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Genetics

Mouse α-globin genes and α-globin-like pseudogenes are not syntenic*

(somatic cell hybrids/recombinant DNA)

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ABSTRACT **A genetic polymorphism for a *Bgl* I endonuclease site near the α-globin-like pseudogene α-4 of C57BL/6 and C3H/HeN mice was used to show that α-4 was not affected by three independent mutations in which the adult globin genes α-1 and α-2 were deleted. These results indicated that α-4 might not be located adjacent to the adult α-globin genes on chromosome 11. Restriction endonuclease analysis of DNA of a primary clone of a Chinese hamster–mouse somatic cell hybrid that had lost mouse chromosomes 11 and 18 showed that this clone lacked the adult murine globin genes α-1 and α-2 but it did contain the α-globin-like pseudogenes α-3 and α-4. These results indicated that the adult α-globin genes and α-globin-like pseudogenes are not located on the same chromosome. Similar analyses of several other Chinese hamster–mouse somatic cell hybrids that had segregated other mouse chromosomes indicated that the α-globin-like pseudogenes α-3 and α-4 are located on mouse chromosomes 15 and 17, respectively. These data explain why α-3 and α-4 were not affected by the three independently induced deletion-type mutations that cause α-thalassemia in the mouse.**

At least seven nonallelic hemoglobin genes are expressed during embryonic development of the laboratory mouse. The α-globin-like x-globin and β-globin-like y- and z-globin genes are expressed only in the nucleated embryonic erythrocytes; two tightly linked α-globin genes are expressed both in the nucleated embryonic and in the enucleated adult erythrocytes; and two tightly linked β-globin genes are expressed in adult erythrocytes (1, 2). The highly homologous structural features of the adult globin genes thus far characterized suggest that they diverged from a common ancestral gene after gene duplication (3–5). Studies on the organization of hemoglobin genes of several mammalian species have shown that the globin genes exist as clusters and are arranged on the chromosome from 5′ to 3′ in the order of their expression during development (6–9). The globin gene clusters also contain one or more DNA segments that are related in nucleotide sequence to the functional globin genes but do not appear to produce functional globin polypeptides; they have been called pseudogenes (5, 7, 9).

Three heritable mutations that cause α-thalassemia have been induced in mice (10–12). Analyses of their hemoglobins showed that the x-globin and both adult α-globin genes, which should have been inherited from the treated males, were not expressed in the original mutants and in their affected progeny (13–16). Restriction endonuclease analysis of genomic DNAs of the mutants indicated that both adult α-globin genes (α-1 and α-2) were deleted but the α-globin-like pseudogene α-3 (and probably α-4) was not affected by any of the three independently induced mutations (17). These results were unexpected in view of the known association of α-globin-like pseudogenes with the functional α-globin genes of humans (7) and chickens (18) and the association of at least four β-globin-like pseudogenes with the functional β-globin genes of mice (9).

In the present study we investigated the chromosomal locations of the α-globin-like pseudogenes α-3 and α-4 described by Vanin *et al.* (19) and Leder and colleagues (20, 21), using Chinese hamster–mouse somatic cell hybrids segregating mouse chromosomes. The data established that these α-globin-like pseudogenes α-3 and α-4 are not located on mouse chromosome 11 as are the α-globin-like embryonic x-globin and the two adult α-globin genes, but are located on chromosomes 15 and 17, respectively.

MATERIALS AND METHODS

Mice. Heterozygous α-thalassemic mice from stocks 27HB and 352HB (10) backcrossed to C57BL/6J for 7–10 generations were from the Oak Ridge National Laboratory; BALB/cJ, C57BL/6J, and *Hba*$^{th\text{-}J}$ (12) mice were from The Jackson Laboratory; and C3H/HeN mice were from the National Institutes of Health.

