Proc. Natl. Acad. Sci. USA
Vol. 77, No. 2, pp. 1087–1090, February 1980
Genetics

Linkage of genes for adult α-globin and embryonic α-like globin chains

(hemoglobin H/thalassemia/embryonic hemoglobin/mice)

J. BARRY WHITNEY III* AND ELIZABETH S. RUSSELL

The Jackson Laboratory, Bar Harbor, Maine 04609

Contributed by Elizabeth S. Russell, November 19, 1979

ABSTRACT **In α-thalassemia, the genetic locus for the α chains of adult hemoglobin is not expressed. We have examined the hemoglobins of a number of individual mouse embryos heterozygous for a particular α-thalassemia (*Hba*$^{th\text{-}J}$) and find no decrease in the proportion of hemoglobins containing the α chain as compared to the hemoglobin containing the α-like embryonic globin chain. This result suggests that the locus for this embryonic α-like chain is inactivated or deleted in these embryos as well. Because a single mutational event inactivated adult and embryonic loci, we conclude that they are probably closely linked to one another on the same chromosome. We also present evidence that an unusual hemoglobin in the blood of these embryos is composed only of an embryonic β-like chain, and is thus analogous to the hemoglobin H (β_4 tetramer) of adult α-thalassemics.**

α-Thalassemia is a genetically determined condition involving reduced production of α-globin relative to β-globin chains. A frequent finding, in addition to a reduction in the amount of $\alpha_2\beta_2$ hemoglobin, is the presence of β_4 hemoglobin. Recent restriction endonuclease mapping studies have shown that some, perhaps all, normal humans inherit two closely linked α-globin genes from each parent (1). The severity of anemia differs among human cases, but expression of at least some of these α-globin genes seems to be deficient in all humans with α-thalassemia or α-thalassemia trait. Deletion of α-chain genes appears to account for some human α-thalassemias, but other cases may better be explained as due to the presence of defective α-globin alleles or to misarrangement of α-globin genes (2).

Structural studies of mouse hemoglobins have shown that homozygous normal mice from many inbred strains produce two nonidentical α-globins, implying that the mouse α-globin locus is also duplicated (3). Results of genetic crosses indicate that the two loci are extremely closely linked, acting as an *Hba* breeding unit, or haplotype. Homozygous normal mice from other inbred strains produce only one kind of α-globin (3), and it is not known whether chromosome 11 of these mice contains one α-globin locus or more. In all, five different kinds of α-globin chains have been distinguished by isoelectric focusing (4). Three different "singlet" *Hba* haplotypes have been described, each being responsible for the presence of one kind of α-globin only. For example, C57BL/6 mice (*Hba*^{a}) produce only the α-globin chain known as "chain 1." In addition, four "doublet" *Hba* haplotypes have been described, each of which leads to synthesis of a different combination of two distinct α-globin chains (4).

One α-thalassemia heterozygote was discovered at The Jackson Laboratory among the progeny of a normal C57BL/6J (*Hba*^{a}) female mouse and a triethylene-melamine-treated male that carried a different, doublet, *Hba* haplotype (5). In this affected α-thalassemic offspring of the treated male, only the *Hba*^{a} haplotype inherited from the C57BL/6J mother was expressed. The hemoglobins of this mouse were found by electrophoresis after cystamine treatment (6) to be unusual in that they contained a lower than normal proportion of the hemoglobin containing the diffuse-major β chain, a condition also reported for the x-ray-induced mouse α-thalassemia discovered at the Oak Ridge National Laboratory (7). Because no littermate of this male was abnormal, it seems reasonable to presume that the α-thalassemia and all associated abnormalities are the result of a single mutational event.

