Volume 14 Number 17 1986 Nucleic Acids Research

$\gamma\delta\beta$-Thalassaemias 1 and 2 are the result of a 100 kbp deletion in the human β-globin cluster

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Received 3 July 1986; Accepted 11 August 1986

ABSTRACT

The DNA spanning two large deletions in the human β-globin gene cluster (γβ-thalassaemia 1 and 2) has been cloned by cosmid cloning and chromosomal walking. The entire region was mapped and analyzed for the presence of repetitive sequences. The results show that the affected loci have lost almost 100kb of DNA in a deletion event not involving homologous or repetitive sequences.

INTRODUCTION

The human β-globin gene family, which contains five expressed genes and a single pseudogene, spans a 60kbp segment of DNA on the short arm of chromosome 11. Within the cluster, the expressed genes are arranged in their order of developmental expression 5'-ε, $^{A}\gamma$, $^{G}\gamma$,δ,β-3' (Fig. 1). Several copies of repetitive elements, including the Alu and Kpn family, have also been found interspersed between the different genes; six Alu and two Kpn family sequences have been reported to date. Several rearrangements involving DNA within the β-globin cluster have been reported. In many cases, the rearrangements involve DNA well outside the cluster and result in the deletion of large segments of the β-globin cluster (for review, see ref. 1). Some of these deletion events have also been shown to alter the expression of the remaining globin genes leading to, for example, γδβ-thalassaemia 1 and a number of cases of hereditary persistence of foetal haemoglobin (HPFH). Until recently, both the mechanism responsible for producing these deletions and the reason for the altered expression of the remaining globin genes was not known.

Recent work (2, 3) has shed some light on the mechanism responsible for some of these deletions. In sequence studies of the two γδβ-thalassaemias, γδβ-thals 1 and 2, (2) it was found that both deletions were the result of a clean non-homologous breakage and reunion event; that is the sequences involved in the recombinational event showed no homology whatsoever and no extraneous bases were added during the event. More

surprisingly, both studies of γδβ-thals 1 and 2 and HPFHs 1 and 2, were able to show that for deletions extending in the same direction from the β-globin cluster, the 5' and 3' breakpoints are in the same order along the DNA and approximately the same distance apart (2, 3). The simplest explanation for these results is that for each pair of deletions, the γδβ-thal pair and the HPFH pair, the amount of DNA removed is approximately the same. Vanin et al. (2) postulated that this constant size deletion phenomenon could be the result of breaks occurring during replication at the site of attachment of DNA to the nuclear matrix. Such a hypothesis predicts that the amount of DNA removed by the deletion event should be the same as the amount of DNA in the loop which normally contains the β-globin gene cluster.

One of these deletions, γδβ-thal 1, also proved to be an interesting example of altered expression of gene(s) remaining after the deletion event. Although genotypically the affected individual has two complete copies of the β-globin gene, yet the patient has haematological symptoms characteristic of a β-thalassaemia heterozygote. Kioussis et al have shown (4) that the β-globin gene on the affected chromosome was less sensitive to DNase I and hypermethylated when compared to the same gene on the normal chromosome (the affected individual was heterozygote). Both the hypermethylation and the lessened sensitivity to DNase I are properties of non-transcribed DNA. They were also able to show that the DNA which is moved next to the β-globin gene, as a result of the deletion event, is normally in a DNase I insensitive region in erythroid chromatin. Therefore, these results suggest that the DNA which has been juxtaposed to the β-globin gene is conferring its inactive chromatin conformation on the β-globin gene which is just downstream of it.

In this paper, we continue our work on the γδβ-thal 1 deletion. The results presented here will show that the rearrangement responsible for this thalassaemia is a simple deletion event. The amount of deleted DNA was found to be 99.4 and 95.9kbp for γδβ-thals 1 and 2, respectively. We also show the distribution of repetitive sequences over the 140kbp of DNA which includes the β-globin gene cluster and 60kbp of 5' and 35kbp of 3' DNA.

