Radiation-induced mutations at mouse hemoglobin loci
(nature of induced mutations/mutation rate/biochemical mutants in the mouse/tandem duplication)

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ABSTRACT In experiments designed to detect new mutations affecting hemoglobin, we irradiated the male or female parent in reciprocal crosses of two mouse strains that differ in alleles at the hemoglobin (Hba, Hbb) loci as well as at five other specific loci. Offspring were analyzed for hemoglobin properties (electrophoretic pattern, solubility, crystal pattern), serum albumin differences, erythrocyte lysis, reticulocyte count, and external appearance. Five hemoglobin variants were found among the mutants. In three, the genetic contribution from the irradiated father was not expressed with regard to the α-chain; one carried a tandem duplication (the first known case in the mouse) involving Hbb; and one probably resulted from double nondisjunction of chromosome 7. The finding that major chromosome aberrations can mimic hemoglobin mutations indicates the need, in similar experiments, to follow F1 screening with thorough cytogenetic analysis. The variants in which there is nonexpression of the α-chain may be the result of small deficiencies or of faults in transcription or translation. Mutation rates based on these three variants are not out of line with earlier specific-locus results, although confidence limits are still wide.

The specific-locus method has been used for years at this laboratory in the course of experiments designed to determine the effects of biological and physical variables on the frequency and nature of induced mutations (1, 2). For several of the loci used in such experiments, the pathways from gene to character are at least partially known; and mutations involving these loci have been analyzed by complementation studies and other means to determine their genetic nature (3, 4).

Specific-locus work has now been extended to two additional loci which control a product that is better defined, chemically, than any other character of the mouse, namely, hemoglobin. Our objectives are as follows: (a) to derive, from analysis of the mutant product, additional clues as to the nature of the mutations; (b) to determine, therefrom, mutation rates for clearly defined biochemical variants; and (c) to discover new variants of intrinsic interest at the hemoglobin loci and possibly at other loci affecting blood characters.

The method has been successful, and several hemoglobin mutants have been recovered, including one that turned out to be a tandem duplication, the first reported in the mouse (5). The broad features and implications of our experiment are described here. More detailed results on individual mutants will be published elsewhere.

MATERIALS AND METHODS

Genetic Material. Mouse hemoglobin consists of two α chains, controlled by the Hba locus on chromosome 11, and two β chains, controlled by the Hbb locus on chromosome 7. Variants are known at both of these loci (6–9), and these are co-dominantly expressed in heterozygotes. Crosses were made between two inbred strains differing in the alleles at both the Hba and Hbb loci, as well as at other loci. The SEC strain is Hba*/Hba*, c-Hbb*/c-Hbb*; a/b; d se/d se (or d+se+). The 101 strain is Hba*/Hba*, c+Hbb*/c+Hbb*, A*+/A*; b*/b+; d+se+/d+se+.

Irradiation and Subsequent Breeding. Either the male or the female parent was irradiated in both of the reciprocal crosses (Table 1). Acute x-irradiation was used in each case (87–91 R/min, 250 kV peak; 30 mA; inherent filtration, 3 mm aluminum; Lucite containers; anterior-body shielding for all males, headshielding for most females) (1 roentgen, R = 2.6 × 10^-4 coulomb/kg). Males were mated immediately after irradiation, and offspring conceived in both the presterile and poststerile periods (derived from poststerigmatlonal and spermatoid stages, respectively) were examined in separate groups. The bulk of these offspring (6914 of 7793) came from irradiations with 600 R; the remainder from irradiations with 300, 400, 500, or 600 + 400 R (24 hr interval). Weighted average doses are shown in Table 1. Females were mated 29 hr after irradiation and remained with a mate until after they had become permanently sterile (after one or two litters). The bulk of the offspring examined (689 of 828) came from irradiations with 500 R; the remainder from 400 or 200 R.

