

Filipino β^0 thalassaemia: a high Hb A₂ β^0 thalassaemia resulting from a large deletion of the 5' β globin gene region

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Abstract

A large novel deletional β^0 thalassaemia mutation associated with unusually high levels of haemoglobin (Hb) A₂ in heterozygotes is described in two unrelated subjects of Filipino background. The deletion was characterised by DNA mapping including pulsed field gel electrophoresis. Filipino β^0 thalassaemia extends for approximately 45 kb beginning approximately 1.5 kb 3' to the δ globin gene. It is the largest deletion to date which gives rise to the β^0 thalassaemia phenotype. This mutation, similar to previously described deletional β^0 thalassaemias associated with high Hb A₂, removes sequences 5' to the β globin gene promoter and emphasises the functional importance of the 5' β globin region in eliciting the unusually high level of Hb A₂. This example also suggests that it is the 3' sequences which are transposed rather than the actual deletion size which are significant in the raised fetal haemoglobin (Hb F) found with some of the thalassaemias.

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The genes on the human β globin gene cluster are arranged in their order of temporal expression during development, 5'- ϵ -G γ -A γ - δ - β -3', on chromosome 11p15. In normal adults the β globin gene encodes the major adult haemoglobin, Hb A ($\alpha_2\delta_2$), which accounts for more than 95% of normal haemoglobin, while the δ globin gene produces the minor haemoglobin, Hb A₂ ($\alpha_2\beta_2$), which is usually less than 3.2% in amount.^{1,2} In heterozygous β thalassaemia, the Hb A₂ is raised approximately two fold, but it is rarely above 6.5%.¹⁻³ Unusually high levels of Hb A₂ have been observed in β thalassaemia heterozygotes with deletional defects that involve the 5' segment of the β globin gene and upstream promoter sequences, but leave the δ globin gene intact.⁴⁻¹⁴

A new, large deletion was detected in two unrelated subjects of Filipino background with the phenotype of high Hb A₂ β^0 thalassaemia. This deletion started 5' to the β globin gene, and 3' to the previously described Dutch β^0 thalassaemia⁶ and extended for approximately 45 kb. The 5' breakpoint of the deletion was localised to an approximately 600 base pair (bp) region 3' to the δ globin gene by restriction endonuclease mapping. The 3' extent of the deletion was not able to be mapped using existing probes 3' to the β globin gene. The size of the deletion (~45 kb) was estimated by pulsed field gel electrophoresis. Previous dele-

tions of similar size are associated with increased fetal haemoglobin (Hb F) and are found in ($\delta\beta$)⁰ thalassaemia or hereditary persistence of fetal haemoglobin (HPFH).¹⁵⁻²⁰ However, in the current deletion the Hb F levels were not remarkably raised. These data show that DNA sequences surrounding this deletion breakpoint have more relevance to the Hb F phenotype than the actual deletion size and confirm the association between loss of the 5' β globin gene specific promoter region and a very high level of Hb A₂.

Materials and methods

PATIENT SAMPLES

Whole blood samples were collected with heparin or EDTA as anticoagulants from a 35 year old female (NP) detected on a routine screen and a 30 year old female (LD) as part of routine antenatal tests. Both subjects were Filipino immigrants.

HAEMOGLOBIN ANALYSES

Haematological data were obtained from freshly collected blood samples using an automated cell counter. Hb electrophoresis was performed at pH 8.9 on cellulose acetate strips in a Tris-EDTA-borate buffer. Hb A₂ was quantitated by elution after electrophoresis on cellulose acetate at pH 8.9.²¹ Hb F was quantitated by a modified Betke method.²²

DNA ANALYSIS

Genomic DNA was prepared from peripheral blood buffy coats by phenol-chloroform extraction,²³ digested with restriction endonucleases, separated by electrophoresis through 0.8% agarose gels, and transferred by Southern blotting.²³ DNA probes were labelled with ³²P dCTP. Membranes were hybridised overnight at 65°C with 10⁶ cpm/ml ³²P labelled probe and washed at 65°C to a stringency of 0.1 × SSC and 0.1% SDS for one hour, followed by autoradiography. Three probes from the β globin gene cluster were used: (1) 2.3 kb *Pst*I δ ; (2) 4.4 kb *Pst*I β ; (3) 2 kb *Bgl*II/*Xba*I fragment of the $\psi\beta$ gene. DNA probes from 3' to the β globin gene cluster used included: (1) pRKR29, a 1.2 kb *Eco*RI fragment approximately 18 kb 3' to β^{24} ; (2) the 3D probe, a 1.0 kb *Bam*HI/*Eco*RI genomic fragment from the 3' end of Negro HPFH-1¹⁶; (3) the H500 probe, a 0.5 kb *Hin*-dIII unique fragment approximately 25 kb 5' to the 3' end of Negro HPFH type 1 obtained

