F0784 contained multiply defective genomes and an inordinate number of G-to-A substitutions. Biased G-to-A hypermutation has previously been described for HIV-1[14,25], but not to the extent found in HIV-2_{F0784}. But in macaques experimentally infected with SIV_{SM}, extensive G-to-A hypermutation was found which correlated with reduced viral pathogenicity[26]. Studies are presently underway to elucidate the molecular basis of G-to-A hypermutation and to determine whether, in the extreme case, it could result in attenuated or even abortive HIV-2 infection.

Finally, a recent suggestion[27] that SIV_{SM} may have been accidentally transmitted to man by inoculation with infected monkey blood probably cannot explain the diversity now recognized for HIV-2. Our results re-emphasize the need to target viruses from feral monkey populations and humans living in remote areas of Africa in a search for the origins of human immunodeficiency viruses and events leading to their recent epidemic spread. □

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A single point mutation is the cause of the Greek form of hereditary persistence of fetal haemoglobin

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IN normal humans the fetal stage-specific γ-globin genes are silenced after birth and not expressed in the adult. Exceptions are seen in cases of hereditary persistence of fetal haemoglobin (HPFH). These are clinically important because the elevated levels of γ-globin can alleviate β-thalassaemia and sickle cell anaemia. One class of mutations is associated with point mutations in the promoter of the γ-globin genes (non-deletion HPFH), whereas others seem to be caused by large deletions 3′ to the γ-globin genes[1]. To test whether the point mutation found in the Greek non-deletion HPFH[2,3] (guanine to adenine at nucleotide position −117) is the cause of the raised γ-globin levels in the adult stage and is not just a linked polymorphism, we engineered this mutation into a γ-globin gene. When this gene was introduced into mice, the presence of the −117 mutation results in persistence of γ-globin expression at a high level and a concomitant decrease in β-globin expression in fetal and adult mice. We show that these changes correlate with the loss of binding of the transcription factor GATA1 to the γ-globin promoter, suggesting that it may act as a negative regulator of the γ-globin gene in adults.

