Physical mapping of the globin gene deletion in hereditary persistence of foetal haemoglobin (HPFH)

R.Bernards

Section for Medical Enzymology and Molecular Biology, Laboratory of Biochemistry, University of Amsterdam, Jan Swammerdam Institute, P.O. Box 60.000, 1005 GA Amsterdam, Netherlands

R.A.Flavell

Laboratory of Gene Structure and Expression, National Institute for Medical Research, Mill Hill, London NW7 1AA, UK

ABSTRACT

        We have mapped the globin gene region in the DNA of two HPFH patients.   In a patient homozygous for the $G\gamma A\gamma$ type of HPFH at least 24 kb of DNA in the globin gene region has been deleted to remove most of the $\gamma - \delta$ intergenic region and the δ and β globin genes.   The 5' break point of the deletion is located about 9 kb upstream from the δ globin gene.   The 3' break point has not been precisely located but is at least 7 kb past the β globin gene.   DNA from an individual heterozygous for the Greek ($A\gamma$) type of HPFH, however, shows no detectable deletion in the entire $\gamma\delta\beta$-globin gene region.   HPFH, therefore, appears to occur in different molecular forms.   These results are discussed in terms of a model for the regulation of globin gene expression in man.

INTRODUCTION

        The major haemoglobin (Hb) in adult humans is HbA $(\alpha_2\beta_2)$, which accounts for more than 95% of the total Hb.   The remainder of the Hb consists of the minor adult form HbA_2 $(\alpha_2\delta_2)$ and HbF $(\alpha_2\gamma_2)$; the latter is restricted to a small fraction of the erythroid cells, called F cells.   (See ref. 1 for a review).

        In normal humans, the expression of the γ-globin genes decreases towards the end of the foetal period, while expression of the adult β- and δ-globin genes increases.   In at least two genetic disorders this $\gamma \rightarrow \delta + \beta$ switch is specifically altered.   First, in adults with homozygous hereditary persistence of foetal haemoglobin (HPFH), no adult globins are produced; instead, HbF is present.   The level of γ globin production is sufficiently high that little or no imbalance in the γ/α ratio occurs, and as a result, the symptoms characteristic of the thalassaemias are essentially absent in HPFH.   In HPFH heterozygotes about 15-30% HbF is pancellularly distributed. In the common form of HPFH ($G\gamma A\gamma$-HPFH), found in Black populations, both $G\gamma$ and $A\gamma$ genes are expressed in the adult;   an uncommon form, $G\gamma$-HPFH, has also been described in Blacks.   In the Greek population, HPFH heterozygotes

are found where the Aγ globin gene is mainly expressed. In the second condition, homozygous $\delta\beta^{o}$ thalassaemia, again HbA and HbA$_2$ are lacking in adults and only HbF is found. In this disease, however, the HbF is restricted to about 30-40% of the erythrocytes and there is a net imbalance of α and γ chains which results in a thalassaemia of moderate severity; in $\delta\beta^{o}$ thalassaemia heterozygotes about 5-20% HbF is heterocellularly distributed in the same way. Finally in homozygous β^{o} thalassaemia, only HbF and HbA$_2$ is present. In this case, however, the $\gamma \rightarrow \delta + \beta$ switch is unaffected and hence the level of γ chain production is only slightly elevated when compared with normal non-thalassaemic humans. As a result a profound imbalance of α and $\delta + \gamma$ globin chains occurs, which results in the severe clinical symptoms associated with this disease. The slight elevation of HbF in β-thalassaemia probably results from the increased proliferation, or longer life span of the F cell population also present in normal individuals (Ref. 1).

Early studies using cDNA titration hybridization showed that the δ- and β-globin genes were at least partially deleted in the black (Gγ + Aγ) type of HPFH and $\delta\beta^{o}$ thalassaemia. The fact that the deletion in HPFH appeared to be greater than in $\delta\beta^{o}$ thalassaemia[2-6] suggested that the extent of the deletion was responsible for the altered expression of the γ genes.

The development of methods for the construction of physical maps of restriction enzyme cleavage sites in and around any given gene[7-9] has made it possible to reinvestigate this matter. This approach and genomic cloning have been used to analyse the β-related globin-gene locus in DNA from normal humans[10, 11, 28, 12, 17, 13], and more recently to analyse the globin gene region in Hb Lepore[10], $\delta\beta^{o}$ thalassaemia[6, 14, 15, 17] and γ-β thalassaemia[16].