METHODS

In situ and Southern hybridization

Normal or γβ thal DNA or chromosome spreads were obtained from white blood cells. DNA isolation and Southern blots were according to standard procedures (11). Repetitive DNA blots were washed to 1.0 and 0.3xSSC. Chromosome preparations and in situ hybridizations were according to

Hagemeijer et al. (5) and Bartram et al. (6). Cosmid libraries were prepared and screened as described previously (10).

RESULTS

Localization of DNA involved in rearrangement

To establish whether the translocated sequences in γδβ-thal 1 were from chromosome 11, two different approaches were used. The first was to use specific DNA sequences to probe a panel of human -A23 hamster somatic cells. The second approach was the use of in situ hybridization to metaphase spread chromosomes from a normal individual. The probes used in these studies are J (2) and Sph 2.5. The J probe corresponds to a 0.2kbp EcoRI-SphI fragment which is located immediately 5' to the breakpoint in γδβ-thalassaemia 1. The Sph 2.5 probe, a 2.5kbp SphI fragment, was isolated from phage λ recombinant clone AN2.1 (2) and is contained within the 4.0kbp EcoRI fragment. This fragment contains the 5' breakpoint for γδβ-thalassaemia 2 and was previously shown to be closely linked, 5 kbp 3', to the J probe. A panel of nine different somatic cell lines were used, each containing a different complement of human chromosomes (6).

Hybridization using either probe to Southern blots of EcoRI digests of DNA from each cell gave the same results and these are summarized in Table I. As can be seen, there is an excellent correlation between the presence of chromosomes 11 and the positive hybridization signal with both probes. The in situ hybridizations (6) were performed using the Sph 2.5 probe and metaphase chromosome spreads from a normal individual. A total of 100 spreads were examined which produced a total of 712 grains. The results are summarized in Fig. 1. 68 of these grains were found on chromosome 11 of which 40 were found on the tips of the short arm of chromosome 11. This value of 40 out of 712 is significantly higher ($p<0.001$) than a merely random number of grains.

Characterization of the rearrangement

In order to better characterize the rearrangement, digests of the γδβ-thal 1 DNA were probed with a number of different sequences. Fig. 2 shows the results obtained using the γ- and β-globin gene probes. The γ-globin gene probe gave the expected three bands in normal DNA; one (2.6kbp fragment) containing the 5' half of the $^{G}\gamma$-gene, one (5.0kbp fragment) containing the 3' half of the $^{G}\gamma$-gene and the 5' half of the $^{A}\gamma$-gene and the other (16.0kbp fragment) containing the 3' half of the $^{A}\gamma$-gene. Each of these fragments was half of its intensity in the thalassaemia DNA, that is, these genes have been

Table I

Cell Line	Chromosome Complement																							Scoring	
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	X	Y	J	Sph2.5
16CB18	-	±	+	+	-	-	±	-	+	-	+	-	-	+	+	+	-	-	-	+	±	+	+	-	+	+
E364.1.5	+	+	+	±	+	+	±	+	+	+	+	+	±	+	-	+	+	+	+	+	-	+	±	-	+	+
E362.9.5	+	+	+	+	-	+	-	±	-	+	-	+	-	+	+	+	-	+	+	-	+	+	+	-	-	-
17CB3B	+	+	-	+	+	+	+	-	+	-	-	+	+	+	+	+	+	-	+	+	+	-	-	-	-	-
17CB2C	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
17CB15B	-	±	+	+	+	-	-	-	-	+	+	+	+	-	-	+	+	±	±	+	+	+	-	+	+	+
17CB8B	-	-	-	+	+	+	-	+	+	+	+	+	±	-	-	+	+	-	+	±	+	+	±	-	+	+
16CB17	+	-	+	+	-	-	-	+	-	-	-	+	-	+	-	+	+	+	+	-	+	+	-	-	-	-

Human chromosome content to human-hamster cell hybrids (Bartram et al., 1983) as determined by karyotype analysis. Columns 1-Y: + and - indicate the presence or absence of human chromosomes 1 to Y. The right hand column shows the J or 2.5Sph probe scoring of the cell lines by Southern blot hybridization.