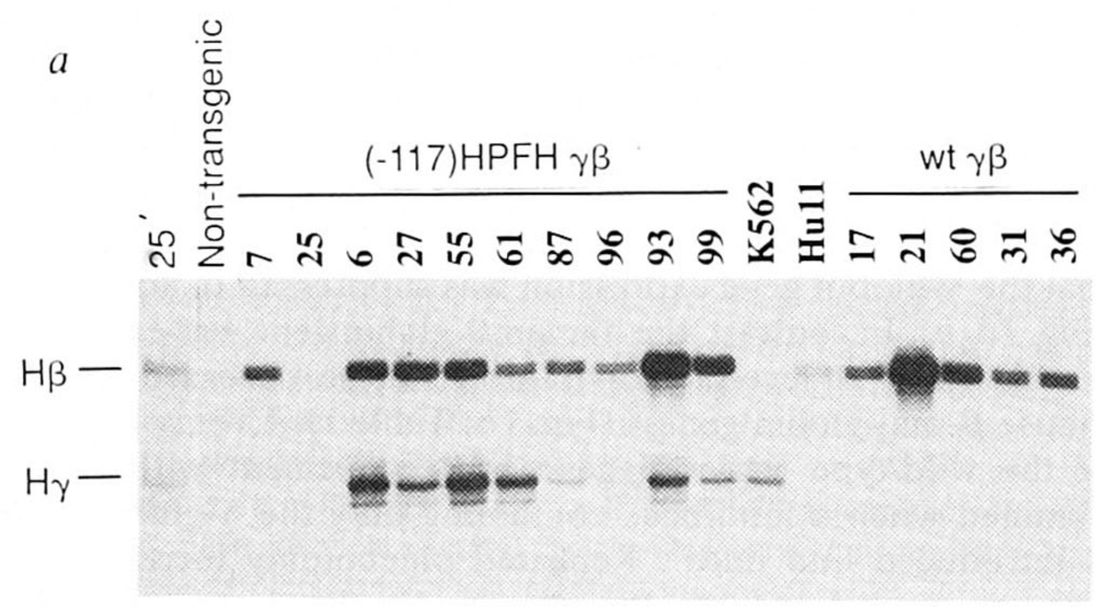
Two globin minilocus constructs were injected into fertilized mouse eggs. The first construct contained a wild-type (wt) γ-globin gene flanked by the entire locus control region (LCR) and a β-globin gene[4] (Fig. 1). The second construct was the same wild-type locus but with a single engineered point mutation at position −117 (G→A) in the promoter of the γ-globin gene. This mutation was verified by sequence analysis (not shown). The β-gene was included as a reference gene for quantitation and to allow rapid analysis of the construct without the need to establish a large number of bred lines. When the wild-type γβ minilocus was introduced into fertilized mouse eggs, five transgenic mice were obtained. Southern blots showed that two of the founders were mosaic (31 and 36) and that all contained the intact γβ minilocus, albeit at different copy numbers (Table 1, and data not shown). S1 nuclease protection analysis showed that the γ-globin gene expression was suppressed in adult mice (Fig. 1*a*, *b*). In contrast, the human β-globin gene was expressed at this stage at levels comparable to those observed for the mouse β-maj-globin genes[5] (Fig. 1*b*; Table 1). The suppression of the wild-type γ-globin gene is in agreement with results obtained when a minilocus containing only the γ-globin gene is introduced into mice[4]. Repeated phlebotomy increases the number of reticulocytes, but even under those conditions the γ-globin gene remains suppressed (Fig. 1*b*). When the −117 mutant γβ minilocus was introduced into mice, nine transgenic mice were obtained and Southern blots showed that they contained intact miniloci at different copy numbers, although a number were mosaic (Table 1, and data not shown). S1 protection analysis showed a completely different result from that obtained with the wild-type γβ minilocus. The −117 mutant γ-globin gene is now expressed at high levels in the adult stage in eight out of nine founder transgenic mice (Fig. 1*a*, *c*). Line 7, which does not express γ at the adult stage, was found to express the γ gene at the embryonic and early fetal stages (not shown). Analysis by polymerase chain reaction (PCR)[6] of the γ-globin gene promoter in line 7 showed that it still contained the −117 mutation, thus ruling out a reversion of the mutation (not shown). The line 7 γ gene was not analysed for other mutations and we have therefore, as yet, no explanation for this exception. The expression level of the γ gene in the other HPFH mice varied considerably (Fig. 1; Table 1), which may be caused by the different arrangement of the transgene loci (P. Fraser and N.D., unpublished results; for example, the three lowest γ/β expressors all have a head-to-head integration) and the fact that the developmental regulation of the γ gene is very sensitive to position effects[4,7,8].

