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Restriction mapping of a new deletion responsible for $^{G}\gamma(\delta\beta)^{o}$ thalassaemia

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ABSTRACT

DNA from individuals heterozygous for $^{G}\gamma(\delta\beta)^{o}$ thalassaemia has been studied by restriction endonuclease analysis. The results reveal a new molecular defect associated with this condition. A total of three defects is now responsible for the one single phenotype, thereby emphasising the complex relationship between genotype and phenotype among the disorders of β-like globin synthesis in man.

INTRODUCTION

Restriction endonuclease studies have demonstrated a number of deletions responsible for disorders of β globin synthesis in man, many of which are associated with varying degrees of persistent γ globin synthesis in adult life. It is possible that further characterisation of these deletions, by adding to the sum total, may provide additional insight into the regulation of γ and β globin synthesis. It is now apparent that similar phenotypes may be produced by a variety of different sized deletions. For example, hereditary persistence of fetal haemoglobin (HPFH), in which there is synthesis of both fetal globins ($^{G}\gamma$ and $^{A}\gamma$) in adults, can result from at least two different deletions which involve the β and δ globin genes and variable amounts of DNA from between the $^{A}\gamma$ and δ globin genes.[1,2,3] $^{G}\gamma(\delta\beta)^{o}$ thalassaemia, which is characterised by the synthesis of only $^{G}\gamma$ globin in adults, is due to two very different rearrangements, which involve deletion of all or parts of the $^{A}\gamma$, δ and β globin genes, while maintaining the same phenotype.[1,4,5,6] We describe here a third type of deletion as the basis for $^{G}\gamma(\delta\beta)^{o}$ thalassaemia in two families of Chinese origin.

MATERIALS AND METHODS

Sources of DNA

DNA from members of two families was studied. Family H contained individuals heterozygous for either $^{G}\gamma(\delta\beta)^{o}$ thalassaemia or $(\beta)^{o}$ thalassaemia, and one individual (AH) heterozygous for both these conditions.[7] The second family, C, contains heterozygotes for $^{G}\gamma(\delta\beta)^{o}$ thalassaemia whose haematological features are virtually identical to those of members of family H heterozygous for the same condition (R. Trent, unpublished observations).

Restriction endonuclease mapping

DNA was prepared from spleen (AH and normal DNA) or from peripheral blood by phenol chloroform extraction.[8] DNA samples were digested with restriction endonuclease and fractionated in 0.8% agarose gel by electrophoresis for 15 hr. at 1.5 V cm^{-1}. DNA fragments were transferred onto nitrocellulose filters[9] and hybridised in the presence of 10% Dextran sulphate[10] to DNA probes labelled with ^{32}P by nick translation.[11] After hybridisation for 15 hr., filters were washed in 0.1 x SSC at 65°C[10] and the bands detected by autoradiography.[12]

The sizes of DNA fragments were calculated from the sizes of fragments in digests of normal DNA.[1,13]

The γ cDNA probe was the γ globin cDNA containing plasmid JW151.[14] Pst δ and Pst β subclones were obtained from T. Maniatis. Plasmid pε1.3 was obtained from F. Baralle. The Sac 3.8, Bam 5.1, Eco 2.3 amd Bgl 4.2 probes were prepared by digestion of either HγG4 or HγG5 lambda clones with Eco Rl, Bam Hl, Bgl II or Sac I, electrophoresis of the digested DNA in low melting point agarose, and elution of the appropriate fragments from the gel by electrophoresis.

Solution hybridisation

A direct comparison of the number of β genes present in normal and AH DNA was made by hybridisation of α or β cDNA to normal or AH DNA with cDNA excess. The preparation of α and β cDNA, and conditions of hybridisation have been described elsewhere.[15]

RESULTS

Deletion of part of the $^{A}\gamma$ globin gene

Various single restriction enzyme digests of DNA from AH and LC, individuals heterozygous for $^{G}\gamma(\delta\beta)^{o}$ thalassaemia, were hybridised to probes containing sequences complementary to a number of regions in the γδβ globin gene complex (Table 1). Among all these, abnormal fragments were detected only in digests with Eco Rl, Hind III and Sac I hybridised to probes containing γ gene sequences (Table 1, Fig. 1). As expected for DNA heterozygotes, DNA fragments of normal size were also present in all the digests. Digest of DNA from members of families C (not shown) and H (Fig. 2) showed that the abnormal fragments were associated exclusively with the $^{G}\gamma(\delta\beta)^{o}$ thalassaemia phenotype.

