Mouse Haemoglobin Beta Chains

SEQUENCE DATA ON EMBRYONIC γ CHAIN AND GENETIC LINKAGE OF THE γ -CHAIN LOCUS TO THE ADULT β -CHAIN LOCUS *Hbb*

By JOHN G. GILMAN*

Department of Genetics, University of Wisconsin, Madison, WI 53706, U.S.A.

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Embryonic haemoglobin γ -chain types were determined by starch-gel electrophoresis for inbred *Mus musculus* strains Sec/ReJ and Au/SsJ (with *Hbb*^s and *Hbb*^p adult β -chain alleles respectively) and for random-bred HA/ICR (Swiss) mice, which had been selected to be homozygous for the adult *Hbb*^d β -chain allele. The strain with the *Hbb*^s allele had γ^1 embryonic β chain and the strain with the *Hbb*^d allele had γ^2 . The strain with the *Hbb*^p allele also had γ^2 chain. A breeding study showed that the maximum recombination frequency between the *Hbb* locus and the γ -chain locus is 5.4% at the 95% confidence level. Embryonic γ^2 chain was sequenced for 39 positions from the N-terminus with an Edman-Begg sequenator. Some tryptic-peptide composition data were obtained on both γ^1 and γ^2 chains. No amino acid substitutions between γ^1 and γ^2 chains were found in the 40 positions for which there are common data on the two chains. γ^2 and adult β chains are different in 12 of the first 21 positions, but show considerable similarity thereafter. It is suggested that homologous but unequal crossing-over between γ -chain and adult β -chain loci could have given rise to the adult β -chain allele *Hbb*^d, which produces structurally different major and minor β chains from two closely linked genes.

Embryonic haemoglobin components different from those of adults are known in a number of mammals, including humans (Huehns *et al.*, 1961), mice (Barrowman & Craig, 1961) and sheep (Hammerberg *et al.*, 1974). The embryonic haemoglobins are produced by nucleated erythrocytes originating in blood islands of the yolk sac. In the best studied mammal, the laboratory mouse (*Mus musculus musculus*), erythropoiesis first occurs in the yolk sac at about day 7–8 of gestation. The nucleated erythrocytes enter the circulation in a wave at about day 9, dividing in the circulation until about day 13 (Bank *et al.*, 1970). The nucleated erythrocytes are then gradually diluted out of the circulation by non-nucleated cells, containing adult haemoglobin, which are given off by the liver at about day 13 (Borghese, 1959; Craig & Russell, 1964).

The embryonic mouse has three embryonic haemoglobins (Craig & Russell, 1964), EI, EII and EIII, among which are distributed three embryonic globin chains x , y and z (Fantoni *et al.*, 1967; Gilman & Smithies, 1968). EI is composed of x and y chains, EII of α and y chains and EIII of α and z chains. Using starch-gel electrophoresis, Gilman & Smithies (1968) reported finding a polymorphic variation in the y chain among various strains of mice. Embryos with the *Hbb*^d adult β -chain allele were found to have

γ^2 embryonic globin (strains C3H/He and DBA/2J). Embryos with the *Hbb*^s adult β -chain allele were found to have γ^1 embryonic globin (strain C57Bl/6J). The *Hbb*^d allele differs from the *Hbb*^s allele in that the *Hbb*^d allele is composed of two linked genes, which produce structurally different β chains in unequal amounts, whereas the *Hbb*^s allele produces only one β chain different from those of the *Hbb*^d allele (Hutton *et al.*, 1962*a,b*). The d (or 'diffuse') haemolysate (from the *Hbb*^d allele) has a major and a minor haemoglobin, β major and β minor; the respective β chains β dmaj and β dmin differ in sequence in at least nine positions (Gilman, 1972, 1973; Popp & Bailiff, 1973). The β s chain, from s haemolysate, differs from the β dmaj chain in at least three positions (Rifkin, 1968; Gilman, 1972, 1973; Popp, 1973).

Steinheider *et al.* (1972) did tryptic-peptide composition studies on embryonic x chain and embryonic y chain, which was originally mistakenly reported as z chain (G. Steinheider, personal communication). They showed that the x chain may be considered an embryonic α chain, similar to embryonic α chains in humans and rabbits (see also Melderis *et al.*, 1974), and that y is an embryonic β chain. The present paper reports sequence data for 39 positions from the N-terminus of γ^2 chain, and amino acid-composition data on some of the tryptic peptides of both γ^1 and γ^2 chains. I also present results of breeding studies, which suggest that the γ -chain and adult β -chain genes are closely linked.

* Present address: Università di Milano, Cattedra di Enzimologia, Via G. Celoria 2, 20133 Milano, Italy.

Experimental

Animals

Mouse strains used in this study were as follows: inbred mice Au/SsJ, Sec/ReJ and C57Bl/6J and the hybrid B₆D₂F₁, offspring of the cross C57Bl/6J × DBA/2J, all from The Jackson Laboratory, Bar Harbor, ME, U.S.A.; and random-bred HA/ICR (Swiss) mice from A R S Sprague-Dawley, Madison, WI, U.S.A. HA/ICR mice have both *Hbb*^s and *Hbb*^d adult β -chain alleles segregating in the population, from which d and s subcolonies were separated by means of haemoglobin typing after cystamine treatment (Wegmann & Gilman, 1970). Cystamine treatment enables one easily to distinguish the *Hbb*^s/*Hbb*^d heterozygote from the *Hbb*^d/*Hbb*^d homozygote by electrophoresis; without such treatment, the one component of s haemolysate and the major component of d haemolysate have virtually identical mobilities. However, they differ in the number of free thiol groups; cystamine treatment, which causes the attachment of the positively charged *S*-ethylamine group to available thiol groups by means of disulphide bonding, thus causes a difference in charge between those two components, permitting their electrophoretic resolution.

