3). The Hba^{th-J} mutation was found among the progeny of a triethylenemelamine-treated male of a stock that had α-globin chains 1 and 4 (genotype Hba^{c}). Isoelectric focusing analysis (5) of the hemoglobin of the mutant individual showed none of the expected chain 4-containing hemoglobin. His mutation-bearing offspring by SEC females had no detectable chain 1, so both adult α-globin loci must be inactive in this third case as well. Test crosses with mice carrying the markers *wa-2* and *sh-2* demonstrate (9) that Hba^{th-J} is located on chromosome 11 at or near the normal location of the *Hba* hemoglobin α-chain locus (10).

By comparison of a mutant DNA to that of its progenitor normal DNA, it should be possible to determine precisely what change was induced by a mutagenic agent to inactivate simultaneously several closely linked structural genes—whether, for each case, it was a deletion or a mutation in a regulatory site necessary for the expression of the entire α-globin gene cluster. Although maps are not yet established for their regions of origin, a number of *Eco*RI fragments of the normal mouse genome are known to carry sequences with homology to adult α-globin mRNA sequences. A 4.7-kilobase (kb) cloned genomic fragment (11) that hybridizes strongly to a mouse α-globin cDNA clone detects genomic *Eco*RI bands of 18.5, 14.7, 12, 10.5, 5.25, 4.7 (self), and 2.4 (or 2.34) kb from noninbred CD1 mice (12). The clone Mα1 of Leder *et al.* (13) shows that the 10.5-kb genomic *Eco*RI fragment carries an adult α-globin gene. The sequence of this clone (14) revealed that α-globin gene intervening sequences are of similar small size and also established that the clone corresponds to the BALB/c adult α-globin chain 2 (with its distinctive serine at amino acid 68).

Determinations of the sequences of portions of the 4.7-kb cloned *Eco*RI fragment and of a comparable 4.5-kb fragment cloned from BALB/c mice (15, 16) reveal that, although its gene or "pseudogene" is closely homologous to the mouse α-globin gene, it completely lacks intervening sequences. Another mouse pseudogene, of the cloned small genomic *Eco*RI fragment, has intervening sequences but also has base substitutions which would make a hemoglobin synthesized from it abnormal (17).

Although homozygous α-thalassemic mouse embryos die too early to provide sufficient DNA for convenient analysis, normal genetic polymorphisms of restriction enzyme cleavage sites in and around the globin loci, characterized in some detail in human DNAs, could be useful for producing informative heterozygous combinations in mice, in which mating can be controlled. One genetic polymorphism in *Eco*RI patterns among mouse strains was found by using mouse globin cDNA or cloned α-globin cDNA as probe (D. Housman and E. Vanin, personal communications). A doublet of bands found in most mouse DNA samples, probably corresponding to the 12- and 10.5-kb bands of Blattner *et al.* (12), were replaced in the DNA of strain AKR by a single band of 10.5 kb plus one or two larger bands, perhaps the same as the 14.7- and 18.5-kb bands of Blattner *et al.* (12). It thus seems likely that the CD1 (pooled) mouse DNA of Blattner *et al.* (12) included DNAs of at least two different genotypes (10.5 + 12 kb, doublet; 10.5 kb, singlet + larger). The absence

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Abbreviations: kb, kilobase(s); NaCl/Cit, standard saline citrate (0.15 M NaCl/0.015 M sodium citrate).

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of the 12-kb band from the "singlet" samples has been used here in the study of the thalassemia mutant mouse DNAs.

The present study of the DNAs of heterozygous α-thalassemic mice complements our study of their mRNAs (18). We find that at least a part of the α-globin gene complex has been deleted by each of the three mouse α-thalsassemia mutations, consistent with the low α-globin synthesis rates and low α-globin mRNA levels in thalassemia trait (heterozygous) individuals.

MATERIALS AND METHODS

Mouse Genomic DNA. Inbred mice were descendants of mice from The Jackson Laboratory. Exotic mice were generously supplied by Michael Potter (National Cancer Institute). Noninbred CD1 Swiss mice were purchased in 1978 and 1979 from Charles River (Wilmington, MA): 3 of 34 from the no. 64 area were heterozygous for an *Hba* haplotype apparently identical to Hba^h (19). Nineteen from area 61 and four each from three other areas had only Hba^c or Hba^f. Single mice with unusual or new *Hba* haplotypes were crossed to inbred mice, and stocks were selected in the following generations to be Hbb^s and homozygous-by-descent for the new *Hba* types. Testing for *Hbb* was carried out by cystamine-electrophoresis (20) and for *Hba*, by isoelectric focusing (5).

