**The EMBO Journal** Vol.18 No.4 pp.949–958, 1999

# Deletion of a region that is a candidate for the difference between the deletion forms of hereditary persistence of fetal hemoglobin and δβ-thalassemia affects β- but not γ-globin gene expression

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**The analysis of a number of cases of β-globin thalassemia and hereditary persistence of fetal hemoglobin (HPFH) due to large deletions in the β-globin locus has led to the identification of several DNA elements that have been implicated in the switch from human fetal γ- to adult β-globin gene expression. We have tested this hypothesis for an element that covers the minimal distance between the thalassemia and HPFH deletions and is thought to be responsible for the difference between a deletion HPFH and δβ-thalassemia, located 5′ of the δ-globin gene. This element has been deleted from a yeast artificial chromosome (YAC) containing the complete human β-globin locus. Analysis of this modified YAC in transgenic mice shows that early embryonic expression is unaffected, but in the fetal liver it is subject to position effects. In addition, the efficiency of transcription of the β-globin gene is decreased, but the developmental silencing of the γ-globin genes is unaffected by the deletion. These results show that the deleted element is involved in the activation of the β-globin gene perhaps through the loss of a structural function required for gene activation by long-range interactions.**

*Keywords*: β-globin gene expression/γβ-thalassemia/HPFH

## Introduction

The human β-globin locus contains five genes that are arranged in the order of their developmental expression. Early during embryonic development the first gene to be expressed is the ε-globin gene, followed by the γ-globin genes during fetal development and finally the δ- and β-globin genes in the adult stage (Stamatoyannopoulos and Nienhuis, 1994). All of these genes are dependent on the locus control region (LCR; Grosveld *et al.*, 1987), which is situated upstream of the locus for their high level of activity (Figure 1). This region has been proposed to act as a holocomplex that appears to interact directly with the genes (and gene local regulatory elements) via a looping mechanism to activate efficient transcription of the genes (Dillon and Grosveld, 1993; Grosveld *et al.*, 1993; Peterson and Stamatoyannopoulos, 1993). It is not known, however, how the expression of the different genes is suppressed at different stages, although it is clear that at least two mechanisms appear to be responsible. The embryonic ε- and fetal γ-globin genes appear to be suppressed autonomously by as yet unidentified proteins acting on the immediate flanking regions of the genes (Behringer *et al.*, 1990; Enver *et al.*, 1990; Shih *et al.*, 1990; Dillon and Grosveld, 1991; Raich *et al.*, 1992; Liebhaber *et al.*, 1996). In contrast, the β-globin gene (largely) appears to be silenced during the embryonic stage by competition with the early genes for the LCR (Behringer *et al.*, 1990; Enver *et al.*, 1990; Hanscombe *et al.*, 1991; Dillon *et al.*, 1997).

Genetic disorders that alter hemoglobin switching have provided naturally occurring molecular models for the study of the regulation of globin gene transcription and the mechanism of switching during development. A number of mutations in the locus lead to a persistence of expression of the γ-globin genes in the adult stage (for review see Forget, 1998), indicating that the genes are suppressed by a complex mechanism that can be easily disturbed. One set of interesting mutations are deletions in the 3′ half of the globin locus, leading to different phenotypes with respect to the expression of the human γ-globin gene. One class of deletions results in a substantial elevation of γ-globin gene expression and are known as hereditary persistence of fetal hemoglobin (HPFH). In other deletions there is much less effect on the expression of the γ-globin genes and this results in a thalassemia. A number of these deletions have been characterized in detail and in some cases (e.g. HPFH1 and HPFH2) the lack of repression of the γ-globin genes appears to be caused by the juxtaposition of distant enhancer elements into the 3′ flanking region of the γ-globin genes (Collins *et al.*, 1987; Feingold and Forget, 1989; Arcasoy *et al.*, 1997). A potentially more interesting set of deletions are those which appear to delineate a small region situated between the γ- and δ-globin genes that may be involved in γ-globin gene repression.

The deletions delineating this region (Figure 1) are the Italian HPFH5 on the 5′ side (Camaschella *et al.*, 1990) and the Eastern European and US black δβ-thalassemias on the 3′ side (Anagnou *et al.*, 1985; Palena *et al.*, 1994), which differ by ~700 bp situated in the region 5′ to the δ-globin gene. This region has long been thought to be involved in globin gene switching, either by mechanisms that invoke chromosomal domains (Bernards and Flavell, 1980; Jones *et al.*, 1981; Smithies, 1982; for review see Flavell *et al.*, 1983) or by the presence of suppressing