Starch-gel electrophoresis

Two-dimensional starch-gel electrophoresis of haemoglobins was as described by Gilman & Smithies (1968), based on the original method of Smithies *et al.* (1966). The gel in the first dimension was at pH 8.6, with 0.085 M-Tris/6 mM-EDTA/0.025 M-boric acid, and was run at 4°C for 20 h at 120 V. The gel in the second dimension contained 0.02 M-Ba(OH)₂, 8 M-urea and 0.05 M-2-mercaptoethanol, and lactic acid was added to the buffer in sufficient quantity to cause the final gel pH to be 4.0. It was run at 4°C for 40 h at 120 V.

The one-dimensional urea/starch gel of Plate 1 (below) is a variation of one described by Niece (1968). It contained 0.05 M-pyridine, 0.056 M-acetic acid, 0.05 M-2-mercaptoethanol and 8 M-urea (pH 5.6) and was run at 4°C for 24 h at 120 V. Samples were either globin or haemoglobin, made 8 M in urea, and acidic with a tiny amount of acetic acid.

Separations of haemoglobins

Embryonic haemoglobin component EII was isolated from embryonic haemolysate by ion-exchange gradient chromatography with DEAE-Sephadex A-50 (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.), the same procedure used by Gilman (1973) to separate mouse adult haemoglobin components d_{major} and d_{minor} from d haemolysate. Washed embryonic erythrocytes were obtained from

14-day embryos, as previously described (Gilman & Smithies, 1968). Cells were frozen for up to 4 weeks. When enough material had been collected, haemolysate (from which cell membranes had been removed by centrifugation) was dialysed against starting buffer, 0.03 M-ammonium acetate to which ammonia had been added to raise the pH to about 9.5 (precise pH was not critical). Haemoglobins were eluted from the DEAE-Sephadex column (2 cm × 12 cm) with a linear gradient of ammonium acetate, made from 350 ml of starting buffer and 350 ml of final buffer containing 0.12 M-ammonium acetate (pH about 9.5). For embryonic haemoglobin from C57Bl/6J mice, three incompletely resolved peaks were obtained. The first to be eluted was embryonic haemoglobin EIII, as judged by starch-gel electrophoresis (pH 8.6). The second peak contained only EII embryonic haemoglobin, and the third was a mixture of EI embryonic and the adult haemoglobin. The second peak (EII) was used for the analyses in this paper, and the other peaks were discarded. A similar chromatographic pattern was obtained for embryonic haemoglobin from HA/ICR mice homozygous for the *Hbb*^d adult β -chain locus. In that case also, the second peak eluted was used for the sequence and peptide analyses described below. However, in that case, the second peak contained not only embryonic EII haemoglobin but also the adult minor haemoglobin component (d_{minor}), as can be seen from the sequence data for that material (Fig. 1).

Sequencing procedures

Before sequencing, haem was removed from the haemoglobin by the method of Rossi-Fanelli *et al.* (1958). Sequencing was carried out in an Edman & Begg (1967) sequenator (Illitron Division, Illinois Tool Works, Chicago, IL, U.S.A.). Sequenator methods and data-processing procedures are those of Smithies *et al.* (1971). Data for the *N*-terminal sequence of the mixture of EII and d_{minor} haemoglobins (Fig. 1) are basically from HI hydrolyses, which were used to convert amino acid thiazolinones into amino acids. The HI-hydrolysis data were corrected for out-of-step sequencing and background amino acids by using an early manual version of the computer-processing procedure described by Smithies *et al.* (1971). The data were plotted manually as the logarithm of the corrected recovery in nanomoles versus position number.

For some positions, the sample was split and NaOH/Na₂S₂O₄ hydrolyses were also performed; data from these hydrolyses were inserted in the semilog plot by using the following rationale. HI hydrolyses convert cysteine thiazolinone, serine thiazolinone and alanine thiazolinone into alanine, and convert tryptophan thiazolinone into a mixture of glycine and alanine. Thus recovery of alanine after

HI hydrolysis implies the presence of either cysteine, serine or alanine at that position (indicated by '&' in the semilog plot). NaOH/ $\text{Na}_2\text{S}_2\text{O}_4$ hydrolyses resolve some of the ambiguities of the HI hydrolyses: NaOH/ $\text{Na}_2\text{S}_2\text{O}_4$ hydrolysis totally destroys cysteine thiazolinone and serine thiazolinone, but converts alanine thiazolinone into alanine, and tryptophan thiazolinone into tryptophan. Thus, where alanine is recovered after HI hydrolysis but not after NaOH/ $\text{Na}_2\text{S}_2\text{O}_4$ hydrolysis, the presence of serine or cysteine is implied at that position (indicated by '\$' in the semilog plot). Where alanine is recovered after both hydrolyses, the presence of alanine is implied at that position (indicated by 'A'). For the data of Fig. 1, both NaOH/ $\text{Na}_2\text{S}_2\text{O}_4$ and HI hydrolyses were performed at positions 3, 5, 6, 8, 9, 10, 14, 16, 27 and 37; at all other positions only HI hydrolyses were performed.

Both NaOH/ $\text{Na}_2\text{S}_2\text{O}_4$ and HI hydrolyses, as described by Smithies *et al.* (1971), convert asparagine thiazolinone and aspartic acid thiazolinone into aspartic acid, and glutamine thiazolinone and glutamic acid thiazolinone into glutamic acid; the milder NaOH/ $\text{Na}_2\text{S}_2\text{O}_4$ hydrolysis, which allows partial recovery of asparagine thiazolinone as asparagine (Gilman, 1974), had not yet been developed at the time of the experiments of this paper. Therefore, in the data of Fig. 1, glutamic acid is not distinguished from glutamine nor is aspartic acid distinguished from asparagine.