Somatic Cell Hybrids. Cell hybrids between Chinese hamster and mouse spleen cells from BALB/c mice were generated and maintained as described (22). Under the conditions employed, hybrid clones retain all the Chinese hamster chromosomes but randomly segregate mouse chromosomes, resulting in the isolation of independent clones of Chinese hamster–mouse cell hybrids that contain different numbers and combinations of mouse chromosomes. Mouse–hamster cell hybrids were analyzed for the mouse chromosome content enzymatically and cytogenetically. Each hybrid clone was analyzed for the expression of 25 enzymes representing linkage groups assigned to 16 of the 19 autosomes and the X chromosome as described (23, 24). Trypsin–Giemsa banding was used to identify each of the mouse chromosomes as described (25). Individual clones were scored positive for a given chromosome if greater than 15% of the metaphases examined contained it; the clone was scored negative for that chromosome if less than 5% of the metaphases contained it. Those chromosomes in the various clones that had been retained with a frequency of between 5% and 15% were not included in the segregation analysis of that chromosome. Each hybrid clone was grown for several passages prior to being characterized enzymatically, cytogenetically, and for the presence of the α-globin-like genes. These analyses were carried out on parallel cultures of each hybrid clone so that the enzyme, chromosome, and globin gene data were correlated.

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Abbreviations: NaCl/Cit, 0.15 M NaCl/0.015 M sodium citrate (pH 7.0) (standard saline citrate); kb, kilobase(s).

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Enzymes and Other Material. The restriction enzymes *Eco*RI, *Bgl* I, and *Sst* I were purchased from New England BioLabs. Agarose was from Seakem (Rockland, ME). Nonradioactive deoxyribonucleoside triphosphates, RNase A, and DNase I were from Sigma, and ^{32}P-labeled dCTP and dGTP were from Amersham. Boehringer Mannheim was the source of DNA polymerase I. Aya Leder generously provided a stock of plasmid pBR322 containing the mouse α-1 globin gene (5).

DNA Extraction. Livers of mice were frozen in liquid N_2 and fragmented into small pieces prior to gentle homogenization in cold Dulbecco's phosphate-buffered saline (minus Ca^{2+} and Mg^{2+}), using a Teflon plunger that fitted loosely in a smooth glass barrel. The homogenate was centrifuged at 2000 × *g* for 5 min and the pellet was washed in Dulbecco's phosphate-buffered saline three times. Approximately 2×10^8 cells from each clone of somatic cell hybrids were similarly washed and pelleted by centrifugation. Each pellet was dissolved in 5.4 ml of 6 M guanidine·HCl, 0.6 ml of 2 M potassium acetate (pH 5.0) was added, and the sample was stirred on a Vortex mixer at high speed for 10 min before 12 ml of 4% Sarkosyl in 0.1 M Tris·HCl (pH 8.0) was added. CsCl (7.2 g) was added and the sample was layered over a 1.6-ml cushion of 5.6 M CsCl in each of two polyallomer tubes (Beckman 331372) and centrifuged at 32,000 × *g* for 60 hr at 20–26°C, using a Beckman SW 41 rotor. After dialysis against 1× NaCl/Cit (0.15 M NaCl/0.015 M sodium citrate, pH 7.0), DNA was treated with RNase A (100 μg/ml) for 1 hr at 37°C. The sample was extracted with an equal volume of phenol/chloroform (1:1, vol/vol) and DNA in the aqueous phase was precipitated overnight from 70% (vol/vol) ethanol and 0.2 M sodium acetate (pH 5) at −20°C. After centrifugation at 10,000 × *g* for 1 hr at −20°C, the DNA pellet was dissolved in 4 ml of H_2O and dialyzed overnight against 0.01 M Tris/0.001 M EDTA (pH 7.5) and stored at 4°C until used for digestion by restriction endonuclease.

Restriction Enzyme Digestion. Fifty to 80 μg of DNA was digested by 100 units of *Eco*RI or 100 units of *Eco*RI plus 100 units of *Bgl* I for 2 hr at 37°C, then a similar quantity of enzyme was added again for an additional 2 hr of digestion. The buffer used was 100 mM Tris·HCl (pH 7.5)/50 mM NaCl/5 mM $MgCl_2$ as suggested by New England BioLabs, and the total reaction volume was 200 μl. After digestion the reaction mixture was extracted with an equal volume of phenol, the phases were separated by high-speed centrifugation in an Eppendorf microcentrifuge, and the aqueous phase was extracted with 2 vol of ether to remove residual phenol.

