ANALYSIS OF A MOUSE α-GLOBIN GENE MUTATION INDUCED BY ETHYLNITROSOUREA

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ABSTRACT

A DBA/2 mouse treated with ethylnitrosourea sired an offspring whose hemoglobin showed an extra band following starch gel electrophoresis. The variant hemoglobin migrated to a more cathodal position in starch gel. Isoelectric focusing indicated that chain 5 of the mutant hemoglobin migrated to a more cathodal position than the normal chain 5 from DBA/2 mice and that the other α-globin, chain 1, was not affected. On focusing gels the phenotype of the mutant allele, Hba^{y9}, was expressed without dominance to normal chain 5, and Hba^{y9}/Hba^{y9} homozygotes were fully viable in the laboratory. The molecular basis for the germinal mutation was investigated by analyzing the amino acid sequence of chain 5^{y9}, the mutant form of α-chain 5. A single amino acid substitution (His → Leu) at position 89 was found in chain 5^{y9}. We propose that ethylnitrosourea induced an A → T transversion in the histidine codon at position 89 (CAC → CTC). This mutation has apparently not been observed previously in humans, mice or other mammals, and its novel occurrence may be indicative of other unusual mutational events that do not ordinarily occur in the absence of specific mutagen exposure.

HEMOGLOBIN has been among the markers used in specific locus tests to estimate the frequency of germinal mutations induced in mice by X rays (RUSSELL *et al.* 1976) and chemicals (JOHNSON and LEWIS 1981; MUROTA, SHIBUYA and TUTIKAWA 1982). The nature of spontaneous mutations and mutations induced by various mutagens can be investigated through comparative analyses of variant hemoglobins and the altered DNA of the hemoglobin genes. The primary structures of the normal hemoglobins of many strains of mice have been determined (HILSE and POPP 1968; POPP 1973; POPP and BAILIFF 1973; GILMAN 1974; POPP *et al.* 1982). Natural hemoglobin variants usually contain amino acid substitutions that are accounted for by base substitutions in the DNA of the hemoglobin genes (KONKEL, MAIZEL and LEDER 1979; NISHIOKA and LEDER 1979). Other hemoglobin variants resulting from frameshifts, deletion of several consecutive bases or nonhomologous crossing over have been found in humans (DAYHOFF 1972) but not in mice. In addition, mutations causing deficiencies of hemoglobin synthesis (thalassemias) occur in

humans (WEATHERALL and CLEGG 1982). A type of α-thalassemia-1, in which both α-globin genes have been deleted from one chromosome, has been induced in mice by X rays and triethylenemelamine (POPP *et al.* 1981; WHITNEY *et al.* 1981). In the present paper we characterize a germinal mutation induced at the hemoglobin α-chain locus (*Hba*) of a DBA/2 male treated with ethylnitrosourea (JOHNSON and LEWIS 1981; WHITNEY 1982).

MATERIALS AND METHODS

The strains of mice analyzed for mutations were C57BL/6J (B6), DBA/2J (D2) and (B6D2)F_1 hybrids. Treatment of males with ethylnitrosourea and electrophoretic screening techniques for detecting mutants have been described (JOHNSON and LEWIS 1981; JOHNSON *et al.* 1981).

For genetic studies, mice with the variant hemoglobin were mated and the blood of parents and progeny typed for hemoglobin by starch gel electrophoresis and in some cases by isoelectric focusing (WHITNEY *et al.* 1979; WHITNEY 1982). Mutant-bearing heterozygotes were intercrossed to produce mutant homozygotes, and a mutant-bearing heterozygote was mated to three α-thalassemic mice of strain 352HB to study the combined effects of the new α-globin mutation on one chromosome with the deletion of both α-globin genes on the other chromosome. An automatic scanning densitometer (Helena Laboratories Auto Scanner) was used to measure the quantity of hemoglobins with the two kinds of α-chains after they had been separated by isoelectric focusing and stained in Coomassie brilliant blue R-250.