Tryptic-peptide composition studies

For the amino acid composition of tryptic peptides, globin was treated with cystamine to cause the attachment of the *S*-ethylamine group to thiol groups (Gibson *et al.*, 1971). The procedure for tryptic digestion, purification of peptides and amino acid analysis was also as described by Gibson *et al.* (1971), with one modification. For the mixture of embryonic EII and adult dminor haemoglobins from HA/ICR mice, the tryptic digest was fractionated on Sephadex G-25 (fine grade) into several crude fractions before the final purification step on cation-exchange resin.

Breeding study

$\text{B}_6\text{D}_2\text{F}_1$ hybrid males were crossed with C57Bl/6J inbred females, and the 14-day embryos resulting from the cross were individually bled, and the haemolysates analysed by two-dimensional starch-gel electrophoresis. The rationale for scoring embryos as to adult β -chain and embryonic γ -chain types is presented in the Results section.

$\text{B}_6\text{D}_2\text{F}_1$ is the hybrid resulting from the cross DBA/2J $\times$ C57Bl/6J. Strain DBA/2J is homozygous for the Hbb^d adult β -chain allele, and C57Bl/6J is homozygous for the Hbb^s allele (Russell & Gerald,

1958). Strain DBA/2J has y^2 embryonic chain and strain C57Bl/6J has y^1 chain. Therefore embryos resulting from the cross $\text{B}_6\text{D}_2\text{F}_1 \times \text{C57Bl/6J}$ were expected to be either Hbb^s/Hbb^s or Hbb^s/Hbb^d at the adult β -chain locus and to have either y^1 chain alone or a mixture of y^1 and y^2 chains. A crossover would be observed as an embryo with Hbb^s/Hbb^s adult β -chain genotype having some y^2 chain, or an embryo of genotype Hbb^s/Hbb^d lacking y^2 chain.

If no crossovers are observed, one may obtain the upper confidence limit for the recombination value r , such that there is a 95% chance that at least one crossover would have been observed in n cases, by solving the equation $(1-r)^n = 0.05$.

Results

Starch-gel studies

Plate 1 shows the results of a urea/starch-gel study (pH 5.6) on haemolysates of 13- and 14-day embryos from several types of mice (slots 2–4). Adult haemolysate is shown for comparison (slot 1). Previous work (Gilman & Smithies, 1968) showed how the various embryonic bands found by urea/starch-gel electrophoresis at pH 4.0 could be classified into components x , y^1 , y^2 and z . The bands of Plate 1 are labelled assuming that the relative electrophoretic mobilities of the various chains are the same at pH 4.0 and pH 5.6. The embryonic z chain cannot be definitely identified in Plate 1, perhaps because it is present in too small an amount to be clearly seen (it is the chain present in lowest quantity at 13 and 14 days) or perhaps because it overlaps one of the other bands. The 13-day embryonic p haemolysate (slot 2) shows a faint band migrating between the α and β chains, close to the β chain; this could be the z chain because this is approximately where it is expected to be (Gilman & Smithies, 1968). In the 14-day embryo samples (slots 3 and 4) there is considerably more adult β chain present, and this may make it difficult to see the z chain in those samples.

In Plate 1, slot 4 shows the embryonic globin chains of the inbred mouse Sec/ReJ, whose adult β -chain genotype is Hbb^s/Hbb^s (Hutton *et al.*, 1962a). This strain has an embryonic y^1 chain, as was the case for strain C57Bl/6J (Gilman & Smithies, 1968), which is also Hbb^s/Hbb^s . In contrast, HA/ICR random-bred mice, selected to be of adult β -chain genotype Hbb^d/Hbb^d , show predominantly or exclusively y^2 chain (slot 3). This was from a pooled sample of blood from 125 embryos. The small amount of band migrating in the position of the y^1 chain in that sample could be y^1 chain, but also could be an adult minor component with the same mobility as y^1 chain that is seen in the adult sample (slot 1). This adult minor band, and the other one migrating between the α and β chains, may be oxidation

products of the β and α chains respectively, since they are seen more distinctly when the sample used is haem-free adult globin stored for some time, and they are seen less distinctly or not at all when fresh adult haemolysate is used.

Slot 2 shows the embryonic globin-chain pattern from one litter of embryos resulting from the cross of an HA/ICR mouse (adult β -chain genotype Hbb^d/Hbb^d) with the inbred mouse Au/SsJ (Hbb^p/Hbb^p). The Hbb^p allele is similar to the Hbb^d allele, the only difference being that the Hbb^p allele produces a variant minor β chain relative to that produced by the Hbb^d allele (Gilman, 1972, 1973). The embryos resulting from this cross, of adult β -chain genotype Hbb^d/Hbb^p , have only y^2 embryonic β chain and no y^1 , demonstrating that the Hbb^p allele, as well as the Hbb^d allele, is associated with the y^2 embryonic β chain.

Breeding study

The preceding evidence shows that mice selected to have the adult β -chain allele Hbb^p had y^1 embryonic β chain, whereas mice selected to have the Hbb^d or Hbb^p alleles had y^2 . This is suggestive of genetic linkage. A breeding study was therefore performed to test this possibility. A preliminary report of the result has already been given (Gilman & Smithies, 1968).

Hybrid mice of type $B_6D_2F_1$, heterozygous for Hbb^d and Hbb^p adult β -chain alleles and for alleles coding for y^1 and y^2 embryonic β chains, were mated with inbred C57Bl/6J mice, homozygous for the Hbb^p allele and for the y^1 embryonic β -chain allele. Embryos of 14 days of age resulting from the cross were individually bled, and their haemoglobin samples were subjected to two-dimensional starch-gel electrophoresis. The first dimension, in alkaline starch gel (pH 8.6), allowed identification of the adult β -chain type. A typical result is shown in Plate 2(a) for haemoglobins from nine embryos.