The DNA from 2 patients with the same type of GγAγ HPFH has been analysed by Fritsch et al.[17] and Tuan et al.[18], and compared by the former authors to the deletion in $\delta\beta^{o}$ thalassaemia[17]. In this paper we characterize a second type of (GγAγ) HPFH with a different deletion to that described and show that no detectable deletion in the $\gamma\delta\beta$ globin gene region has occurred in a heterozygous case of Greek (Aγ) HPFH.

MATERIALS AND METHODS

DNA samples

HPFH DNA was obtained from cultured lymphocytes derived from

patients originally described elsewhere[19, 20] and obtained from the Human Genetic Mutant Cell Repository, Camden, New Jersey 08103, U.S.A. The DNA was prepared as described previously[10].

Blot hybridization studies

The method used was the same as that described[9, 10], except that 1g of dextran sulphate[21] was added to 9 ml of hybridization solution. Hybridization was for 12 h at 65° with a ^{32}P probe (0.5-4 x 10^8 cpm/μg concentration of 1-5 ng/ml. Washing of filters was as described[10]. The probes used were isolated from the pHγG1 or pHβG1 plasmid by Hha I cleavage, agarose gel electrophoresis and extraction of the globin gene fragment by the method of Tabak and Flavell[22]. The 0.5 kb RIH fragment is located 4 kb upstream from the δ-globin gene and is originally derived from a genomic clone (see ref. 17). The RIH probe was recovered in the same way after Eco RI cleavage of the pMB9-RIH subclone, kindly provided by T. Maniatis and his colleagues.

RESULTS

Abnormal globin gene fragments in GγAγ HPFH

To examine the extent of the deletion in HPFH, DNA from cultured lymphocytes derived from a GγAγ-HPFH homozygote was digested with various restriction enzymes, fractionated on agarose gels and blotted onto nitrocellulose filters according to Southern[7]. The filters were then hybridized with probes for either the β- (pHβG1 DNA) or γ- (pHγG1 DNA) globin gene as described in Materials and Methods.

No β-globin specific bands were detected in HPFH DNA (Fig. 1a). Instead, faint bands of 2.6 and 3.7 kb were obtained when the filters were examined after low stringency washes. Washing the filters in low salt removed these bands completely (Fig. 1a) which shows that the β- and δ genes have been entirely deleted. These components have also been seen in DNA of the same patient by Mears et al.[11] and interpreted as β-specific sequences. The low stability of these hybrids and the fact that normal DNA also exhibits the same components in addition to the β and δ components makes it more likely that these DNA fragments are derived from other globin genes (for example the ε and γ genes).