Blood Sampling. F1 offspring derived from the above groups were stored (six like-sexed ones per cage) at weaning age. At a later date, 75–100 μl of blood was drawn from each into a heparinized capillary tube by orbital sinus puncture (10) for subsequent analysis of electrophoretic pattern, phosphate solubility, crystal pattern, and erythrocyte lysis. For the electrophoretic analysis, blood samples were pooled in pairs (25 μl from each animal) in order to decrease the number of screening test runs. (This procedure was based on preliminary experiments in which deletion mutants were simulated—see next paragraph.) Where an exceptional result was obtained with the two-animal sample, the blood from each animal was then re-tested separately. All other blood analyses were performed on single-animal samples, some requiring repeated bleeding. Exceptional animals were mated in appropriate crosses for genetic follow-up, and their offspring were individually analyzed for the same blood characters.

Electrophoresis of Hemoglobin and Serum Albumin. Blood was lysed, mixed with iodoacetic acid and ferricyanide, and electrophoresed on starch gels (11). The hemoglobins of strains SEC (Hbb*/Hbb*) and 101 (Hbb*/Hbb*) are distinguishable on the basis of the amino acid present at position β13: glycine in strain SEC, and cysteine in strain 101. Iodoacetic acid adds to the cysteine to form a carboxymethyl derivative, which imparts an extra charge (Fig. 1). It is clear that any mutant with an altered net charge of hemoglobin would have been detected. A deletion mutant was simulated by mixing equal parts of F1 (Hbb*/Hbb*) and either 101 or SEC bloods (Fig. 1A). In these mixtures, one or the other of the normal F1 bands was clearly reduced.

Serum albumin was detected on the same gels by staining for
protein, using 0.05% buffalo black in 10% acetic acid–50% methanol, and destaining in 10% acetic acid.

**Solubility, Crystal Pattern, and Erythrocyte Lysis.** All analyses were performed on single-animal samples. Samples (100 μl) of whole blood were added to 0.6 ml of water; lysis was found to be incomplete for samples with high reticulocyte counts. Hemoglobin solubility was analyzed by 2.98 M potassium phosphate buffer. Under these conditions, SEC hemoglobin is quite insoluble (supernatant absorbance, A, at 575 nm, 0.01) and the precipitate is composed of fine needles; 101 hemoglobin has an intermediate solubility (A, 0.30) and the precipitate is amorphous; and hemoglobin from the F1’s has a high solubility (A, 0.45) and the precipitate contains many small, irregularly shaped aggregates of hemoglobin crystals.

**Chemical Analysis of Abnormal Hemoglobins.** Blood from certain F1 animals (or their offspring) that had yielded exceptional electrophoretic patterns and abnormal results in the solubility and crystal tests was used for further chemical analysis of hemoglobin, as described elsewhere (6–9). Briefly, the α and β chains were separated by chromatography over carboxymethyl-cellulose. Each chain was subsequently digested by trypsin, and the soluble peptides in the tryptic digests were separated by chromatography over Dowex 50-X2. Further purification was by paper chromatography and/or paper electrophoresis (fingerprinting). Analyses were made primarily for those purified tryptic peptides that are known to have different amino acids in the hemoglobins of SEC and 101 mice. (927 and 9352 have not, as yet, been analyzed by this method.)

### RESULTS

In addition to mutants involving the hemoglobin loci, a number of exceptional F1’s of various other types were recovered. All are summarized in Table 2.

#### Hemoglobin variants

The hemoglobins of all of the exceptional F1 animals discovered had both an abnormal electrophoretic pattern and abnormal phosphate solubility and crystal structure. Of the five variants detected, four exhibited a diminished fast band (Fig. 1B), and one a diminished slow band (Fig. 1C). Among the former, three are very similar in all features of their phenotype and will be described as a group.

927, 972, 9352. Hemoglobin from all of these F1 animals showed, in addition to the abnormal electrophoretic pattern (fast band diminished), a higher than normal solubility (A, 0.55). The precipitate contained relatively few large, round aggregates of crystals. Subsequent chemical analysis of the hemoglobins of 972 and 9352 indicated that the genetic contribution from the irradiated SEC parent was not being expressed with regard to the α chain. The evidence for this is as follows. Position α68 of the αT-9 peptide is occupied by aspartagine in 101 mice, and by serine and threonine in the two kinds of α chain of SEC mice. Analysis of αT-9 showed that asparagine was the only amino acid at position 68 in hemoglobins from 972 and 9352.