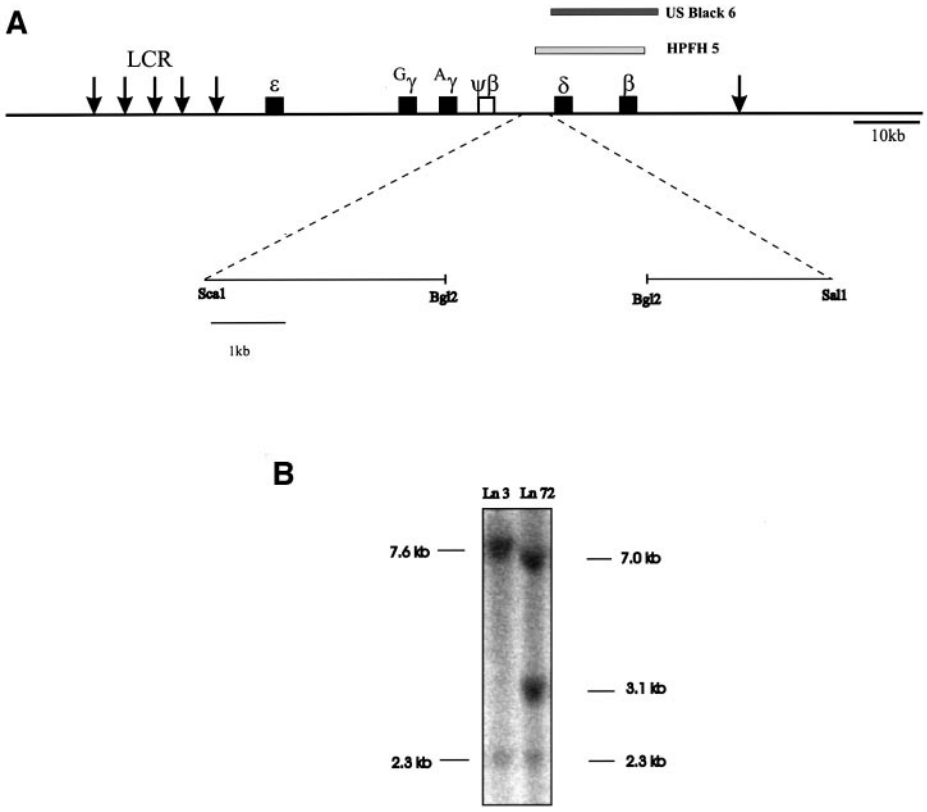


**Fig. 1.** Map of the human β-globin locus and characterization of the deletion. (**A**) Map of the human β-globin locus with the 5′ breakpoints of the US black thalassemia 6 and HPFH 5 deletions (grey bars). The bottom line shows the deleted 2.5 kb *Bgl*II fragment and its position covering the area between the 5′ breakpoints of the US black thalassemia 6 and HPFH5. The coordinates of the *Bgl*II fragment are 52724–55228; the breakpoints of the thalassemia and the HPFH are 55172 and 54394. (**B**) Southern blot analysis of the –2.5*Bgl*II deletion line 3 and the wild-type locus in line 72 (Strouboulis *et al.*, 1992). The normal 7.0 and 3.1 kb *Eco*RI fragments present in the normal locus have been replaced by a 7.6 kb fragment due to the deletion that removes an *Eco*RI site. The 2.3 kb *Eco*RI fragment containing the 5′ end of the δ-gene has remained the same.

sequences (Huisman *et al.*, 1974; Mears *et al.*, 1978; Ottolenghi *et al.*, 1982; Tuan *et al.*, 1983). More recent experiments have shown that these sequences have a negative effect on the expression of the γ-genes in reporter assays in erythroid cell transfection experiments (Vitale *et al.*, 1994; Anagnou *et al.*, 1995; Kosteas *et al.*, 1997), while transgenic mouse experiments have shown that the γ-genes can be silenced autonomously in the absence of these sequences (Dillon and Grosveld, 1991). However, the latter experiments were not carried out in the context of the complete locus and it remained unclear what role these sequences played in γ-gene silencing and/or β-gene activation *in vivo*. This paper directly examines the role of this region in the switching process, in the context of the whole β-globin locus, by using transgenic mice carrying a yeast artificial chromosome (YAC) with a deletion of this region.

## Results

### *Generation of transgenic mice with the mutant locus*

The minimal region between the 5′ deletion endpoints of the HPFH5 (Camaschella *et al.*, 1990) and the US black δβ-thalassemia (Anagnou *et al.*, 1995) is located between ~2.5 and 3.1 kb 5′ of the initiation codon of the δ-globin gene (Figure 1A). An 8 kb *Sca*1–*Sal*1 fragment containing this region was subcloned for the deletion of a 2.5 kb *Bgl*II fragment that encompasses the region which is located between the 5′-ends of the HPFH5 and black δβ-thalassemia (Figure 1A). This fragment had previously been shown to have a negative effect on γ-globin gene transcription in reporter gene transfection experiments (Vitale *et al.*, 1994). The resulting 5.5 kb *Sca*I–*Sal*I fragment was cloned next to a Lys2 gene for selection purposes in yeast. The resulting plasmid was recombined into the β-globin YAC220 (Figure 1B; Gaensler *et al.*, 1991) by homologous recombination and followed by selection to remove the Lys2 sequences (Burke *et al.*, 1987; Schiestl and Gietz, 1989). The DNA from individual recombinant yeast colonies was purified and analysed by Southern blot hybridization. Both electrophoresis of *Bam*HI and *Eco*RI digests and pulse-field gel electrophoresis (PFGE) of *Sfi*I digests (140 kb fragment) showed that the entire locus was identical to that of the wild type except for the deletion of the 2.5 kb (data not shown).

The Δ-2.5 kb YAC was purified by preparative PFGE (Gnirke *et al.*, 1993), checked for integrity by PFGE and injected into fertilized mouse eggs, resulting in two transgenic mice carrying the human β-globin locus. The integrity of the transgenic locus was analysed as described for the isolated YAC above. In transgenic line 3 one copy of the entire locus could be detected, while in transgenic line 13 three to four copies of the locus were detected. At least one of these copies contained the complete 140 kb *Sfi*I fragment while two copies had a