GγAγ HPFH DNA exhibits different γ-globin gene fragments than normal DNA (Fig. 1b). Bam HI digests showed the normal 5.0 and 2.7 kb fragments but gave a 14 kb fragment, containing the 3' regions of the Aγ gene, rather than the 15 kb fragment seen in normal DNA (Fig. 1b). Likewise, Xba I digests showed the normal 5.0 kb and 3.7 kb gene fragments,
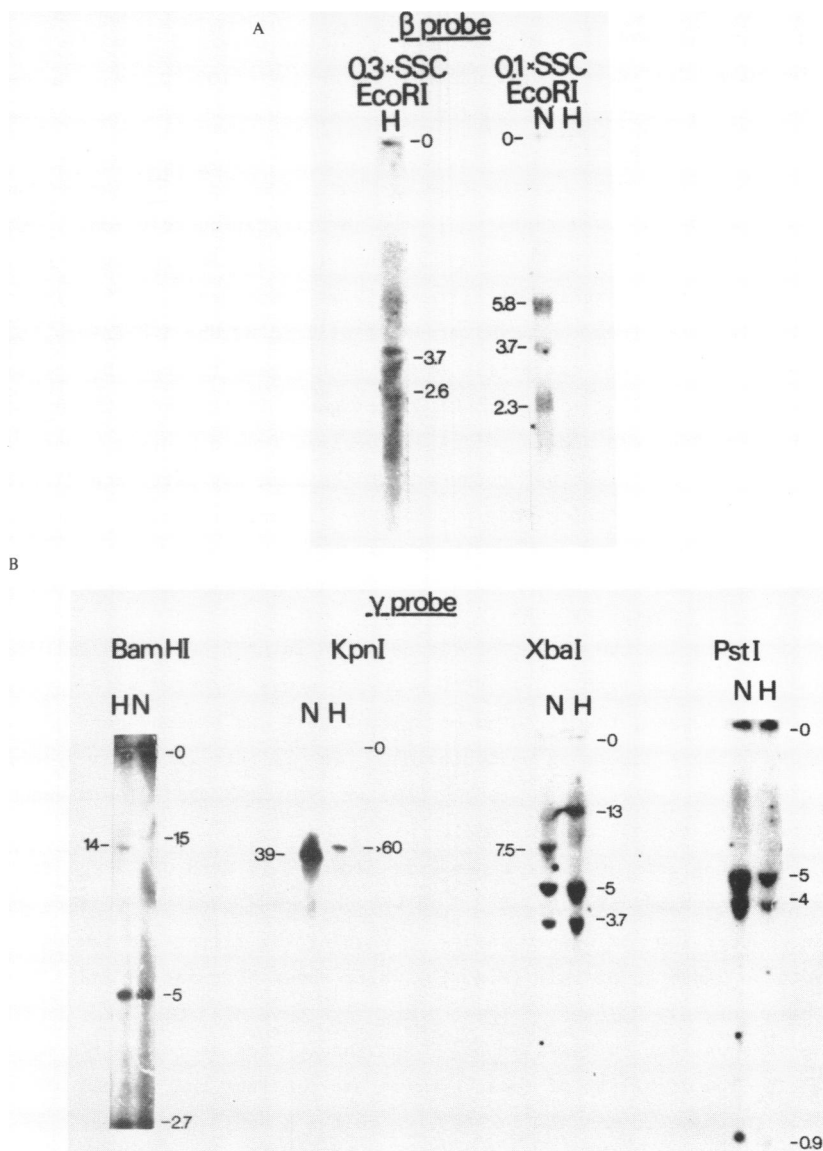
Fig. 1. Abnormal globin-gene fragments in GγAγ HPFH

Normal placental DNA or HPFH DNA was digested with the restriction endonucleases indicated, and the globin-gene fragments detected in blot-hybridizations using a) pHβG1 DNA[10] or b) pHγG1 DNA[12] as hybridization probes. In a) the filters were washed in 0.3 x SSC 0.1% SDS, which permits detection of heterologous ε-β and γ-β hybrids, or 0.1 x SSC 0.1% SDS where only the δ-β and β-β hybrids are seen; in b) only 0.1 x SSC 0.1% SDS washes were used. N is normal DNA and H is HPFH DNA. The numbers refer to the size of the fragments detected in kb.

but a 13 kb 3' Aγ-fragment was seen instead of the 7.5 3' Aγ-fragment.
These data suggest that the HPFH deletion eliminates the Bam HI site in
the δ gene (that is, the 3' terminus of the 15 kb Bam HI-Aγ fragment) and
the Xba I site about 7 kb to the 3' side of the Aγ-gene.   In both cases a
new fragment results.   Similarly, the normal 39 kb Kpn I fragment which
contains the Gγ, Aγ, δ and β genes is missing in HPFH DNA and is instead
replaced by a very large DNA fragment of about 60 kb.   Other γ-gene
digests gave normal patterns.   Thus, Pst I showed the normal 5.0, 4.0 and
0.9 kb fragments (Fig. 1b) and Bgl II the normal 14 kb fragment (not
shown).   This establishes that the Bgl II site 5 kb to the 3' side of the
Aγ gene is present and this is confirmed by the fact that HPFH DNA also
has the normal 6 kb 3' Aγ Bam HI + Bgl II double digest fragment (Fig. 3).
The 5' break point of the deletion would therefore appear to lie between
this 'normal' Bgl II site and the (deleted) Xba site;   that is, in a
region 5 to 6.5 kb in the 3' direction from the Aγ gene.