Two of the three presumed mutants in this group, 927 and 9352, have transmitted their abnormal phenotype by a simple dominant mode of inheritance. Further studies on these stocks will be published elsewhere. In spite of repeated matings with seven females of two different stocks, 972 yielded no offspring. Histological examination of one of his testes indicated that spermatogenesis was blocked in pachytene. Chromosome banding analysis of cells from kidney culture revealed that 972 carried a reciprocal autosomal translocation involving chromosomes 3 and 16. Since this rearrangement was the probable cause of his sterility (15), and since the Hba and Hbb loci are not located on either of the chromosomes involved, it appears unlikely that there is any causal connection between the sterility and the abnormal blood phenotypes. In view of the great similarity of 972 to 927 and 9352, he is assumed to be a hemoglobin mutant, despite the fact that transmission of the mutation could not be tested.

986. Abnormalities in the hemoglobin of 986 were found for all the parameters studied. The animal was, further, of small stature and reproduced rather poorly. The hemoglobin of 986 differed from that of normal F1’s by having lower solubility (A, 0.15), and the precipitate contained small granular crystals. Since, in the electrophoretic pattern, the fast-moving component appeared diminished, the mutation was at first assumed to be an alteration in the Hbbα allele (contributed by the 101 parent). Subsequent genetic and cytological tests, however, revealed that the abnormal phenotype results from a tandem duplication involving about 20% of the SEC-contributed chromosome 7, with the duplicated segment including the Hbb and c loci and, presumably, Mod-2 between them (5). The abnormal hemoglobin phenotype may be explained by the overproduction of βSEC, which causes the fast-moving band in the electrophoretic pattern to appear relatively fainter. Quantitative testing of the amino acid composition of the βT-3 and βT-14 peptides of 986 bears out the hypothesis of overproduction. Results from more extensive studies with this duplication will be published elsewhere.

9732. In only one F1 animal was the slow-moving band found to be diminished (Table 2); the electrophoretic pattern (Fig. 1C)
and solubility of hemoglobin from Y732 were identical to that of the parent 101 strain. Thirty-five offspring of F1 Y732 × SEC d were analyzed for the various hemoglobin characters. By all criteria, all 35 behaved like normal (101 × SEC)d, with regard to β-chain-related characters. Furthermore, all 35 of them were wild type with regard to c-locus phenotype (i.e., no cch allele or c-locus deficiency was transmitted). On the other hand, approximately 50:50 segregation occurred, as expected, with regard to a, b, d, se, and Hba loci. Cytological analysis of mitotic metaphases from ear culture showed only normal karyotypes. We conclude that Y732 has two chromosomes 7 derived from her 101 parent, and no chromosome 7 derived from her SEC parent. With regard to chromosomes 2, 4, 9, and 11 (and probably all the rest), she is a normal F1 animal. Double nondisjunction of a chromosome, which would explain the origin of Y732, has earlier been invoked in several cases involving chromosome 9 (13).

Serum albumin

Of the 8621 F1 mice tested by electrophoresis, none revealed a detectable alteration in mobility of serum albumin, nor a quantitative alteration in the stained band on the starch gel, as judged visually.

Other blood characters

Four animals were observed to be anemic on the original test and subsequent retest, but showed no abnormality in hemoglobin characters (Table 2). Thus, the electrophoretic and crystal patterns and the phosphate solubility were typical of anemia but not otherwise disturbed. More detailed analysis was carried out for 861 and 8122. Fingerprints of tryptic peptides of both the α and β chains appeared normal. An analysis of many of the larger tryptic peptides for possible amino-acid substitutions not altering charge gave negative results. On the basis of these various findings, it was concluded that the anemias were not caused by structural alterations of hemoglobin. Erythrocyte lysis was incomplete, and the reticulocyte counts were abnormally high.