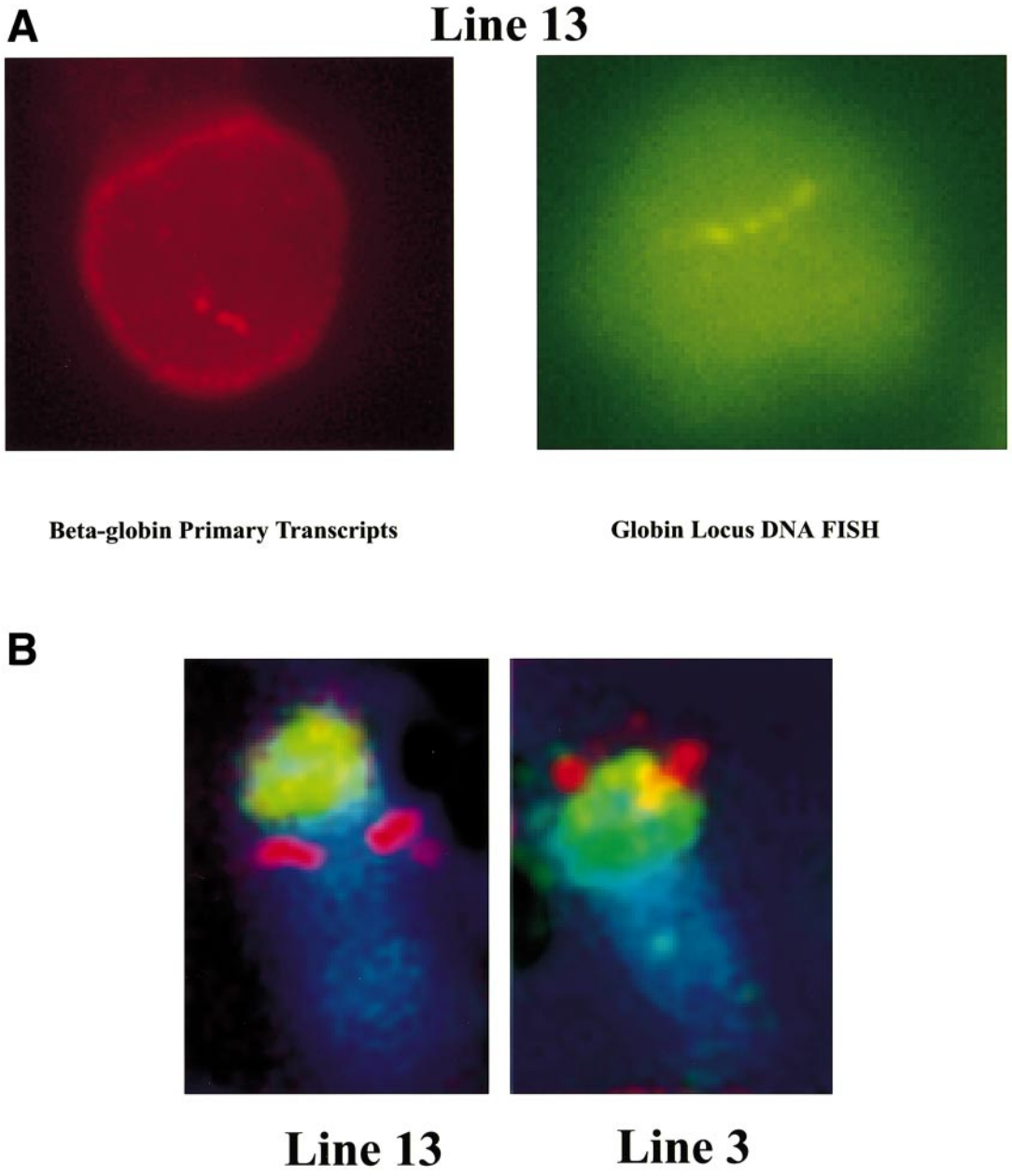


**Fig. 2.** Fluorescence light microscopy of DNA and RNA FISH. (**A**) Left panel, β-globin primary transcript signals in line 13. Fetal liver cells were fixed and hybridized to a set of oligonucleotide probes to detect the first exon and second intron of the β-globin primary RNA transcript (Wijgerde *et al.*, 1995). The signals (Texas Red) were detected by light microscopy and recorded with a CCD camera. Right panel, DNA FISH (N.Custódio, M.Carmo-Fonseca, F.Geraghty, J.Hurst, S.Pereira, F.Grosveld and M.Antoniou, manuscript submitted). The transgenic β-globin loci were detected using cosmid 28 which spans the region from 5′ of the γ-globin genes to 3′ of the β-globin gene (Grosveld *et al.*, 1981). The signals (FITC) were detected by light microscopy and recorded by CCD camera. (**B**) Detection of the integration sites of the human β-globin locus in transgenic lines 3 and 13. The chromosomes were prepared from cultured peripheral blood cells and hybridized as described by Mulder *et al.* (1995). The probes were the human LCR-labelled with biotin and the mouse α satellite labelled with digoxygenin. The labels were immunochemically visualized with FITC (green) or Texas Red. The DNA was counterstained with DAPI.

deletion 3′ to the β-globin gene. All the normal *Eco*RI and *Bam*HI fragments were present with the exception of one *Bam*HI fragment corresponding to the 3′ part of the β-globin gene (data not shown). Since the normal 3′ *Eco*RI fragment was observed, we conclude that this also represents a complete copy of the globin locus, even though it contains a deletion close to the 3′ end of the β-globin gene. At least 3.4 kb of the β-globin 3′ flanking region is intact, including the 3′ enhancer sequence (Strouboulis *et al.*, 1992).

The copy numbers determined by Southern blot hybridization were confirmed by *in situ* hybridization (Mulder *et al.*, 1995) with a probe against the complete human LCR. Line 3 shows one hybridizing spot while line 13 shows three to four spots (Figure 2A). Significantly, when the same cells are hybridized with a centromeric satellite DNA probe, it shows that the single copy locus of line 3 is integrated in centromeric heterochromatin, while the multicopy locus in line 13 is integrated away from the centromere on a chromosome arm (Figure 2B).

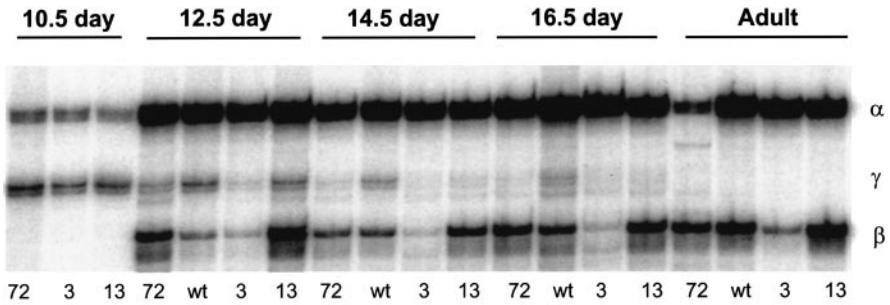


**Fig. 3.** mRNA levels by S1 nuclease protection. RNA was collected from line 72 (Strouboulis *et al*., 1992), a YAC single copy transgenic (Peterson *et al*., 1993), line 3 and line 13 on 10.5 d.p.c. (yolk sac), 12.5, 14.5 and 16.5 d.p.c. (fetal liver), and from adult blood. S1 analysis was carried out as described (Wijgerde *et al.*, 1996). The position of the protected bands from the α, β and γ probes are indicated on the right. The signals were quantitated from independent experiments by PhosphorImager (Molecular Dynamics) and are presented in Table I.