Embryos were expected to have either a mixture of s and d adult haemoglobins or only s haemoglobin. The leading minor band, which migrates faster than any of the other adult or embryonic haemoglobins on starch gel at pH 8.6, is found only in haemolysates that contain d haemoglobin. It could be due to disulphide bonding of an acidic thiol-group-containing substance, such as glutathione, to the cysteine at position 13 of the β chain of the major haemoglobin of d haemolysate, d_{major} (Gilman, 1973). The one β chain of s haemolysate has glycine at position 13, and the leading minor band is not seen for s haemolysate. Thus those embryos showing the leading minor band in Plate 2(a) must be of adult β -chain genotype Hbb^d/Hbb^p , whereas those embryos whose haemolysates do not show the leading minor band must be Hbb^p/Hbb^p . Embryos whose haemoglobins were run

in slots 1, 2, 3, 5, 6 and 7 were therefore of adult β -chain genotype Hbb^p/Hbb^d , and those embryos whose haemoglobins were run in slots 4, 8 and 9 had genotype Hbb^p/Hbb^p .

Plate 2(b) shows the globin-chain separations of component EII in the urea/starch gel (pH 4.0). For each embryonic sample, the haemoglobin component EII was cut out of the original alkaline starch gel and placed in the acidic starch gel containing 8M-urea. Haemoglobin EII from slots 1, 2, 3, 5, 6 and 7 has both y^1 and y^2 embryonic chains. Thus, those embryos were heterozygous for both the adult β -chain alleles Hbb^d and Hbb^p and for the alleles for embryonic β chains y^1 and y^2 . Haemoglobin EII from slots 4, 8 and 9 shows only y^1 chain and no y^2 , so that those embryos were homozygous for both the adult allele Hbb^p and for the embryonic allele for y^1 chain. Therefore for these nine embryos whose haemoglobins were analysed in the gels of Plate 2, there is no evidence for a crossover between the adult β -chain and embryonic y -chain loci. Some 56 embryos were analysed in this manner, and no crossovers were found, indicating a maximum recombination frequency of 5.4% at the 95% confidence level between the adult and embryonic β -chain loci.

Sequence studies

Haemoglobin was taken from 14-day embryos of strain HA/ICR, whose parents had been selected to be of adult β -chain genotype Hbb^d/Hbb^d . Plate 1 showed that these embryos have predominantly or exclusively the y^2 type of y chain. The haemolysate was fractionated on DEAE-Sephadex, and the embryonic haemoglobin EII was obtained as a mixture with the adult minor haemoglobin, d_{minor}. That mixture, which was expected to be composed of three globin chains α , β d_{minor} and y^2 , was subjected to sequencing with an Edman & Begg (1967) sequenator for 39 positions from the N-terminus. The semilog plot of corrected amino acid recoveries in nanomoles (Smithies *et al.*, 1971) is shown in Fig. 1. There are, at most, three residues at any position, corresponding to residues of α , β d_{minor} and y^2 chains. Since the α -chain sequence is known (Popp, 1972), as well as the sequence of the β d_{minor} chain in that region (Gilman, 1972, 1973), the sequence of the y^2 chain could be deduced. The corresponding interpretive sequences of α , β d_{minor} and y^2 chains are shown beneath the semilog plot. The sum of the three interpretive sequences is consistent with the data of the plot.

One should note that at position 25 both glycine and valine are attributed to the α chain. The yield of valine is too low to be wholly due to the y^2 chain, and, in some other strains of mice, Hilse & Popp (1968) have shown that there are at least two α chains, a major one with glycine at 25 and a minor one with

EXPLANATION OF PLATE I

Urea/starch-gel electrophoresis (pH 5.6) of embryonic haemolysates from several mouse strains

Adult α -major globin was also included for comparison. Experimental details are as described in the text. Slot 1, adult globin from the α -major haemoglobin component [purified from haemolysate of Hbb^d/Hbb^d mice, as described by Gilman (1973)]. Slot 2, 13-day embryonic globin. The blood sample was pooled from one litter of embryos resulting from the cross of Au/SsJ (adult β -chain genotype Hbb^p/Hbb^p) by HA/ICR (adult β -chain genotype Hbb^d/Hbb^d). Slot 3, 14-day embryonic globin, from a pooled blood sample from 125 embryos of HA/ICR mice (adult β -chain genotype Hbb^d/Hbb^d). Slot 4, 14-day embryonic globin, from a pooled blood sample from embryos of Sec/ReJ mice (adult β -chain genotype Hbb^p/Hbb^p).

EXPLANATION OF PLATE 2

Determination of adult β -chain genotype and embryonic y -chain genotype for nine embryos from the cross $Hbb^s/Hbb^s \times Hbb^d/Hbb^s$

Experimental details are described in the text for two-dimensional starch-gel electrophoresis. The individual haemolysates from each embryo were first run on starch gel (pH 8.6) without urea (a). The presence of the leading minor band shows that the embryonic sample has the d type of adult haemoglobin, implying that the adult β -chain genotype of that embryo was Hbb^d/Hbb^s ; the absence of that leading minor band implies that the embryo was of adult β -chain genotype Hbb^s/Hbb^s (see the text for elaboration of this point). The band containing embryonic haemoglobin component EII was cut out of the first starch gel and placed in a gel containing 8M-urea and 0.05M-2-mercaptoethanol (pH 4.0). [For this reason EII is not shown in (a).] This urea gel allowed direct determination of y^1 and y^2 embryonic y -chain types. Samples 1, 2, 3, 5, 6 and 7 were from embryos of adult β -chain genotype Hbb^d/Hbb^s , as shown by the presence of the leading minor band in (a); they had both y^1 and y^2 chains, as shown in (b). Samples 4, 8 and 9 were from embryos of adult β -chain genotype Hbb^s/Hbb^s , as shown by the absence of the leading minor band in (a); they had only y^1 chain, as shown in (b).