Thalassemia mutations—Hba^{th-J} from The Jackson Laboratory and 27HB and 352HB from The Oak Ridge National Laboratory—were maintained by repeated backcross to the C57BL/6J strain. For the Hba^{th-J}, 27HB, and 352HB mutations on this inbred background, 39%, 9%, and 37%, respectively, of the third (and higher) backcross generation weanling offspring of male thalassemia heterozygotes and normal females proved to be thalassemia carriers. A final cross of C57BL thalassemics to CE/J mates produced heterozygotes with each thalassemia and a CE/J normal chromosome 11.

Genomic DNA was prepared from liver as described by Blin and Stafford (21) but with proteinase K digestion times of 6 hr or more and dialysis of the RNase-digested DNA as described by Blattner *et al.* (12). Samples were digested with the indicated restriction endonuclease (New England BioLabs) and then were phenol-extracted and ethanol-precipitated.

Gel Electrophoresis and Transfer. Gels of 0.7% agarose (Bio-Rad or Seakem) were prepared and run in 40 mM Tris·HCl, pH 7.8/5 mM sodium acetate/1.2 mM EDTA. Electrophoresis was for 18 hr at 20 V (or an equivalent combination) at 15–16°C in a Bio-Rad vertical slab gel apparatus, with buffer recirculated. Southern transfer (22) was with 1.8 M sodium chloride/0.18 M sodium citrate, pH 7.0, for 20 hr [this is 12 × standard saline citrate (NaCl/Cit)].

Hybridization Probes. A 3-kb *Sac* I fragment of clone Mα1 (13, 14) was prepared by electroelution from a 6-mm agarose gel. A pBR322 subclone bearing the 4.7-kb *Eco*RI insert of Charon 3A Mm30.5 (15) was a gift of Elio F. Vanin. Probes were prepared by nick-translation (23) using DNA polymerase I (Boehringer Mannheim), nonradioactive deoxyribonucleotide triphosphates and DNase I (Sigma), and [^{32}P]dCTP (NEG013X, New England Nuclear).

Hybridization. Prehybridization of tne BA85 membrane was in a sealed bag for 2–4 hr at 65°C in 50 mM potassium phosphate at pH 6.8 containing per 200 ml: 60 ml of 20× NaCl/Cit, 4 ml of polyvinylpyrrolidone (Sigma PVP-360; 4% in 3× NaCl/Cit), 4 ml of Ficoll (Sigma, 4% in 3× NaCl/Cit), 0.1 g of bovine serum albumin (Miles) (24), 0.2 g of Na pyrophosphate, 10 ml of 10% $NaDodSO_4$, 1 ml of poly(rA) (Miles, 10 mg/ml), and 1 g of glycine. The probe (4×10^7 cpm) was double-boiled for 10 min in 0.02 M Tris·HCl (pH 7.5) with 2 mg of poly(rA). The 40-ml hybridization solution included, at prehybridization concentrations, 6× NaCl/Cit, polyvinylpyrrolidone, Ficoll, bovine serum albumin, and $NaDodSO_4$. After 2 days at 65°C, the BA85 membrane was incubated an additional 60 min at 65°C in prehybridization solution. It was then washed at 65°C twice each with 3× NaCl/Cit, NaCl/Cit, and 0.3× NaCl/Cit (each containing 0.5% $NaDodSO_4$ and 0.1% Na pyrophosphate) and once with 0.3× NaCl/Cit, dried, and autoradiographed with Kodak XR1 film and Hi-plus intensifying screens at −70°C.