Agarose Gel Electrophoresis and Southern Blot Analysis. Enzyme-digested DNA was subjected to electrophoresis in 1% agarose gels (40 mM Tris·HCl (pH 7.8)/5 mM sodium acetate/1 mM EDTA). The DNA fragments were transferred to nitrocellulose paper (Schleicher & Schuell, BA85) with 10× NaCl/Cit for 24 hr (26).

Probe for Hybridizations. A pBR322 stock with the 9.7-kilobase (kb) *Eco*RI genomic fragment of the BALB/c α-1 gene was obtained from Aya Leder. Recombinant DNA was handled as prescribed by the National Institutes of Health guidelines. The plasmid was grown in *Escherichia coli* K-12 C-600 and harvested by centrifugation in CsCl containing ethidium bromide. A 3-kb *Sac* I fragment (5) was recovered by digestion with *Sst* I and electroelution from a preparative agarose gel. The 3-kb fragment contains the entire α-1 gene, and this probe was labeled with [^{32}P]dCTP and [^{32}P]dGTP by nick-translation (27).

Hybridization. Each nitrocellulose filter containing the transferred DNA fragments was prehybridized (28) for 3 hr at 65°C in a sealable plastic bag containing 40 ml of 5× NaCl/Cit, 5× Denhardt's solution [1× Denhardt's solution is 0.02% Ficoll 400 (Pharmacia), 0.02% polyvinylpyrrolidone (Sigma PVP-360), and 0.02% bovine serum albumin (Sigma)], 50 mM sodium phosphate, (pH 7.5), 1% glycine, sonicated salmon sperm DNA (Sigma) at 50 μg/ml, and 0.5% sodium dodecyl sulfate. After the above solution had been decanted the nitrocellulose paper was incubated for 1 hr at 65°C in 40 ml of 5× NaCl/Cit/5× Denhardt's solution/20 mM sodium phosphate (pH 7.5)/sonicated salmon sperm DNA at 100 μg/ml/10% dextran sulfate (Pharmacia)/0.5% sodium dodecyl sulfate. The probe, which contained 4×10^7 cpm, was dissolved in 1 ml of 5× NaCl/Cit and heated in a double-boiler for 10 min before being added to the above 40 ml of prehybridization mixture. Hybridization then proceeded for 12 hr at 65°C. The nitrocellulose filters were then washed at 65°C in 40 ml each of 3× NaCl/Cit/2× Denhardt's solution/0.5% sodium dodecyl sulfate; 1× NaCl/Cit/2× Denhardt's solution/0.5% sodium dodecyl sulfate; 0.5× NaCl/Cit/0.5% sodium dodecyl sulfate; and finally in 0.3× NaCl/Cit. The nitrocellulose filters were then dried at 80°C under reduced pressure and autoradiographed with Kodak XR-2 film with intensifying screens at −70°C.

RESULTS

A normal genetic polymorphism in a *Bgl* I restriction cleavage site in or near the α-globin-like pseudogene α-4 was observed between genomic DNAs extracted from livers of C57BL/6J and C3H/HeN mice (data not shown). The *Bgl* I restriction cleavage site polymorphism is demonstrated better in DNAs digested with both *Eco*RI and *Bgl* I (Fig. 1, lanes 1 and 2, respectively). The DNA fragment in an *Eco*RI digest of C57BL/6J and C3H/HeN genomic DNAs is 2.4 kb (Fig. 2, lanes 1 and 4). Although the α-globin-like pseudogene α-4 in the *Eco*RI/*Bgl* I digest of C57BL/6J DNA remained in a 2.4 kb DNA fragment, α-4 in the *Eco*RI/*Bgl* I digest of C3H/HeN was in a DNA fragment of approximately 1.8 kb. Fig. 1, lane 3, shows an *Eco*RI/*Bgl* I digest of genomic DNA from a (C3H/HeN × C57BL/6J)F_1 hybrid; this digest shows the presence of α-4 in both the 2.4- and the 1.8-kb fragments as expected. Lanes 4 and 6 in Fig. 1 show *Eco*RI/*Bgl* I digests of genomic DNAs from (C3H/HeN × 352HB)F_1 α-thalassemic mice and lane 8 shows a similar