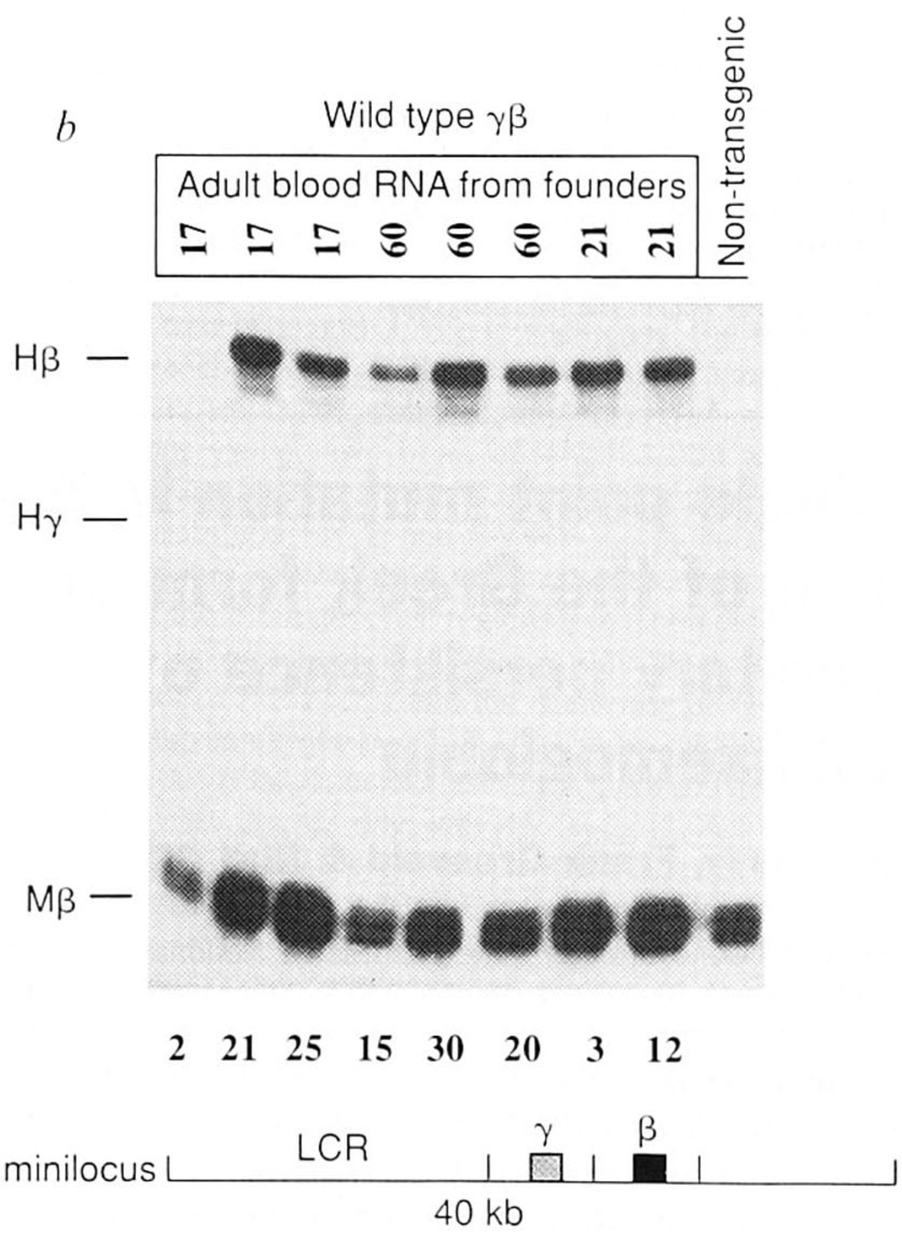
To determine whether expression of the γ-globin gene at the adult stage in the HPFH mice leads to a partial suppression of the linked human β-globin gene, all RNA samples of the wild type and HPFH γβ minilocus-containing mice were compared

directly using probes of comparable specific activity for all the genes in the same experiment (Fig. 1, and data not shown). Quantitation of the signals (Table 1) suggests that the expression of the human β-globin gene in HPFH mice is reduced significantly per gene copy and that the total output per locus is slightly reduced in the adult bred mice (99 and 61) when compared with either the mouse β-maj-globin gene or the human β-globin gene in the wild-type $\gamma\beta$ mice. This provides further evidence that the γ gene competes with the β gene for the LCR[9-11] and correlates well with the lower levels of expression of the β-globin gene that is allelic with the HPFH gene in humans[12,13].

The effect of the HPFH mutation at earlier stages of development was also examined by analysing staged embryos from lines 99 and 61 (Fig. 2). In embryonic yolk sac the pattern of expression for both the wild-type and HPFH constructs was similar to that previously reported for LCR $\gamma\beta$ constructs, with γ expressed and β suppressed[9,10]. In 13.5-day fetal liver there is a striking difference between the wild-type and HPFH constructs. The wild-type $\gamma\beta$ mice gave similar levels of γ and β expression, but in HPFH $\gamma\beta$ mice the expression of the human β gene is almost completely suppressed ($\gamma > 96\%$ of $\gamma+\beta$). Because this is significantly different from the $\gamma/\gamma+\beta$ ratio observed in adult animals (Table 1), it is further evidence that the balance of transcription factors is substantially different at the fetal liver stage than at the adult stage of mouse erythropoiesis[4]. Our data are different from the results of Morley *et al.*[14], who did not obtain expression of an HPFH gene in the adult mouse. Although we cannot explain this difference at present, it should be pointed out that their construct[14] contained only site 2 and lacked the LCR elements that are most active in the adult (P. Fraser and F.G., unpublished results).