The absence of new DNA fragments in digests with Pst I, Xba I and Bam HI is best explained by proposing a deletion in

TABLE 1: **Restriction fragments identified in the region of the γ, δ and β globin genes in $^{G}\gamma(\delta\beta)^{o}$ thalassaemia.**

	Sac 3.8	Eco 2.3	Bgl 4.2	δPst + βPst
Bam HI	2.6, 5.1, 15.5		15.5	15.5, 4.4, 1.8, 8.3
Bcl I	18.0	18.0		
Bgl II	13.0	13.0		7.7, 5.8
Eco RI	7.2, 2.7, 3.0*, 1.6	2.3	7.2	2.2, 1.7, 5.2, 3.6
Hind III	8.0, 3.5, 3.2*		18.0	18.0, 7.5
Hpa I	24, 5.1, 14			
Pst I	4.0, 5.1, 0.9	2.7	4.0 4.8	
Sac I	2.7, 3.8, 6.0*	1.1	6.0	
Xba I	3.7, 5.1, 7.5			

DNA from members of families H or C, heterozygous for $^{G}\gamma(\delta\beta)^{o}$ thalassaemia, was digested with the restriction endonuclease indicated. The sizes of DNA fragments identified in blot-hybridisation with the Sac 3.8 probe and a mixture of the δPst and βPst probes (families H and C) and with the Eco 2.3 and Bgl 4.2 probes (family H) are shown in kilobases. Abnormal fragments associated with the $^{G}\gamma(\delta\beta)^{o}$ phenotype are marked by an asterisk.

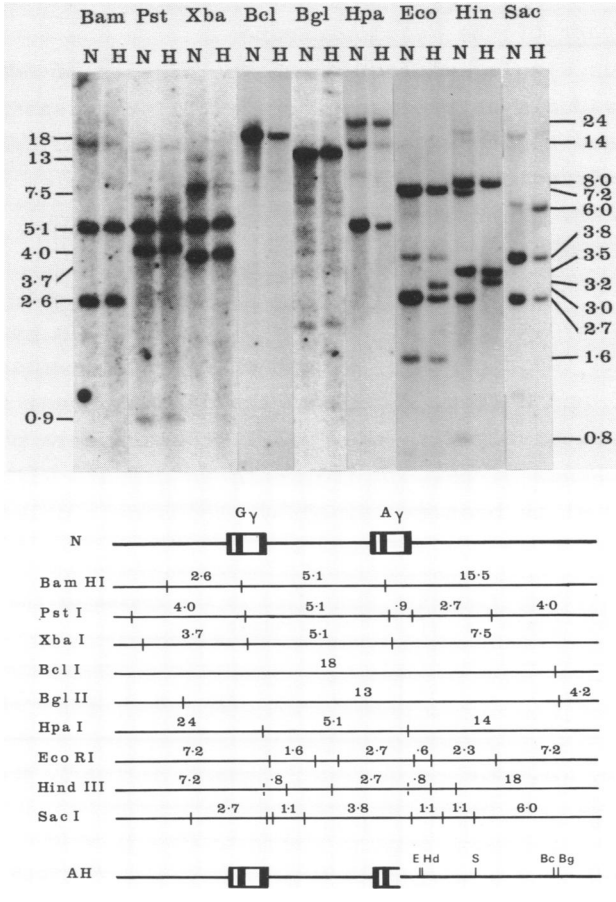

Figure 1

Single enzyme digests of normal (N) and AH (H) DNA were digested with Bam Hl (Bam), Pst I (Pst), Xba I (Xba), Bcl I (Bcl), Bgl II (Bgl), Hpa I (Hpa), Eco RI (Eco), Hind III (Hin), and Sac I (Sac), and hybridised to the Sac 3.8 probe which contains sequences complementary to the G_γ and A_γ genes. The sizes of DNA fragments, in kilobases, is shown. Below the autoradiograph are shown normal fragments detected with the Sac 3.8 probe as shown above and some of those detected by the Eco 2.3 and Bgl 4.2 probes as listed in table 1. New restriction sites identified in AH DNA are shown below this (E, Eco Rl; Hd, Hind III; S, Sac I; Bc, Bcl I; Bg, Bgl II).