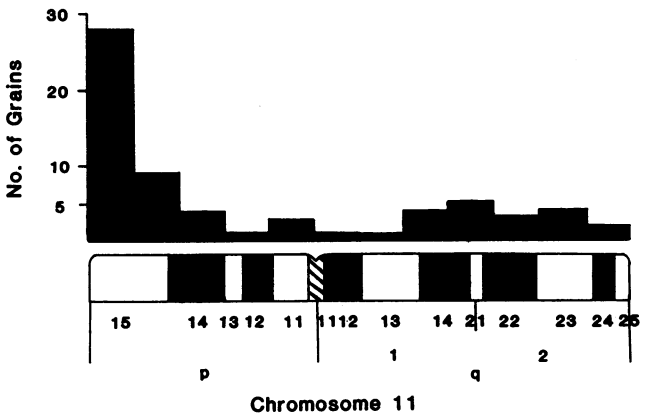

Figure 1.
Histogram of grains present on chromosome 11 after in situ hybridization. The number of grains are indicated above a schematic drawing of chromosome 11.

deleted from the affected chromosomes. The β-globin gene probe also produced the expected pattern. The 2.25kbp EcoRI fragment, which contains the 5' half of the δ-globin gene, is less intense in the thalassaemic DNA, indicating that the δ-globin is not present on the affected chromosome. The two remaining bands in the normal DNA contain the 5' half (5.2kbp fragment) and the 3' half of the β-globin gene (3.6kbp fragment). As can be seen in the affected individual, the 5.2kbp band is half the intensity of that observed in the normal DNA, while the intensity of the 3.6kbp band is the same in both. An additional band, 4.2kbp, is observed in the affected individual and corresponds to the fragment which spans the deletion breakpoint. The results obtained using the 5' and 3' breakpoint probes are consistent with the rearrangement being a simple linear deletion, as had been previously suggested (2). The 5' breakpoint probe is not present on the affected chromosome, as the band obtained in the affected individual is half the intensity of that in normal DNA, while the 3' breakpoint is present on the affected chromosome.

We also probed for the rasHI and insulin genes (all of which are on the short arm of chromosome 11) (7, 8, 9), in order to determine their fate on the affected chromsome. As can be seen from Fig. 2, all those genes are present in two copies in the affected individual's DNA. The rasHI probe gave two bands on BamHI digests and three on TaqI digests of the patient's DNA. These bands are similar to those previously observed (7) and are due to restriction site polymorphisms. The insulin probes gave the normal (diploid)