Our data show that a mutation in the distal CAAT box can override the adult suppression of the γ-globin gene. This could result from the loss of a bound suppressor or the gain of a bound activator. The 13-base-pair deletion[15] that removes the distal CAAT box (Fig. 3) would favour the first possibility. Previous attempts to determine whether the -117 mutation affects the binding of transcription factors have been hampered by lack of good adult-stage human erythroid cell lines. Some differences have been reported in the binding of factors and CDP (ref. 16), NFE3 (ref. 17) and CP1 (refs 16, 18) have all been considered as candidates for a role in γ suppression through binding to this region (Fig. 3). Because the wild-type





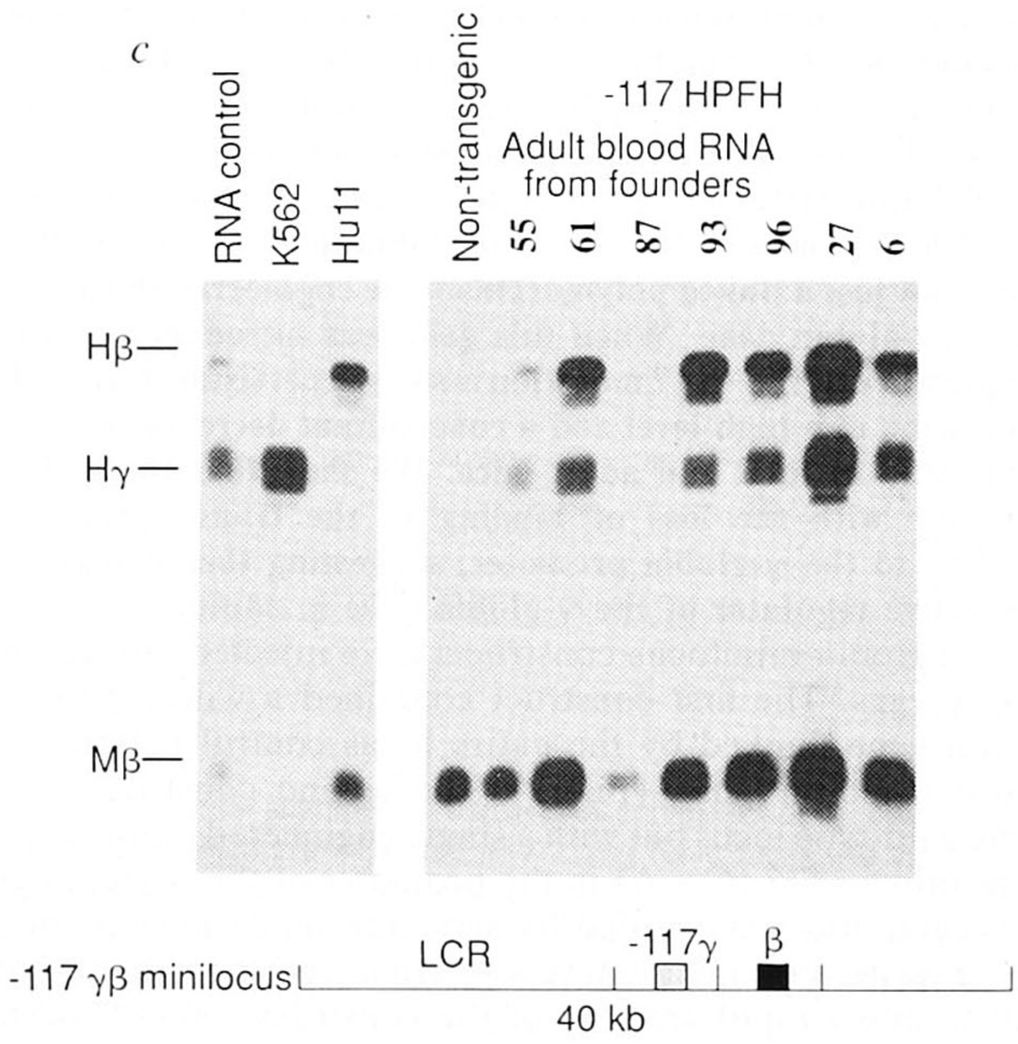


FIG. 1 Expression of wild-type (wt) $\gamma\beta$ and -117 HPFH $\gamma\beta$ minilocus constructs in transgenic mice. Schematic representation of the wild-type $\gamma\beta$ and Greek HPFH $\gamma\beta$ minilocus constructs are shown in *b* and *c*. The wild-type or Greek HPFH minlocus constructs were made by cloning a 4.98-kb *Bgl*II fragment containing the human β-globin gene into the LCR γ-globin construct[5]. The Greek HPFH $\gamma\beta$ minilocus was made by replacing the wild-type promoter with the equivalent mutagenized promoter containing the G→A base change at position -117 upstream of the γA CAP site. *a*, S1 nuclease analysis of all trasgenic mice (numbered as in Table 1), using probes of equal specific activity for human β and γ RNA (Hβ, Hγ). Controls were; γ-globin RNA from K562 cells; β-globin RNA from Hu11 cells. Line 99 was bred from founder 27 and has a single transgene integration site. Founder 27 had multiple integrations. Leftmost lane (25′) shows a higher exposure for mouse 25. *b*, S1 nuclease analysis of three wild-type $\gamma\beta$ mice after phlebotomy. Probe specific activities (Hγ/Hβ/Mβ) were 1/1/5. The reticulocyte count (%) is indicated below the lanes. *c*, S1 nuclease analysis of seven -117 HPFH $\gamma\beta$ mice. Probe specific activities (Hγ/Hβ/Mβ) were 0.5/0.8/1. RNA controls were a mixture of γ and β RNA: γ RNA from K562 cells and β RNA from Hu11 cells.