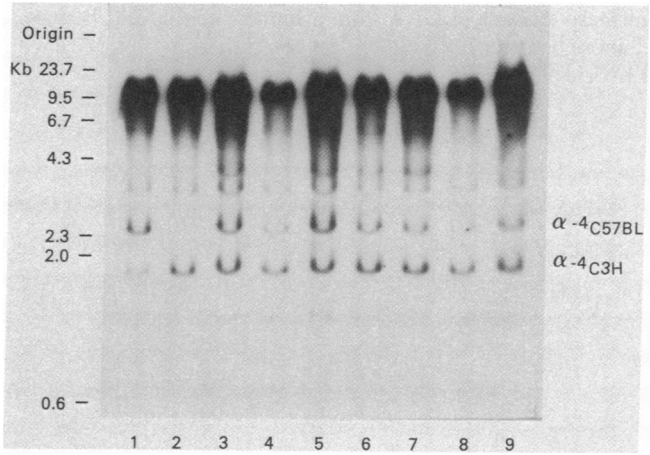


FIG. 1. α-Globin genomic DNA fragments of normal and thalassemic mice. Southern blots of *Eco*RI/*Bgl* I fragments were hybridized with a radiolabeled 3-kb *Sac* I fragment containing BALB/c α-1 globin. Lanes 1, 2, and 3 contained DNA from C57BL/6J, C3H/HeN, and (C3H/HeN × C57BL/6J)F_1 mice, respectively. Lanes 4, 6, and 8 contained DNA from α-thalassemic mice, and lanes 5, 7, and 9 contained DNA from normal littermates (see text). The α-globin-like pseudogene α-4 is in a 2.4-kb fragment in digests of C57BL/6J DNA and in a 1.8-kb fragment in digests of C3H/HeN DNA. Both forms of α-4 are present in normal and α-thalassemic F_1 hybrids.

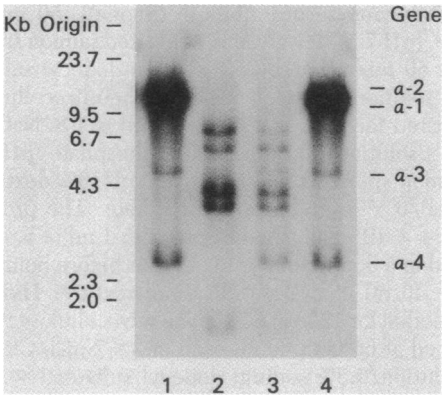


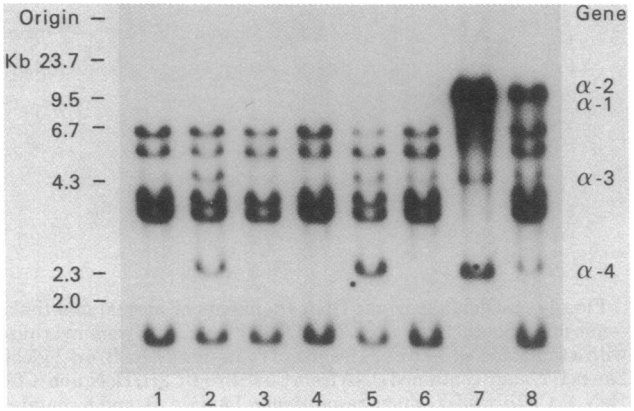
FIG. 2. α-Globin genomic DNA fragments of mouse liver, Chinese hamster V79 cells, and a somatic cell hybrid. Southern blots of *Eco*RI fragments were hybridized with a radiolabeled 3-kb *Sac* I fragment containing BALB/c α-1 globin. Lanes 1 and 4 contained DNA from C3H/HeN and C57BL/6J mice, respectively. Lane 2 contained DNA from a V79 Chinese hamster cell line E36, and lane 3 contained DNA from a Chinese hamster–BALB/c mouse spleen somatic cell hybrid clone EBS-5. Note the absence of the adult α-globin genes α-1 and α-2 and the presence of α-3 and α-4 in the somatic cell hybrid in lane 3.