The relative rates of synthesis of the two types of α-chains were determined using ^{3}H-leucine incorporation into the globin chains in reticulocytes that had been induced by multiple injections of phenylhydrazine (MARTINELL *et al.* 1981). The radiolabeled globin chains were separated by carboxymethylcellulose chromatography (CLEGG, NAUGHTON and WEATHERALL 1966), and aliquots were counted in a Packard 3225 liquid scintillation spectrometer. The primary structure of the mutant hemoglobin induced by ethylnitrosourea was analyzed using procedures described to analyze genetic variants of the α-chains of mouse hemoglobins (POPP *et al.* 1982). Briefly, the β-chain and the two forms of α-chains were separated by carboxymethylcellulose chromatography. The isolated α-chains were digested by trypsin, the tryptic peptides were separated by ion exchange chromatography over Dowex 50-X2 and Dowex 1-X2 resins and the amino acid content of each tryptic peptide was determined. Tryptic peptide αT-9, which contained an amino acid substitution of leucine for histidine, was further digested by pepsin, and the peptic fragments were separated by chromatography over Dowex 1-X2 resin (POPP *et al.* 1979). An automated amino acid sequencer programmed for peptide sequencing using dimethylallylamine buffer and 5 mg of polybrene (Pierce Chemical Company) added to the spinning cup was used to perform two cycles of Edman degradation on the tetrapeptide representing residues 87–90 to determine which histidine residue was substituted by leucine. All tryptic peptides and the peptic fragments of αT-9 and αT-12 were also compared by two-dimensional "fingerprinting" on paper by chromatography and high voltage paper electrophoresis (POPP 1965).

RESULTS

The original F_1 mutant animal (JOHNSON and LEWIS 1981) was identified because of a variant hemoglobin pattern on starch gel as shown in Figure 1A. An extra band (identified by an arrow) is seen trailing the single/diffuse-major band in the electropherogram of the mutant Hbb^s/Hbb^d heterozygote. A single trailing band is also seen in Hbb^s/Hbb^s mutant carriers (not shown). Thus, the mutant allele specifies a hemoglobin which migrates slower than the parental form.

The isoelectric focusing pattern of the mutant heterozygote's hemoglobin has been described (WHITNEY 1982). We confirm these findings. The density of the hemoglobin bands following isoelectric focusing suggests that some of