METHODS. *Sal*I fragments containing either the wt $\gamma\beta$ minilocus or Greek HPFH $\gamma\beta$ minilocus were injected into mouse oocytes to generate transgenic mice. RNA was prepared from blood of adult transgenic mice and human γ, β and mouse β-maj globin RNA levels were compared by S1 nuclease protection analysis using a 5′ end-labelled 525-bp *Acc*I fragment of the human β-globin gene, a 190-bp *Bst*NI fragment of the Aγ gene and a 700-bp *Nco*I–*Hin*dIII fragment from the mouse β-maj gene[4]. The 5′ end of human γ, β and mouse β-maj globin mRNA protect 140, 160 and 95 nucleotides of these probes respectively. All Southern blot and S1 nuclease protection signals were quantitated by scanning on a phosphorimager.

TABLE 1 Copy numbers of transgenic mice and relative expression of human and mouse globin gene

Mouse	Copy number	Human β/ mouse β	Human $\gamma+\beta$/ mouse β	Human γ/ human $\gamma+\beta$
HPFH				
6	3–4†	9	20	55
25	3	3	6	50
27	14†	6	10	40
61*	1	30	90	65
87	<1†	20	30	40
93	3	30	45	30‡
96	2	20	30	30‡
55	5	25	60	60
99*	3	40	60	30‡
7*	2	80	80	<1
Wild type				
17	<1†	>70	>70	<1
60	5–6	100	100	<1
21	2	100	100	<1
31	13†	5	5	<1
36	3†	10	10	<1

First column: mouse number; *, bred lines. These lines are the only lines guaranteed not to be mosaic. Line 99 was generated from founder 27 (see Fig. 1*a*). Second column: number of intactly integrated transgenic loci; †, mice mosaic for the transgene based on Southern blot analysis, not all mosaics can be detected this way. Third column: per cent human β-globin RNA of mouse β-globin RNA per gene copy. Fourth column: per cent total human globin RNA of mouse β-globin RNA per locus. Fifth column: per cent human γ-globin RNA of total human globin RNA in adult mice; ‡, head-to-head integration of injected fragments. Results are the average of three S1 nuclease protection experiments. All S1 nuclease protection and Southern blot signals were quantitated on a phosphorimager.

