# THE STRUCTURE AND EXPRESSION OF GLOBIN GENES IN RABBIT AND MAN

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ABSTRACT The rabbit and human β-related globin genes have been analysed using genomic 'Southern blotting' and molecular cloning. The rabbit β-globin gene structure has been worked out in detail and its transcripts have been characterized by $S_1$ nuclease transcription mapping.

The arrangement of the human γδβ-globin gene locus has been largely elucidated. The gene order is (5' to 3') GγAγδβ and the intergene distances are Gγ-Aγ, 3.5 kb; Aγ-δ, 13.5 kb; δ-β, about 6 kb. All these β-related globin genes are transcribed from the same DNA strand. Several abnormal globin genes have been characterized by the same methods. Thus, δβ°-thalassaemia is the result of a deletion which begins approximately in the δ-globin gene large intron and extends beyond the β-globin gene. A form of β°-thalassaemia has been mapped where a 600 bp deletion, including the 3' exon of the β-globin gene, has occurred.




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## INTRODUCTION

In the past two years our ideas on gene structure have undergone a dramatic revision (see e.g. ref. 1). The structure of a large number of eukaryotic viral and cellular genes has been elucidated [2-11]. In this paper we shall discuss current knowledge on the structure of the rabbit and human non-α-globin genes, their transcription products and the structure of various abnormal globin genes.

## THE STRUCTURE AND TRANSCRIPTION OF THE RABBIT β-GLOBIN GENE

Initial experiments, using Southern blotting and filter hybridization, showed that the rabbit β-globin gene consisted of at least two non-contiguous blocks of coding sequences separated by an intron [for a definition of intron, see ref. 12] of about 600 bp [6]. Analysis of a cloned rabbit β-globin gene has shown that in fact two introns are present, one of 126 bp in length present between the DNA sequences coding for amino acids 30 and 31, and the one already mentioned above of 573 bp present between the sequences coding for amino acids 104 and 105 [8; Van Ooyen, A. and Grosveld, G.C., unpublished].

The mouse β-globin gene has an essentially identical structure [7,9]. A mouse β-globin pre-mRNA of about 1200-1500 nucleotides has been described; this RNA forms a hybrid co-linear with the mouse β-globin coding regions and intervening sequences [13]. This RNA is presumably a precursor to β-globin mRNA; both intervening sequences must, therefore, be removed from this RNA and the mature mRNA obtained by splicing the exonic sequences.

We have investigated β-globin pre-mRNA in more detail in the rabbit β-globin gene system. Rabbit bone-marrow RNA is hybridized to segments of the cloned rabbit β-globin gene and then the hybrids are analysed by the $S_1$ nuclease transcription mapping of Berk and Sharp [14]. These results, which have been briefly reported in a recent Symposium volume [15] and will be described in detail elsewhere, can be summarized as follows:

1. The largest transcript detected up to now is 1250 nucleotides long. Its 5' and 3' termini map at the same position as the termini of the mature β-globin mRNA. This RNA is, therefore, a precise transcript of the β-globin exon-intron region; transcripts of the extragenic regions cannot be

detected on this RNA (resolution about 10-20 nucleotides).

2. A second major species is detected. From the mapping data we deduce that this RNA lacks the small intron but contains the large intron and the exons. This RNA is presumably a processing intermediate where the small intron has been removed by an excision-splicing mechanism. The alternative expected intermediate, lacking the large intron but containing the small one, cannot be detected in these experiments. This suggests that the major splicing pathway for the rabbit β-globin gene transcripts is first the removal of the small intron and subsequently the large intron. Whether this pathway is obligatory or not cannot be deduced from these experiments it is also possible that the rate of removal of the small intron is simply intrinsically greater than that for the large intron.

## REPETITIVE DNA AROUND THE RABBIT β-GLOBIN GENE

We have previously localized a segment of repetitive DNA in a region between 640 and 2400 bp to the 3'-side of the rabbit β-globin gene [16,17]. This study also showed that less than about ten copies of the regions immediately flanking the gene at the 5'- and 3'-sides, as well as the intronic regions, were present in the rabbit genome.

The globin genes constitute a gene family in which simultaneous expression of the α- and e.g. β-globin genes must be coordinated; within a family such as the β-related globin genes, simultaneous expression of two globin genes occurs in man (δ and β) and probably also in rabbit [18]; alternative expression of β-related genes occurs during embryogenesis (e.g. in man $\varepsilon \longrightarrow \gamma \longrightarrow \delta+\beta$) of most organisms. One might envisage that such coordination of these and perhaps other genes expressed in erythroid cells, would be mediated via homologous DNA regions closely linked to these genes. Such homology would conceivably be only partial, but if these hypothetical sequences were long enough, they should be detectable by hybridization under non-stringent conditions. Our previous experiments excluded the presence of large numbers of such homologous sequences ($\sim$10-20), but less than ten copies would be difficult to distinguish from a single copy in such experiments (see refs. 16, 17).