**Table I.** mRNA levels as determined by S1 nuclease protection

| | | Developmental stage (d.p.c.) | | | | |
|---|---|---|---|---|---|---|
| | | 10.5 | 12.5 | 14.5 | 16.5 | Adult |
| Wt YAC | γ | n.d. | 51.4 ± 2.96 | 26.4 ± 2.5 | 10.4 ± 1.5 | 0 |
| | β | n.d. | 38.6 ± 2.27 | 54.26 ± 3.7 | 85.2 ± 4.24 | 100 ± 1.7 |
| Line 3 | γ | 98.17 ± 3.5 | 11.2 ± 3.75 | 3.84 ± 1.98 | 4.26 ± 2.21 | 0 |
| | β | 0 | 27.34 ± 4.9 | 7.68 ± 2.30 | 10.94 ± 0.83 | 32.68 ± 3.06 |
| Line 13 | γ | 57.45 ± 2.14 | 9.14 ± 1.79 | 4.47 ± 0.64 | 2.6 ± 0.6 | 0 |
| | β | 0 | 37.54 ± 0.99 | 37.26 ± 1.7 | 39.66 ± 1.60 | 74.4 ± 4.15 |
| Line 72 | γ | 90.50 ± 0.71 | 17.9 ± 0.14 | 6.4 ± 0.56 | 2.2 ± 0.28 | 0 |
| | β | 0 | 72.1 ± 0.14 | 70.4 ± 0.56 | 44.4 ± 0.56 | 0.99 ± 1.41 |

This table shows the levels of γ and β mRNA at different days of development from a mouse line containing a single copy YAC of the β-globin locus (Peterson *et al*., 1993), line 72 (Strouboulis *et al*., 1992), line 3 and 13. The numbers are expressed as a percentage of expression of the mouse α-globin genes. The calculations were made on the basis of three copies of a complete transgenic locus for line 13. The numbers (and standard deviations) given are the average of three independent experiments.

**Table II.** Primary transcript quantitation by *in situ* hybridization[a]

| | | Developmental stage (d.p.c.) | | | |
|---|---|---|---|---|---|
| | | 10.5 | 12.5 | 14.5 | 16.5 |
| Line 3 | γ | 74.33 ± 0.58 | 12.4 ± 0.69 | 1.67 ± 0.57 | 1 ± 1 |
| | β | 0 | 38.66 ± 2.30 | 19.67 ± 1.53 | 19.83 ± 0.29 |
| Line 13 | γ | 70.33 ± 0.58 | 27 ± 1.41 | 11.7 ± 0.42 | 1 ± 1 |
| | β | 0 | 76 ± 2.82 | 63.5 ± 0.71 | 69 ± 1.41 |
| Line 72 | γ | 94 ± 1.73 | 20.33 ± 0.58 | 1 | 0 |
| | β | 0 | 81.67 ± 2.89 | 81.67 ± 2.89 | 81.67 ± 2.89 |

The table shows the number of cells expressing the human γ- or β-gene on different days of development. The numbers are expressed as a percentage of cells showing a mouse β-globin signal. More than 300 human cells were counted per time point. The numbers (and standard deviations) are the average of three independent experiments.

### *Expression of the Δ-2.5 kb YAC during development*

Total RNA was isolated at different time points of development from embryonic yolk sac [10.5 days post-coitus (d.p.c.)], fetal liver (from 12.5 to 16.5 d.p.c.) and adult blood and probed for the level of γ- and β-globin mRNA, using RNA from a transgenic mouse carrying the same undeleted YAC (Peterson *et al.*, 1993) or a much shorter β-globin locus (an 80 kb locus integrated as a single copy in a euchromatic region and known as line 72; Strouboulis *et al.*, 1992) as the controls (Figure 3). Three independent experiments showed no difference between the level of expression of the γ-globin genes from the locus carrying the Δ-2.5 kb deletion or the wild-type loci at 10.5 d.p.c. However, a reduction in expression of both the γ- and the β-globin genes is apparent in the mice carrying the deletion YAC from 12.5 d.p.c. onwards using the level of endogenous mouse α-globin RNA as a control. The wild-type YAC control also shows some decrease in total output during the fetal period when the γ-genes are suppressed and the β-gene becomes fully active, but total output recovers in adult blood (from 90 to 100% γ + β RNA per copy of mouse α-globin). However, the drop in expression from the deletion locus is more severe and only partially recovers in adult blood (in line 3 from 38 to 12%, recovering to 33% in adult blood; in line 13 per