γ-globin transgene is expressed at the early fetal stage and suppressed at late stages in mice, we used nuclear protein extracts from embryonic yolk sac, fetal liver, a mouse erythroid leukaemia (MEL) cell line (adult stage) and from non-erythroid HeLa and F9 cells to investigate which protein factors are bound to the distal CAAT box region, and which of those would show a correlation with developmental stage-specific expression and the HPFH mutations. Oligonucleotides corresponding to the wild type and the −117 mutation were used in gel retardation experiments (Fig. 3, and data not shown). First the wild-type and −117 HPFH probes were used to determine the number of complexes at different developmental stages. Figure 3 shows that three prominent bands and several weak bands were formed at the different developmental stages. As shown previously, the slowest migrating complex corresponds to the CAAT box binding factor CP1 (ref. 18), which shows no increase in binding during development and no significant difference in binding to the wild-type or −117 HPFH CAAT boxes. It therefore does not correlate with the developmental expression of the wild-type gene and the lack of suppression of the −117HPFH γ gene and is unlikely to be the factor providing stage specificity, although it may be part of a suppressor complex involving multiple factors. A fast-migrating band (NF-E6) also does not increase significantly during development. This band represents the principal complex formed when the β-globin CAAT box is used for binding *in vitro* (not shown). It is absent from HeLa and F9 cells and therefore appears to be an erythroid-specific protein complex. It does not correlate with changes in transcription of the wild-type gene and binding is not affected by the HPFH mutations. CDP also fails to show the change in levels during development (not shown) that would be expected of a suppressor. The only complex that does show correlation with the expression patterns of the wild-type and HPFH γ-globin genes is the complex migrating between CP1 and NF-E6, which is formed by GATA1 as shown by competition (although it self-competes much less effectively than a perfect GATA1 binding site) and antibody binding experiments (Fig. 3). As reported