The Hind III digest of the normal DNA shows fragments due to the presence of each of the two Hind III polymorphic sites in the γ genes in addition to fragments lacking these sites. There is no evidence for the presence of these polymorphic sites in AH DNA.

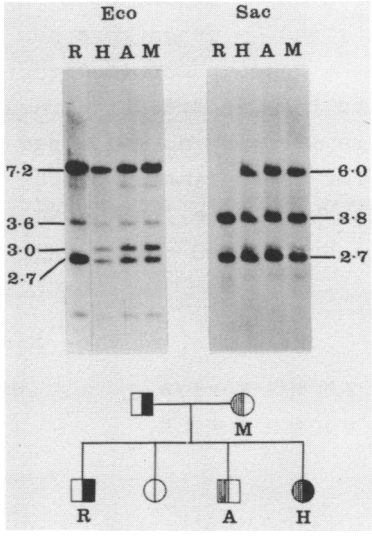

Figure 2
DNA from members of family H, including AH (H), were digested with either Eco Rl (Eco) or Sac I (Sac) and hybridised to the Sac 3.8 probe. The sizes of DNA fragments, in kilobases, is shown. The 3.6 kb fragment is due to cross-hybridisation to the Eco Rl fragment of this size which contains the ε gene.
Also shown are the near relatives of AH, with their associated phenotypes: hatched - $^{G}\gamma(\delta\beta)^{o}$ thalassaemia; solid - β^{o} thalassaemia.[7]

the affected chromosome which removed the Hind III, Eco Rl and Sac I sites at the 3' end of the $^{A}\gamma$ gene, while leaving intact the Bam Hl, Xba I and Pst I sites at its 5' end (Fig. 1). The latter enzymes would generate abnormal fragments extending downstream from the $^{A}\gamma$ gene, but there would be insufficient sequence complementary to the γ probes for these to be detected. The same argument cannot be applied to a deletion downstream from the equivalent point in the $^{G}\gamma$ gene since this would require new Eco Rl and Hind III fragments larger than those observed. In this model the 5' ends of the 3.2 kb Hind III and 3.0 kb Eco Rl fragments should be in normal positions and so when cut by Bam Hl and Xba I, fragments of normal size should result. Double digests with these enzymes demonstrated only the normal pattern (not shown) supporting the model.

Similarly the new 6.0 kb Sac fragment must extend from a normal site between the $^{G}\gamma$ and $^{A}\gamma$ genes to a new site 3' to the remaining part of the $^{A}\gamma$ gene. Double digests with Sac I and Xba I hybridised to the Sac 3.8 probe resulted in the 6.0 kb Sac I fragment being replaced by normal sized fragments, providing further evidence for the retention of the 5' end of the $^{A}\gamma$ gene together with its Xba 1 site, in AH DNA (Fig. 3). However, in Sac I/Hpa I digests hybridised to the same probe the 6.0 kb