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from the p3'N10R²⁵; (4) the 3'IH probe, a 0.75 kb *HinfI-EcoRI* fragment from a plasmid containing a 1.1 kb *BamHI-BglII* genomic fragment obtained from the 3' end of an Indian HPFH deletion, approximately 30 kb 3' to the β globin gene¹⁷; and the 3'VH probe, a 873 bp *SacI-BamHI* fragment from the 3' breakpoint of Vietnamese HPFH (Motum *et al*, in preparation). The positions of these probes are illustrated in fig 1B.

PULSED FIELD GEL ELECTROPHORESIS

DNA for pulsed field gel electrophoresis was prepared from fresh lymphoblastoid cells from a Filipino β^0 thalassaemia heterozygote (NP) and a normal subject. DNA was isolated in agarose blocks and digested with the restriction enzyme *SfiI*.²⁷ Specimens were then electrophoresed on the Biorad CHEF-DRII sys-

tem (Biorad, Richmond CA) for 24 hours at 200 V with pulse times ranging from 20 to 50 seconds. DNA was transferred onto nylon filters (Hybond, Amersham) and hybridised with the *PstI* δ , *PstI* β , and the 3'VH probes labelled by the random hexamer primer method.²⁸

Results

HAEMATOTOLOGY

Both subjects had a microcytic hypochromic anaemia and haematological parameters consistent with β thalassaemia trait but had unusually high levels of Hb A₂ of 7.7 and 7.5% (table 1). The Hb F level was normal in LD and raised in NP at 4.0%. The latter is only slightly higher than that normally seen in heterozygous β thalassaemia.²⁹

RESTRICTION ENDONUCLEASE ANALYSIS

DNA from NP and LD was digested with various restriction enzymes and hybridised to the $\psi\beta$, *PstI* δ , and *PstI* β probes from the β globin gene cluster to define the 5' breakpoint of the deletion. Subsequently these digests were hybridised to the pRK29, 3'VH, 3'IH, H500, and 3D probes to characterise the 3' breakpoint. In addition to the normal bands, restriction fragments of abnormal size were also present with *PstI* δ probe (table 2, fig 2). Since NP and LD are heterozygotes, the normal bands are derived from the wild type β globin allele, while the abnormal fragments are from the mutant allele. Hybridisation with the $\psi\beta$ and *PstI* β probes did not show any abnormal bands although the intensity of hybridisation for PD with the *PstI* β probe was significantly reduced. Normal bands were detected with all the 3' globin cluster probes. Reduced intensity of hybridisation was shown with the pRK29, 3'VH, and 3'IH probes. Hybridisation with the H500 and 3D probes was entirely normal. Using this information the 5' breakpoint was localised between the *AccI* site (present) and its nearby 3' *EcoRI* site (deleted) downstream from the δ globin gene (fig 2). The 3' breakpoint could not be mapped precisely on restriction enzyme analysis using the probes available. However, it extended beyond the 3'IH probe which was deleted but did not