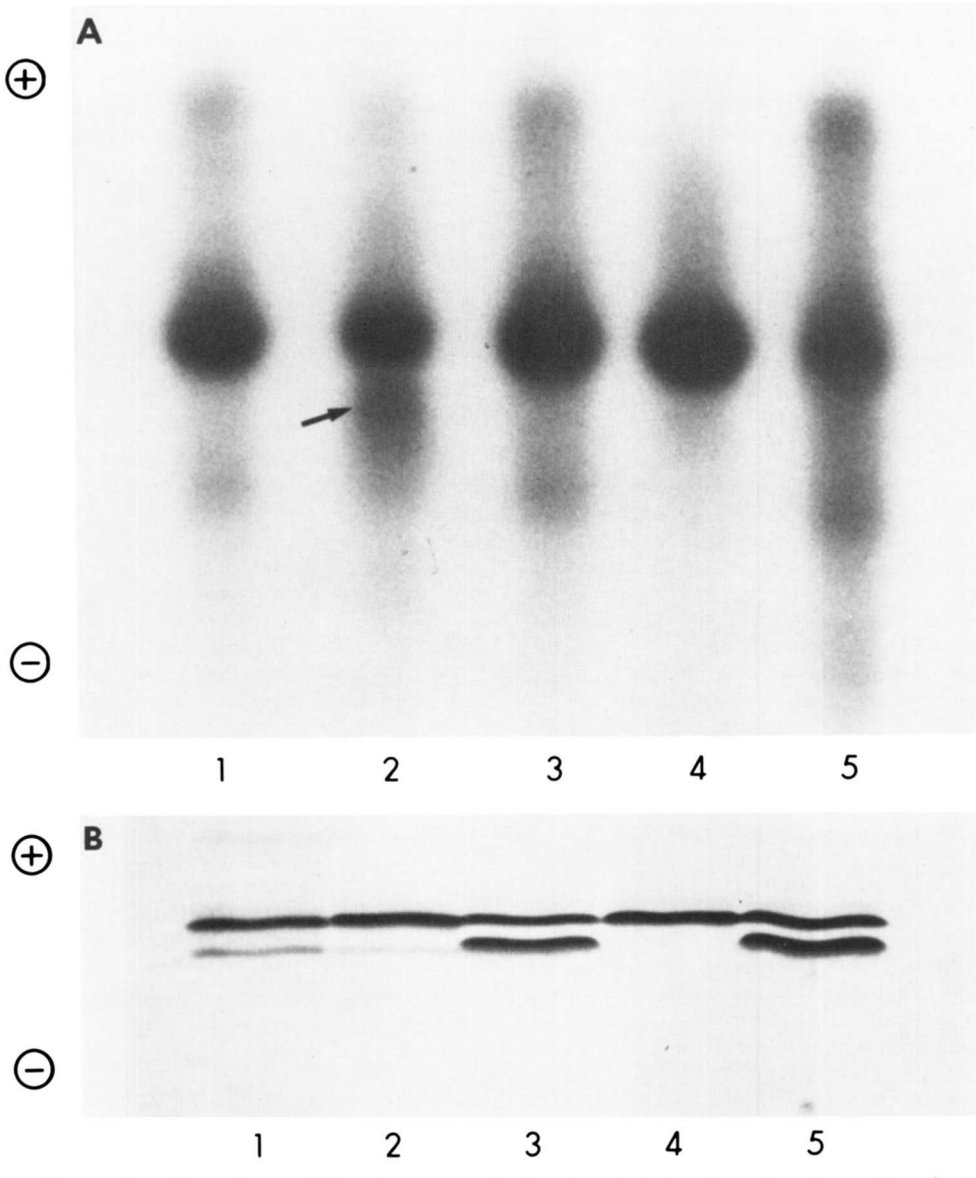

FIGURE 1.—Mouse hemoglobin phenotypes. A, Starch gel electrophoresis patterns. Samples 1 and 3 were from normal B6D2F1 mice (Hba^a/Hba^g; Hbb^s/Hbb^d), sample 2 was from the original B6D2F1 mutant (Hba^a/Hba^{x9}; Hbb^s/Hbb^d) and samples 4 and 5 were from parental mice, C57BL/6J (Hba^a/Hba^a; Hbb^s/Hbb^s) and DBA/J2 (Hba^g/Hba^g; Hbb^d/Hbb^d), respectively. B, Isoelectric focusing patterns. Samples 1 and 4 were from parental mice, DBA/2J and C57BL/6J, respectively, sample 2 was from a normal B6D2F1 mouse and samples 3 and 5 were from the B6D2F1 mutant.

the hemoglobin from normal B6D2F1 mice is shifted to a more cathodal position in the mutant (Figure 1B). Higher resolution gels (not shown) indicate that α-chain 5, not α-chain 1, is altered in the mutant heterozygote's hemoglobin.

Backcrosses of mutant heterozygotes produced 33 mutant carriers out of 65 total progeny (Table 1). Thus, the mutant segregates as a simple Mendelian trait. Furthermore, the mutant trait assorted randomly from the *Hbb* alleles in backcrosses to both parental strains. As judged by the density of the slower migrating band of hemoglobin in starch gels, one-fourth of the F_2 progeny (six of 24) were determined to be homozygous for the hemoglobin mutation (Table 2). The homozygotes were fully viable, bred well and produced normal-sized litters. Twenty-four progeny were also raised from a mating of a heterozygous mutant-bearing male mated with three α-thalassemic females of strain 352HB. Thirteen offspring were α-thalassemic heterozygotes, and six of these carried the ethylnitrosourea-induced mutant hemoglobin as well (Table 2). The six mice showed no clinical symptoms other than those typical of α-thalassemia.