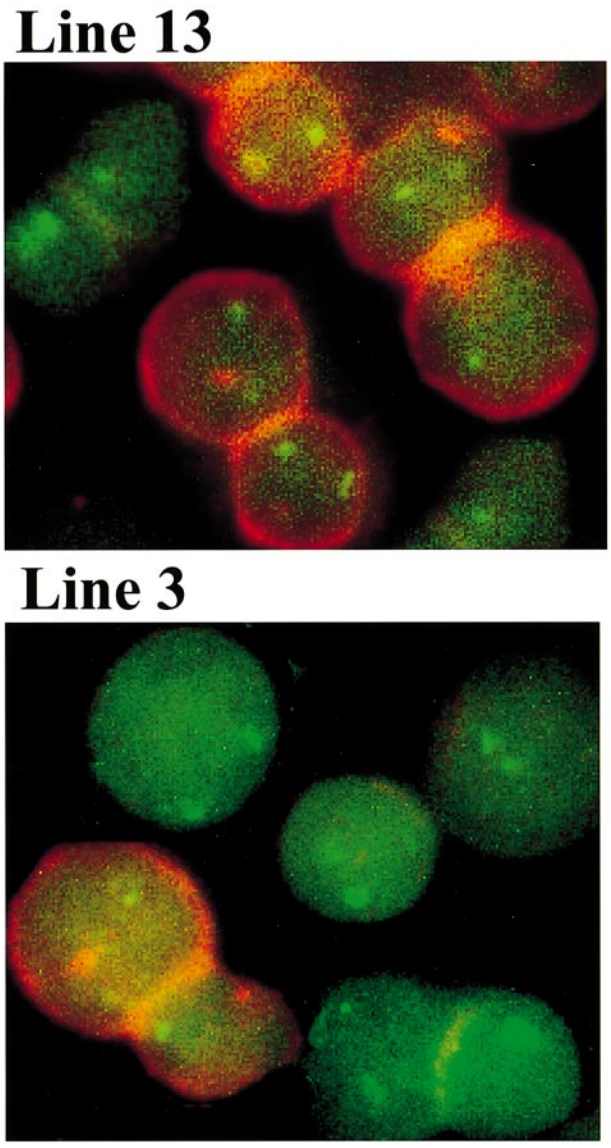


**Fig. 4.** RNA *in situ* hybridization. Fetal liver nuclei (14.5 d.p.c.) were hybridized (Wijgerde *et al.*, 1996) with gene-specific probes corresponding to the human β-globin first exon and both introns (red) and the mouse β-globin gene introns (green). The pictures were taken with a CCD camera. Only the cells with a human primary transcript signal show a halo of cytoplasmic mRNA. Cells were counted in three independent experiments and are presented in Table II.

copy of the locus from 46 to 41%, recovering to 74% in adult blood), suggesting that both of the transgenic loci may be subject to position effects (Table I).

### *Primary transcript analysis by in situ hybridization*

In order to distinguish between possible position effects and/or a general lowering of the transcription efficiency in the Δ-2.5 kb YAC transgenic mice, we performed an *in situ* hybridization to the primary γ- and β-globin transcripts from 12.5 to 16.5 d.p.c. (Figure 4). Individual cells in fetal liver samples from heterozygous lines 3 and 13 were scored for the presence of primary transcription spots in the nucleus and a halo of hybridizing mRNA in the cytoplasm. In each case at least 300 cells that contained an endogenous mouse β-globin transcript signal were counted. Analysis of the numbers shows that only a fraction of the mouse β-globin-expressing cells contain primary human γ- or β-globin signals (Table II). Since the mouse β-globin signal is present in almost all of the erythroid cells (Trimborn *et al.*, 1999), this indicates that the human locus in both of the transgenic lines suffers from a position effect. We have observed such a position effect before in transgenic lines that carried a defect in the LCR (Milot *et al.*, 1996).

Two types of position effects were observed in those experiments: position effect variegation (PEV) in which some of the cells express, while the others never do; and a timing-dependent position effect in which all of the cells express the genes, but they only do so during a particular short period of the cell cycle (Milot *et al.*, 1996; E.Milot, T.McMorrow, M.de Bruijn, M.von Lindern and F.Grosveld, submitted). Figure 4 shows that in the mice containing the Δ-2.5 kb YAC, only those cells containing a primary human globin transcription signal also show a cytoplasmic human globin mRNA (halo). We did not find cells containing a halo without a primary transcription signal in the nucleus. We therefore conclude that part of the erythroid cell population never expresses the transgenic loci. Thus both Δ-2.5 kb YAC transgenic loci show PEV; it is low in the embryos at 10.5 d.p.c. when most cells express the human locus (70–75%) when compared with the wild-type control and only slightly increases in line 13 during the fetal liver period, but line 3 shows a very significant increase to only 20% of the cells expressing (Table II). If only 20% of the cells transcribe the human β-globin gene it should result in a substantial decrease in the DNase hypersensitivity of the β-globin promoter in the deletion lines and a loss of general hypersensitivity. We therefore carried out a DNase hypersensitivity assay on 14.5 d.p.c. fetal liver nuclei (Figure 5), which shows that the hypersensitive site at the β-globin promoter is indeed much weaker than in the wild-type control 72. The fact that the parent restriction fragments (5.5 kb for β, 2.6 kb for γ and 8 kb for the γδ intergenic region; Figure 5) are digested at a similar rate with increasing DNase I concentrations suggests that general DNase sensitivity does not appear to have been affected (Figure 5), indicating that although 80% of the cells in line 3 do not transcribe the β-globin gene the locus has retained its DNase sensitivity even though it is integrated in or near the centromere (Figure 2B). Hence the presence of the complete LCR appears to be able to induce an open chromatin structure despite the absence of transcription.

Perhaps even more interesting is the observation that the number of cells that show a primary transcription signal does not correlate with the levels of mRNA as determined by S1 analysis (Figure 3). In previous analyses of the line 72 wild-type control (Wijgerde *et al.*, 1995), the number of cells showing a primary transcription signal correlated with the levels of mRNA. This has also been seen in loci which show a change in the level of transcription of the human β-globin gene due to the introduction of a competing gene (Dillon *et al.*, 1997), a position effect (Milot *et al.*, 1996) or a change in the level of the rate-limiting transcription factor EKLF (Wijgerde *et al.*, 1996). Hence we concluded that, when transcribed, the gene is always transcribed at the maximum level. The same phenomenon is also observed at 10.5 d.p.c. for the expression of the γ-globin genes, when the wild-type locus is