To test whether multiple copies of the flanking sequences exist in the rabbit genome we have

hybridized Southern blots of rabbit DNA which had been cleaved with a variety of restriction endonucleases, with probes for the 5' extragenic regions (a $^{32}$P-labelled HaeIII fragment of RβG1 DNA [8] extending from -1600 to -70 bp [O is defined as the 5' nucleotide of globin mRNA]) and the 3' extragenic regions (a $^{32}$P-labelled HaeIII fragment extending from +1160, the coding site for amino acid 137 of β-globin, to +2200 bp). We also hybridized filter strips to a $^{32}$P-labelled probe (a HaeIII fragment, extending from +2200 to +3400 bp), which contains the reiterated sequence previously characterized [16].

After hybridization the filters were subjected to increasingly stringent salt washes exactly as described in ref. 19. Washing of filters at low salt concentrations causes mismatched hybrids to melt and as a result, only well base-paired hybrids remain bound to the filter. On filters washed only in high salt, poorly base-paired, heterologous hybrids can also be detected (e.g. human β-γ [20]). As seen in Fig. 1, under stringent washing conditions, both the 5' or 3' extragenic probes only detect a single band. Both HaeIII probes only detect their corresponding genomic fragments in HaeIII-digested rabbit DNA; the 5' extragenic probe also detects the characteristic EcoRI (2.6 kb) and PstI x KpnI double digest fragments (1.3 kb and a faint band at 3.6 kb) predicted to be the 5' extragenic regions of the rabbit β-globin gene [19]. A similar conclusion may be reached for the fragments detected by the 3' extragenic probes. Essentially identical results are obtained for the filters washed at high salt (not shown) except that one or two extra components are detected faintly. These probably correspond to the rabbit β-related globin genes, since three components can be detected in Southern blots of rabbit DNA hybridized with cDNA probes and washed under non-stringent conditions [19]. We conclude that the 5' and 3' extragenic regions of the β-globin genes studied here are essentially single-copy DNA.

When the probe for the repeated sequence is used, this forms hybrids with DNA spanning the entire molecular weight range generated by PstI, KpnI or EcoRI (Fig. 1). This repetitive DNA, present in thousands of copies per cell, shows therefore the properties expected of interspersed repeated sequences rather than those of a 'satellite' DNA - in the latter case we would expect to find bands