     Fritsch et al.[17] have shown recently that the 5' break point of 2
apparently unrelated (GγAγ) HPFH patients is located about 4 kb upstream
from the δ-globin gene, in a region to the 3' side of a small Eco RI frag-
ment (called RIH), whereas the deletion discussed above would be 9 kb up-
stream from the δ globin gene.   To exclude that the 5' break point of the
deletion in our HPFH case was at the same site, we hybridized Bam HI and
Xba I digests of HPFH or normal DNA with ^{32}P-labelled RIH probes: the RIH
fragment is derived initially from a cloned δ + β globin gene fragment[28]
(Fig. 2).   While normal DNA gave the same fragments as those described by
Fritsch et al.[17] the RIH probe did not hybridize with the HPFH DNA digests.
We conclude that the RIH region, located 4 kb to the 5' side of the δ-
globin gene, is deleted in this HPFH DNA, as would be expected from the
size of the 3' Aγ Xba I fragment discussed above.

Fine mapping of the 5' break point of the deletion in (GγAγ) HPFH

     To confirm the location of the 5' break point in HPFH DNA, we
analysed double digest of Bam HI in conjunction with various other res-
triction enzymes (Fig. 3).   Bam HI + Xba I digests still show the 14 kb
Xba I fragment in HPFH DNA.   Bam HI, therefore, does not cut the Xba I-3'
Aγ fragment.   Bam HI plus Bcl I double digests show the 6 kb 3' Aγ double
digest fragment in both normal and HPFH DNA (Fig. 3).

     Bgl II cleaves at least 3 times in the DNA region between the Aγ
and δ genes[17, 12].   The position of these sites cannot be determined
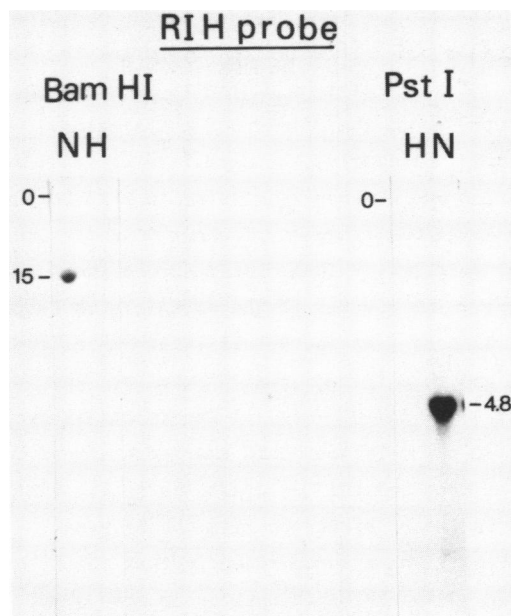directly in total Bam HI + Bgl II double digests.   They can, however, be

Fig. 2. Globin gene fragments detected with the RIH probe.

Normal placental DNA or HPFH DNA was assayed for sequences homologous to ^{32}P-labelled RIH probe[17] in blot-hybridizations. N is normal DNA, H is HPFH DNA. It can be seen that the RIH probe does not form hybrids with HPFH DNA.

visualized in partial Bgl II digests on DNA that has been digested to completion with Bam HI. Using this approach we have previously mapped these Bgl II sites in normal DNA[13]