RESULTS

DNAs from normal mice of various inbred strains and partially inbred stocks of diverse origins were digested with *Eco*RI and subjected to agarose gel electrophoresis and Southern transfer. Three of the normal patterns obtained after hybridization to α-globin gene specific probe are shown in Fig. 1. The pattern in lane C is the one most often found; this or a similar pattern was obtained with mouse strains C57BL/6J, SEC/1Re, SWR/J (Hba^c), and BXD-29 (which inherited its hemoglobin α-genes from DBA/2J) and one stock with an α-genotype (Hba^k) from wild American *Mus musculus domesticus*. Within this group is characteristically found a doublet of hybridizing bands at 10.5 and 12 kb, with the 10.5-kb band always more intense. The pattern in lane E is common in mice that express α-globin chain 5—strains CE/J, AKR/J, and three stocks with *Hba* genotypes from *M. m. molossinus* (Hba^i) (6), BDP/J (Hba^h), and a Swiss mouse (Hba^h)—but was also found in one noninbred stock homozygous for a genotype that specifies no chain 5. Within this group there is no 12-kb *Eco*RI band (which in SEC presumably carries the globin gene that encodes α-globin chain 3). A band, of variable intensity, of ≥25 kb is characteristic of these stocks and a weaker band of about 15 kb is often detectable. A third pattern, shown in lane A with strain SM/J DNA, is probably different, with a strong band at 10.5 kb and a second band at ≤15 kb.

The presence of the 12-kb band in samples of the first ("doublet") group and its absence from all other samples is invariant. This difference has been used to analyze whether the 12-kb band that was present before mutation in the SEC chromosome

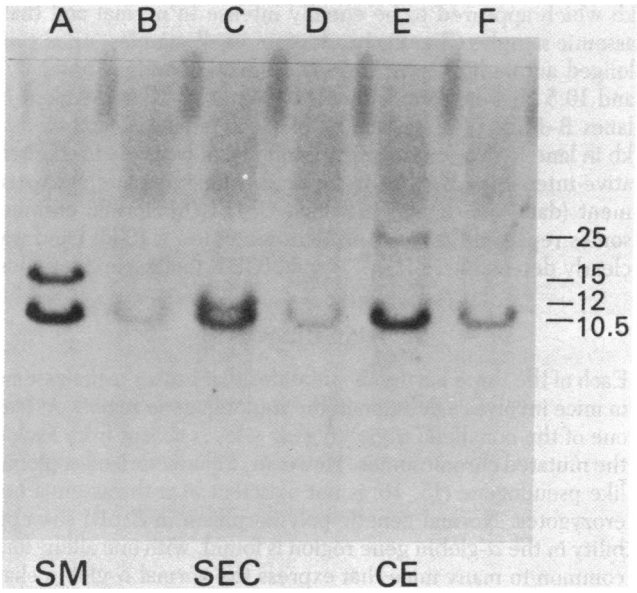

FIG. 1. α-Globin genomic DNA fragments of normal and thalassemic mice. Southern blots (30 μg per lane) of *Eco*RI fragments were hybridized with a radiolabeled 3-kb fragment which contains the cloned BALB/c gene for α-globin chain 2. DNA from normal mice was in lanes A (strain SM/J), C (strain SEC/1Re), and E (strain CE/J). DNA in lanes B, D, and F came from mice that carried one normal CE/J chromosome and, respectively, the mutated chromosome Hba^{th-J}, 352HB, or 27HB. Sizes are shown in kb.

(and presumably in the Hba^c progenitor of the Hba^{th-J} chromosome) is still present in any of the mutant chromosomes of thalassemic mice. Heterozygotes bearing one mutated chromosome from a strain that originally had the 12-kb fragment and one normal chromosome from a strain that did not (CE/J) should have the 12-kb fragment only if it was not deleted by the mutational event. Additionally, they will have only half of the normal amount of the 10.5-kb band (from their normal CE chromosomes) if that segment was also deleted.

Lanes B, D, and F of Fig. 1 show patterns from thalassemic mice, each carrying one normal CE/J chromosome and one mutated chromosome (Hba^{th-J}, 352HB, or 27HB, respectively). The same amount of total genomic DNA was applied to each lane as was applied to each normal/control lane. A band at 10.5 kb (but not at 12 kb) in each of the thalassemia sample lanes is visible. This also was true at longer times of autoradiography. In lanes E and F a weak band at ≥25 kb was visible in the original. In each of the thalassemia sample lanes, the 10.5-kb band is notably less intense than in each normal DNA lane. This has been true in every comparison of these DNAs and implies that at least the bulk of both of the nonallelic α-segments homologous to the probe is deleted.

These six DNA samples and other normal mouse samples have also been analyzed with *Hin*dIII, with the same probe and hybridization conditions. In all normal and thalassemic *Mus musculus* samples analyzed, genomic bands of approximately 14.7 kb (the more intense band) and 3.7 kb were found (data not shown) (the 10.5-kb *Eco*RI segment in clone Mα1 has no *Hin*dIII site and therefore must be contained within the 14.7-kb genomic *Hin*dIII region). In each sample from the thalassemic mice, both of the *Hin*dIII bands were notably weaker than the corresponding bands from normal samples, on the order of half of normal intensity or less. No extra band of abnormal size was found.