digest of DNA from ($Hba^{th\text{-}J}$ × C3H/HeN)F_1-α-thalassemic mice. Lanes 5, 7, and 9 in Fig. 1 are *Eco*RI/*Bgl* I digests of DNAs of normal littermates of the α-thalassemic mice shown in lanes 4, 6, and 8, respectively. Both genetic forms of DNAs were observed in the F_1 hybrids whether or not they inherited the deletion of the adult functional globin genes α-1 and α-2 that causes murine thalassemia. This deletion is evident in Fig. 1, lanes 4, 6, and 8, by the reduced hybridization of DNA 10- to 12.5-kb fragments. These data established that α-4 was not deleted by the induced mutations causing α-thalassemia. Either the mutations caused a partial deletion of the α-globin complex that did not include α-4 or α-4 might not be located near the adult α-globin genes α-1 and α-2, which are located on chromosome 11 (29).

These alternatives were investigated by restriction endonuclease analysis of DNA from a somatic cell hybrid between Chinese hamster E36 cells and spleen cells from BALB/c mice. A hybrid clone (EBS-5) was found to contain all the mouse chromosomes except 11 and 18. Restriction endonuclease analysis showed that this hybrid clone did not contain the adult globin genes α-1 and α-2 but it did contain the α-globin-like pseudogenes α-3 and α-4 (Fig. 2, lane 3). The 3-kb *Sac* I probe of the mouse α-1 globin gene did cross-hybridize with five *Eco*RI DNA fragments of Chinese hamster E36 cells (Fig. 2, lane 2) but their sizes were distinct from the 4.7-kb DNA fragment containing α-3 and the 2.4-kb fragment containing α-4 for both C57BL/6J and C3H/HeN DNAs (Fig. 2, lanes 1 and 4, respectively). The possibility that EBS-5 contained a fragment of chromosome 11 was ruled out because it did not contain α-1 and α-2 genes, which are located on the proximal region of chromosome 11, and it did not express galactokinase, which is located on the distal region of chromosome 11 (30, 31).

A series of Chinese hamster–mouse somatic cell hybrids was examined for the presence of the α-globin-like genes (Fig. 3). Restriction endonuclease analysis of these clones demonstrated that none of the hybrid clones contained the adult α-globin genes α-1 and α-2. However, all of the hybrids, except one hybrid clone, were positive for pseudogene α-3, and six hybrids were positive and three hybrids were negative for α-4. These data confirm that the adult α-globin genes are not syntenic with the pseudogenes α-3 and α-4. The data further indicate that these pseudogenes segregated independently of each other, suggesting that they are on separate chromosomes.

The segregation of the α-globin-like genes in the hybrid clones was compared with the segregation of mouse chromosomes as determined enzymatically and cytogenetically on parallel cultures of each hybrid clone (Table 1). As was expected, none of the clones contained chromsome 11, because all clones were negative for α-1 and α-2, thus confirming the location of α-1 and α-2 on mouse chromosome 11. Pseudogene α-4 segregated concordantly with chromosome 17. Six clones retained chromosome 17 and α-4 and three clones lost chromsome 17 and α-4. There were no exceptions and all other chromosomes segregated discordantly with α-4. Moreover, α-4 appeared in reduced quantities in two clones (Fig. 3 *Left*, lane 3; Fig. 3 *Right*, lane 2), and cytogenetic analysis of these clones demonstrated that chromosome 17 was present in 20–30% of the cells in each clone. Pseudogene α-3 segregated concordantly with chromosome 15 in eight of the nine clones (Table 1). The one exception was positive for α-3 but negative for an intact chromosome 15. This clone, however, did contain a fragment