Fig. 3 shows the partial Bgl II digests on Bam HI cut normal or HPFH DNA. In normal DNA, partials of 10.5 and 8 kb are seen in addition to the uncut 15 kb Aγ–δ fragment and the 6 kb Aγ–gene total digestion product. In HPFH DNA both the 10.5 kb and the 8 kb partials are absent; instead, a 6.7 kb partial digestion product is visible in addition to the 6 kb total digestion product. This shows that a new Bgl II site is located 6.7 kb from the Bam HI site in the Aγ gene. Since this site is not present in normal DNA, the 5' break point of the HPFH deletion must lie in the 0.7 kb region between the 3' Aγ–Bgl II/Bcl I sites (which are present about 4.7 kb to the 3' side of the Aγ globin gene in both DNAs) and the new Bgl II site found only in HPFH DNA.
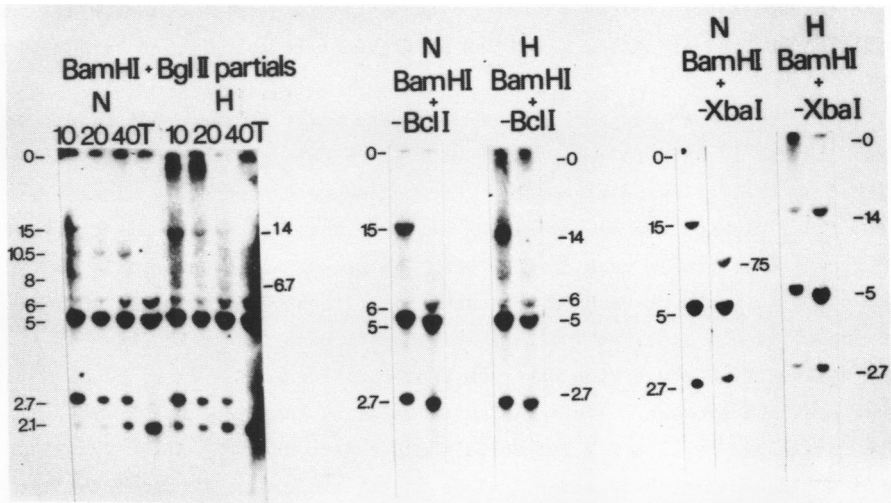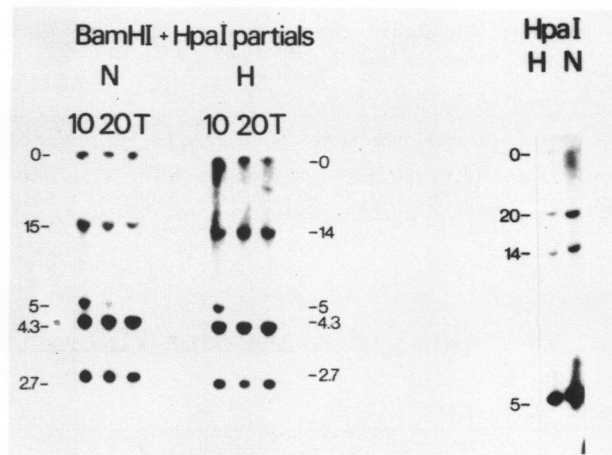
Fig. 3. Detection of globin gene fragments in double digests of HPFH DNA.

Bam HI digested normal, or HPFH DNA was digested with Bgl II for the time period indicated (in minutes) to obtain partial digestion products or for 3h with all the enzymes to obtain limit digests (T). The samples were analysed for γ-globin gene fragments using blot-hybridizations with pHγG1 DNA probes as described in methods. The sizes of the fragments detected is indicated in kb.

The 3' break point of the deletion in HPFH

In Fig. 1 it can be seen that HPFH DNA contains a large Kpn I γ-fragment. This suggests that the 3' break point of the deletion extends

past the Kpn I site to the 3' side of the β-globin gene in normal DNA. In
addition we found that the 14 kb Bam HI fragment is not cleaved by Hpa I
(Fig. 3).

These data suggest that the 3' break point of the deletion extends
past the Bam HI site to the 3' side of the β-globin gene. This is estab-
lished by the following argument. Let us assume that the Bam HI site 3'
from the β globin gene in normal DNA were the same as the Bam HI site found
3' from the Aγ globin gene in HPFH DNA. Since we know that the 14 kb
Bam HI-3' Aγ fragment in HPFH DNA contains at least 6 kb directly adjacent
to the Aγ globin gene, we would expect in this case that 8 kb (14 − 6) of
DNA derived from the region upstream from the 3' Bam HI site of the β globin
gene would be present. This region contains at least one Xba I site, one
Kpn I site and one Hpa I site, so we would expect that the 14 kb Bam HI frag-
ment would contain these sites. This is not the case. We conclude that
the 3' break point lies downstream from the 3' Bam HI-β-globin site and,
therefore, that the deletion in HPFH DNA comprises at least 24 kb. Fig. 4
compares the maps of the globin gene region of this GγAγ HPFH DNA and normal
DNA.