Embryonic hemoglobins in the mouse (8) are found in nucleated erythrocytes of yolk sac origin (9). Beginning around day 12 of gestation, these large cells containing the three embryonic hemoglobins are joined in the circulation by smaller, nonnucleated cells of fetal liver origin, which contain adult-type hemoglobin (9–12). Mice apparently have no true fetal hemoglobin (13). By chromatography (11) and electrophoresis (14), the globin compositions of the embryonic hemoglobins have been found to be: EI, x- and y-globins; EII, α- and y-globins; and EIII, α- and z-globins. Because they combine with α chains, both y and z chains are presumed to be β-like. Because the x-globin combines with the β-like y, it must be α-like. Some sequence data (15) and other structural data (16) support these assumptions. Further, it has been established by direct linkage studies (14, 15) and strain distribution analysis (17) that the locus for the β-like y chain (*Hby*) is intimately associated with the adult β-chain locus (*Hbb*) on mouse chromosome 7.

The lowering of the number of active adult-α genes in an α-thalassemia heterozygote to half (those inherited from the untreated parent) provides a unique opportunity to study the nature of the mutation itself and thereby to better understand the structure and workings of the genetic region in which it arose. We have reasoned that if the locus for the α-like x embryonic globin chain (*Hbx*) was *not* inactivated when The Jackson Laboratory α-thalassemia was induced, then the amount of the EI (x_2y_2) embryonic hemoglobin, which contains the x chain, should be normal in α-thalassemia heterozygous fetuses, whereas the amounts of the adult ($\alpha_2\beta_2$), EII (α_2y_2), and EIII (α_2z_2) bands containing the α chain should be reduced. This would cause an apparent increase in the proportion of EI relative to the other hemoglobins in heterozygous thalassemia embryos, when compared to normal controls. Conversely, simultaneous inactivation or deletion of the adult α-chain locus (*Hba*) and the *Hbx* locus (no proportional increase in EI) would support the concept that they were closely linked to one another

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* Present address: Laboratory of Molecular Hematology, National Heart, Lung, and Blood Institute, Building 10, Room 7D-18, National Institutes of Health, Bethesda, MD 20205.

on chromosome 11. The results we present support the second alternative.

METHODS

Mice. C57BL/6J mice (*Hba*^{a}, *Hbb*^{s}) were bred from C57BL/6J mice supplied from The Jackson Laboratory Foundation Stocks or obtained from the Animal Resources Department of The Jackson Laboratory. Our original α-thalassemic male No. 15975 was the offspring of an untreated C57BL/6J female and a triethylenemelamine-injected male of a stock (PosA) derived from the mating of *Mus musculus poschiavinus* and "Swiss" mice (5); this stock carried *Hbb*^{d} and probably *Hba*^{c} (4). The mutation induced in male 15975 has been designated *Hba*$^{th\text{-}J}$. Three or more additional backcrosses to the C57BL/6J strain produced the α-thalassemic fathers of the embryos used for this study. These embryos, normal +/+ and heterozygous *Hba*$^{th\text{-}J}$/+, were collected from C57BL/6J females 14 or 14½ days after the appearance of a mating plug.

Isolation of Embryonic Hemoglobins. Individual embryos were allowed to bleed into small petri dishes containing 1–2 ml of 0.9% NaCl/1% heparin. The blood cells were pelleted with a Clay-Adams Serofuge and resuspended in a small volume of 0.9% NaCl. The suspension was drawn into nonheparinized 20-μl Drummond Microcaps, which were sealed and then centrifuged inside larger tubes for 5 min in a hematocrit centrifuge. Erythrocyte columns were lysed in CO-saturated 5 mM ammonium acetate at 150 μl/11 μl of packed cells. Electrophoresis on Mylar-backed cellulose acetate strips was performed as described (13) for 30 min or longer. In some cases, Ponceau S-stained strips were cleared and scanned in a Helena Laboratories Auto Scanner recording densitometer.