Fig. 1. Sequenator data for 39 positions from the N-terminus of a mixture of α chain, β dmin and γ^2 embryonic β chains

Corrected semilog plot of amino acid recoveries in nanomoles versus position after automatic sequencing of a mixture of EII embryonic and dminor adult haemoglobins from HA/ICR mice (adult β -chain genotype *Hbb^d/Hbb^d*). Sequenator and data-processing procedures are those of Smithies *et al.* (1971). The results are predominantly from HI hydrolyses of amino acid thiazolinones to amino acids; NaOH/Na₂S₂O₄-hydrolysis data were inserted at positions where they were available, as described in the text. Interpretive sequences, sorting out the data into residues due to α , β dmin and γ^2 chains, are shown beneath the plot; this interpretation was possible because the sequences of α and β dmin chains are known, as discussed in the text. In the semilog plot, residues interpreted as being wholly or in part due to γ^2 chains are circled. At one position in the interpretive sequence, tryptophan (W) is shown in parentheses; it was not positively identified by the data of the semilog plot, and is assigned by homology to β dmin chain, but its presence is consistent with the data of the semilog plot (see the text). The one-letter codes for the amino acids are: A, alanine; B, aspartic acid or asparagine; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; P, proline; R, arginine; T, threonine; V, valine; W, tryptophan; Y, tyrosine; Z, glutamic acid or glutamine. Two additional symbols are used: \$, serine or cysteine; &, serine, cysteine or alanine. About 350 nmol of protein were used for this experiment.

valine at 25 (with additional differences toward the middle of the chain). One should also note that the interpretive sequence of the γ^2 chain shows tryptophan in parentheses at position 15, although only glycine and alanine are found in the semilog plot. This assignment is consistent with the data of the plot, since only HI hydrolysis was performed at this position, and HI hydrolysis converts tryptophan thiazolinone into glycine and alanine (Smithies *et al.*, 1971). Tryptophan is suggested at position 15 by homology with β dmin (Gilman, 1973) and other β chains, and was therefore placed in the interpretive sequence of the γ^2 chain in parentheses.

Tryptic-peptide studies

The sequenator study described above did not use all of the sample of haemoglobin EII mixed with dminor haemoglobin from HA/ICR embryos; the remainder was digested with trypsin. Some clearly embryonic peptides were obtained in pure form after the purification step on cation-exchange resin. Table 1 shows the amino acid compositions of four peptides from the γ^2 chain. Peptide γ^2 T-1 covers positions 1-8, and peptide γ^2 T-3 covers 18-30. Their compositions are consistent with the sequence obtained from the sequenator study (Fig. 1). Peptide γ^2 T-9a is homologous with the corresponding

peptide from the β dmin chain, with at least one difference from it (see Fig. 2). The validity of assigning the name 'y²T-10a' to the remaining peptide depends to some extent on tryptic-peptide data on embryonic y¹ chain.

Embryonic EII haemoglobin containing y¹ chain was isolated from haemolysate of 14-day C57Bl/6J embryos. Since these mice have the *Hbb*^s adult β -chain allele, which does not produce a minor haemoglobin β chain, EII haemoglobin was obtained in pure form uncontaminated with adult components. Table 2 shows the amino acid compositions of six peptides found in relative purity after tryptic digestion and fractionation of peptides on cation-exchange resin. Four of the peptides are equivalent to those obtained from y² chain (Table 1). A fifth is homologous to peptide T-9b of β dmin chain. A sixth, peptide y¹T-10b, has the same composition as the region 88–95 of β dmin chain (see Fig. 2). Since peptide T-10 of β dmin chain covers the region 83–95, an amino acid difference must exist between y¹ and adult β chains at position 87 to cause trypsin to cleave at 87 in y¹ chain but not in β dmin chains.

The peptide that I have called 'y¹T-10a' (with the same composition as 'y²T-10a') has the correct length of five residues to allow it to cover the region 83–87. It is different from the adult β chain at positions 83, 84, 85 and 87, but the differences are plausible: glycine and threonine in the adult β chain replaced by alanine and serine in the y chain; phenylalanine in the adult β chain replaced by leucine in the y chain; serine in the adult β chain replaced by lysine in the y

chain. The assignment of this peptide to the region 83–87 therefore seems justified.

One should note that, for both y¹ and y² chains, peptide T-9a was hydrolysed for 24h or less, yet shows nearly complete recovery of valine and isoleucine (one residue each). This contrasts with

Table 1. *Amino acid compositions of several tryptic peptides of y² chain from HA/ICR mice*

The sample was from the same batch of protein used in the sequenator experiment of Fig. 1, namely a mixture of α , β dmin and y² chains. Several clearly embryonic peptides were purified as described in the text. Hydrolyses were for 24h in constant-boiling HCl at 105°C. Background amino acids below 0.2 residue are not shown. Names were assigned to each peptide by using the rationale given in the text.

Peptide ...	Composition (residues/mol)			
	y ² T-1	y ² T-3	y ² T-9a	y ² T-10a
Lys	1.12		1.00	1.05
Arg		1.00		
Asx	1.15	1.05		
Thr	0.90		1.12	
Ser			1.06	0.87
Glx	2.08	2.87	1.17	
Gly		3.07	1.25	
Ala	1.07	1.19	1.15	2.11
Val	0.90	2.78	0.90	
Ile			1.03	
Leu		1.11	1.12	1.00
Phe	1.00		1.21	

Table 2. *Amino acid compositions of several tryptic peptides of y¹ chain from C57Bl/6J mice*

Hydrolyses were for 16h in constant-boiling HCl at 105°C. Background amino acids below 0.15 residue are not shown. Names were assigned to each peptide by using the rationale given in the text.