Genomic DNAs from the same group of strains and thalassemic mice were also digested with *Eco*RI and hybridized to a 4.7-kb α-globin-like pseudogene probe. The genomic segment homologous to the probe itself gave a strong band at 4.7 kb which appeared to be equally intense in normal and thalassemic samples (Fig. 2). In addition, in all samples, upon prolonged autoradiography, cross-hybridizing bands of about 2.7 and 10.5 kb were found. Bands of 6 and 7.5 kb are evident in lanes B–F, of 12 kb in lane C, of 15 kb in lane A, and of ≥25 kb in lane E. No convincing argument can be made for the relative intensities of these weak bands but, in a parallel experiment (data not shown) in which C57BL/6J normal chromosomes replaced CE/J normal chromosomes, a 12-kb band was clearly detectable in Hba^{th-J} and 352HB thalassemic samples.

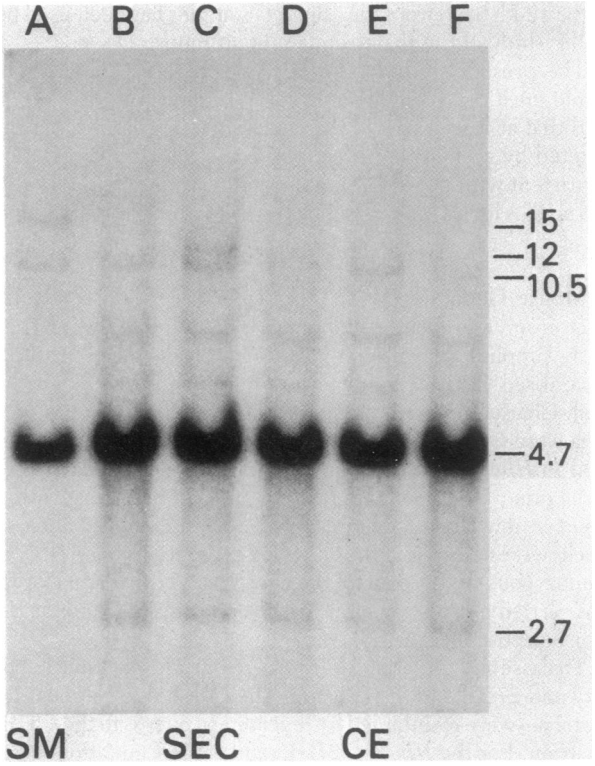

FIG. 2. Genomic DNA fragments of normal and α-thalassemic mice. Southern blots of 50 μg of *Eco*RI-digested DNA per lane were hybridized with a nick-translated plasmid with a 4.7-kb insert which contained a mouse α-globin-like pseudogene. DNA from normal mice was in lane A (strain SM/J), lane C (strain SEC/1Re), and lane E (strain CE/J). DNA in lanes B, D, and F came from mice that carried one normal CE/J chromosome plus, respectively, the mutated chromosome Hba^{th-J}, 352HB, or 27HB. Sizes are shown in kb.

DISCUSSION

Each of the three heritable mutations that causes α-thalassemia in mice involves a deletion in the α-globin gene region. At least one of the nonallelic α-globin gene sites is absent from each of the mutated chromosomes. However, a characterized α-globin-like pseudogene (15, 16) is not deficient in α-thalassemia heterozygotes. Normal genetic polymorphism in *Eco*RI susceptibility in the α-globin gene region is found, with one allelic form common to many mice that express the normal α-globin chain 5.

Various different DNA patterns have been found in humans with α-thalassemias. Normal individuals have two closely linked adult α-globin genes per haploid genome (25). Analyses of the DNAs of some persons with the relatively mild α-thalassemia-2 reveal no deletion of α-globin genetic material [samples from Turkish, Italian, Israeli (26), Chinese (27), and Saudi Arabian (28) populations]. A deletion of the 5′ α-globin gene (α2) can also account for α-thalassemia-2, both in Chinese (29, 30) and American Black populations (L. B. Wilson, personal communication). Finally, α-thalassemia-2 is caused in other cases, in Chinese and Algerian and in Jamaican and American Black populations, by the presence on the mutant chromosome of a single α-globin gene thought to have arisen via a fusion of the 5′ and 3′ genes in an unequal crossover event (30–32). Both of these deletions have also arisen during the propagation of bacteriophages carrying cloned normal human α-genomic inserts (33).