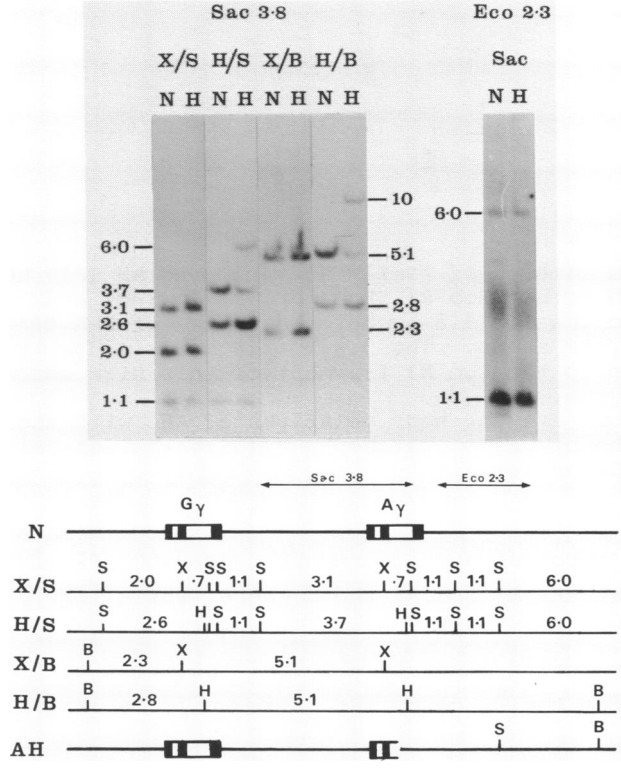

Figure 3
Double digests of normal (N) and AH (H) DNA with Bgl II (B), Hpa I (H), Sac I (S), and Xba I (X) were hybridised to the Sac 3.8 probe. Single enzyme digests of normal (N) and AH (H) DNA with Sac I (Sac) were hybridised to the Eco 2.3 probe. The extent of normal sequences contained in these probes is shown below the autoradiograph. Also shown is the distribution of normal restriction sites in the region of the γ genes, and the abnormal Sac I and Bgl II sites identified in AH DNA.

Sac I fragment persisted demonstrating the loss of the Hpa I site at the 3' end of the $^{A}\gamma$ gene (Fig. 3). Loss of the Hpa I site in the $^{A}\gamma$ gene is also supported by the result of Hpa 1/ Bgl II double digests hybridised to the Sac 3.8 probe (Fig. 3). In addition to the normal bands, a new fragment of 10 kb was generated. This suggests that the abnormal chromosome in AH DNA contains a 13 kb Bgl II fragment, the same size as the normal Bgl II fragment, but lacking the Hpa I site in the $^{A}\gamma$ gene, and that it is therefore cut only once at the Hpa I site in the $^{G}\gamma$ gene to produce 2.8 kb and 10 kb fragments. In Xba I/Bgl II digests hybridised to the same probe, only fragments of normal size were seen, which is again consistent with retention of the 5' end of the $^{A}\gamma$ gene in the abnormal 13 kb Bgl II fragment (Fig. 3).

Loss of the Hpa I site in the $^{A}\gamma$ gene should have generated a new fragment in digests of AH DNA with Hpa I alone, but no new band was seen (Fig. 1). This apparent contradiction can be reconciled since the intensities of the normal 5.1 and 14 kb fragments are reduced relative to the intensity of the 24 kb fragment in Hpa I digests of AH DNA hybridised to the γ probe. The most probable explanation of this is that the loss of the Hpa I site in the $^{A}\gamma$ globin gene in AH DNA leads to the loss of the 5.1 and 14 kb Hpa I fragments in the affected chromosome, and these are replaced by a new fragment which is 24 kb in length and masked by the normal fragment of this size. Hybridisation of Hpa I/Kpn I double digests to γ(Bam Hl), ε(pE 1.3) and δ Pst probes demonstrated an abnormal 24 kb fragment in the digest of AH DNA hybridised to the γ probe, but not in the digests hybridised to the other two probes (Fig. 4). This DNA fragment is therefore distinct from the normal 24 kb Hpa I fragment which lies upstream from the $^{G}\gamma$ globin gene, and must instead run downstream from the Hpa I site in the $^{G}\gamma$ globin gene to a new Hpa I site on the 3' side of the $^{A}\gamma$ globin gene.

The extent of the deletion

The absence of new fragments in Bgl II and Bcl I digests hybridised to the γ probe (Fig. 1) was surprising, since any extensive deletion in the region of the two γ genes would be expected to give new fragments in digests with these enzymes.