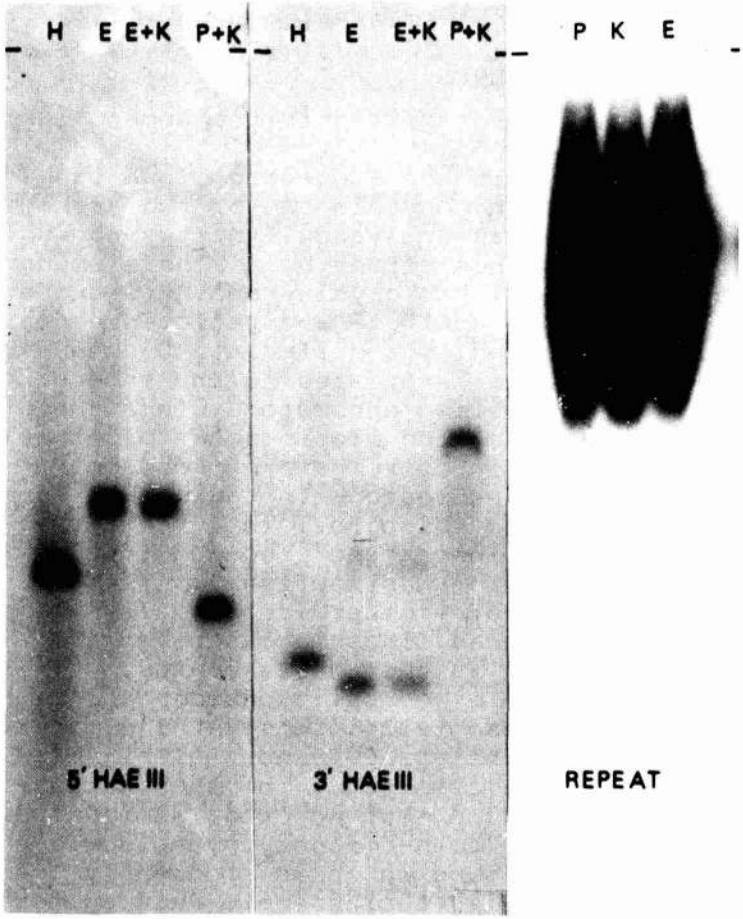


Fig. 1. Reiteration of the 5' and 3' extragenic regions of the ß-globin gene in the rabbit genome. Rabbit DNA was cleaved with the restriction endonucleases indicated and Southern blots prepared of this DNA as described [19]. The filter strips were hybridized with $^{32}$P-labelled, nick-translated probe (50-100 x $10^6$ cpm/µg) as described [16,20]. The probes were prepared by HaeIII cleavage of 20 µg RßG1 DNA [8], followed by acrylamide gel electrophoresis to separate the fragments. The map positions of the probes used were 5' extragenic HaeIII, -1600 to -70; 3' extragenic HaeIII, +1160 to +2200; 'repeat' HaeIII, +2200 to +3400. The restriction endonucleases used were: HaeIII (H), EcoRI (E), PstI (P) or KpnI (K). The gel used to prepare the blot for the 'repeat' experiment was run for a shorter time than that used for the other two samples.

rather than a general smear along the filter.

## THE HUMAN GLOBIN GENES

We have been interested in the human globin genes for two reasons:

1. Genetic evidence suggested close linkage of the non-α-globin genes in one of two arrangements, either $G\gamma A\gamma \delta\beta$ or $A\gamma \delta\beta G\gamma$ (the two non-allelic foetal γ-globin genes differ by a single amino acid at position 136; Gγ has glycine and Aγ alanine at this position; the adult δ- and β-globin genes are also closely related, but differ at 10 amino acid positions). We were interested in the intergene distance on a mammalian chromosome since the linkage of the β-related globin genes might well play a role in their coordinate expression.

2. Well-defined lesions in the globin genes have been described which fall into two class:

a) Abnormal globins are produced (e.g. as a result of amino acid substitutions).

b) The level of - otherwise normal - haemoglobin can be reduced, in some cases to zero, in the diseases known as the thalassaemias.

In this article we shall consider the β-thalassaemias which also fall into several classes. In some types of $\beta^{\circ}$-thalassaemia no β-globin mRNA can be detected in the cell [21]; in other nuclear β-globin RNA is found, but no cytoplasmic globin mRNA [22]; in still other cases, cytoplasmic β-globin mRNA is found which is apparently inactive in translation. In $\beta^{+}$-thalassaemia reduced levels of β-globin mRNA are found with a parallel decrease in the levels of β-globin in the cell.

We have constructed physical maps of the normal human globin genes using the blotting procedure [23,6] and then compared these with the corresponding physical maps of the globin genes in DNA of patients with various thalassaemias. This makes it possible to detect large deletions in DNA regions within or around the globin genes.

## THE STRUCTURE OF THE HUMAN γδβ-GLOBIN GENE LOCUS: THE USE OF GLOBIN-PROTEIN VARIANTS FOR GENE IDENTIFICATION

We have constructed maps of both the δβ region [20] and the γ genes [24], using cloned β-globin cDNA probes which detect the β- and δ-globin genes and a cloned Gγ-globin cDNA which detects both γ-globin genes. The fact that a

single probe detects two genes poses a problem - How do we distinguish the two genes and identify them? To do this unequivocally we have made use of globin protein variants.

The β-globin gene was identified using DNA from a patient with Hb (O-Arab). This abnormal haemoglobin has lysine instead of glutamic acid at position 121. In the normal human β-globin gene amino acids 121-122 are coded for by GAATTC - the intragenic EcoRI site. In DNA from Hb (O-Arab) patients, however, this site is lost because of the G ⟶ A transition which gives AAATTC. Indeed, EcoRI digested DNA of an Hb (O-Arab) patient showed a novel globin-gene fragment of a size equal to the sum of two of the four EcoRI fragments. This established that these two fragments contained the β-globin gene [20].

The δ-globin gene was identified in DNA from patients with Hb Lepore [20]. This protein is a fusion product of the N terminal regions of δ-globin with the C terminal regions of β-globin. Likewise, the Hb Lepore gene is a fusion product of the δ- and β-globin genes. Fig. 2 shows the physical map of the normal δ+β locus and that present in the DNA of Hb Lepore patients.