The globin gene region in Greek (Aγ) HPFH

We have performed the same type of blot-hybridization analysis,
described above for the Black (Gγ-Aγ) form of HPFH, with DNA from a Greek
HPFH heterozygote. No difference is detectable, however, when this DNA is
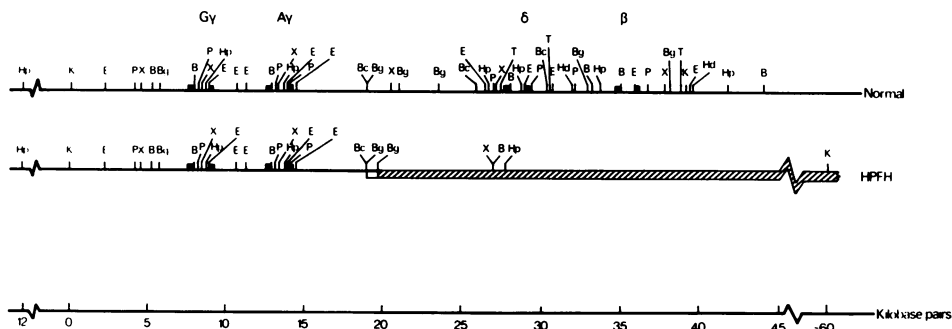

Fig. 4. A physical map of the globin gene region in GγAγ-HPFH-3 DNA

The normal map is derived from refs. 10-13, 17 and 28 and the
HPFH map from the present data.   The hatched area indicates the region
which has replaced the normal γδβ region in HPFH DNA and the unfilled box
indicates the uncertainty of this measurement.   The enzymes used were
Bam HI (B), Bgl II (Bg), Eco RI (E), Hpa I (Hp), Kpn I (K), Taq I (T) and
Xba I (X).

compared with normal DNA using either the β-, γ or RIH probes (Fig. 5). All
fragments detected are the same as those seen in normal DNA (see refs. 10, 11,
12, 17). We conclude that Greek Aγ-HPFH must either result from a mutation
elsewhere in the genome or from a point mutation or small deletion, below
our detection level, in the γδβ globin gene region.

DISCUSSION

        In this paper we describe the mapping of the γδβ-globin gene region
in DNA from a Black (Gγ Aγ) HPFH patient and from a heterozygous patient with
the Greek (Aγ) form of HPFH. One form of GγAγ HPFH has been analysed recent-
ly[17,18] which results from a deletion similar to that described by us. In
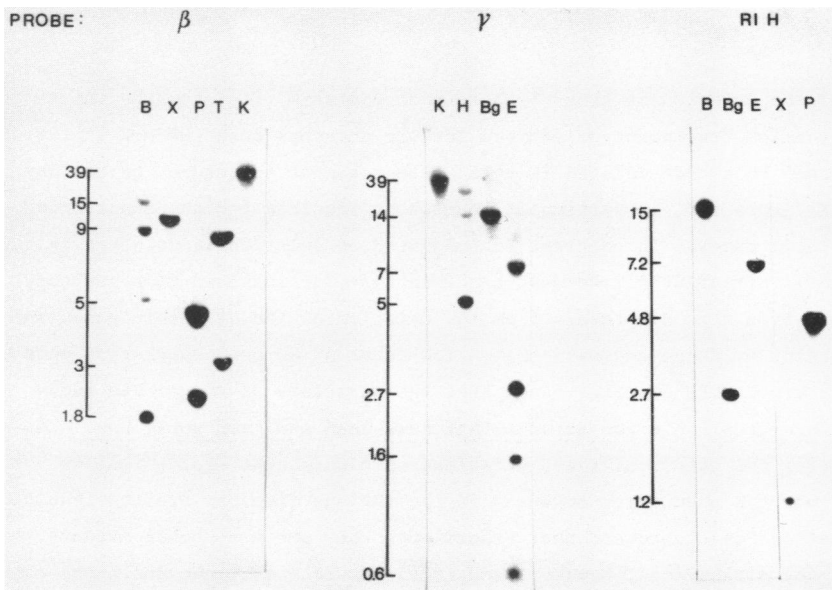the case (HPFH-3 in Fig. 6) considered in this paper, however, the 5' break