One of the anemic F1’s, Y585, died at about 12 months of age without producing offspring. (She was, unfortunately, not mated until 9.5 months old, at which time her abdomen appeared distended.) The other three were fully fertile and did not transmit the anemia. Because of the possibility that there

<table>
<thead>
<tr>
<th>Proband parent</th>
<th>Irradiated germ stage</th>
<th>Variant phenotype</th>
<th>Solubility* (A)</th>
<th>Crystal pattern</th>
<th>Reticulocyte %</th>
<th>Heritability</th>
<th>Probable cause</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Electrophoretic pattern</td>
<td></td>
<td></td>
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</table>
| Blood characters

A. Hemoglobin variants

<table>
<thead>
<tr>
<th>Proband</th>
<th>Irradiated germ stage</th>
<th>Variant phenotype</th>
<th>Solubility* (A)</th>
<th>Crystal pattern</th>
<th>Reticulocyte %</th>
<th>Heritability</th>
<th>Probable cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y27 SEC5 Spermatozids</td>
<td>Diminished fast band</td>
<td>~0.55</td>
<td>Abnormal</td>
<td>5.3</td>
<td>Yes</td>
<td>Mutation or deficiency causing nonexpression of α-chain</td>
<td></td>
</tr>
<tr>
<td>Y352 SECd Spermatozids</td>
<td>Diminished fast band</td>
<td>~0.55</td>
<td>Abnormal</td>
<td>2.9</td>
<td>?†</td>
<td>Tandem duplication involving Hbb</td>
<td></td>
</tr>
<tr>
<td>Y86 SECd Mature oocytes</td>
<td>Diminished fast band</td>
<td>0.15</td>
<td>Abnormal</td>
<td>0.9</td>
<td>Yes</td>
<td>Double nondisjunction of chromosome 7</td>
<td></td>
</tr>
<tr>
<td>Y732 SECd Early spermatoocytes</td>
<td>Diminished slow band</td>
<td>0.30</td>
<td>Abnormal</td>
<td>4</td>
<td>No</td>
<td>Double nondisjunction of chromosome 7</td>
<td></td>
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</table>

B. Anemic variants

<table>
<thead>
<tr>
<th>Proband</th>
<th>Irradiated germ stage</th>
<th>Variant phenotype</th>
<th>Solubility* (A)</th>
<th>Crystal pattern</th>
<th>Reticulocyte %</th>
<th>Heritability</th>
<th>Probable cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y312 SECd Spermatogonia</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal(?)</td>
<td>10, 6</td>
<td>No</td>
<td>Environmental</td>
<td></td>
</tr>
<tr>
<td>Y122 SECd Spermatogonia</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>11, 14</td>
<td>No</td>
<td>Environmental</td>
<td></td>
</tr>
<tr>
<td>Y585 101d Spermatogonia</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>n.t.</td>
<td>?†</td>
<td>Environmental</td>
<td></td>
</tr>
<tr>
<td>Y779 101d Spermatogonia</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>n.t.</td>
<td>No</td>
<td>Environmental</td>
<td></td>
</tr>
</tbody>
</table>

Specific-locus and miscellaneous variants

<table>
<thead>
<tr>
<th>Proband</th>
<th>Irradiated germ stage</th>
<th>Variant phenotype</th>
<th>Solubility* (A)</th>
<th>Crystal pattern</th>
<th>Reticulocyte %</th>
<th>Heritability</th>
<th>Probable cause</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y152 101d Spermatogonia or spermatids</td>
<td>Coat color</td>
<td>Yes</td>
<td>b-locus allele, viable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y288 101d Spermatogonia</td>
<td>Coat color</td>
<td>Yes</td>
<td>d-locus allele, prenatally lethal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y281 101d Spermatogonia</td>
<td>Coat color</td>
<td>Yes</td>
<td>c-locus allele, viable</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Y1015 101d Spermatogonia</td>
<td>Coat quality</td>
<td>Yes</td>
<td>Ca-locus, dominant</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Y59 101d Spermatogonia</td>
<td>Scattered white hairs</td>
<td>Yes</td>
<td>New(?) dominant</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Y98 101d Spermatogonia or spermatids</td>
<td>Dark coat, lop-ear</td>
<td>Yes</td>
<td>?</td>
<td></td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

n.t., not tested.
* Measured as absorbance at 575 nm. A is 0.01 for SEC, 0.30 for 101, and 0.45 for the F1.
† Proband sterile.
might be late expression of the anemic phenotype, 21 offspring of 6122 and 22 offspring of 681 were retested at ages ranging from 12 to 19 months. With the exception of two offspring of 681 that had incomplete lysis and high reticulocyte counts, all others were normal. The two exceptional animals were mated together, and 52 of their progeny were tested, about half of them at over a year of age. All were normal.