In the case of the γ-globin gene, a similar problem arises: How do we distinguish the Gγ- and Aγ-globin genes? Fortunately, the mutation causing the only amino acid difference between these two proteins results in a PstI site being present in the Aγ-globin gene, but absent in the Gγ gene. This enabled us to identify these two genes. The γ--globin gene map [24] is shown in Fig. 3.

## DETERMINATION OF THE γ-δ GENE DISTANCE

In our initial studies [20,24] we noted that a 15 kb BamHI fragment hybridized with probes for both β- and γ-globin genes under conditions where no cross hybridization between these two gene sequences occurs. This suggested the possibility that the 15 kb BamHI fragment of the Aγ globin gene in fact terminated at the BamHI site in the 5' region of the δ-globin gene. The alternative possibility would be that there are in fact two different 15 kb BamHI fragments, one containing the 3' regions of the γ-globin gene and one containing the 5' regions of the δ-globin gene. Several lines of evidence now show that the former model is the correct one.

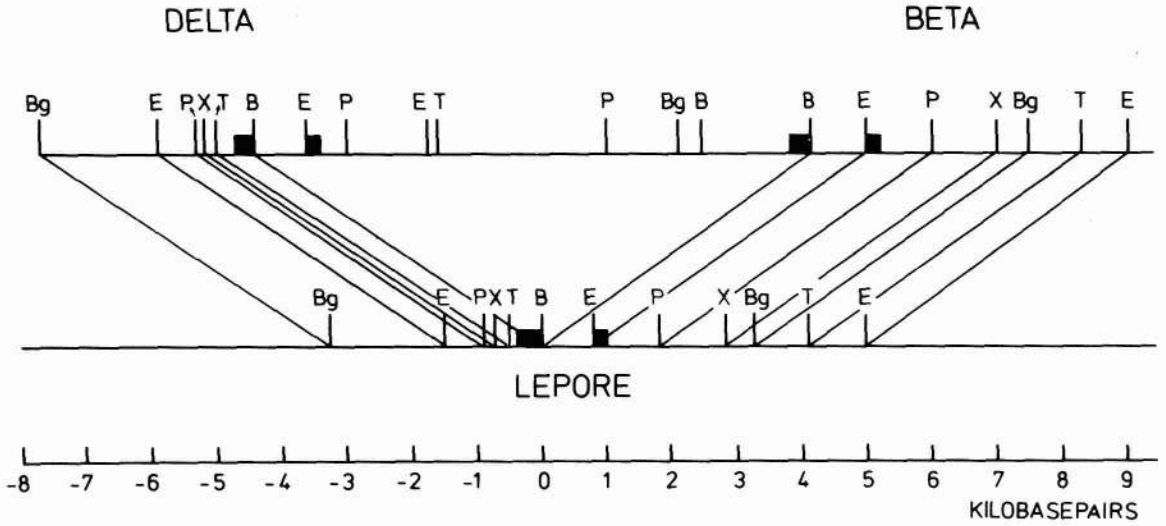




Fig. 2. A physical map of the ß- and δ-globin genes in normal and Hb Lepore DNA. The probable positions of the coding regions of the two globin genes are shown as filled boxes. It should be stressed that the only extragenic cleavage sites which can be detected for a given enzyme by this analysis are those closest to the gene examined. Although the ß- and δ-genes are presented as being composed of two coding segments in each case, the possibility that these segments are further split cannot be excluded from these data. BamHI, B; BglII, Bg; EcoRI, E; PstI, P; TaqYI, T; XbaI, X. Reproduced from ref. 20 with permission.

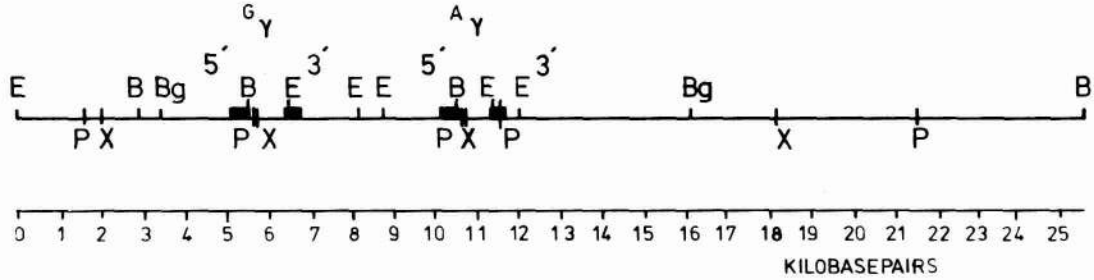


Fig. 3. A physical map of the γ-globin genes. The general comments in Fig. 2 apply here also. EcoRI, E; BamHI, B; BglII, Bg; PstI, P; XbaI, X.