Globin Electrophoresis. For second-dimensional analysis of the subunits of hemoglobins separated in the first dimension by cellulose acetate electrophoresis, the region containing each hemoglobin band of interest was removed and mixed with noncystamine/8 M urea globin gel running buffer (12). The EIII material whose second dimension analysis is shown in Fig. 2 came from the pooled EIII bands in first dimension preparations from three normal 14-day embryos, as did each of the EII, EI, and adult materials. In Fig. 3, the EII material is the same as in Fig. 2, and the abnormal band was pooled from first-dimensional preparations from two heterozygous α-thalassemic littermates. After 1 hr at room temperature each sample was mixed with 0.5 vol of 0.2 ml acetic acid: 1.0 ml of deionized 40% sucrose in 8 M urea. Globin electrophoresis on polyacrylamide disc gels (without cystamine, for 18 hr), staining with amido black, and gel scanning were as described (12).

RESULTS

Fig. 1 depicts the electrophoretic pattern of the hemoglobins from 14½-day normal embryos (samples b and e) and α-thalassemic littermates (samples c, d, and f). In the normal embryo samples, migration of adult-type hemoglobin is fastest, followed in order by EI, EII, and EIII (14). An abnormal hemoglobin band in preparations from the thalassemic embryos, which migrates slightly slower than EII, is indicated by an arrow.

In spectrophotometer scans (not shown) of normal and heterozygous thalassemic mouse hemoglobin patterns similar to those shown in Fig. 1, it is clear that the unusual band is the most intense hemoglobin band in preparations from 14-day thalassemic embryos (the adult band becomes the major band soon thereafter). Further, the proportion of EI hemoglobin, which contains the x chain, is not markedly increased in the thalassemics relative to the other hemoglobins (adult, EII, and EIII), which contain the α chain.

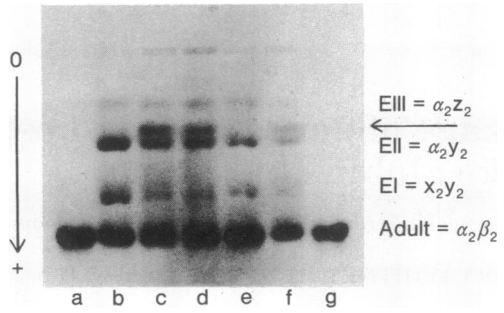

FIG. 1. Electrophoresis of the hemoglobins of C57BL/6J mice. Channels a and g show samples from normal adult mice; channels b and e, from normal 14½-day embryos; and channels c, d, and f, from heterozygous α-thalassemic embryos (*Hba*$^{th\text{-}J}$/+). An arrow indicates the abnormal band of the thalassemic embryos. O, origin. These animals are all homozygous "single" (*Hbb*^{s}), but the abnormal band of thalassemic embryos can also be observed in samples from "diffuse" (*Hbb*^{d}) mice. In diffuse embryos, the D-minor adult-type hemoglobin lies between the embryonic hemoglobins EI and EII under these conditions.

It should be noted that no additional phenotypes were observed among embryonic offspring of matings between heterozygous *Hba*$^{th\text{-}J}$/+ parents and that the number of all 11- to 14-day offspring was abnormally small. The clear implication is that homozygous severely affected *Hba*$^{th\text{-}J}$/*Hba*$^{th\text{-}J}$ embryos were not present.

Fig. 2 shows the globin compositions of the normal adult, EI, EII, and EIII hemoglobin bands. Note that baselines are offset on the vertical axis for ease of interpretation. As previously reported (11, 14), the adult hemoglobin contains α and β globins, EI is composed of x and y, EII has α and y, and EIII contains α and z. Although the proportions of the α-like and β-like chains must have been equal in the hemoglobins, the α and α-like bands are stained more intensely than are the β and β-like globins in each of these preparations of normal hemoglobins.