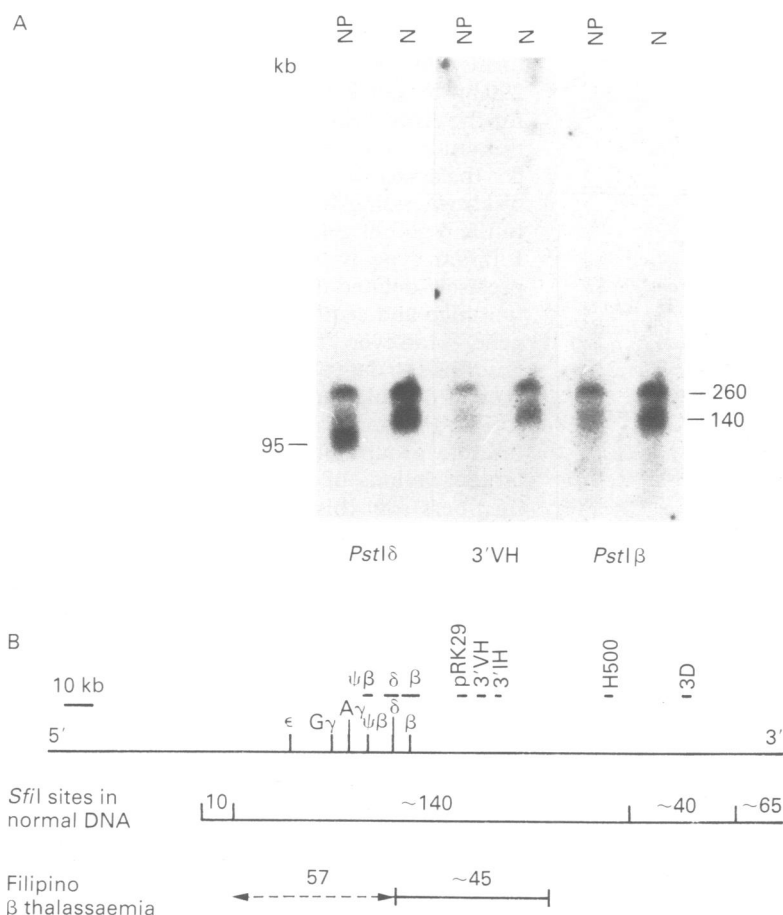


Figure 1 (A) Pulsed field gel electrophoresis of *SfiI* digested DNA in a normal (N) and a Filipino β^0 thalassaemia heterozygote (NP). There was a 260 kb band in all samples which probably represents a partial band fragment $\sim 140 + 40 + 65$.²⁶ In addition to the normal ~ 140 kb band there was a novel 95 kb band detected with the *PstI* δ probe, but no abnormal bands were detected with either the *PstI* β or the 3'VH probes. (B) The position of the probes from chromosome 11 used in the restriction mapping analysis are indicated: ($\psi\beta$); (δ *PstI* δ); (β) *PstI* β ; pRK29; 3'VH; 3'IH; H500; and 3D (see text for details). The normal *SfiI* sites in the β globin gene cluster region of chromosome 11²⁶ and the *SfiI* pulse field map of the ~ 45 kb deletion of Filipino β^0 thalassaemia are illustrated.

Table 1 Haematological parameters in heterozygous Filipino β^0 thalassaemia.

Subject	Sex/age	Hb (g/dl) (12.5-16.5)	MCV (fl) (76-96)	MCH (pg) (27-31)	Hb A ₂ (%) 1.5-3.7	Hb F (%) (<1.0)
NP	F/35	12.7	72	22	7.7	4.0
LD	F/30	10.8	67	23	7.5	1.0

The normal ranges are indicated below the parameter in parentheses.

Table 2 Comparison of restriction fragment lengths in normal subjects and heterozygous Filipino β^0 thalassaemia.

Probe	Enzyme	DNA fragment size (kb)	
		Normal DNA	NP & LD DNA*
<i>PstI</i> δ	<i>AccI</i>	3.5, 2.4	3.5, 2.4
	<i>AvaII</i>	4.4	4.4, <u>4.0</u>
	<i>BamHI</i>	15.4, 4.7	15.4, 4.7, <u>9.8</u>
	<i>BglI</i>	~ 30	~ 30 , <u>16.5</u>
	<i>BglIII</i>	8.2, 5.0	8.6, 8.2, <u>5.0</u>
	<i>EcoRI</i>	2.3, 1.8	<u>2.3</u> , 1.8, <u>8.6</u>
	<i>EcoRV</i>	15.5	15.5, <u>8.7</u>
	<i>HpaI</i>	7.5, 2.0, 1.5	7.5, 2.0, 1.5
	<i>HindIII</i>	17.5, 7.8	17.5, 7.8, <u>16.0</u>
	<i>NcoI</i>	8.9, 4.0	8.9, 4.0, <u>7.4</u>
	<i>PvuII</i>	12.8	12.8, <u>16.0</u>
<i>SacI</i>	16.4	16.4, <u>18.0</u>	
<i>XbaI</i>	11.1	11.1, <u>4.3</u>	

* Abnormal bands in the probands are underlined.