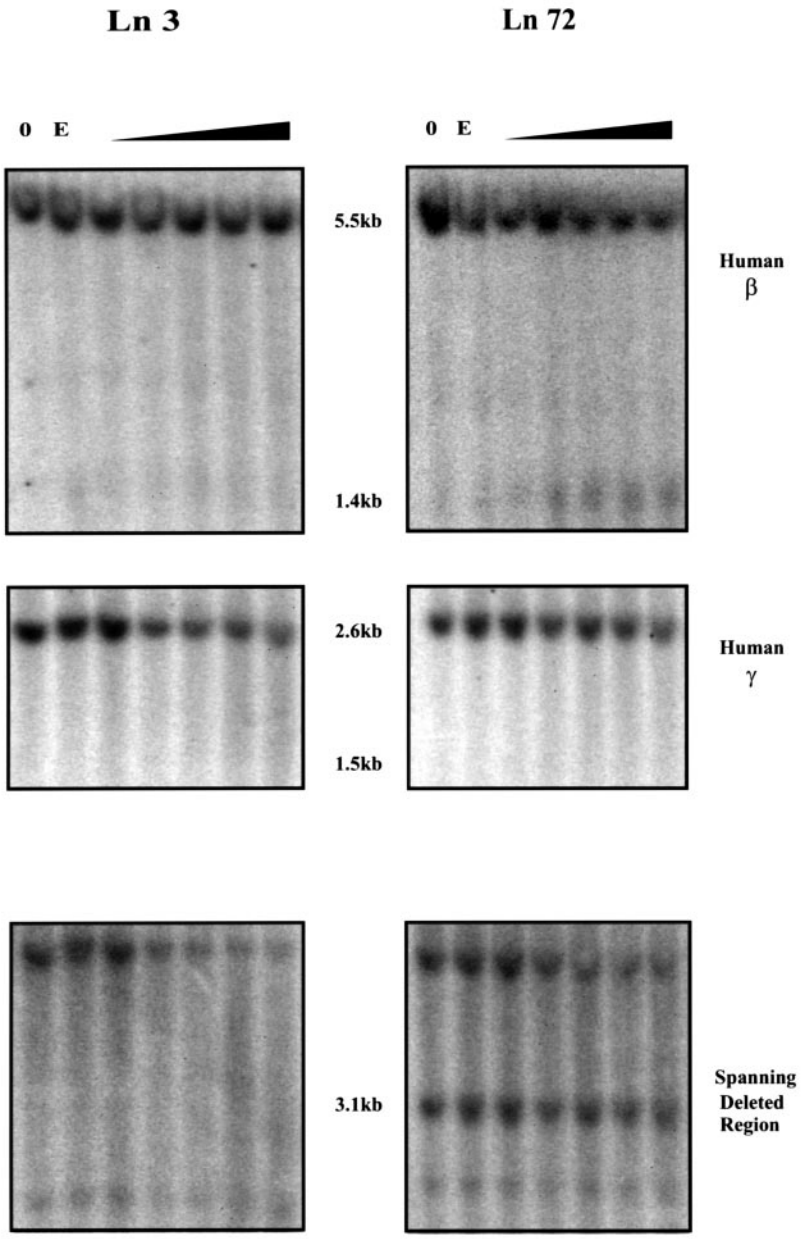


**Fig. 5.** DNase I analysis of 14.5 d.p.c. fetal liver cells from line 3 and line 72. Nuclei were isolated from 14.5 d.p.c. fetal liver cells and treated with an increasing amount of DNase I, followed by isolation of the DNA restriction digestion with *Eco*RI and Southern blotting as described (Wijgerde *et al.*, 1996). Top panels show the sensitivity of the 5.5 kb *Eco*RI human β-globin promoter fragment. This fragment covers the β-globin promoter region and the gene into the third exon. The fragment is normally cleaved to a 1.4 kb fragment corresponding to the proximal part of the promoter measured from the third exon with $^{32}$P-labelled end probe containing intron 2. Middle panels show the sensitivity of the 2.6 kb *Eco*RI human γ-globin promoter fragment. This fragment covers the equivalent region of the β-globin gene as described for the β-globin gene and normally shows a similar cleavage fragment when the γ-globin genes are active (probe intron 2). Bottom panels show the *Eco*RI fragments of the γ/δ intergenic region (probe 8 kb *Sca*I–*Sal*I fragment) covering the deleted fragment as shown in Figure 1A.

compared with the two Δ-2.5 kb YAC transgenic lines (Tables I and II). However, the data obtained for the fetal liver show that the number of primary transcript signals is approximately double the number expected from the mRNA levels. For example, the rate of γ-globin gene transcription in the transcribing cells of line 3 at 12.5 d.p.c. appears not to have been affected, the RNA quantitation by S1 analysis shows a level of 11% γ-globin level (γ per copy/mouse α-gene per copy) and matches well to 12% of cells with a primary transcript signal for γ (wild-type control shows 18% expression RNA level and 20% of cells). However the β-globin gene already appears to be somewhat affected, showing a 27% expression RNA level, but with 38% of the cells showing a primary *in situ* transcription signal (wild-type control 72% expression RNA level and 80% of cells). This difference increases 2-fold at 14.5 (8% expression RNA versus 20% of cells) and 16.5 d.p.c. (11% expression RNA versus 20% of cells). Thus the number of loci transcribing is much higher than normally required for the observed levels of RNA and we conclude that less RNA is produced per transcribing locus when compared with the normal locus. Similarly, the number of transcribing cells in line 13 (76%) is much higher than expected for a 3–4 copy line expressing ~18%

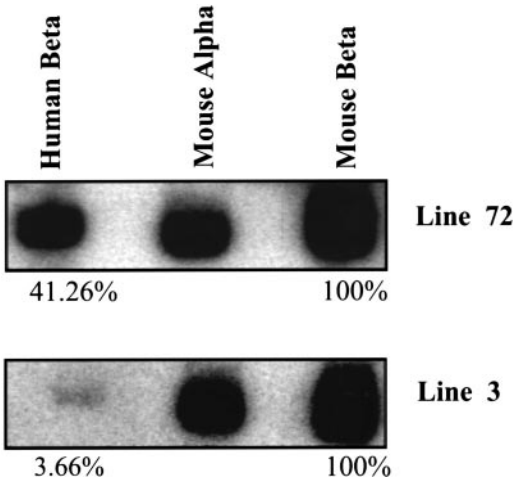


**Fig. 6.** Nuclear RNA synthesis. Filters containing denatured plasmid DNA with the human β-globin gene, the mouse α- and β-globin genes, were hybridized with the total yield of $^{32}$P-labelled nuclear RNA from 14.5 d.p.c. fetal liver. The signals were quantitated by PhosphorImager and expressed as a percentage of mouse α- or β-globin RNA synthesis. Plasmids on the filter contained: human β-globin gene from *Acc*I to *Pst*I (2 kb), mouse βmaj-globin gene *Hin*dIII fragment (1 kb), mouse α-globin gene *Bam*HI fragment (0.3 kb).

per gene copy and the majority of cells expressing three of the tandem integrated loci simultaneously (Figure 2A). Again this suggests that the initiation of transcription of the β-globin gene in these loci has been affected in the fetal liver.