1. BamHI-digested DNA has been fractionated by RPC-5 chromatography (which does not separate DNA primarily on the basis of molecular weight [25]) and then analysed by Southern blotting. The 15 kb 'bands' produced by hybridization with both β and γ probes are found in the same fraction, while all the other γ- and β BamHI fragments found partition differently over the column.

2. Both BclI and BglII cleave at the same position 5.4 kb to the 3'-side of the Aγ globin gene BamHI site. BclI and BglII also cut close to the δ-globin gene (2.1 kb and 3 kb upstream, respectively). If we perform a partial BclI or BglII digest or a limited BamHI digest, in both digests we detect a partial fragment of 9.2 kb which hybridizes with probes for the 5' regions of the δ-globin gene and which disappears in a limit BclI or BglII digest (BglII shows an additional partial of 4.9 kb, indicating a third BglII site between the Aγ and δ-globin genes). These two partials are of the size expected if the cleavage site closest to the 3'-side of γ has been cleaved, but that the respective sites to the 5'-side of the δ-gene are uncleaved. This approach of course selects these partials because a δ probe is used.

3. The 15 kb BamHI -globin gene fragment is cleaved by HpaI to give a 1.3 kb 5' δ-globin gene fragment. The corresponding 15 kb γ-globin gene fragment is trimmed approximately the same amount to give a 14 kb 3' Aγ-globin gene double digest fragment. No other HpaI sites are detected on these γ and δ BamHI 'fragments' in partial digests. In addition, neither KpnI nor BstEII cleave the 15 kb BamHI 'fragments' as measured with either γ or β hybridization probes. Thus either there is a single BamHI fragment linking the γ- and δ-globin genes, or alternatively - but less likely - there are two BamHI fragments, one containing the γ-gene region, the other containing the δ-gene, which contain BclI, BglII and HpaI sites at the same respective positions in both fragments.

4. KpnI cleaved human DNA contains a 46 kb fragment which hybridizes with probes for both the γ- and β-globin genes. The size of this fragment was estimated in 0.5% and 0.7% agarose gels using internal phage lambda DNA and EcoRI-cleaved DNA markers. A KpnI site close to the β-globin gene has been mapped by double digestion with BamHI, HpaI, HindIII or EcoRI to be 3.8 kb to the 3'-side of the end of the β-globin gene. In addition, we

have shown that KpnI does not cleave any of the XbaI, BamHI or EcoRI fragments in the γ, δ, β region except those fragments to the 3'-side of β described above. Assuming that the 15 kb BamHI fragment does link the γ- and δ-genes, the minimal length of this KpnI fragment possible on a hypothetical linkage map which is consistent with the lack of cleavage of the 5' EcoRI fragment of the Gγ--globin gene is 37 kb. The objection to this linkage model (see above) would, therefore, be that two tandem 15 kb BamHI fragments exist, one containing the 5' region of the δ-globin gene and one containing the 3' region of the γ-globin gene. In this case, however, this minimal distance becomed 53 kb (37 + 15 kb). This size is significantly larger than the size of the fragment (46 kb) estimated from the gel with the internal markers.

Taken together these results show that the best measure of the Aγ- to δ-globin gene distance is 13.5 kb although final proof of this point requires cloning of this region as a recombinant DNA. Fig. 4 shows the linkage map of the γδβ locus derived from our blotting data.

This map shows:

a) All four globin genes are transcribed from the same DNA strand.

b) The Gγ-globin gene is 3500 bp to the 5'-side of the Aγ-globin gene; the Aγ gene is 13500 bp to the 5'-side of the δ-globin gene; the δ-globin gene is about 6000 bp to the 5'-side of the β-globin gene.

c) All genes contain the large intron, previously found for the mouse and the rabbit β-globin genes, at the same position within the resolution of the Southern blotting analysis, i.e. between the codons for amino acids 101-120. The structure of the δ-β-gene locus has also been elucidated by Mears et al. [26] and Lawn et al. [27]. In the latter case [27], the linkage of the δ- and β-globin genes has also been demonstrated.

## THE STRUCTURE OF β-THALASSAEMIC GLOBIN GENES: δβ°-THALASSAEMIA

δβ°-Thalassaemia is a rare condition in which the β- and δ-globin chains are completely absent. From cDNA titration-hybridization experiments it has been concluded that at least partial deletion of the β- or δ-globin genes has occurred, although the extent of the deletion could not be defined.