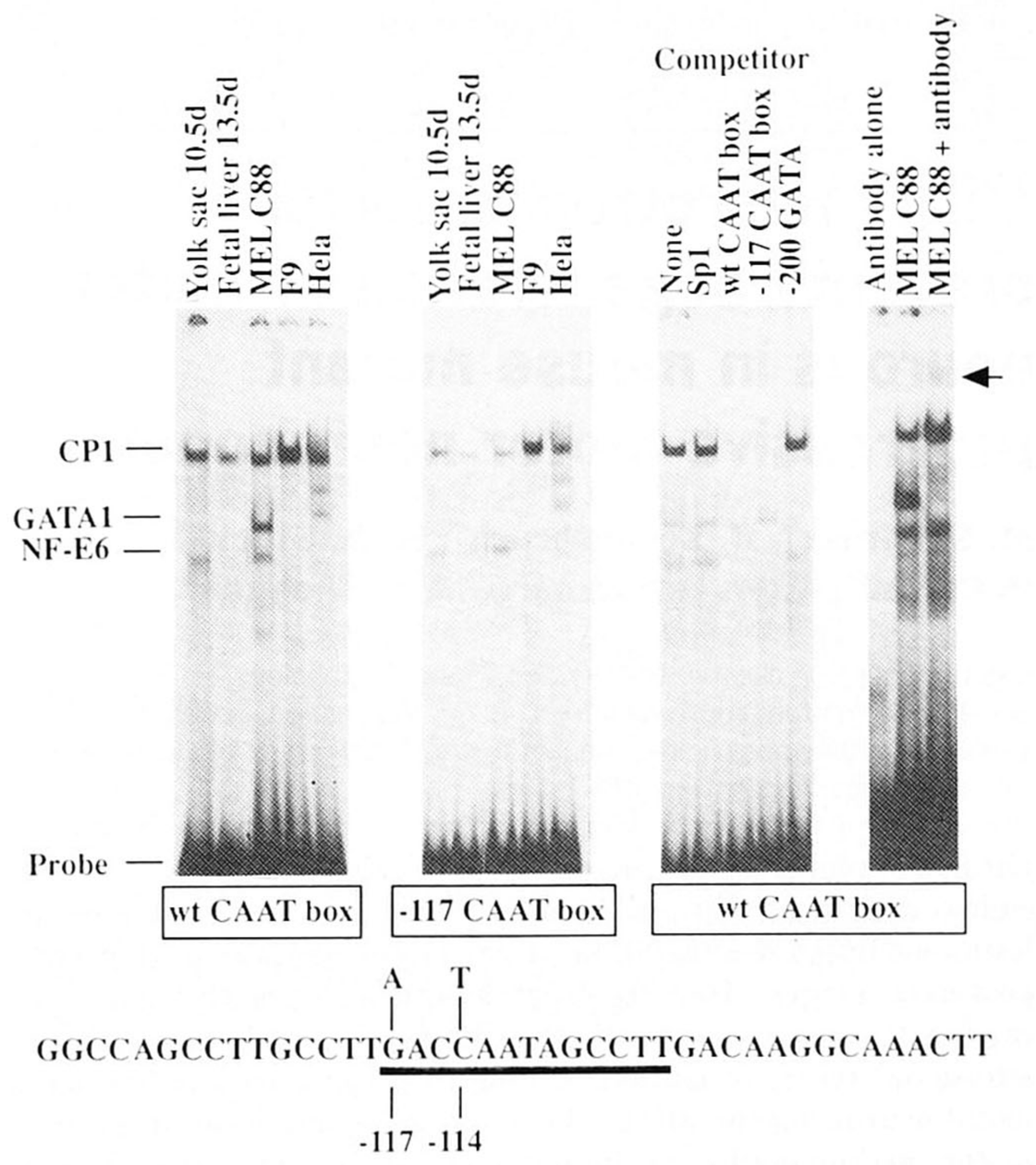


FIG. 3 Gel retardation analysis using wild-type (wt) and −117 γ-globin CAAT box. The γ-globin wild-type (left panel) or −117 G→A distal CAAT box (second panel) was subjected to gel retardation analysis using nuclear extracts prepared from embryonic yolk sac (10.5 d.p.c.), fetal liver (13.5 d.p.c.), adult mouse erythroleukaemic cell line (MEL, C88), non-erythroid uninduced F9 cells and HeLa cells. The third panel shows a competition assay using the wt CAAT box as the probe. The competitors were an Sp1 binding site, wt or −117 γ-globin CAAT box and a consensus GATA1 binding site from the human β-globin promoter[23]. The right-hand panel shows a supershift with a GATA1 monoclonal antibody (arrowed). The first lane contains only the antibody, lane 2 only MEL extract, and lane 3 has both extract and antibody. The sequence of the CAAT box oligonucleotides is shown at the bottom. The numbers indicate the position relative to the γ globin CAP site. The solid bar indicates the 13-bp deletion[15].
METHODS. Nuclear extracts from mouse tissues or cell lines were prepared as described[23–25]. Each gel retardation assay (10 μl) contained ~5 μg nuclear protein, 2 μg poly(dI · dC) and 0.5 ng of 5′ ^{32}P end-labelled double-stranded wild type or −117 HPFH oligonucleotides, and was done as described[23]. Extracts used in each assay had similar levels of Sp1 transcription factor as judged by gel retardation using a CACC double-stranded oligonucleotide[25].

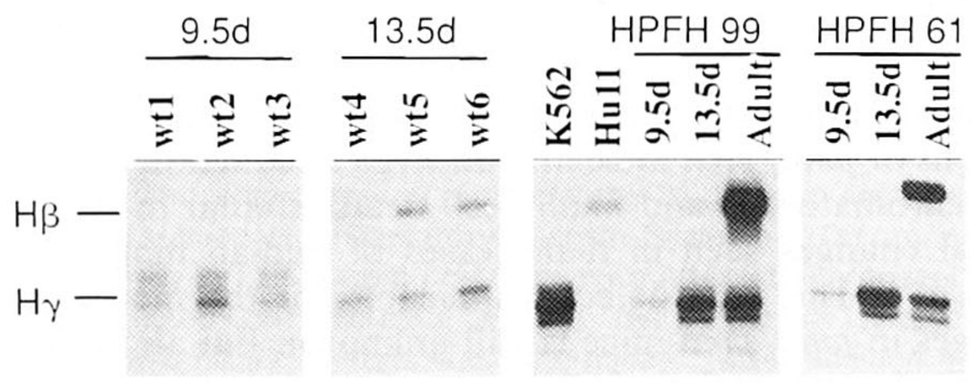


FIG. 2 S1 nuclease mapping analysis of minilocus wild-type $\gamma\beta$ and Greek HPFH $\gamma\beta$ mice during development. RNA was prepared from transgenic 9.5-day yolk sacs, 13.5-day fetal livers and from blood of adult mice. Yolk sacs and fetal livers containing minilocus wild-type $\gamma\beta$ (wt 1–6) were analysed directly after microinjection; yolk sac, fetal liver and adult blood containing the Greek HPFH $\gamma\beta$ construct were obtained by breeding lines 99 and 61. Control RNAs were from K562 and Hu11 cells. The specific activity of the γ and β probes was 1:1, except in panel HPFH 61, for which the ratio γ:β is 1:2.

previously[19], the relative amount of GATA1 binding activity increases during development. Figure 3 shows that this GATA1 binding is not observed with extracts from the yolk sac or fetal liver when the wild-type γ gene is still expressed (Fig. 2). In extracts from adult-stage cells, when the HPFH but not the wild-type gene is expressed, GATA1 binds to the wild-type but not to the HPFH CAAT box (Fig. 3). This suggests that GATA1, in contrast to its reported transactivation properties[20,21], may also function as a component of stage-specific suppression of the human γ-globin genes.