In the more severe form of α-thalassemia in man (α-thalassemia-1), the mutant chromosome has no functional α-globin gene. At least four types have been distinguished at the DNA level. First, in some Mediterranean individuals, although the 3′ end of the more 3′ of the normal α-globin genes (α1) is present, the 5′ end of this gene and a vast region 5′ to it are deleted (26, 34, 35). Second, both of the adult α-globin genes can be entirely absent but both embryonic ζ-globin genes are intact, the situation found in a Thai homozygote. Third, the more 3′ ζ-globin gene as well as both of the adult α-globin genes can be deleted, the configuration deduced for a Greek homozygote whose 5′ ζ-globin gene remained functional (28). Finally, an α-thalassemia-1 chromosome may have a complete, nonfunctional α-globin gene (36).

The mouse α-thalassemia mutations most closely model the human α-thalassemia-1 mutations, although the exact extent of each mouse deletion remains to be determined. The data imply that both nonallelic mouse α-genes were simultaneously deleted at the DNA level, which suggests that they must be physically closely linked to one another on the same chromosome. The finding that deletion of an α-globin structural gene is associated with the thalassemia blood disorder that maps on chro-

mosome 11 establishes that the α-globin structural genes are indeed located at the *Hba* site, as had been inferred from studies of the α-globin polypeptides. Because embryonic α-globin-like x-globin expression is affected in heterozygous α-thalassemic embryos (9, 37), it seems probable that each deletion extends at least into the vicinity of the linked x-globin gene(s). That the α-globin-like pseudogene is not deleted (Fig. 2) suggests that it lies on a different chromosome [recently confirmed by using hybrid somatic cells by Popp *et al.* (38) and E. F. Vanin (personal communication)]. That the three mouse mutations affect the viabilities of their C57BL strain carriers to different degrees suggests that the total length of the deletion is different for each. Their early embryonic lethality when homozygous (39) suggests that the mouse mutant chromosomes, like the 2.6-kb *Eco*RI fragment from the human α-thalassemia chromosome, may lack sequences in addition to the ζ- or x-globin loci which are essential for normal embryonic development. If α-thalassemic mice are to be used as models for genetic therapy, the viability of heterozygotes suggests that replacing half of the missing complement of gene products should be sufficient to rescue otherwise-doomed homozygous thalassemic embryos.

Although we have concluded here that the α-globin genes are actually deleted from each of the three mutant chromosomes, the α-/β-globin synthesis ratios and α-/β-globin RNA level ratios are not so low in heterozygous carriers as might have been anticipated (18). In each case, the α/β ratio is about 75% of normal, in spite of the fact that the carriers have only half the normal complement of α-globin genes. The proportion of Hb H (β_4 tetramers) found in the blood of Hbb^s α-thalassemia-1 heterozygous mice (6–8%) is consistent with this synthesis ratio if there is little loss of Hb H from the erythrocytes (although anisocytosis is observed) (40).

The polymorphism among inbred mouse strains in α-globin-related *Eco*RI fragment patterns (DNA doublet vs. singlet types) could indicate that the mouse α-globin locus complex has undergone gross rearrangement(s) during the course of evolution. Alternatively, the differences could be the result of single nucleotide substitution mutations having removed or added sites susceptible to *Eco*RI cleavage while the *Hin*dIII sites have remained unchanged. Neutral polymorphisms of this type have been reported in or near the human $^{A}\gamma$-, $^{G}\gamma$-, δ-, and β-globin genes (41–45). There seems to be no correlation between the DNA doublet vs. singlet types and whether a mouse expresses one or two different adult α-globins: all combinations are seen.

The fact that most of the tested mice that express α-globin chain 5 share the DNA singlet type need not necessarily indicate a special functional significance to this pattern: it might simply reflect a close linkage of the chain 5 gene site to the variant *Eco*RI site, so that the two sites are not frequently separated by normal genetic recombination. Nonetheless, the existence of chromosomes that encode chains 5 and 4 (Hba^h), chains 5 and 1 (Hba^g), and chains 1 and 4 (Hba^c) suggests that recombination must have taken place within the mouse α-globin complex at some time after α-globin-gene duplication and divergence.

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