Specific-locus and miscellaneous variants

The two parent strains differ in five of the seven loci that have in the past been used in large-scale mutation-rate experiments, and induced mutations at these loci are detectable where the wild-type parent (101 strain) is irradiated. Three such were, in fact, found: a viable b-locus repeat, a prenatally lethal d-locus allele, and an intermediate, viable, allele at the c locus (see Table 2). In addition, external observation of the F1 led to the discovery of two dominants, one at Ca (caracul) and one at tagenesis experiments lies in the detailed knowledge, already available, about the normal product of these genes and therefore the relative ease with which alterations in the product can be characterized. It is hoped that such characterization will allow conclusions to be drawn about the nature of mutations induced by various mutagens. Several laboratories are also embarking on studies of mutation rates for biochemical variants, which include the hemoglobin loci. The results of the present experiment, in which two different major chromosome aberrations (duplication and double nondisjunction) mimicked hemoglobin mutations, illustrate the need for caution in interpreting F1 findings unless these are followed by thorough cytogenetic studies.

The tandem duplication is, to our knowledge, the first found in the mouse. The genetic imbalance caused by such duplications would ordinarily not be detectable in specific-locus experiments, where wild-type chromosomes are treated with mutagens. The new tandem duplication is of fundamental interest because of the information it will supply with regard to (a) correlations of the cytological and genetic maps, and (b) gene dosage effects in a chromosomal region for which many deficiencies and several X-autosome translocations are already available from earlier work.

The other three hemoglobin mutants resemble each other in nonexpression of the δ chain. It is not yet known whether this nonexpression is the result of a small deficiency in chromosome 11, or whether it represents a fault in transcription or translation. The characteristics of the ultimate defect caused by these three mutations make it likely that they will provide extremely valuable animal models for human α thalassemia.

No cases of base-pair substitution were detected in the present series. However, it should be noted that only one of the mutants was derived from the irradiation of spermatogonia, the cell stage that yields the lowest frequency of chromosomal aberrations. Expansion of the experimental series should provide more critical information in the future.

The numbers of induced mutations so far observed, from irradiated spermatogonia and post-spermatogonial stages, at the Hba and Hbb loci are too small for reliable estimates of mutation rates. However, it is worth noting that, as they stand, the rates are not out of line with those obtained in past experiments with other loci. For spermatogonia, the rate, based on one mutation in 6918 offspring and a weighted mean dose of 583 R, is $1.2 \times 10^{-7}$/R per locus, with 90% confidence limits of (0.1 and 5.6) $\times 10^{-7}$. For post-spermatogonial stages, the rate, based on the two observed mutations (excluding the presumed double nondisjunction) in 875 offspring for a weighted mean dose of 585 R, is 19.5 [90% confidence limits of (5.2 and 58.4) $\times 10^{-7}$/R per locus]. The two rates are approximately one-half and three times the mean rates obtained earlier at seven specific loci (a, b, c, p, d, s), for spermatogonia and post-spermatogonial stages, respectively (1, 14). The differences, even from the mean values for the seven loci, are clearly not statistically significant, and there is, of course, a considerable range of variation in mutation rates among the seven loci themselves. (The limited mutational information on five of these seven loci in the current experiment is not at variance with the results from the earlier work.)

We wish to express our gratitude to Kathryn Stelzer and Elizabeth Phiggs for irradiating the mice; to Savanna Maddux and Georgia Guinn for the breeding of irradiated animals and subsequent maintenance of mutant stocks; and to N.L.A. Cacheiro for the cytogenetic studies on 572, 586, and 5732. Research was sponsored by the U.S. Energy Research and Development Administration under contract with the Union Carbide Corp.