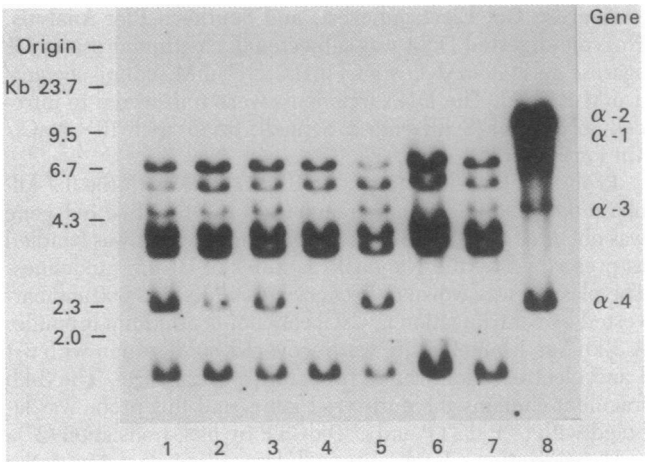


FIG. 3. α-Globin genomic DNA fragments of mouse liver, Chinese hamster V79 cells, and several somatic cell hybrids. Southern blots of *Eco*RI fragments were hybridized with a radiolabeled 3-kb *Sac* I fragment containing BALB/c α-1 globin. (*Left*) Lane 1, E36; lane 2, EBS-1; lane 3, EBS-4; lane 4, E36; lane 5, EBS-5; lane 6, EBS-12; lane 7, BALB/c; and lane 8, 3:1 mixture of E36 and BALB/c. (*Right*) Lane 1, EBS-15; lane 2, EBS-17; lane 3, EBS-51; lane 4, E36; lane 5, EBS-5; lane 6, EBS-63; lane 7, EBS-74; and lane 8, BALB/c.

Table 1. Distribution of α-globin genes, *GLO*, and mouse chromosomes in mouse–Chinese hamster cell hybrids

Cell hybrid	Marker				Chromosome																		
	α-1/α-2	α-3	α-4	*GLO**	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	X
EBS-63	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	Frag.†
EBS-12	−	+	−	−	−	−	+	−	−	−	−	−	−	+	−	−	−	−	(−)†	(−)†	−	+	+	+
EBS-4	−	+	+	+	−	−	−	−	−	−	−	−	−	−	−	−	+	−	+	−	+	−	−	+
EBS-17	−	+	+	+	+	+	−	−	+	−	+	−	+	−	−	−	+	−	+	−	+	−	−	+
EBS-51	−	+	+	+	−	+	−	−	+	−	+	+	+	−	−	+	−	−	+	+	+	+	+	+
EBS-1	−	+	+	+	+	+	+	+	−	+	+	+	+	+	−	+	+	+	+	+	+	+	+	+
EBS-15	−	+	+	+	−	+	+	+	−	+	+	+	−	+	−	+	+	−	+	+	+	−	+	+
EBS-74	−	+	−	−	−	+	+	−	−	+	−	−	−	−	−	+	−	+	+	+	−	−	−	+
EBS-5	−	+	+	+	+	+	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	−	+	+

Restriction endonuclease analysis was performed on DNA extracts of hamster–mouse hybrid clones; duplicate cultures of the same passage were used for enzyme and karyotypic analyses. Twenty-five metaphase spreads were analyzed per hybrid clone. Whether a particular chromosome was scored as − or + was determined as described in *Materials and Methods*. The presence or absence of mouse enzyme markers assigned to 16 of the 19 mouse autosomes and the X chromosome agreed with the presence or absence, respectively, of the particular mouse chromosome.

* Glyoxylase-1; the structural gene for this enzyme is located on chromosome 17 (ref. 32).

† A fragment, but not the complete chromosome, was present.

of chromosome 15. All other chromosomes segregated discordantly with α-3 (Table 1). These data allow us to assign α-4 to chromosome 17 and α-3 to chromosome 15.