Fig. 3 compares the globin profile of the normal EII, also

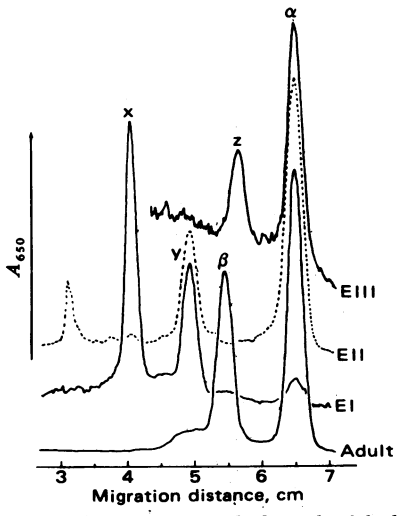

FIG. 2. Globin analysis on acrylamide disc gels of the hemoglobins of normal 14-day C57BL/6J embryos. The individual hemoglobins were prepared by cellulose acetate electrophoresis, as shown in Fig. 1, then subjected to electrophoresis in 8 M urea. Horizontal axes are offset upward for clearer representation. The vertical scale has been expanded as necessary to give an approximately equal deflection to the highest peak of each sample. Individual hemoglobins and globin peaks are identified. The leftmost, unlabeled, peak in the EII sample may be carbonic anhydrase.

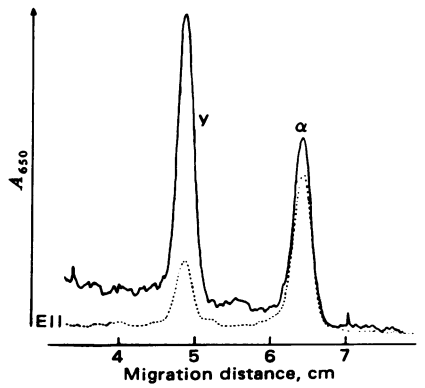

FIG. 3. Electrophoretic analysis of the globin of the abnormal hemoglobin of heterozygous α-thalassemic embryos (—). All of the α-globin and some of the y-globin in this sample probably comes from the EII hemoglobin that contaminated this preparation (see Fig. 1). Globins from the EII band of normal 14-day C57BL/6J littermate embryos gave the profile shown by - - -.

shown in Fig. 2, to the pattern of the globin from the abnormal hemoglobin band of the α-thalassemic embryos. Because of the incomplete resolution of this band from EII in the first dimension (Fig. 1), we anticipated some contamination of this abnormal hemoglobin with EII, the globin of which serves as a useful marker. The α chain found in this preparation presumably comes entirely from the EII contamination. A small part of the y-globin in this sample must therefore also come from this EII. Nonetheless, the great majority of the y-globin in this sample must be derived from the abnormal thalassemic band. Further, no other globin band is evident.

DISCUSSION

The evidence that is available (8) has supported the arrangement of all of the genes for the β and β-like chains of mammalian hemoglobins in a tightly linked group on one chromosome, and the genes for the adult α chains in another cluster, unlinked to the β cluster. In humans, the β cluster includes the genes for the adult β chain of hemoglobin A, and δ chain of hemoglobin A_2, and the γ chains of the fetal hemoglobins (18). This cluster has recently been shown to lie on the short arm of human chromosome 11 (19). Direct evidence has not yet been published to suggest that the locus for the human ε chain, a β-like embryonic chain (20), is a part of this cluster. In mice, however, direct linkage studies have shown that the gene for one β-like embryonic globin is in fact on the same chromosome as and linked to the adult β-chain locus (*Hbb*), on mouse chromosome 7 (14, 15). Because an extensive study of inbred strains of mice found one allele for the embryonic y globin (Hby^1) always associated with the Hbb^s "allele," or haplotype, and the alternative Hby^2 always associated with the Hbb^d haplotype, the linkage is inferred to be tight (17).