The variant hemoglobin is considered to be the result of an induced mutation by virtue of its occurrence as a unique event among more than 50 progeny of an ethylnitrosourea-treated male in matings with several females. In the total experiment 1974 progeny were examined from ethylnitrosourea-treated parents.

The density of hemoglobin in each of the two bands in starch gels and upon isoelectric focusing suggested that the mutant form of chain 5, henceforth to be identified as 5^{y9}, represented more than one-half of the total hemoglobin in homozygous mutants (Figure 1B). Quantitative measurements on the two forms of hemoglobin following isoelectric focusing revealed that Hba^{y9} homozygotes contained 57.73 ± 0.51% (ten samples), Hba^{y9} heterozygotes that were also α-thalassemic contained 58.33 ± 0.24% (nine samples), and Hba^{b}/Hba^{y9} heterozygotes contained 33.00 ± 1.14% (four samples) hemoglobin in the form of chain 5^{y9}.

Separation of the α- and β-globin chains by carboxymethylcellulose chromatography showed that chain 5^{y9} separated from chain 1 (Figure 2). Thus, the relative rates of synthesis of chains 1 and 5^{y9} could be measured via incorporation of ^{3}H-leucine into the globin chains. Such studies showed that more chain 5^{y9} than chain 1 was synthesized in homozygotes for the new mutation as well as in double heterozygotes for the mutation and α-thalassemia (Table 3).

Ion exchange chromatography of the tryptic peptides from each of the two kinds of α-chains gave similar elution profiles as shown in Figure 3. However, when each fraction from the Dowex 50-X2 chromatography was rechromatographed on Dowex 1-X2, we found that αT-9 of chain 5^{y9} had eluted from the Dowex 50-X2 column with αT-1 in fraction 1, whereas αT-9 of chain 1 had eluted with αT-2 in fraction 2 (Figure 3). The amino acid compositions of αT-9 from chain 1 and chain 5^{y9} differ in that the latter contained one less histidine and glycine and one more alanine and leucine (Table 4). The amino acid compositions of each of the other tryptic peptides of chains 1 and 5^{y9} were indistinguishible from those of C57BL/6 (POPP 1965; new data not shown). Moreover, fingerprints of the tryptic peptides, as well as the peptic peptides of αT-9 and T-12, revealed no acid-amide substitutions (data not shown). Pepsin was used to digest αT-9 into smaller fragments that were sep-

TABLE 1

Segregation and assortment of Hba *and* Hbb *genotypes in progeny of* Hba *mutant mice*

Hba and *Hbb* genotypes*	Mutant carriers			Homozygous normal		
	s/d	*s/s* or *d/d*	Total mutant	*s/d*	*s/s* or *d/d*	Total normal
Backcross to DBA/2J Hba^{y9}/Hba^{a}, Hbb^{s}/Hbb^{d} × Hba^{g}/Hba^{g}, Hbb^{d}/Hbb^{d}	6	11	17	11	6	17
Backcross to C57BL/6J Hba^{y9}/Hba^{a}, Hbb^{s}/Hbb^{d} × Hba^{a}/Hba^{a}, Hbb^{s}/Hbb^{s}	6	10	16	7	8	15
	12	21	33	18	14	32

* Hba^{y9} designates the mutant allele, Hba^{a} the allele of C57BL/6 and Hba^{g} the allele of DBA/2 mice. Hbb^{s} designates the single and Hbb^{d} the diffuse alleles of C57BL/6 and DBA/2 mice, respectively.