The pseudogenes α-3 and α-4 appeared in reduced quantities in some of the hybrids (Fig. 3 *Left*, lanes 3 and 6; Fig. 3 *Right*, lanes 2 and 7). These reduced quantities result from the fact that less than 50% of the cells in the hybrid clone contain the chromosome in question. There was an appropriate correlation between the percent of cells of a clone retaining chromosome 15 or 17 and the quantity of α-3 or α-4 observed (data not shown).

DISCUSSION

Each of the three heritable α-thalassemia mutations in mice is associated with a deletion of a segment of chromosome 11 that includes the adult globin genes (16, 17). We found that these deletions did not simultaneously affect the α-globin-like pseudogenes α-3 and α-4 (ref. 17 and Fig. 1). DNAs of several independent clones of Chinese hamster–mouse spleen cell somatic cell hybrids were analyzed to identify the mouse chromosomes that contained α-3 and α-4. Segregation of mouse chromosomes in such hybrids allows for the possibility of genetically dissecting complex polygenic systems such as the hemoglobin system by isolating its component parts. The somatic cell hybrid strategy has been used extensively for mapping mouse genes (23, 24), and recently Owerbach *et al.* have demonstrated the usefulness of combining somatic cell hybrid and restriction enzyme cleavage techniques to map genes that are not expressed by cells in culture. They mapped the genes coding for growth hormone and chorionic somatomammotropin to chromosome 17 and insulin to chromosome 11 in humans (33, 34).

In the present study, techniques of somatic cell hybridization and restriction enzyme cleavage have been combined to confirm that the functional α-globin genes are located on mouse chromosome 11 and to show that the mouse α-globin-like pseudogenes α-3 and α-4 are located on chromosomes 15 and 17, respectively (Table 1 and Figs. 2 and 3).

The discovery that the pseudogenes α-3 and α-4 are physically separated from the adult α-globin genes was quite unexpected because of the close association of α-globin-like pseudogenes with the functional α-globin genes of chicken (18) and humans (7) and because several β-globin-like pseudogenes are tightly linked with the adult β-globin genes of mice (9). However, Jahn *et al.* (9) did not find the embryonic z-globin gene within a 70-kb segment of DNA that contained the two adult β-globin genes, four β-globin-like pseudogenes, and one embryonic y-globin gene. Translocation can be invoked to explain how the α-globin-like pseudogenes became separated from the other α-globin genes of mice, but additional studies will be required to establish whether or not the z-globin gene is on the same chromosome as the β-globin genes. Howe *et al.* (35) have demonstrated that the locus for a B-lymphocyte antigen (*Lyb-4*) on chromosome 4 of DBA/2J mice is not located on chromosome 4 in C3H/HeJ mice, indicating that the location of a gene might not always be constant within a species.

The possible origin and significance of the pseudogenes have been discussed by others (19, 20, 36, 37). Globin pseudogenes may regulate the level of globin message by diverting transcription into a degradative pathway, and they may participate in intergenic recombinational events to conserve DNA sequences of adjacent globin genes. The fact that at least some α-globin-like pseudogenes of the mouse are physically separated from the embryonic and adult α-globin genes weakens but does not negate the possibility that they regulate the expression of the embryonic x-globin and adult α-globin genes because pseudogenes could still regulate splicing, diversion, or message degradation in a *trans* position. Polypeptides of polymeric proteins are often controlled by genes on different chromosomes—e.g., hemoglobin (29) and lactate dehydrogenase (38).

We are grateful to Dr. Aya Leder, National Institutes of Health, who provided us with the pBR322 containing the α-1 globin gene. We recognize the excellent technical assistance of L. S. Clawson and J. Diaz. The EBS hybrids used in this study were originally isolated in the laboratory of Dr. John D. Minna, National Institutes of Health. This research was sponsored jointly by the Office of Health and Environmental Research, U.S. Department of Energy, under contract W-7405-eng-26 with the Union Carbide Corporation; the National Institutes of Health; and National Cancer Institute Contract YO1-CP-90208.

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