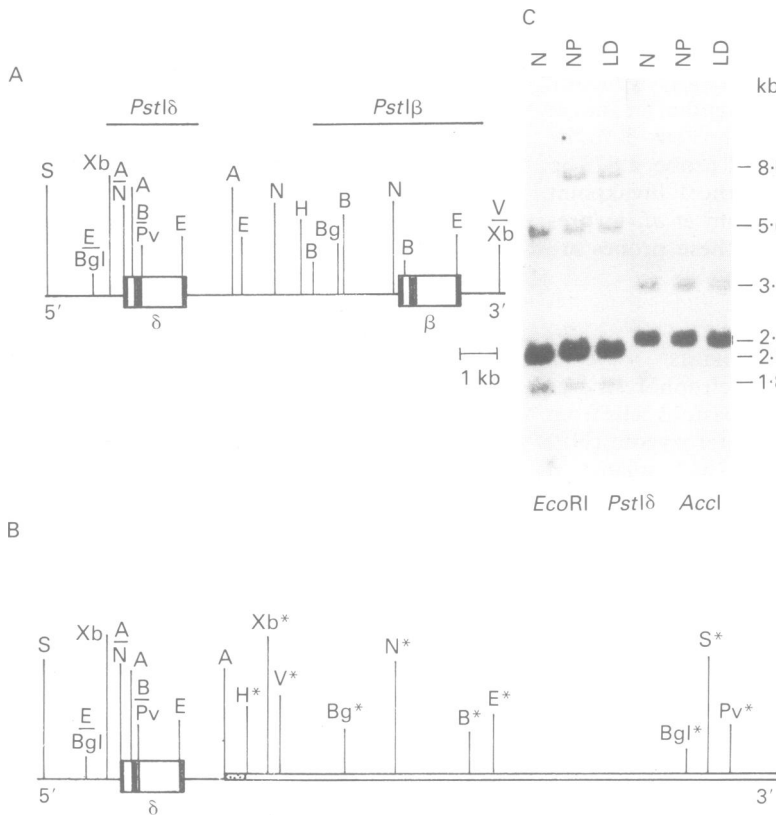


Figure 2 Restriction map of the normal (A) and abnormal (B) chromosomes surrounding the 5' breakpoint of Filipino β thalassaemia using the *PstI* δ and *PstI* β probes. Restriction enzyme sites are: (N) *NcoI*, (B) *BamHI*; (X) *XmnI*; (Xb) *XbaI* (Bg) *BglII*; (H) *HindIII*; (Bgl) *BglI*; (E) *EcoRI*; (S) *SacI*; (V) *EcoRV*; (Pv) *PvuII*; (A) *AccI*. The normal restriction map was derived from the GenBank databank.³⁰ In the Filipino β^0 thalassaemia allele the *AccI* site at 57450 was present and the *EcoRI* site at 58035 was absent (both detected with the *PstI* δ probe). The approximately 600 bp region of uncertainty is indicated by the hatched area. The restriction sites 3' to the breakpoint in the new DNA are indicated (*). (C) Autoradiograph showing the bands detected with the *PstI* δ probe in a normal subject (N) and the subjects heterozygous for Filipino β^0 thalassaemia (NP and LD) following digestion with the restriction enzymes *EcoRI* and *AccI*.

involve the H500 probe which was present (fig 1).

SIZE OF THE DELETION ON PFGE

DNA from NP was digested with *SfiI* and hybridised consecutively with probes *PstI* δ , *PstI* β , and 3'VH. In addition to the normal ~140 kb band there was a new 95 kb band detected with the *PstI* δ probe (fig 1). There were no abnormal bands detected with the *PstI* β or 3'VH probes consistent with deletion of these loci. These data indicate that the deletion is ~45 kb in size.

Discussion

There are currently over 100 mutations associated with β thalassaemia.³¹ The majority involve single base substitutions producing transcription, RNA modification, and translation mutants. There are only eight deletional forms of β thalassaemia.^{4-14,32} These range from 290 bp to 12.6 kb in size, and are rare except for the Asian Indian deletion type³² (fig 3). In this study we have defined a new Filipino type β^0 thalassaemia deletion of approximately 45 kb extending from a region 1.1 to 1.7 kb 3' to the δ globin gene. The 3' breakpoint of the Filipino type β^0 thalassaemia could not be precisely defined owing to the limited restriction map and sequence data 3' to the β globin gene. However, the deletion is the largest described to date which still retains the phenotype of β^0 thalassaemia.

The Filipino β^0 thalassaemia defect joins a discrete groups of thalassaemias which have large deletions of 30 to 50 kb in size. Other members of this group include German $G\gamma(A\gamma\delta\beta)^0$ thalassaemia,¹⁹ Belgian $G\gamma(A\gamma\delta\beta)^0$ thalassaemia,²⁰ Turkish $G\gamma(A\gamma\delta\beta)^0$ thalassaemia,¹⁶ Black $G\gamma(A\gamma\delta\beta)^0$ thalassaemia,¹⁵ Indian HPFH (HPFH-3),¹⁷ Italian HPFH (HPFH-4),¹⁸ and Vietnamese HPFH (Motum *et al*, in preparation). All except the Filipino β^0 thalassaemia are characterised by significantly raised Hb F levels. Although some increase in Hb F was observed in one of the two affected subjects, it was only a modest rise (4.0%) and lower than those usually found in heterozygotes with deletion HPFH and $G\gamma(A\gamma\delta\beta)^0$ thalassaemia (ranges in Hb F of 10 to 30% and 4 to 19% respectively). These observations would suggest that the functional nature of the sequences transposed to the β globin gene cluster rather than deletion size is an important determinant of Hb F phenotype.