TABLE 2

Summary of matings with producing homozygous and hemizygous mutant mice

	Distribution of genotypes*								
	Normal			Mutant heterozygotes			Mutant homozygotes	Thalassemic	
Matings	*a/a*	*a/g*	*a/b*	b/y^{9}	g/y^{9}	a/y^{9}	y^{9}/y^{9}	*a/dl*	y^{9}/dl
Hba^{a}/Hba^{y9} × Hba^{a}/Hba^{y9}	7					11	6		
Hba^{b}/Hba^{dl} × Hba^{a}/Hba^{y9}			11	(genotypes not separated)				7	6

a = Hba^{a} (homozygous in C57BL/6; *b* = Hba^{b} (homozygous in SEC); *g* = Hba^{g} (homozygous in DBA/2); y^{9} = Hba^{y9} (the mutant allele); *dl* = Hba^{dl} (deletion of *Hba* that causes α-thalassemia in mice). The Hba^{dl} mutation is maintained on the inbred SEC/1Re background.

arated by chromatography on Dowex 1-X2 resin. Amino acid analyses of the peptic fragments showed that fragment C contained the naturally occurring polymorphism of alanine rather than glycine at α78 as previously reported for chain 5 (POPP *et al.* 1982) and that fragment F of chain 5^{y9} had one residue each of lysine, histidine, alanine and leucine and chain 1 had two residues of histidine and one residue each of lysine and alanine (Table 4). Eight-five nanomoles of that tetrapeptide of chain 5^{y9} was subjected to two cycles of Edman degradation to establish the position of the histidine → leucine substitution. Step 1 yielded 55 nmol of phenylthiohydantoin-histidine, and step 2 yielded 20 nmol of phenylthiohydantoin-alanine. The residue left in the cup after these two cycles of Edman degradation contained 13 nmol of lysine and leucine, 9