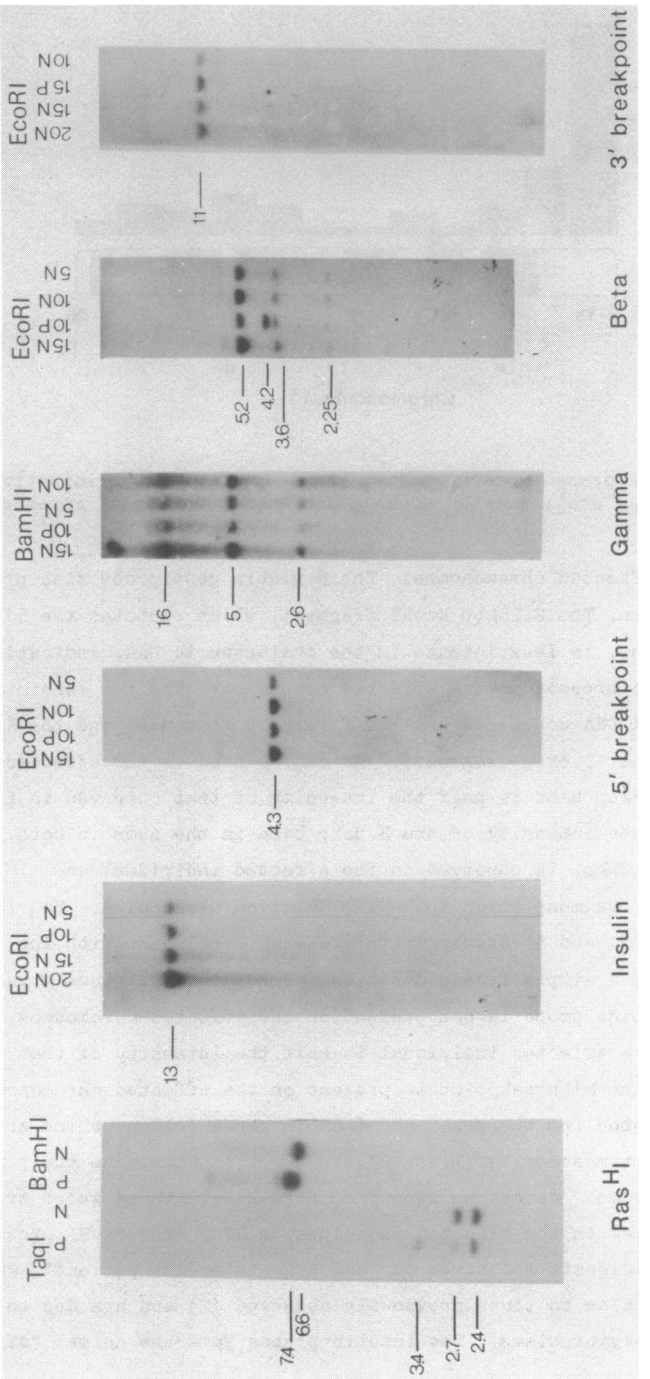

Figure 2.
Southern blot hybridization to normal DNA and γβ-thalassaemia 1 DNA. Varying amounts of normal DNA, 5, 10, 15, or 20μg (5N-20N) or thalassaemia DNA (P) were hybridized with a variety of probes, as indicated in the figure. Probe copy numbers in the thalassaemia DNA were estimated from the comparison of band intensities.

intensity of bands. In addition, we probed for the presence of the PTH gene which is present at the other side of the β-globin locus (10). This probe also showed the normal diploid intensity of hybridization (not shown). These results therefore show that closely linked genes on either side of the globin cluster have not been affected in the thalassaemia.

Linking 5' normal DNA with β-globin gene cluster

All the data obtained to date indicate that the rearrangement which resulted in the γδβ-thal 1 was a simple, local deletion. Therefore, we decided to attempt to link the DNA from λ-AN2.1, which contained the breakpoint for γδβ-thals 1 and 2, to the β-globin gene cluster by "chromosome walking". A normal DNA cosmid library was simultaneously screened with the Sph 2.5 probe (contained in λ-AN2.1 (2) and a 3.3 kbp Eco probe from cosmid HG16 (11, Fig. 3). Several clones were isolated and two of these are denoted as HG4 and HG9 in Fig. 3.

The cosmid HG9 which hybridized only to the 3.3 kbp EcoRI probe, contained a total of 34 kbp of human DNA. Its restrictions showed that it has an overlap of 22 kbp with the cosmid HG16 (Fig. 3). The clone HG4 contained 40 kbp of human DNA and hybridized to both the Sph 2.5 probe and the Eco 3.3 probe. The restriction maps show the restriction sites surrounding the region hybridizing to the 3.3 kbp probe were the same as seen in cosHG16 and cosHG9 (Fig. 3). Furthermore, orientation of restriction sites indicated that this was the 3' end of the clone. Similarly, the DNA surrounding the regions hybridizing to the 2.5kbp probe had the same restriction map as now seen in λ-AN2.1. As before, the orientation of the restriction sites was consistent with this being the 5' end of cosHG4.

The DNA shown 5' to AN2.1 in Fig. 3 was actually isolated from a cosmid library of the γδβ-thal 1 patient's DNA with a β-globin gene probe. The cosmid clone isolated, cos γβ5, contained a total of 39kbp of DNA, of which approximately 30 kbp were 3' end β-globin cluster DNA. Of the nine kbp which is not β-globin cluster DNA, 0.1 kbp is contained within the 5' end of AN2.1 and is part of the J probe. The remaining 8.9 kbp of DNA is 5' upstream to AN2.1 as shown in Fig. 3.