It has been presumed that the α-like embryonic globins are encoded by loci closely linked to the loci for the adult α-globin genes. However, because no genetic variant of embryonic α-like globin chains has been described in any species, despite an extensive search by electrophoresis for EI variants of the mouse (17), no direct evidence has supported this assumption. The evidence presented here strongly suggests that the assumption is correct. Because the α-thalassemia mutation we describe arose as a single step, it seems reasonable to attribute all of the three gene inactivations to the same primary cause, especially after the mutation has been maintained intact when transferred by several additional generations of backcrossing to normal C57BL/6J mice. That single mutational event may indeed have been a deletion of all three of these globin genes, or, alternatively, an intolerable alteration in some genetic region critical to the expression of each of them.

Fig. 1 shows that some fetuses fathered by heterozygous α-thalassemic males ($Hba^{th\text{-}J}/+$) have a major hemoglobin component never seen in normal fetuses. These fetuses make up about half of the total and must be the α-thalassemia heterozygotes. It is clear that their proportion of EI hemoglobin is not markedly increased, as it would be if the *Hbx* locus for the embryonic α-like globin were fully active while only half of the usual number of adult α genes were active. Fig. 3 shows that the abnormal band near EII in preparations from heterozygous thalassemic embryos contains much y (β-like) globin. (Some contamination with EII hemoglobin could not be avoided.) We conclude that the new abnormal hemoglobin band is a tetramer of y chains comparable to the human hemoglobin H (β_4 tetramer) of adults and the hemoglobin Bart's (γ_4) of fetuses and neonates with relatively severe α-thalassemia.

The absence of recognizable $Hba^{th\text{-}J}/Hba^{th\text{-}J}$ embryos among 11- to 14-day offspring of crosses between heterozygous $Hba^{th\text{-}J}/+$ parents suggests that homozygous affected embryos die prior to the 11th day of development. Deletion or inactivation of the embryonic x-chain gene would almost certainly cause an early lethality by preventing the synthesis of x_2y_2, the earliest to be formed of the three mouse embryonic hemoglobins.

Heterogeneity among human α-thalassemias has been described: in some cases of α-thalassemia the globin genes are actually deleted whereas in other affected individuals the genes are present though inactive (2). In Asian adult-gene-deletion cases, the presumed homozygous individual, severely affected, is observed as an infant with hydrops fetalis. This class is rarely seen in non-Asian pedigrees of α-thalassemia (2). One possible explanation of this difference between east and west would be that, in at least some non-Asian populations, the α-thalassemia mutation involves "deletion of essential DNA sequences, possibly embryonic α-like genes," (2) which would lead to early embryonic lethality. Our finding no homozygous affected mouse embryos when the embryonic x-like globin locus is inactivated certainly supports this possibility.

Although the hemoglobins of different inbred strains of mice have been studied extensively (4, 8, 17), no spontaneously occurring abnormalities of mouse hemoglobin structure or expression have been described. In an attempt to discover a hereditary mouse hemoglobinopathy, we examined the hemoglobins of approximately 500 offspring of mutagen-treated males; we recovered only the single thalassemia we describe here. Two other heritable x-ray-induced α-thalassemias of the mouse have been described at the Oak Ridge National Laboratory (21). After measuring incorporation of radiolabeled amino acids into EI and EII hemoglobin tetramers, Popp *et al.* (22) had concluded that the *Hbx* locus was still active in the Oak Ridge 352HB mutant, although the adult-α loci were totally inactivated (21). However, application of our cellulose acetate electrophoretic method to the embryonic hemoglobins of Oak Ridge thalassemia heterozygotes (23) gives results like those shown in Fig. 1. It thus seems that the *Hbx* loci of the Oak Ridge mutants are inactivated, the original incorrect conclusion (22) having been based upon the failure of their method to resolve the EIII hemoglobin from the abnormal y_4 band (23). Parallel analysis of the α-globin gene DNAs of the three mutants should provide a better understanding of the genetic bases of their nonexpression of α and α-like globin genes.