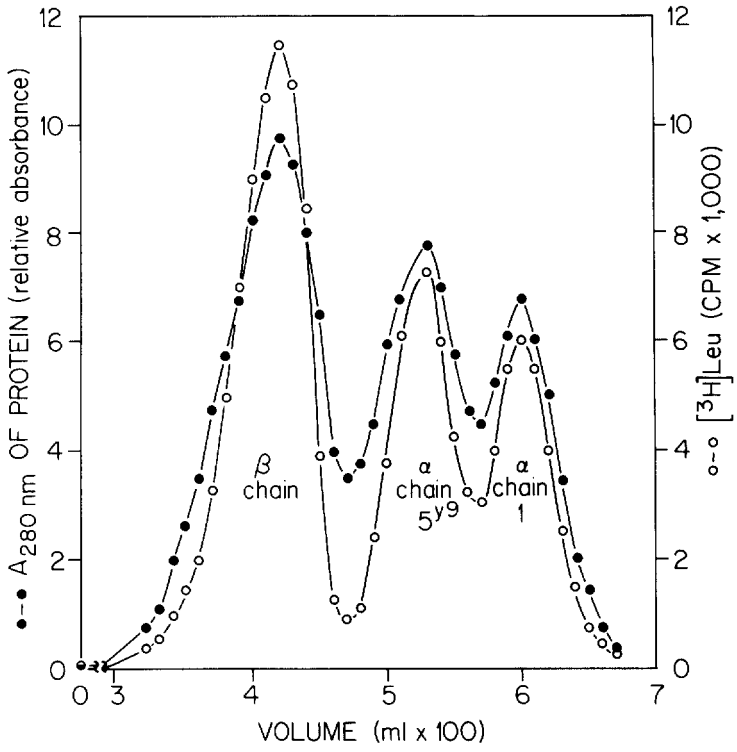

FIGURE 2.—Separation of globin chains by carboxymethylcellulose chromatography. Freshly prepared reticulocytes (0.5 ml of packed cells containing 60–75% reticulocytes) were incubated in 5 ml of minimal essential medium (minus leucine) that also contained 0.5% $NaHCO_2$, 0.6 mg of human transferrin, 1.25 mg of ferrous ammonium sulfate and 0.25 mCi of ^{3}H-leucine, pH 7.4. After incubation for 90 min at 37°, the reticulocytes were washed several times in saline, lysed by adding 4 volumes of H_2O, and globin was prepared by precipation in acidified acetone. Globin chains were separated by chromatography over carboxymethylcellulose (Whatman CM 23) using a 5–30 mM gradient of sodium phosphate in 8 M urea, pH 6.7. The A_{280} of the protein in the eluate was recorded, and aliquots of each fraction were analyzed for ^{3}H-leucine in a scintillation counter.

nmol of alanine and 4 nmol of histidine. Thus, the sequence was His-Ala-Leu-Lys for residues 87–90.

DISCUSSION

Hemoglobin is a well-characterized protein. Two α-globin genes and two β-globin genes are expressed in adult mice (HILSE and POPP 1968; LEDER *et al.* 1981; KONKEL, MAIZEL and LEDER 1979; WEAVER *et al.* 1979), although some mice such as C57BL/6 apparently produce the same kind of α-chain and β-chain polypeptides from each of their two α- and β-globin genes. Changes in the expression of the hemoglobin genes are easily analyzed and are potentially of great use for determining mechanisms by which mutations are induced by mutagens.

TABLE 3

Synthesis of α- and β-globin chains in reticulocytes of normal and Hba^{y9}*-bearing mice*

Hba genotype*	Total cpm				
	β-chain	α-chain 5^{y9}	α-chain 1 (B6) or 1 + 5 (D2)	α/β	5^{y9}/1
Hba^{y9}/*Hba*^{y9}	138,422	87,883	69,909	1.10†	1.19†
Hba^{y9}/*Hba*^{dl}	142,524	60,024	41,170	0.69†	1.38†
Hba^{a}/*Hba*^{a}	180,870		199,242	1.10	
Hba^{a}/*Hba*^{dl}	189,714		138,369	0.73	
Hba^{g}/*Hba*^{g}	204,596		216,259	1.06	

* *Hba*^{y9} designates the mutant allele, *Hba*^{a} the allele of C57BL/6, *Hba*^{dl} the deleted *Hba* locus of α-thalassemic mice and *Hba*^{g} the allele of DBA/2. All mice carried *Hbb*^{s}/*Hbb*^{s}.

† Corrected for difference in the number of leucine residues per chain.