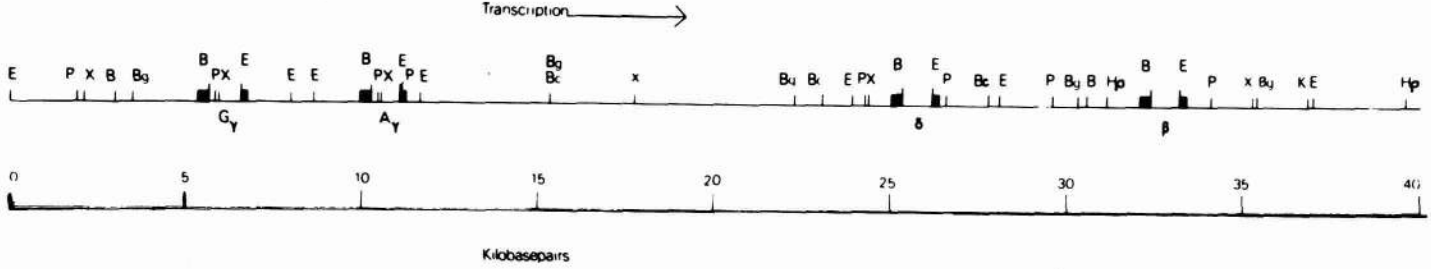


Fig. 4. A physical map of the normal human γδβ locus. See Fig. 2 for general comments. BamHI, B; BclI, Bc; BglII, Bg; EcoRI, E; PstI, P; XbaI, X; HpaI, Hp; KpnI, K.

Recently we have constructed a physical map of the 'δβ°-thalassaemia' globin gene. Two (sibling) homozygous δβ°-thalassaemia patients (Italian) and two unrelated δβ° heterozygotes (one Greek and one Italian) have been examined and shown to have the same deletion. This map (Fig. 5) shows that a large deletion of DNA has occurred to give the δβ°-thalassaemia genotype. The 5' break point of the deletion is in the δ-globin gene. The δ-globin gene up to the intragenic BamHI site in the second exon (coding for amino acids 31-104) is present. The deletion has occurred somewhere between this position and a HindII site which is present close to the 3'-end of the large intron of the δ-globin gene and which is absent in the corresponding gene in δβ°-thalassaemia patients. The β-globin gene seems to be entirely deleted in δβ°-thalassaemia: we have not yet identified the 3' break point of the deletion. Other very faint bands are seen in δβ°-thalassaemia DNA, but these probably derive from cross-hybridization to other globin genes such as ε. The regions containing the γ-globin genes show the normal γ-globin gene fragments already described [24] up to and including the 15 kb BamHI fragment which links the A- and δ-globin genes. This shows that no gross deletions have occurred in the foetal globin gene region in δβ°-thalassaemia. The same structure for the δβ°-thalssaemia gene has recently been deduced independently by T.Maniatis and his colleagues (see this Volume).

## β-THALASSAEMIA: A SECOND CASE WHERE AN EXONIC DELETION HAS OCCURRED

Blotting hybridizations show that in the majority of cases of $\beta^{\circ}$- or $\beta^{+}$-thalassaemia gross deletions of the DNA regions in or around the β-globin gene have not occurred. In PstI (Fig. 6), EcoRI or XbaI digests no differences in the fragment pattern can be seen between normal and β-thalassaemic DNA. In one exceptional case (No. 11 in Fig. 6) a deletion of about 600 bp in the β-globin gene region has occurred to give an abnormal PstI band. This clinically homozygous patient is apparently a compound heterozygote for two forms of β°-thalassaemia: the common type where no deletion can be seen and the deletion form described above.

We have mapped the deletion in this DNA by performing the relevant double digests and comparing