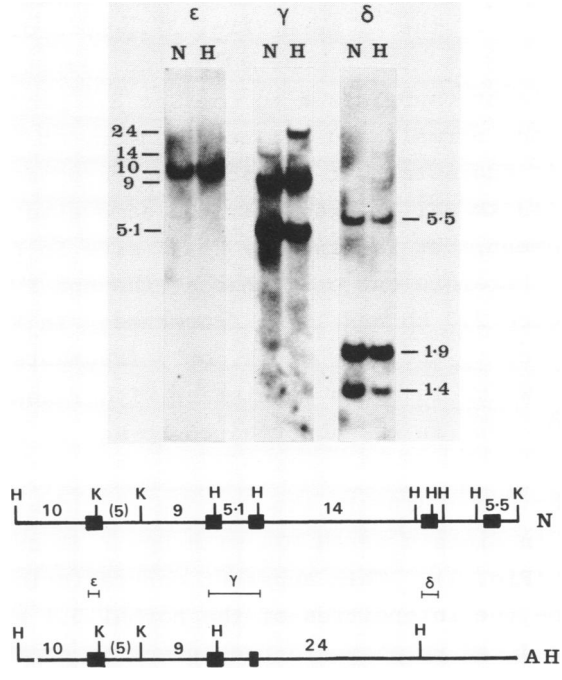

Figure 4
Double digests of normal (N) and AH (H) DNA with Hpa I and Kpn I were hybridised to the pε 1.3 (ε), the Bam 5.1 (γ), and Pst δ (δ) probes. The extent of normal sequences contained in these probes is shown beside the autoradiographs. Also shown is the distribution of Hpa I (H) and Kpn I (K) sites in normal and AH DNA.

It is possible that this is due either to a small deletion removing the Hpa I, Eco Rl, Hind III and Sac I sites at the 3' end of the $^{A}\gamma$ gene, or to a more extensive rearrangement within the Bgl II and Bcl I fragments containing this region which leaves the size of these latter fragments unaltered. A small deletion would mean that the 3' end of the abnormal 6 kb Sac I fragment should hybridise to the Eco 2.3 probe, but this DNA fragment was not detected by this probe (Fig. 3). A more extensive rearrangement within the Bgl II and Bcl 1 fragments containing the γ genes, but leaving a normal DNA sequence downstream, should have generated a 19 kb Hpa I fragment, but instead a new 24 kb Hpa I fragment is generated which

does not conform to the normal arrangement of Hpa I sites in the region of the δ and β genes, and which does not contain sequences complementary to the δ Pst probe (Fig. 4). Similarly, the absence of abnormal fragments in a number of digests hybridised to the Bgl 4.2 and δ Pst probes supports the loss of the latter sequences from the abnormal chromosome (Table 1).

Absence of β and δ globin genes from the affected chromosome

The most likely interpretation of the data from restriction enzyme analysis of AH DNA is therefore the model proposed in which there is an extensive deletion starting between the Xba 1 and Hpa I sites in the $^{A}\gamma$1VS-2 and extending downstream through the β globin gene. Independent support for this comes from solution hybridisation data. This shows that while normal and AH DNA contain the same complement of α globin genes, there is a substantial reduction of δ and β globin gene sequence in AH DNA, consistent with the absence of both these genes from the chromosome responsible for the $^{G}\gamma(\delta\beta)^{o}$ thalassaemia phenotype.

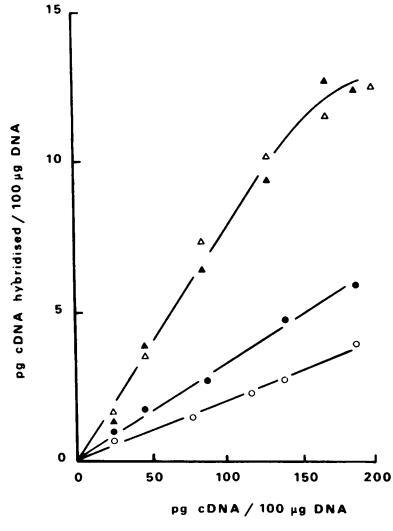

Figure 5
Hybridisation of cDNA α to normal (▲) and AH (△) DNA, and of cDNA β to normal (●) and AH (○) DNA, with cDNA excess.