Fig. 5. Globin-gene fragments in Greek Aγ-HPFH.
        DNA from cultured lymphocytes from a heterozygous Aγ-HPFH
patient was digested with the restriction enzymes indicated and the genomic
fragments containing the δ and β   (β), Gγ and Aγ (γ) or RIH regions de-
tected using blot-hybridizations with the pHβG1, pHγG1 or RIH probes, res-
pectively.   The enzymes used were Bam HI (B), Bgl II (Bg), Eco RI (E),
Hpa I (Hp), Kpn I (K), Pst I (P), Taq YI (T), Xba I (X).   All the frag-
ments detected in this DNA are also seen in normal DNA (see the normal
map of Fig. 4 and refs. 10-13, 17 and 28).   The size of the fragments are
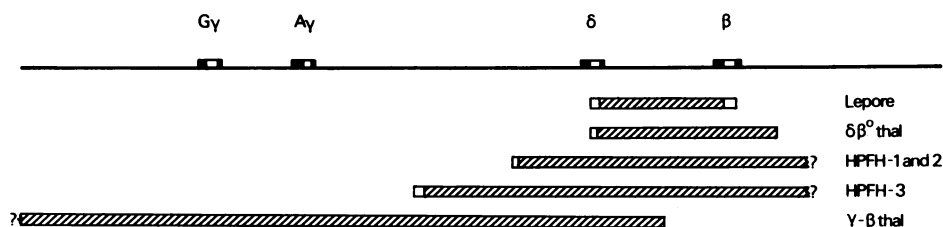indicated in kb.

Fig. 6.
        Schematic map of the deletions in the β-related globin-gene region
in various diseases. The genes are indicated as filled boxes and the
intergene distance drawn to scale. The hatched boxes show the established
deletions and the open areas of these boxes indicate the uncertainty in
these measurements. A question mark indicates that the break point at
that end is unknown. HPFH-1 and 2 are from refs. 17 and 18 and HPFH-3
is the Ghanaian GγAγ-HPFH patient in this paper. The remaining data
are from: Lepore[10], δβ° thalassaemia[15, 17, 6], γ-β thalassaemia[16].

point of the deletion is about 5 kb further upstream (i.e. towards the Aγ
gene) from the breakpoint determined for the previous case (HPFH-2 in Fig. 6).
The extra 5 kb of DNA deleted in this patient cannot therefore contain any
essential genes and, in particular, cannot be required for the functioning of
the γ-globin genes. In contrast to GγAγ-HPFH no deletion is detected in the
Greek (Aγ) form of HPFH. We must conclude that, at least in this patient,
this condition does not result from the deletion of the Gγ globin gene. In-
deed, recent evidence suggests that a low level of Gγ-gene expression does
occur in Greek HPFH[23]. Fig. 6 compares the structure of the globin gene
region in several deletion mutants that have been analysed until now. In
considering the effect of these deletions on globin gene expression, we
assume that the phenotypes observed in the various diseases are the result of
the deletions described and that, therefore, they are not double mutants.
This is not established, however, and it is possible that the deletions map-
ped are not responsible for these phenotypic effects.   These effects can be
summarized as follows (see Ref. 1 for a general discussion of this problem):
        1)   deletion of the δ - β intergenic region in Hb Lepore[1, 10]
results in a marginal enhancement of γ gene expression (1-5% in heterozy-
gotes)
        2)   extension of the deletion in the 3' direction causes a sig-
nificant enhancement of γ-gene expression (5-20% HbF in heterozygotes).
In δβ° thalassaemia the 3' break point is 1.8 kb to the 3' side of the β-
globin gene[15].   The HbF is not distributed evenly among erythrocytes;

rather, about 30-40% of the erythrocytes contain HbF.

    3) extension of the deletion in the 5' direction to eliminate
the regions to the 5' side of the δ-globin gene causes a still greater
elevation of HbF levels in HPFH by causing all erythrocytes to contain HbF.
The 5' break point of the deletion can be 4 kb (ref. 17, 18) or 9 kb (this
paper) upstream from the δ-globin gene.

    4) still further extension of the deletion to remove the Aγ-
globin gene causes a reversion to the heterocellular distribution of HbF
in Gγ-δβ^{o} thalassaemia[17].