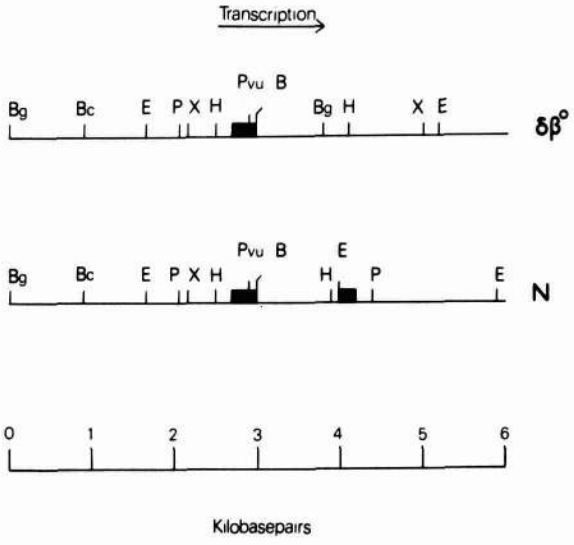


Fig. 5. A physical map of the δ-globin gene region in DNA from a patient homozygous for δβ°-thalassaemia. The DNA was isolated from a lymphocyte cell line, digested with the restriction enzymes indicated in single and in various double digests. The map is based upon the following fragments. BamHI: + BglII, 3.0 kb; + BclI, 2.1 kb; + EcoRI, 1.35 kb; + PstI, 0.95 kb; + XbaI, 0.85 kb; + HindII+III, 0.5 kb. BglII: + EcoRI, 2.1 kb; + PstI, 1.7 kb; + XbaI, 1.65 kb; + HindII+III, 1.3 kb. PvuII + XbaI, 0.8 kb. BglII, 3.8 kb. EcoRI, 3.5 kb. XbaI, 2.9 kb. HindII+III, 1.6 kb. In normal DNA, the δ-derived BamHI double digest fragments are as mentioned above. In addition, the following fragments were used to construct this map: EcoRI, 2.3 kb and 1.9 kb; HindII+III, 1.4 kb; EcoRI + HindII+III, 1.4 kb; PstI, 2.3 kb; PvuII + XbaI, 0.9 kb. BamHI, B; BclI, Bc; BglII, Bg; EcoRI, E; HindII+III, H; PstI, P; PvuII, Pvu; XbaI, X.

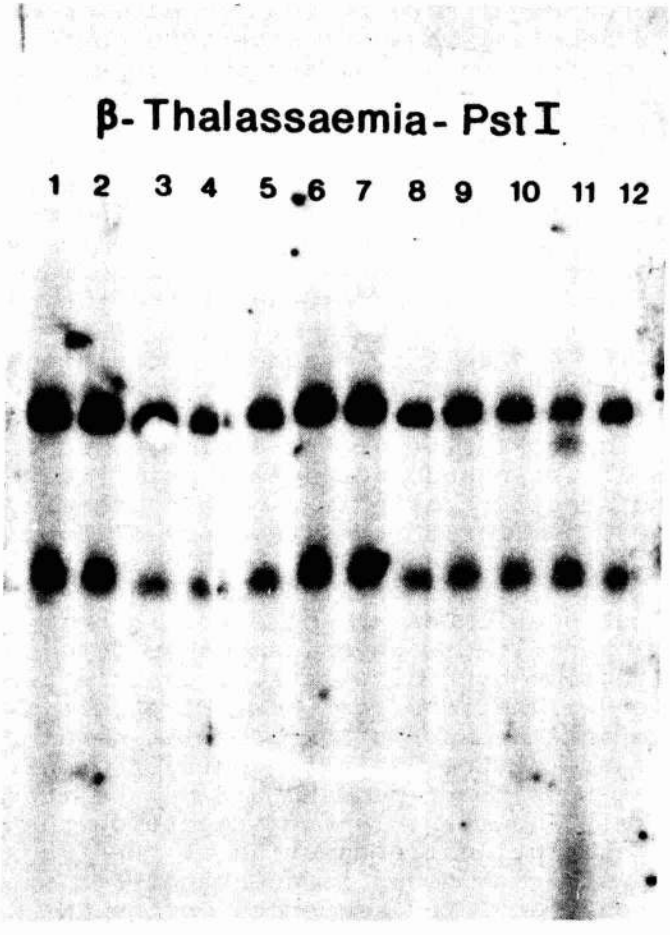


Fig. 6. β- and δ-globin gene fragments in digests of DNA from patients homogyzous for β-thalassaemia. DNA was isolated from β-thalassaemia as described [19] and digested to completion with PstI. The samples were analysed for the β-δ-globin genes as described [20]. Patient 5 is described by Comi et al. [22]: patient 11 in ref. 21. Patients 2, 3 and 10 have been diagnosed as $\beta^{\circ}$-thalassaemics (Southern Italian; Ottolenghi, S., personal communication) and the remainder as homozygous $\beta^{+}$-thalassaemics. 1, 4, 8 and 12 show control normal DNA (derived from a placenta from a Dutch individual).

the fragments obtained with the pattern obtained in normal DNA (see Fig. 7).

These data show that the 600 bp deletion lies between the BamHI site in the second exon of the β-globin gene and the PstI site 1000 bp to the 3'-side of the end of the β-globin gene. Two convincing lines of evidence suggest that the third exon (containing the sequences coding for amino acids 105-146 plus the 3' non-translated region of the mRNA) has been deleted in this DNA.