Distribution of repetitive sequences

The distribution of repetitive sequences over the complete region was also determined (Fig. 4). A total of four different probes were used, one AluI family sequence probe, two KpnI family sequence probes (one for the 5' half and one for the 3' half) and total human genomic DNA. The probe used to identify the AluI family sequences was a 0.55kbp EcoRI fragment located

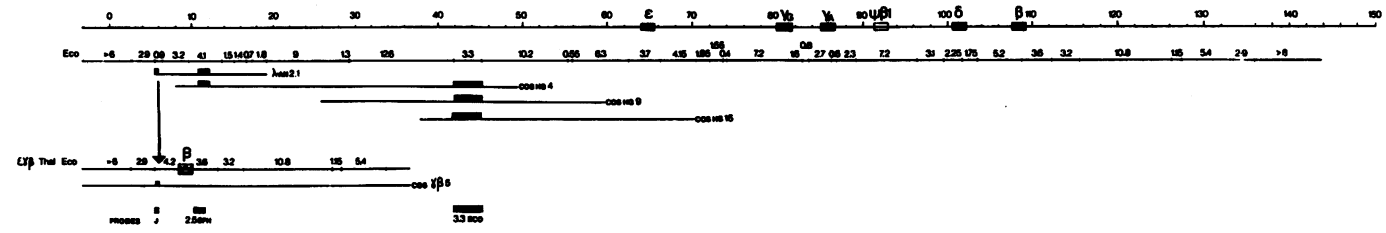

Figure 3.
Mapping of the γβ-thalassaemia 1 by chromosomal walking.
The overlapping cosmid clones cos HG16, 9 and 4 and λ clone AN2.1 are indicated. cosγβ5 was isolated from a γβ-thalassaemia 1. Probes J, 2.5 Sph and 3.3 Eco are indicated as shadowed boxes. The map is in kilobase pairs, showing the globin genes and the EcoRI restriction map of the region.

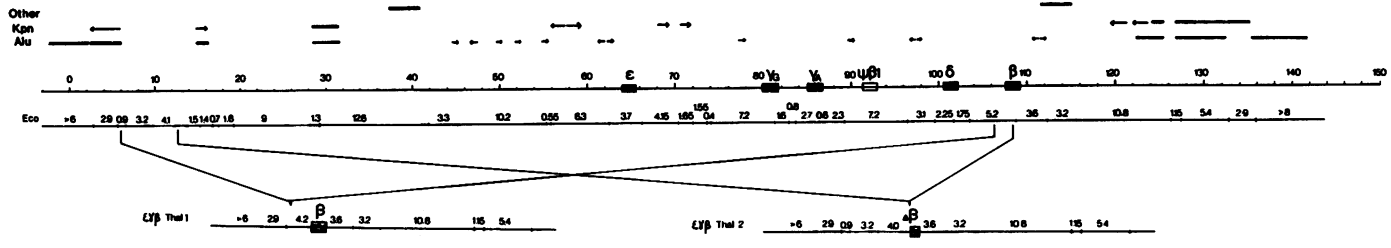

Figure 4.
The top part of the figure shows the localization of Kpn, Alu and other repetitive sequences. Open boxes indicate restriction fragments containing a repeat. Short arrows indicate both direction and exact localization of the repeat sequence (see text). The comparison of γβ-thalassaemia 1 and 2 are indicate in the lower half of the figure.

approximately 8kbp 5' to the ε-globin gene. This probe hybridized to a total of 14 different regions over the entire stretch of DNA, that is there is one hybridising region per 10kbp of DNA. The location of KpnI family sequences was achieved using two different probes, one corresponding to the 5' half (1.65kbp EcoRI fragment) and the other to the 3' half (1.55bkp EcoRI fragment). These two fragments comprise the KpnI family sequence found approximately 6kbp 5' to the $^{G}\gamma$-globin gene. The 1.65kbp fragment was found to hybridize to eight different regions, while the 1.55kbp fragment hybridized to seven different regions. As expected, the majority of times the hybridization was either to the same fragment or to a neighbouring fragment.