Peptide ...	Composition (residues/mol)					
	y ¹ T-1	y ¹ T-3	y ¹ T-9a	y ¹ T-9b	y ¹ T-10a	y ¹ T-10b
Lys	0.89		1.00	??	1.00	1.19
His				??		0.92
Arg		1.00		??		
Asx	1.29	1.09	0.51†	2.05		1.04
Thr	0.97		1.05		0.26†	
Ser			1.08		0.90	1.02
Glx	2.02	2.92	0.84			0.96
Gly		2.98	1.11			0.31†
Ala	1.00	1.07	0.95		1.76	0.40†
Cys						+‡
Val	1.10	2.75	0.90		0.24†	
Ile			1.00			
Leu		1.02	1.26	2.00	1.04	2.00
Phe	1.03		1.00			

* The analyses of lysine, histidine and arginine were accidentally lost for peptide y¹T-9b.

† These amino acids are assumed to be background contamination.

‡ Cysteine was detected in low yield, since no measures were taken to prevent oxidation, so that its presence is indicated but not its quantity.

results on peptide β T-9a from β dmin chain (Gilman, 1973). In β dmin chain, valine and isoleucine are adjacent at positions 67–68, and 24h hydrolysis of peptide β dminT-9a, which covers positions 67 and 68, yields only about 0.5 residue each of valine and isoleucine, because of the low rate of hydrolysis of valine–isoleucine bonds. This difference between corresponding peptides of β dmin and γ chains suggests the possibility that the sequence at 67–68 of the γ chain is not valine–isoleucine.

Discussion

Structural studies

In *Mus musculus musculus*, three alleles are known at the *Hbb* adult β -chain locus, *Hbb^s*, *Hbb^d* and *Hbb^p* (Russell & McFarland, 1974). Hutton *et al.* (1962a, b) showed that the *Hbb^d* allele produces two β chains in unequal amounts, and that the genes coding for the two β chains are closely linked. Gilman (1972, 1973) showed that the two β chains β dmaj and β dmin differ in sequence in at least nine positions. Gilman (1972, 1973) also showed that the *Hbb^p* allele is similar to the *Hbb^d* allele in producing major and minor β chains, β pmaj and β pmin. Although no structural differences were found between β pmaj and β dmaj chains, two amino acid substitutions were found between β pmin and β dmin chains. The *Hbb^s* allele produces only one β chain (Hutton *et al.*, 1962a). Bonaventura & Riggs (1967) and Rifkin (1968) showed that β s chain differs structurally from β dmaj chain, Rifkin (1968) finding a minimum of three amino acid substitutions between them.

Gilman & Smithies (1968) showed that there are variant embryonic γ chains γ^1 and γ^2 , γ^1 being more positively charged at acidic pH. Data of Gilman & Smithies (1968) and of the present paper show that the γ^2 embryonic chain is associated with the *Hbb^d* adult β -chain allele, and the γ^1 embryonic chain is associated with the *Hbb^s* allele. Stern *et al.* (1976) have extended this correlation to many additional strains of mice. The results of the present paper also show that the *Hbb^p* allele, which differs only slightly from the *Hbb^d* allele, is like the *Hbb^d* allele in that it is associated with the γ^2 embryonic β chain. Table 3 illustrates the association between the adult and embryonic β -chain types.

The results of the breeding study reported in the present paper suggest a maximum recombination frequency of 5.4% (at the 95% confidence level) between the *Hbb* adult β -chain locus and the embryonic γ -chain locus. Stern *et al.* (1976) report additional data which, taken together with the data in the present paper, lower the estimate of the maximum recombination frequency to 1.2%. Given the persistent association of adult β -chain and γ -chain alleles in both inbred and random-bred mice, it is conceiv-

able that there may actually be a much lower crossover frequency or that selection may be acting to preserve the observed association.

Sequence and tryptic-peptide composition data on γ^1 and γ^2 chains are summarized in Fig. 2. Steinheider *et al.* (1972) have already reported tryptic-peptide composition data on the γ chain of Balb/c-mice, and these data are also included in Fig. 2. [Stern *et al.* (1976) have shown that Balb/c-mice have the γ^2 chain.] In Fig. 2, the embryonic data are compared with the sequence of β dmin chain reported by Gilman (1972, 1973). Tryptic-peptide composition data on γ chain are arranged to be homologous to the embryonic γ^2 -chain sequence data of Fig. 1 for 39 positions from the N-terminus, and with adult β -chain sequence of β dmin chain (Gilman, 1972, 1973) and β s chain (Popp, 1973) beyond position 39.

Fig. 2 shows that the amino acid substitutions leading to the electrophoretic difference between γ^1 and γ^2 chains do not occur in the 40 residues for which there are comparative data on the two chains. Such a substitution most likely involves a difference of histidine in γ^1 chain versus a neutral residue in γ^2 chain, since γ^1 chain is more positively charged than γ^2 chain at acidic pH, yet, at alkaline pH, the haemoglobin EII that has γ^1 chain has almost the same electrophoretic mobility as the haemoglobin EII containing γ^2 chain, as can be deduced from the two-dimensional starch-gel data of Plate 2.

Comparing sequences of β dmin and γ chains, one sees that they differ at a minimum of 20 positions, at 2, 3, 5, 6, 9, 10, 11, 12, 13, 16, 20, 21, 72, 77, 83, 84, 85, 87, 104 and 135. (The amino acid difference assigned to position 135 could have equally well been assigned to positions 138, 140 or 142.) Sequence data on β dmaj chain (Rifkin 1968; Popp & Bailiff, 1973; Gilman, 1973) show that it and γ chain differ at four

Table 3. Correlation of the known laboratory-mouse adult β -chain 'breeding unit alleles' with the known embryonic γ -chain types

For a summary of the sequence differences among the various adult β chains see Gilman (1972). The term 'breeding unit allele' is from Russell & McFarland (1974).