Two observations suggest that, if $Hba^{th\text{-}J}$ is a deletion, it must be small. First, cytogenetic studies (M. T. Davisson, personal

communication) have not detected any chromosomal abnormality associated with the α-thalassemia. Secondly, the recombination rate between flanking chromosome 11 markers *wa-2* and *sh-2* in α-thalassemia heterozygotes is quite normal in the limited number of potential recombinants we have studied thus far and establishes conclusively that the mutation we call *Hba*$^{th\text{-}J}$ actually resides at or near the *Hba* locus complex on mouse chromosome 11.

We are grateful to T. H. Roderick and N. Hawes for providing us with the offspring of mutagen-treated males. We thank R. A. Popp for making information on the Oak Ridge thalassemic embryos (22) available to us before publication. We thank Dorothy Chapman for able technical assistance. This investigation was supported by Research Grants CA-01074 from the National Institutes of Health, VC58 from the American Cancer Society, and 3264 from the Department of Energy. The Jackson Laboratory is fully accredited by the American Association for Accreditation of Laboratory Animal Care.

1. Orkin, S. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5950–5954.
2. Orkin, S., Old, J., Lazarus, H., Altay, C., Gurgey, A., Weatherall, D. J. & Nathan, D. G. (1979) *Cell* **17,** 33–42.
3. Hilse, K. & Popp, R. A. (1968) *Proc. Natl. Acad. Sci. USA* **61,** 930–936.
4. Whitney, J. B., III, Copland, G. T., Skow, L. C. & Russell, E. S. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 867–871.
5. Whitney, J. B., III & Russell, E. S. (1978) *Mouse News Letter* **58,** 47–48.
6. Whitney, J. B., III (1978) *Biochem. Genet.* **16,** 667–672.
7. Popp, R. A., Stratton, L. P., Hawley, D. K., Tierney, E. C. & Hirsch, G. P. (1976) *Genetics* **83,** s58–s59.
8. Russell, E. S. & McFarland, E. C. (1974) *Ann. N.Y. Acad. Sci.* **241,** 25–38.
9. Craig, M. L. & Russell, E. S. (1964) *Dev. Biol.* **10,** 191–201.
10. Barker, J. E. (1968) *Dev. Biol.* **18,** 14–29.
11. Fantoni, A., Bank, A. & Marks, P. A. (1967) *Science* **157,** 1327–1329.
12. Whitney, J. B., III (1977) *Cell* **12,** 863–871.
13. Whitney, J. B., III, McFarland, E. C. & Russell, E. S. (1978) *Dev. Biol.* **65,** 233–237.
14. Gilman, J. G. & Smithies, O. (1968) *Science* **160,** 885–886.
15. Gilman, J. G. (1976) *Biochem. J.* **155,** 231–241.
16. Steinheider, G., Melderis, H. & Ostertag, W. (1975) *Nature (London)* **257,** 712–715.
17. Stern, R. H., Russell, E. S. & Taylor, B. A. (1976) *Biochem. Genet.* **14,** 373–381.
18. Fritsch, E. F., Lawn, R. M. & Maniatis, T. (1979) *Nature (London)* **279,** 598–603.
19. Jeffreys, A. J., Craig, I. W. & Francke, U. (1979) *Nature (London)* **281,** 606–608.
20. Huehns, E. R., Dance, N., Beaven, G. H., Keil, J. V., Hecht, F. & Motulsky, A. G. (1964) *Nature (London)* **201,** 1095–1097.
21. Popp, R. A., Stratton, L. P., Hawley, D. K. & Effron, K. (1979) *J. Mol. Biol.* **127,** 141–148.
22. Popp, R. A., Bradshaw, B. S. & Hirsch, G. P. (1979) in *Cellular and Molecular Regulation of Hemoglobin Switching,* eds. Stamatoyannopoulos, G. & Nienhuis, A. W. (Grune & Stratton, New York), pp. 227–235.
23. Popp, R. A., Skow, L. C. & Whitney, J. B., III (1980) *Ann. N.Y. Acad. Sci.,* in press.