Lastly, we hybridized the complete region with nick translated human DNA, a procedure that detects middle and highly repetitive DNA. In addition to the Alu and Kpn repeat sequences, this procedure detected a number of additional repetitive sequences. It should be noted that the actual number of either family or repetitive sequences within the entire region will be higher than indicated in Fig. 4, because only a minimum number of sites can be detected by hybridization studies. For example, a fragment which hybridizes may contain multiple sequences of that particular repetitive sequence. One possible explanation for the creation of the deletion could be a crossover involving repetitive sequences. However, when the region around the 5' end of the region is aligned with the δ- and β-gene region using the deletion-junction points as the fixed position, none of the repetitive sequence from the two regions lineup.

DISCUSSION

Previous work on both γδβ-thals 1 and 2 indicated that both were the result of a non homologous breakage and reunion event. These studies also showed that the 5' breakpoints for both were approximately the same distance apart, and in the same order along the DNA, as their respective 3' breakpoints. Therefore, they suggested that these thalassaemias had resulted from simple linear deletions and that if this were the case, their results indicated that the amount of DNA deleted in both γδβ-thals 1 and 2 was approximately the same. The possibility still remained that these thals could have been the result of a more complex rearrangement, for example, translocation in conjunction with deletion. In order to test this possibility we used two probes, J (located immediately 5' to the breakpoint of γδβ-thal 1) and Sph 2.5 (normal 5' DNA containing the breakpoint for γδβ-thal 2) in somatic cell hybrid studies as well as *in situ* hybridization studies. As can

be seen (Table 1), the hybridization pattern for both probes is consistent with the DNA being located on chromosome 11. In order to further localize this DNA, the Sph 2.5 was used as a probe for in situ hybridization studies (Fig. 1). The results obtained localized this DNA to the tip of the short arm of chromosome 11. As the human β-globin gene cluster has been localized to the short arm of chromosome 11, these results strengthen the view that these thalassaemias are in fact the result of simple deletion events.

The results obtained when probing the γδβ-thal 1 patient's DNA were also consistent with the recombination event being a simple deletion. Previous data (2) indicated that the breakpoint for γδβ-thal 2 (contained within the Sph 2.5) was 3' to the breakpoint for γδβ-thal 1. Therefore, if γδβ-thal 1 was the result of a simple deletion, then the γδβ-thal 2 brekpoint must be closer to the β-globin gene cluster. If this were the case, one would predict that the DNA corresponding to the Sph 2.5 would not be present on the affected chromosome of the patient, as it is between the two breakpoints. The results which are shown in Fig. 2 are consistent with this prediction.

Both the Sph 2.5 and the γ-globin gene probe (the γ-globin genes had been shown to be deleted (12)) gave similar results, that is the DNA corresponding to each of these probes was present in only one copy (the individual is a heterozygote). Other genes (rasH, insulin and PTH) (8) which were known to be on the short arm of chromosome 11 were also used as probes to determine whether any large deletions or complex rearrangement had taken place on the affected chromosome before attempting a chromosomal "walk". All genes were present in two copies as was the DNA corresponding to the normal 3' breakpoint for HPFH 1 (2). These results indicate that large rearrangements did not occur.

All the data obtained at this point strongly supported the idea that the DNA removed in γδβ-thal 1 was the result of a simple deletion event. However, this cannot be proven unless the normal 5' and 3' DNAs for γδβ-thal 1 are linked. By screening a cosmid library with two different probes (Fig. 3) we were able to link the 5' breakpoint for γδβ-thal 1 with the β-globin gene cluster. Therefore, these results conclusively show that the γδβ-thal 1 was produced by a simple deletion event at approximately 99kbp.

As a result of this chromosomal walking, we now have approximately 150kbp of DNA surrounding the β-globin gene cluster. We then proceeded to determine the distribution of repeated sequence elements using an AluI family probe, two KpnI family probes and human genomic DNA as a probe (Fig. 4). This

analysis detected a number of additional repeats to the previously mapped Alu and Kpn repeats (13, 14, 15, 16, 17).