In order to confirm that this is indeed the case, we carried out transcriptional run-on experiments to measure RNA synthesis from the β-globin gene. Nuclei from 14.5 d.p.c. fetal liver cells from line 72 and line 3 were incubated in the presence of radioactive CTP (Ashe *et al.*, 1997) and the resulting RNAs were hybridized to filters containing DNA from the mouse α-, mouse β- and human β-genes. The result (Figure 6) shows that the incorporation into primary human β RNA transcripts (41% heterozygous human β versus 100% per homozygous mouse α or β control) closely correlates with the mRNA levels (35% heterozygous human β per homozygous mouse α-genes) and the number of transcriptional spots observed (41% heterozygous human β versus 100% homozygous mouse β) in line 72. However, whereas the incorporation for line 3 (4%) correlates with the RNA levels (4%), it does not agree with the number of primary *in situ* signals (20% heterozygous human β). Hence we conclude that the rate of transcription of the β-globin gene has been affected by the deletion of the 2.5 kb fragment.

## Discussion

In this paper we have examined the role of a small region just 5′ of the δ-globin gene in the switching process of the human β-globin locus. From the analysis of patients, this region appears to mark the difference between the silencing and persistent expression of the human γ-globin genes in the adult. Two explanations have been offered to explain this phenotypic difference: either the region contained sequences that are involved in γ-globin gene suppression; or they are required for the maintenance of an active gene domain in the adult stage of development. In support of the first possibility are transfection experiments with reporter constructs which showed these sequences to have a negative effect on the expression of the γ-genes (Vitale *et al.*, 1994); support for the second possibility is more indirect. Perhaps the best indication comes from the fact that, although at a low level, the β-genes in the mouse are already activated at the embryonic stage of expression, while the human gene is still completely silent (Trimborn *et al.*, 1999). Importantly, the mouse locus does not contain any substantial homology to the region that was deleted in this study (Margot *et al.*, 1989; Shehee *et al.*, 1989), even when the comparison was carried out for much smaller homologies (data not shown). Therefore, this leaves the possibility that it may play a role in β-gene accessibility in humans but not in mice.

Two separate effects are observed when the region is deleted from a complete locus in the experiments described here. The first is PEV and the second is a loss of β-globin transcription efficiency in the population of expressing cells. No effect is seen on the silencing of the γ-globin genes in the mouse fetal liver. The latter agrees with earlier results which showed that a single transgenic γ-globin gene, when coupled as a small fragment to the LCR, is silenced autonomously (Dillon and Grosveld, 1993). We therefore conclude that this element is not involved in γ-globin silencing.

The PEV, we observe, is low or absent at early embryonic stages of development when the γ-globin genes are expressed highly, but is very clear in line 3 in the fetal liver when normally the β-globin gene becomes expressed highly. Significantly, in that mouse line, the locus has been integrated into a centromere, a heterochromatic region of the mouse genome. It is known that this renders an integrated locus carrying deletions in the LCR subject to position effects (Festenstein *et al.*, 1996; Milot *et al.*, 1996). Line 13, which shows a much milder PEV, appears to have a locus that is not integrated in a heterochromatic environment, although this cannot be excluded completely. In addition it is possible that there is some effect due to the presence of multiple copies. Nevertheless, it is tempting to speculate that particularly when the locus is integrated in a heterochromatic environment, the PEV observed with the Δ-2.5 kb YAC is due to the deletion of the 2.5 kb region, since both lines show the same phenomenon and therefore this region would be directly involved in the activation of the β-globin gene. However, general DNase sensitivity in the deleted locus is maintained, implying that the 2.5 kb region is not important for chromatin accessibility, but that it is involved in the maintenance of a transcriptionally competent structure. Thus the LCR would be capable of maintaining an accessible chromatin structure in the absence of transcription.

The second effect is that the efficiency of transcription in the expressing population of cells is different from that observed previously. Normally the efficiency of transcription is not affected in the expressing cells as characterized for CD2 by FACS analysis of the expressing cells (Festenstein *et al.*, 1996) or for globin by primary transcript *in situ* analysis (Milot *et al.*, 1996).

At present, it is not clear why the efficiency of transcrip-

tion in the expressing cells of line Δ-2.5 kb YAC mice would be affected, but it suggests that these sequences play a role in the recruitment of the initiation complex. This could be a direct role as part of a larger LCR–gene complex or perhaps more probably, an indirect role as a purely structural component of the locus that would be required for accessibility of the adult genes to allow an (efficient) interaction between the LCR and the genes. Previously we have proposed that the LCR forms a complex that directly interacts with the genes, giving rise to alternate transcription of the genes during the switch from γ to β (Wijgerde *et al.*, 1995; Dillon *et al.*, 1997; Gribnau *et al.*, 1998). During this process the LCR continuously forms interactions with one of the genes. Such interactions decay and new complexes are formed. Since there are very few cells in which the gene is 'off', the formation of interactions must be very efficient and may require a number of structural components such as the 2.5 kb region 5′ of the δ-gene. If this were the case, it would explain why the expression of the early genes is not affected in embryonic stages: the region 3′ to the γ-genes is not yet important and the adult genes are not yet accessible. Once adult erythropoiesis commences, involving the β-globin gene, efficient looping and accessibility of the region between γ and β would be required and the region 5′ of the δ-gene may be important in this process.

## Materials and methods

### *Deletion of 2.5 kb fragment in the β-globin YAC*

The cosmid 28 (Grosveld *et al.*, 1981), containing the *Sma*I–*Cla*I fragment (coordinates 33949–69470), was digested with *Sca*I–*Sal*I restriction enzymes (coordinates 49600–57615).

The fragment of 8015 bp was blunt-ended with Klenow and cloned in the *Sma*I site of Bluescript KS (Promega). The resulting plasmid was digested with *Bgl*II (coordinates 52723–55227) to remove the 2.5 kb intergenic region. A 5 kb *Sal*I fragment, containing the *Lys2* gene, was cloned in the *Sal*I site of the plasmid described above. The 2.5 kb deletion plasmid was recombined into the β-YAC220 (Gaensler *et al.*, 1991) by the 'pop in/pop out' method of homologous recombination in yeast containing the wild-type β-YAC. Before the transformation, 5 μg of plasmid were linearized with *Avr*II (which cuts asymmetrically relative to the *Bgl*II deletion) and transformed into spheroplasted *Saccharomyces cerevisiae* AB1380 containing the β-globin YAC. The transformants were selected in minimal broth lacking uracyl, tryptophan and lysine.