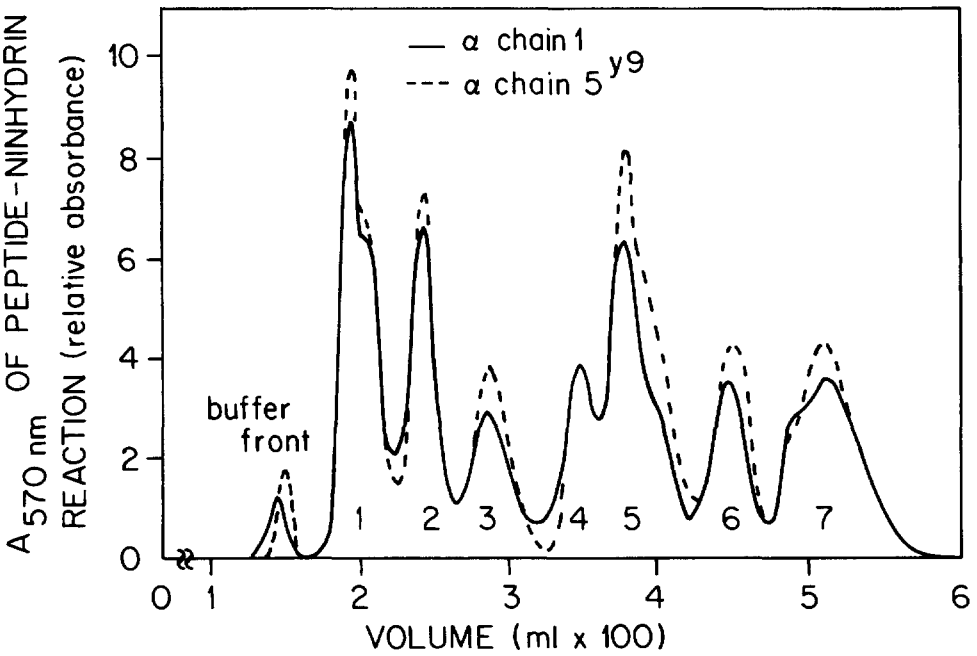

FIGURE 3.—Separation of tryptic peptides by Dowex 50-X2 chromatography. The A_{570} of the peptide-ninhydrin reaction was monitored continuously using the split stream assembly of the amino acid analyzer. After rechromatography of each fraction on Dowex 1-X2, the tryptic peptide in each fraction was identified by amino acid analysis.

Several variations in the expression of the hemoglobin genes have been induced by X rays: (1) deletion of the compound α-globin locus in chromosome *11* (WHITNEY *et al.* 1981), (2) tandem duplication of a portion of chromosome *7* that included the compound β-globin locus (RUSSELL *et al.* 1976), (3) nondisjunction of chromosome *7* (RUSSELL *et al.* 1976) and (4) a somatic cell mosaic (R. A. POPP, unpublished results). Chemicals have also induced several mutations at the hemoglobin loci: (1) deletion of the α-globin locus by triethylene-

TABLE 4

Amino acid analyses of αT-9 and peptic fragments of αT-9 in the two chains of α-globin from Hba^{y9} *mice*

	Residues per molecule													
			αT-9 peptic fragments											
	αT-9		A		B		C		D		E		F	
	1	5^{y9}	1	5^{y9}	1	5^{y9}	1	5^{y9}	1	5^{y9}	1	5^{y9}	1	5^{y9}
Lysine	1.18	1.04											1.14	1.14
Histidine	*2.81*	*2.25*			1.00	1.07							*1.86*	*0.82*
Aspartic acid	4.93	5.34	1.09	1.11	1.12	0.96	1.71	1.98			0.99	1.02		
Serine	2.02	2.00							0.95	0.96	0.95	0.95		
Proline	1.29	0.87					1.00	0.93						
Glycine	*1.98*	*0.98*			0.90	1.07	*1.09*							
Alanine	*7.42*	*8.85*	2.11	2.01	2.81	2.85	*1.02*	*1.98*	1.04	1.13			1.00	0.94
Valine	1.18	0.87	0.80	0.96										
Leucine	*6.18*	*6.79*	1.00	0.93	1.17	1.05	2.18	2.10	1.01	0.92	1.06	1.03		*1.10*

Values for amino acid residues that are different in chains 1 and 5^{y9} are italicized.

melamine (WHITNEY *et al.* 1981) and (2) electrophoretic mobility changes in an α-chain (JOHNSON and LEWIS 1981) and in a β-minor chain (MUROTA, SHIBUYA and TUTIKAWA 1982) have been induced in germinal cells by exposure to ethylnitrosourea.