We found that there is at least one AluI family sequence per 7.5kbp of DNA, which would correspond to approximately 300,000 such copies per hybrid genome. As these sequences are an average of 300bp in length, this represents 3% of the total human genome. This value is an underestimate because we based our calculation on hybridization studies which would not detect multiple AluI family sequences close to one another. With the globin gene cluster there are three examples of two AluI family sequences in close proximity (see ref. 1), while there are seven regions with only single AluI family sequences, the other eight regions could have one or two Alu repeats (Fig. 4). The KpnI family sequence results indicate that there is on average one KpnI family sequence every 13kbp. Therefore, from these results one would estimate that there are approximately 200,000 copies per haploid genome.

This value is in agreement with previous estimates (18). As before, the hybridization results may be misleading. For example, multiple hybridizing regions may actually be due to a single KpnI family sequence which is split due to some other rearrangement event. Nevertheless, we can distinguish at least 12 separate Kpn repeats in the β-globin cluster.

As previously mentioned, (see Introduction), γδβ-thal 1 was shown to be related to γδβ-thal 2, in that the 5' and 3' breakpoints of both thalassaemias are in the same order along the DNA and approximately the same distance apart. This constant size deletion relationship, seen between γδβ-thals 1 and 2, led Vanin et al. (2) to postulate the involvement of DNA loops in these deletion events. They hypothesized that the two thals were the result of the deletion of a single DNA loop at different times during the replication cycle. Therefore, in linking the breakpoints of γδβ-thal 1, we have also linked the 5' and 3' breakpoints for γδβ-thal 2. The amount of DNA between the breakpoints will also help in establishing whether DNA loops are actually involved. The amount of DNA deleted in γδβ-thal 2 was found to be approximately 96kbp, in comparison to γδβ-thal 1, in which the deletion event had removed approximately 99kbp of DNA (Fig. 4). If the hypothesis of Vanin et al. (2) is correct, then this small 3% difference in the amount of DNA deleted between the two thals could be the results of differential rates of replication at the respective attachment points. Attachment points of DNA to the nuclear matrix have been shown to be sites at which DNA replication occurs (19). DNA loops have been reported to range in size from 30kbp to 200kbp,

although the average size in mammalian cells seems to be approximately 80kbp (20). As can be seen, the amount of DNA deleted in these two thals is within the range of DNA loop sizes and is, in fact, close to the average size of loops in mammalian nuclei. Although this does not conclusively prove that DNA loops are involved, it does add strength to the hypothesis. In the future, we intend to determine the position at which this 150kbp stretch of DNA is attached to the nuclear matrix. If, as we expect, the DNA loops are an integral part of the deletion mechanism, then one would expect the 5' and 3' breakpoints of either thal to be equidistant from their closest attachment points. The involvement of DNA loops in chromosomal rearrangements has also been suggested by Feinberg and Coffey (21). They stated that multiple loops could be transposed during chromosomal translocations as a result of the transposition of their attachment points.

γδβ-thal 1 has also proved to be interesting from the point of view of gene expression. When originally reported (12), it was found that the affected individual (who was heterozygous for the deletion) had two complete copies of the adult β-globin gene, yet phenotypically had symptoms of a β^{o}-thalassaemia heterozygote. Kioussis et al. (4) studied this phenomenon and found that the DNA containing the 5' breakpoint for γδβ-thal 1 is DNaseI insensitive in normal erythropoetic cells. They were also able to show that when juxtaposed 5' to the β-globin gene, this same DNA was able to confer its DNaseI insensitive state to the adult β-globin gene 2.5kbp 3' to it, thereby inactivating the β-globin gene. This implies that one border of the active domain surrounding the β-globin gene cluster must be within the deleted DNA described in this paper. Although we do not know the location of this region activating the β-globin cluster in erythropoetic cells, a good candidate might be the DNase hypersensitivity region recently described by Tuan et al. (3) upstream from the ε-globin gene, which was deleted in this patient.

ACKNOWLEDGEMENTS

We are grateful to Cora O'Carroll for the preparation of this manuscript. RT was the recipient of a long-term EMBO fellowship, F.G. the recipient of a short-term EMBO fellowship. This work was supported by the British Medical Reearch Council.

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