1. The intragenic EcoRI site (amino acids 121-122) is absent in this β-thalassaemic gene; this results in the presence of an abnormal β-globin EcoRI fragment of 9 kb instead of the two normal fragments of 6 kb and 3.5 kb.

2. The 4.4 kb PstI fragment containing this abnormal globin gene only hybridizes with probes containing the regions to the 5'-side of the intragenic EcoRI site of the β-globin gene. Probes for the 3' regions do not detect this fragment.

The structure of this β-globin gene is, therefore, similar to the structure of the δ-globin gene in δβ°-thalassaemia. In both cases the 3' terminal exon, together with part of the intron and 3' extragenic DNA are missing; in both cases, transcripts of these genes cannot be detected in cDNA titration hybridizations. There are two possible explanations for this. First, it is possible that transcription of split genes requires the presence of the 3' (extragenic) regions. In this case these part-genes could not be transcribed. More likely, however, is the suggestion that the post-transcriptional processing of the transcripts of these abnormal genes is aberrant. For example, the 3' non-translated sequences on the RNA which are required for polyadenylation are deleted in these DNAs. Since polyadenylation seems to precede splicing in both globin [28] and adenovirus [29], it is possible that these hypothetical transcripts could not be spliced. In any case, it is unlikely that the large intron could be excised since the 3' intron-exon junction has certainly been deleted in δβ°-thalssaemia and probably also been deleted in this type of β°-thalassaemia. The net result of these processing defects might be the intranuclear degradation of the RNA. This would account for the low level of β-globin RNA sequences in the cell in this form of β°-thalassaemia and δ-globin RNA sequences in δβ°-thalassaemia.

One final word of caution is relevant. It is

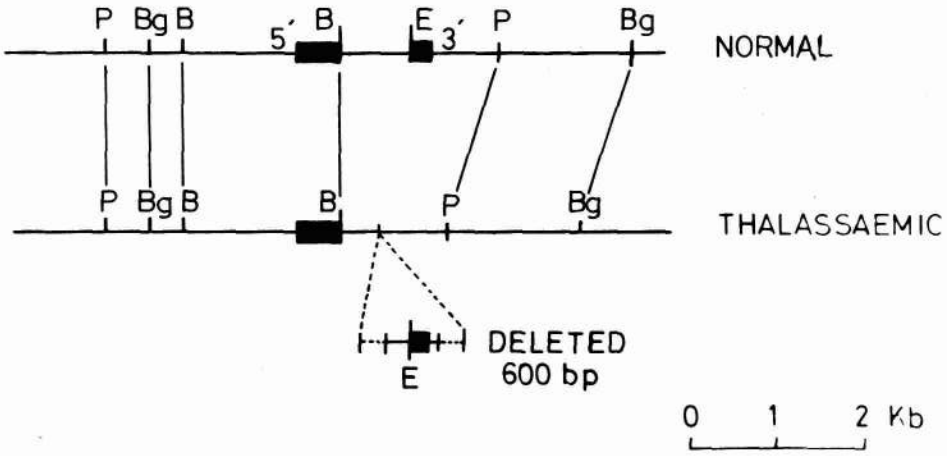


Fig. 7. A physical map of the ß-globin gene region in a normal individual and a patient with ß°-thalassaemia. The map shows a 600 bp deletion centered around the ß-globin intragenic EcoRI site which is missing in this ß°-thalassaemia gene. The 600 bp deletion is arbitrarily drawn centered on this EcoRI site. Also shown are the possible limites of the deletion which stretch 600 bp either side of this site. EcoRI, E; PstI, P; BamHI, B; BglII, Bg.

not established that the deletions which we have characterized here are the primary events which generated the two respective forms of thalassaemia. It is also possible that the initial lesion which produces the thalassaemic genotype was a point mutation or small deletion. A subsequent deletion could occur in an already defective gene with no further phenotypic consequences. Since the three cases of $\delta\beta^{\circ}$-thalssaemia described here all show identical deletions and since more recently others have found similar types of $\delta\beta^{\circ}$-thalssaemia with a deletion like that described above (Weatherall, D., personal communication), we consider this unlikely.

It is not yet clear what the lesion is in the other forms of β-thalassaemia where no deletions have been detected by Southern blotting. It is possible that different genetic defects have caused various forms of β-thalassaemia; alternatively, they may all turn out to be less extensive deletions of the type described above. Molecular cloning of these thalassaemic globin genes will provide the material with which to test this. In turn, the phenotypic analysis of these cloned thalassaemic genes can be performed in systems such as the $TK^{-}$ mouse L-cell system to elucidate the nature of the defect at a molecular level.

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