Natural genetic variations at *Hba* encode for substitutions of neutrally charged amino acids (HILSE and POPP 1968; POPP *et al.* 1982) that are not detectable by zone electrophoresis but are detectable by isoelectric focusing (WHITNEY *et al.* 1979). The ethylnitrosourea-induced α-globin mutation described in this report is detectable by zone electrophoresis using low ionic strength buffer in starch gel and by isoelectric focusing (Figure 1) but not by zone electrophoresis using standard ionic strength buffer in starch gel and on cellulose acetate plates. Most electrophoretic separations of proteins depend on the amount of surface charge at the pH of the buffer used, although starch gel has a small enough pore size to cause some molecular sieving. Isoelectric focusing indicates that chain 5^{y9} has a higher isoelectric point than chain 5 (Figure 1). The $\alpha 89^{His \rightarrow Leu}$ substitution reduces the number of positively charged amino acids by 1, which should theoretically lower, rather than increase, the isoelectric point. The possibility that additional charge differences exist, *i.e.*, acid-amide substitutions, has been ruled out by the common ion exchange properties of each tryptic peptide over Dowex 50-X2 and Dowex 1-X2 resins and by comparison of all tryptic peptides, as well as the peptic peptides of αT-9 and α-12, by two-dimensional paper chromatography and high voltage paper electrophoresis (data not given). Thus, the $\alpha 89^{His \rightarrow Leu}$ substitution may affect the tertiary structure of chain 5^{y9} in low ionic strength buffer that approaches denaturing conditions in a manner that either increases net positive charge or partially unfolds the molecule to impede migration in starch gel.

Hba^{y9} does not cause any hemoglobinopathy, and homozygotes appear to be completely viable under laboratory conditions (Table 1). We also found that significantly more chain 5^{y9} than chain 1 is present and is synthesized (Table 3) in Hba^{y9} homozygotes and in Hba^{y9}/Hba^{dl} double heterozygotes, even though chains 1 and 5 are present in nearly equal quantities in DBA/2 mice (POPP *et al.* 1982), the strain in which the germinal mutation was induced by ethylnitrosourea. On the other hand, mice of a *M. m. molossinus* stock, which also expresses α chains 1 and 5, have four times as much chain 1 as chain 5 (POPP *et al.* 1982). Although the genetic basis for the quantitative differences in the expression of these two adult globin genes in these mice is unknown, the three permutations, *i.e.*, $1 < 5^{y9}$, $1 = 5$ and $1 > 5$, suggest that the regulation is not polar in nature.

Histidine is the usual amino acid at $\alpha 89$ in mammalian hemoglobins (DAYHOFF 1972). If mutations occur naturally in the codon for $\alpha 89$, the rare expression of variant amino acids would suggest that mutant forms are selectively eliminated in nature. Since the histidine $\rightarrow$ leucine substitution is not associated with deleterious effects, the absence of this amino acid variant at $\alpha 89$ may simply indicate that without mutagen exposure the spontaneous mutation rate is practically zero. However, this substitution may cause detrimental effects under unknown environmental conditions. Had the leucine substitution oc-

curred at $\alpha 87^{His}$, which is the site of heme binding (PERUTZ 1976), the mutant would have been expected to form methemoglobin, a generally severe, deleterious condition.

Histidine at $\alpha 89$ in BALB/c, and most probably also in DBA/2, mice is encoded by the CAC codon (NISHIOKA and LEDER 1979). The simplest way to change the CAC codon into a codon for leucine is via an A → T transversion in the second letter to form a CTC codon (CRICK 1968). An A → T transversion is the most plausible mechanism for generating the Hba^{y9} allele because coding of all amino acids adjacent to $\alpha 89$ is normal (Table 4). Thus, we propose that ethylnitrosourea induced an A → T transversion in the α-globin gene that normally codes for chain 5 in DBA/2 mice.

The y9 alteration in chain 5 is to our knowledge the first example of a newly induced germinal mutation resulting in a specifically identified amino acid substitution in a mammalian gene product. That this induced mutation is not found naturally suggests other novel genotypes may also be recoverable after mutagen exposure. As additional induced mutations are characterized, comparisons between newly induced and naturally occurring mutants in the mouse may provide insight into potential genetic hazards to which man may be subjected from exposures to mutagens in the environment. Although genetic risks may be incurred in part from elevated rates of induction of naturally present mutations that cause familar inherited disorders, another component to risk may result from unexpected genetic alterations that are new to nature. A number of the electrophoretically identified induced mutations in mice also appear to have no known counterpart in inbred strains or wild populations (LEWIS and JOHNSON 1983), but most of these mutations have so far not been characterized in detail.

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