    The interpretation of the consequences of these deletions must
remain, at present, speculative.   From a number of observations, it has
been suggested that the deletions discussed only act in cis (see Ref. 1).
A deletion model for the persistence of γ-gene expression in adults was
first proposed by Huisman et al.[24], where a regulatory gene was postulated
to lie between the Aγ and δ-globin genes.   Its deletion would cause the
HPFH phenotype.   The mapping of the deletions in δβ^{o} thalassaemia[15, 17]
and in HPFH (ref. 17, 18 and this paper) have suggested that 'regulatory'
regions lie both at the 3' side of the β-globin gene and at the 5' side of
the δ-gene, respectively, far from the γ genes, where the mutations act.

    To explain this we have recently considered an 'equilibrium'
model for the expression of the Gγ plus Aγ and δ plus β globin genes[15].
Specifically, we suggest that the foetal (Gγ and Aγ) and adult (δ and β)
gene-pairs form two mutually exclusive chromosomal domains.   Thus, when
the γ-region is active on a given chromosome, the δ plus β region is not;
and vice versa.   Evidence in favour of this idea has previously been
presented by Kabat[25].

    It follows that each domain must have two boundaries; trans-
acting components would presumably interact with the boundaries of the
domains to effect a transition from a closed to open transcriptional state;
subsequently, the transcription apparatus would utilize the exposed tem-
plate.   In foetal life, the trans component would favour the foetal genes;
in adult life a (different?) component would favour activating the adult
genes.   In both cases, however, an equilibrium between active foetal,
and adult genes would occur.   In normal adults the cells (F cells) that
contain significant amounts of HbF, and the reciprocally low level of ex-
pression of the adult genes during foetal life (Ref. 1), would reflect
this equilibrium.

    We suggest that the 'regulatory' regions deleted in the diseases

considered here are in fact the boundaries of these domains and that their deletion influences this equilibrium. These deletions can give three types of result:-

1) The deletion of a complete domain shifts the equilibrium entirely to the active configuration for the alternative domain. Thus, in GγAγ HPFH, the δ + β domain is deleted and the γ domain is expressed in every adult erythroid cell.

2) If a functional domain requires the presence of two boundary regions, then the deletion of one of these boundaries creates a defective domain. Thus, deletion of the 3' extragenic regions of the β-globin gene in δβ° thalassaemia[15] would result in a defective δ + β domain. In this case the equilibrium would shift in favour of γ-globin gene expression: this partial transition from δ + β -> γ would be reflected in the hetero-cellular response seen in δβ° thalassaemia. Similarly, in Gγ-δβ° thalass-aemia, the entire δ + β domain is deleted and a similar defective γ-domain would be created by the deletion of the 3' regions of the γ gene locus[17]. Again, the defective γ-domain would only function heterocellularly and a thalassaemia syndrome, rather than HPFH would result.

Finally in γ - β thalassaemia the entire γ domain and δ-globin gene region has been deleted[16]. This would remove the 5' boundary region of the δ + β-globin gene domain and, again, create a defective domain. This can explain why the β-globin gene, which is still present in these patients, is thalassaemic.

3) The fusion of the γ and δ + β domains would occur in Hb Kenya. Here, the Aγ and β genes are fused with the (presumptive) deletion of the 3' boundary region of the γ-domain and the 5' boundary region of the δ + β domain. This creates a single hybrid domain which would therefore func-tion pancellularly to produce Gγ and Hb Kenya globins. The rarer Gγ HPFH and Gγβ+ HPFH cases could be similarly explained, but mapping data have not yet been obtained in these cases.

The Greek case of HPFH described in this article shows no detect-able deletion, so it is not yet possible to discuss this in terms of this model. Possibly the mutation is a small lesion located in the boundaries discussed.

The equilibrium idea for the γ -> δ + β switch is supported by the fact that the HbF levels per F cell in δβ° thalassaemia and HPFH are essentially the same. The difference in gene expression, therefore, does not seem to be the level of γ-gene expression per cell, but the probability

that a cell expresses the genes at a high level.

Possible candidates for the regulatory regions are perhaps the DNase I-sensitive regions of chromatin that have been shown to flank certain Drosophila genes[26], or the regions complementary to small RNAs[27] that are found in the regions proposed as boundary sites here.

It is clear that any model proposed at this stage must contain a large element of conjecture. The model described here is, however, easily tested by analysing other HPFH and $\delta\beta^{o}$ thalassaemia deletions and by analysing the expression of suitably mutagenized cloned DNA segments upon their reintroduction into animal cells.

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