Adult 'breeding unit allele'	Mouse strains	Major adult β chain	Minor adult β chain	γ chain
<i>Hbb^s</i>	C57Bl/6J Sec/ReJ	β s	None	γ^1
<i>Hbb^d</i>	C3H/He DBA/2J HA/ICR	β dmaj	β dmin	γ^2
<i>Hbb^p</i>	Au/SsJ	β pmaj	β pmin	γ^2

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	
1	βdmin	Val-His-Leu-Thr-Asp-Ala-Glx-Lys-Ser-Ala-Val-Ser-Cys-Leu-Trp-Ala-Lys-Val-Asn-Pro-																			
2	y ² (a)	Val-Asx-Phe-Thr-Ala-Glx-Glx-Lys-Thr-Leu-Ile-Asx-Gly-Leu-***-Syr-Lys-Val-Asx-Val-																			
3	y ² (b)	(Val,Asx,Phe,Thr,Ala,Glx,Glx,Lys/Thr,Leu,Ile,Asx,Gly,Leu,***,Ser,Lys/Val,Asx,Val,																			
4	y ¹	(Val,Asx,Phe,Thr,Ala,Glx,Glx,Lys)																	(Val,Asx,Val,		
	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
1	Asp-Glx-Val-Gly-Gly-Glx-Ala-Leu-Gly-Arg-Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Glx-Arg-Tyr-																				
2	Glx-Glx-Val-Gly-Gly-Glx-Ala-Leu-Gly-Arg-Leu-Leu-Val-Val-Tyr-Pro-Trp-Thr-Glx																				
3	Glx,Glx,Val,Gly,Gly,Glx,Ala,Leu,Gly,Arg/Leu,Leu,Val,Val,Tyr,Pro,***,Thr,Glx,Arg/Tyr,																				
4	Glx,Glx,Val,Gly,Gly,Glx,Ala,Leu,Gly,Arg)																				
	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60	61	62
1	Phe-Asp-Ser-Phe-Gly-Asp-Leu-Ser-Ser-Ala-Ser-Ala-Ile-Met-Gly-Asn-Pro-Lys-Val-Lys-Ala-																				
2																					
3	Phe,Asx,Ser,Phe,Gly,Asx,Leu,Ser,Ser,Ala,Ser,Ala,Ile,Met,Gly,Asx,Pro,Lys/Val,Lys/Ala,																				
4																					
	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83
1	His-Gly-Lys-Lys-Val-Ile-Thr-Ala-Phe-Asn-Glu-Gly-Leu-Lys-Asn-Leu-Asp-Asn-Leu-Lys (Gly,																				
2	(Val,Ile,Thr,Ala,Phe,Ser,Glx,Gly,Leu,Lys)																			(Ala,	
3	His,Gly,Lys/Lys/Val,Ile,Thr,Ala,Phe,Ser,Glx,Gly,Leu,Lys/Lys/Leu,Asx,Asx,Leu,Lys/Ala,																				
4	(Val,Ile,Thr,Ala,Phe,Ser,Glx,Gly,Leu,Lys/ ? Leu,Asx,Asx,Leu, ? /Ala,																				
	84	85	86	87	88	89	90	91	92	93	94	95	96	97	98	99	100	101	102	103	104
1	Thr,Phe,Ala,Ser,Leu,Ser,Glx,Leu,His,Cys,Asx,Lys/Leu,His,Val,Asx,Pro,Glx,Asx,Phe,Arg)																				
2	Ser,Leu,Ala,Lys)																				
3	Ser,Leu,Ala,Lys)																				
4	Ser,Leu,Ala,Lys/Leu,Ser,Glx,Leu,His,Cys,Asx,Lys)																				
	105	106	107	108	109	110	111	112	113	114	115	116	117	118	119	120	121	122	123	124	125
1	Leu-Leu-Gly-Asn-Ala-Ile-Val-Ile-Val-Leu-Gly- ? - ? -Leu-Gly- ? -Asp-Phe (Thr,Pro,Ala,																				
2																					
3																					
4																					
	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	141	142	143	144	145	146
1	Ala,Glx,Ala,Ala,Phe,Glx,Lys/Val,Val,Ala,Gly,Val,Ala,Thr,Ala,Leu,Ala,His,Lys/Tyr,His)																				
2																					
3	Ala,Glx,Ala,Ala,Phe,Glx,Lys/Val,Val,Ser,Gly,Val,Ala,Thr,Ala,Leu,Ala,His,Lys/Tyr,His)																				
4																					

Fig. 2. Comparison of partial sequences of adult β dmin and embryonic y^1 and y^2 chains

The sequence data on β dmin chain are from Gilman (1973). Where it is shown in parentheses, the sequence of β dmin chain was inferred by homology with the sequence of β s chain (Popp, 1973), because only tryptic-peptide composition data were available. The data labelled $y^2(a)$ and y^1 are from the present paper. The data labelled $y^2(b)$ are from Steinheider *et al.* (1972). Where only tryptic-peptide composition data are available on y^1 or y^2 chains, they are shown in parentheses, and the sequence within parentheses has been arranged so as to maximize homology with the sequence of β dmin chain. The data on positions 9-17 of $y^2(b)$ were originally interpreted by Steinheider *et al.* (1972) as belonging in a different region of the polypeptide. I have interpreted that tryptic peptide as being from positions 9-17, because its composition is consistent with the sequence data on y^2 chain (see Fig. 1), if one assumes that tryptophan was present in that peptide but was destroyed by the hydrolysis. G. Steinheider (personal communication) indicated that their method of hydrolysis did indeed destroy tryptophan. Two unusual symbols are used: ***, probably tryptophan, by homology with β dmin chain; Syr, either serine or cysteine. A slash (/) is used to delineate individual tryptic peptides.

additional positions, 58, 73, 76 and 80, where β dmaj chain has alanine, aspartic acid, asparagine and serine respectively. Thus it appears that, of the adult β chains, β dmin is more closely related to γ than is β dmaj, with 20 differences between γ and β dmin and

24 between γ and β dmaj. β s chain also has 24 differences from γ chain, being like γ chain and unlike β dmaj and β dmin chains in having glycine at position 13, and being unlike γ , β dmaj and β dmin chains in having alanine instead of threonine at position 139.