β thalassaemia heterozygotes with Filipino β^0 thalassaemia have unusually high levels of Hb A₂ (mean 7.6%) similar to other examples of deletional β thalassaemia which remove the 5' β globin gene and its associated promoter sequences (fig 3). Family studies in heterozygotes for β thalassaemia and a δ chain variant have shown that the increased Hb A₂ in β thalassaemia is derived from δ chains in *cis* and *trans* to the β thalassaemia gene.^{33,34} However, a more recent study has shown that the excess Hb A₂ is derived from the δ gene in *cis* to the deletional β thalassaemia allele.³⁵ The common

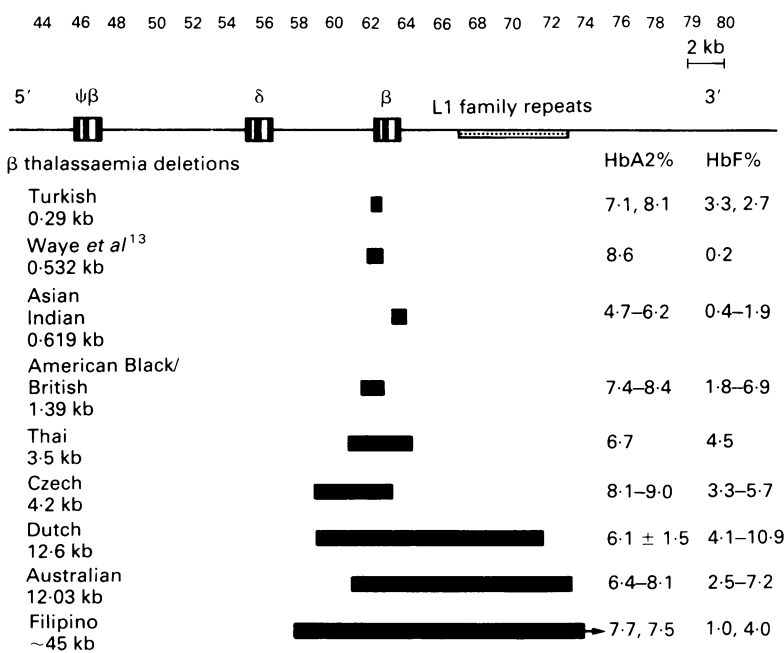


Figure 3 Summary of the β thalassaemia deletions with the corresponding levels of Hb A₂ and Hb F in heterozygotes.^{4-14,32} The numbers correspond to the GenBank coordinates³⁰ and the sizes of the deletions are in kilobases (kb).

molecular feature of the high Hb A₂ producing deletions is their 5' breakpoint regions which lie upstream from the β mRNA cap site.

Thus the β globin gene promoter TATA, CCAAT, and CACCC boxes which are involved in regulation of transcription are deleted.^{36,37} The mechanism(s) by which sequences in the 5' β globin gene might influence the δ and γ globin gene expression have not been fully elucidated. Deletions removing the β globin gene promoter regulatory sequences could alter competition for limiting transcription factors and make the latter more available to the δ globin promoter to increase transcription of the δ globin gene. If this were the mechanism for the raised Hb A₂ it should affect both the δ gene in *cis* and in *trans* to the β thalassaemia allele.

Alternatively it has been suggested that the transcription of the δ globin gene promoter could be influenced by loss of the 5' β promoter, if both are affected by the same 3' β enhancer.⁵ Enhancers have been identified downstream from the A γ ³⁸ and β globin genes³⁹ and on either side of the β globin gene cluster.⁴⁰ In the latter may be found the locus control regions (LCRs) which consist of five DNase I hypersensitive sites 5' to the ϵ globin gene and one site 21.3 kb (HS VI) 3' to the β globin gene. Transgenic and transfection experiments have confirmed the critical role played by the LCR in globin gene regulation.^{40,41} The LCR is thought to represent one mechanism by which deletions of the β globin cluster can have *cis* acting effects over considerable distances.⁴⁰ In the deletional β^0 thalassaemias the absence of a functional β globin gene promoter might permit an enhancer such as the LCR to interact with the δ globin gene in *cis*. Thus the 3' breakpoint in itself does not appear to play a role in the generation of the high Hb A₂ β thalassaemia phenotype, but it may influence the degree of γ chain compensation.

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