DISCUSSION

Three distinct lesions, including that reported here, are now known to be associated with $^{G}\gamma(\delta\beta)^{o}$ thalassaemia.[1,4,5,6] In all three, the $^{A}\gamma$, δ and β globin genes have been lost in all or part, but otherwise the deletions or rearrangements show considerable heterogeneity. However,all three share a similar phenotype.[7,16,17] The level of γ-globin synthesis is virtually the same as in $^{G}\gamma^{A}\gamma(\delta\beta)^{o}$ thalassaemia, and greater than that found in $(\beta)^{o}$ thalassaemia. This ehnancement of γ globin synthesis must therefore be a feature of the molecular lesions responsible for $^{G}\gamma(\delta\beta)^{o}$ thalassaemia other than the loss of function of the β globin gene, and although the level of γ globin synthesis is less than is achieved in HPFH, this may be due to the presence of only a single γ globin gene. The change in phenotype, from $^{G}\gamma^{A}\gamma(\delta\beta)^{o}$ thalassaemia to HPFH which accompanies the loss of a small region of DNA near the 5' end of the δ gene suggests that this region contains a sequence responsible for the suppression of γ globin synthesis in adult erythropoiesis, and that its deletion accounts for persistent γ globin synthesis in HPFH.[1,2,3,4,5,18,19] This same sequence has been lost in two of the three cases of $^{G}\gamma(\delta\beta)^{o}$ thalassaemia; and is inverted in the third. Loss of this sequence, or loss of its function by inversion, may be responsible for the level of $^{G}\gamma$ globin synthesis in this condition.

An alternative explanation of the complex relationship between phenotypes and genotypes in disorders of globin gene expression in the $\gamma\delta\beta$ cluster is that deletions interfere with the ordered structure of the chromosome in this region.[3,20] However, the variety of deletions and rearrangements responsible for the single phenotype of $^{G}\gamma(\delta\beta)^{o}$ thalassaemia argues against some features of a model of this kind. In one case the sequence downstream from the partially deleted $^{A}\gamma$ globin gene is known,[6] and involves an inversion and deletions, with some features of normal chromosome architecture, such as the relative position of repetitive sequences,[21] being retained. The other two cases consist of simple deletions, but one retains the 5' end of the $^{A}\gamma$ globin gene, while the other

has lost in addition much of the DNA between the γ globin genes, as well as all of the $^{A}\gamma$ gene.[1] These rearrangements must involve the introduction of at least two, if not three different DNA sequences 3' to the $^{G}\gamma$ gene, but without any effect on the level of $^{G}\gamma$ globin synthesis.

We have previously proposed a model in which the regulation of gene expression in the γδβ globin gene cluster entails a mechanism which is polar in nature, and involves sequential activation of these genes in a 5' - 3' direction by a process acting in cis through the chromosome.[6,22] Support for such a mechanism includes the arrangement of the $^{G}\gamma$, $^{A}\gamma$, δ and β globin genes on the chromosome in the same sequence as they are expressed during both development and erythropoiesis.[21,23,24] Also deletion upstream from an intact β globin gene in $(\gamma\delta\beta)^{o}$ thalassaemia appears to be responsible for the inactivity of the latter gene,[20] while by contrast deletions downstream from the γ globin genes tend to be associated with some degree of enhanced γ globin synthesis in adult life.

In this model, interruption of the normal continuity of the DNA in the gene cluster, for example by deletions, would halt sequential gene inactivation at the last intact gene: the $^{G}\gamma$ gene in $^{G}\gamma(\delta\beta)^{o}$ thalassaemia; the $^{A}\gamma$ gene in HPFH. The fact that the phenotypes of these two conditions are related primarily to the retention and loss of functional genes, and are relatively insensitive to the precise form of the deletion or rearrangement responsible, suggests that the process involved in propogating through the chromosome a change in its structure necessary for gene activity is sensitive to the presence of genes or their regulatory sequences. Thus this process would advance stepwise from gene to gene, so that the genes themselves may assume a regulatory role, and sequential gene expression would be a prerequisite for the normal expression of the γδβ globin gene cluster during erythropoiesis.

However, two observations conflict with this hypothesis as it stands. One is the clearly different behaviour of this gene cluster during fetal compared with adult erythropoiesis,

which strongly suggests that this behaviour is modified by factors external to the chromosome. The second is the change in phenotype from HPFH to $^{G}\gamma^{A}\gamma(\delta\beta)^{o}$ thalassaemia which suggests the presence of a regulatory sequence near the 5' end of the δ gene. The site of this regulatory sequence is close to one of the repeat sequences in the γδβ gene cluster.[21] There is evidence that this sequence is potentially transcribable, and the suggestion has been made that it may have a cis-active role in the regulation of the fetal to adult globin switch.[25] In the context of a mechanism involving the sequential activation of the β-like globin genes, additional transcribable sequences such as these could participate in the stepwise transmission of gene activation, and act as regulatory sequences modifying this process, possibly under the influence of factors external to the chromosome.

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