Fig. 3. An evolutionary scheme to account for the patterns of adult β -chain genes seen in various species of mice

It is proposed that basically homologous but unequal crossing-over may be the means by which the different species of mice evolved different numbers of genes for the adult β chain. The present-day β -chain patterns of species *M. musculus*, *M. cervicolor* and *M. caroli* are summarized by Gilman (1974), along with the available sequence information on the β chains of the several species. The evolutionary scheme shown in this Figure is consistent with the available data and is presented as suggestive of how the various β chains might have evolved; no attempt has been made to compute the probability of this scheme relative to other plausible schemes (some of which may not invoke unequal crossing-over). It is proposed in this scheme that homologous but unequal crossing-over between the ancestral γ -chain gene (the 'hat' over the chain indicates that it is ancestral) and the ancestral adult β -chain gene led to the creation of an ancestral polymorphism involving a 'doublet' locus (such as *Hbb^d*) coding for two adult β chains and a 'singlet' locus (such as *Hbb^s*) coding for only one adult β chain. For reasons discussed in the text, it is suggested that this crossover took place between positions 21 and 58. In the line leading to *M. cervicolor*, it is suggested that unequal crossing-over led to the creation of a doublet locus coding for β s and β dmin chains. That crossover is postulated to have taken place somewhere before position 13, to account for the parallel polymorphism in major β chains in both *M. musculus* and *M. cervicolor* that involves a difference at position 13 of glycine [in β s and β cmaj (s-like) chains] versus cysteine [in β dmaj and β cmaj (d-like) chains], as shown by Gilman (1974). In the line leading to *M. caroli*, the unequal crossover is assumed to have taken place between positions 58 and 73, for reasons discussed in the text and in Gilman (1972, 1974).

Evolutionary possibilities

Gilman (1974) described haemoglobins of two species of mice, *Mus cervicolor* and *Mus caroli*, which are evolutionarily somewhat distant from *M. musculus*. DNA hybridization studies have shown that the three species diverged from each other one-third as long ago as mouse diverged from rat (Rice, 1972). In *M. cervicolor*, Gilman (1974) found a polymorphism for the major β chains, which parallels the β s/ β dmaj difference seen in *M. musculus*. However, in *M. cervicolor*, both the β s-like chain and the β dmaj-like chain are produced by doublet loci of the *Hbb^d* type, which produce a minor β chain as well as a major β chain.

The other non-*musculus* species examined, *M. caroli*, has only one β chain, the *N*-terminal region of which resembles β dmin chain (through position 58) and the *C*-terminal region of which (beyond position 72) resembles β dmaj chain. Gilman (1972, 1974) suggested that β caroli may be a Lepore type of β chain, having arisen by means of a homologous but unequal crossover between the genes coding for ancestors of β dmin and β dmaj chains. Gilman (1972, 1974) also pointed out that one could imagine other possibilities not involving unequal crossing-over to account for the evolution of the three chains, β caroli, β dmin and β dmaj.

Homologous but unequal crossing-over offers a means of generating additional genes when two similar closely linked genes are already present (Smithies *et al.*, 1962). The existence of several types of human Lepore β chains suggests that such crossover events are not unusual in the course of evolution. It is thus tempting to speculate that unequal crossing-over gave rise to the cluster of at least three embryonic and adult β -chain genes of *M. musculus*, after some unspecified initial duplication event had produced the first two closely linked genes. Fig. 3 presents one such scheme, which could account for the various situations found in the three species of mice whose β chains have been extensively examined, *M. musculus*, *M. caroli* and *M. cervicolor*. This is meant to be a general scheme suggestive of how the various species could have evolved the different numbers of genes. It is consistent with the sequence data on the β chains of the various mouse species (Gilman, 1973, 1974), but is not necessarily the most probable scheme. Other general patterns of evolution consistent with the data on mouse β chains have also been devised (Gilman, 1973; G. Steinheider, personal communication).

The hypothesis of unequal crossing-over suggests a reason for the greater similarity between y and β dmin chains than between y and β dmaj or β s chains. y chain differs extensively from all of the adult β chains up to position 22; beyond that point, y and β dmin chains are alike at four positions, where both differ

from β dmaj and β s chains (at positions 58, 73, 76 and 80). If the unequal crossover that is postulated in Fig. 3 had taken place between positions 21 and 58, y and β dmin chains would automatically have been alike at position 58 and beyond. (It would have to be assumed that the differences that do exist between β dmin and y chains beyond 58 arose as substitutions in the y chain after the occurrence of the crossover event.)

The hypothesis of Fig. 3, that the ancestral gene for β dmin chain was formed as the result of unequal crossing-over between the ancestral genes for y and β dmaj chains, is thus a parsimonious explanation of the greater sequence similarity between y and β dmin chains than between y and β dmaj or β s chains. It is also an evolutionarily reasonable description of how the duplicate adult β -chain locus *Hbb^d* could have been generated.

Note Added in Proof (Received 2 March 1976)

Steinheider *et al.* (1976) have suggested alternative crossover hypotheses to account for the evolution of mouse embryonic and adult β chains.

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