The recombinant colonies were grown for 48 h in minimal broth, lacking uracyl and tryptophan but containing lysine, to allow spontaneous excision of the plasmid via homologous recombination between the duplicated regions. Then, $10^6$ yeast cells were plated on AA plates to select for spontaneous plasmid excision events (Chatoo *et al.*, 1979).

### *Purification of the Δ-2.5 kb β-globin YAC and micro-injection*

Growth of yeast strain AB1380, containing the human β-globin locus YAC (β-YAC) and gel purification and concentration were performed as described previously by Gnirke *et al.* (1993).

Agarose blocks containing yeast DNA were prepared as described and separated in preparative 1% low melting point agarose (SeaPlaque GTG, FMC) gels by PFGE in 0.25× TAE at 13°C. The gels were run at 15–5 s switching time, 180–160 V for 36 h.

An unstained YAC-containing gel slice was excised after ethidium bromide staining of marker lanes on both sides of the gel to locate the YAC in the gel. The gel slice was equilibrated in microinjection buffer for 2 h at room temperature (rt) and then digested with agarose (Epicentre) (1 U/100 mg of gel) at 42°C for 1 h. Undigested agarose residues were removed by centrifugation at 12 500 r.p.m. for 5′ at rt and the supernatant was stored at 4°C. The YAC was concentrated by loading 400 μl of supernatant into an Ultrafree-MC filter unit (nominal molecular weight limit 100 000; Millipore) and centrifuged at 3000 r.p.m. for 10–15 min. The remaining solution containing the DNA, was incubated at rt for 1 h in the filter, pipetted gently a few times and then transferred to a clean tube.

The quality of DNA was tested by PFGE and the concentration was estimated in a 0.8% agarose gel. Prior to injection into fertilized mouse eggs, the DNA was diluted to 1 ng/μl with micro-injection buffer.

### *Structural analysis of the Δ-2.5 kb YAC*

Transgenic mice were identified by Southern blot analysis of genomic DNA prepared from tail biopsies according to standard procedures (Strouboulis *et al.*, 1992). Intactness of the human β-globin locus in the Δ-2.5 kb YAC transgenic mice was determined by detailed examination of the 140 kb *Sfi*I fragment encompassing most of the locus.

Agarose plugs, containing high molecular weight DNA, were prepared from mouse spleen cell suspension. The plugs were incubated overnight in LDS solution at 37°C (LDS = 1% LDS, 100 mM EDTA pH 7.5, 10 mM Tris–HCl pH 8). The plugs were rinsed twice in NDS at rt for 30 min (NDS = 0.2% laurylsarcosinate, 100 mM EDTA, 2 mM Trizma pH 9.5) and twice with TE at rt for 30 min (Gnirke *et al.*, 1993). One plug was equilibrated with *Sfi*I digestion buffer for 1 h on ice and then digested overnight with 100 U of enzyme at 50°C. The digestion was stopped with EDTA and the digested DNA was fractionated on PFGE.

### *DNA FISH analysis*

Peripheral blood cells were cultivated for 24 h in RPMI 1640 medium (Gibco-BRL). Chromosome preparations were made according to standard procedures. FISH was carried out as described by Mulder *et al.* (1995). The probes used were the human LCR and the mouse α satellite, labelled with biotin and digoxygenin and immunochemically detected with fluorescein or Texas Red. The DNA was counterstained with DAPI.

### *mRNA and primary transcript in situ hybridization*

Primary transcript and mRNA *in situ* hybridization to detect transcription of the human and mouse globin genes in the yolk sac and fetal liver cells were performed as described by Wijgerde *et al.* (1995) and Milot *et al.* (1996).

### *Preparation of RNA and S1 nuclease protection assay*

RNA was prepared from whole, frozen, transgenic 10.5 d.p.c. embryos and fetal livers (from 12.5, 14.5 and 16.5 d.p.c.) and adult blood and subjected to S1 nuclease protection assays as described previously (Wijgerde *et al.*, 1996). The probes used were those described by Milot *et al.* (1996). Protected bands on the S1 gels were quantitated via PhosphorImager analysis (Molecular Dynamics).

### *HS assay*

Ten transgenic fetal livers (14.5 d.p.c.) for each line were treated as described by Forrester *et al.* (1990). Suspension of nuclei was performed by 20 strokes of a Dounce pestle (type B) and 100 μl aliquots were digested for 3 min at 37°C with increasing amounts of DNase I. Reactions were stopped and treated with proteinase K and extracted with phenol-chloroform. After ethanol precipitation, the pellet was resuspended in water. An aliquot was digested with *Eco*RI and followed by Southern blot analysis. Hybridizations were performed with the human probes for β-IVS II, γ-IVS II and the 8 kb *Sca*I–*Sal*I fragment from the γ–δ intergenic region.

### *Nuclear run-on*

Transcription in isolated 14.5 d.p.c. fetal liver nuclei and isolation of $^{32}$P-labelled nuclear RNA was performed as described by Ashe *et al.* (1997). The labelled RNA was hybridized to prehybridized filters containing denatured plasmids with the human β-, mouse β- and mouse α-globin genes. The hybridization signals were quantitated by PhosphorImager (Molecular Dynamics).

## Acknowledgements

We are grateful to Ken Peterson for generously supplying RNA from YAC transgenic mice, for helpful advice from Peter Fraser, technical support from Joost Gribnau, Mark Wijgerde, Gaetano Zafarana and Lien Braam. R.C. was supported by the CNR progetto 95.04669.ST75 and the Fondazione Italiana Leonardo Giambrone per la guarigione della Talassemia. T.M. was supported by the EC. The work is supported by the EC and NWO (The Netherlands).

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*Received December 1, 1998; revised and accepted December 18, 1998*