In conclusion, we have provided evidence that a single point mutation in the γ-globin promoter is the cause of the HPFH phenotype and that the suppression of the γ-globin gene in mice closely mimics the suppression observed in humans. The only qualitative difference that we have detected in mice is the absence of γ-globin reactivation in response to erythroid stress, which is observed in humans[22]. For therapy in β-globin-related haemoglobinopathies, a specific reactivation of the γ-globin genes would be more useful than the stress-related reactivation. Our results showing that an HPFH mutation is functional in mice and that this correlates with loss of binding of a transcription factor, indicates that procedures to achieve specific γ-globin reactivation could be developed in the absence of the stress-related pathway using our mouse model system. □

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Ciliary neurotrophic factor prevents degeneration of motor neurons in mouse mutant progressive motor neuronopathy

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CILIARY neurotrophic factor (CNTF) supports the survival of embryonic motor neurons *in vitro*[1,2] and *in vivo*[3], and prevents lesion-mediated degeneration of rat motor neurons during early post-natal stages[4]. Here we report that CNTF greatly reduces all the functional and morphological changes in *pmn/pmn* mice[5], an autosomal recessive mutant leading to progressive caudo-cranial motor neuron degeneration. The first manifestations of progressive motor neuronopathy in homozygous *pmn/pmn* mice become apparent in the hind limbs at the end of the third post-natal week, and all the mice die up to 6 or 7 weeks after birth from respiratory paralysis. Treatment with CNTF prolongs survival and greatly improves motor function of these mice. Moreover, morphological manifestations, such as loss of motor axons in the phrenic nerve and degeneration of facial motor neurons, were greatly reduced by CNTF, although the treatment did not start until the first symptoms of the disease had already become apparent and substantial degenerative changes were already present. The protective and restorative effects of CNTF in this mouse mutant give new perspectives for the treatment of human degenerative motor neuron diseases with CNTF.

We have evaluated the effects of CNTF in the *pmn/pmn* mouse, which is an animal model for human spinal motor neuron disease[5]. In contrast to two other mouse mutants, *wobbler*[6–8] and *mnd*[9,10], the manifestations of motor neuron degeneration in *pmn/pmn* mice appear earlier and progress more rapidly. In 4-week-old *pmn/pmn* mice, the number of axons of the phrenic nerve is already highly reduced, indicating that at this time the disease has already reached an advanced stage. The motor neurons of *pmn/pmn* mice first undergo a reduction in cell size, then chromatolysis and finally cell death, similar to the pathological changes seen in many cases of human motor neuron diseases[11]. The gene defect responsible for the motor neuron changes in *pmn/pmn* mice is still unknown. But an insufficient or defective expression of CNTF does not seem to be responsible for the degenerative changes. Northern blots of sciatic nerve reveal CNTF transcripts with similar size and intensity to those of the healthy controls. Western blots from sciatic and facial nerve extract are indistinguishable from those of controls and the same extracts contain comparable CNTF biological activity, as determined in the embryonic chick ciliary neuron survival assay (data not shown).

Because intravenously injected CNTF has a half-life of only a few minutes (F. Dittrich and M.S., unpublished results) and

TABLE 1 Effect of CNTF treatment on the number of facial motor neurons and phrenic nerve axons in *pmn/pmn* mice

	Number of facial motor neurons	Number of phrenic nerve axons
pmn mice (40–50 days old)	1,881 ± 199* ($n=6$)	87 ± 4* ($n=7$)
CNTF-treated *pmn* mice (40–48 days old)	2,679 ± 108* ($n=7$)	144 ± 22* ($n=7$)
Healthy control mice (littermates)	3,108 ± 153 ($n=5$)	ND

The brain stem of mice perfused with 4% formaldehyde was embedded in paraffin, serial sections 7-μm thick were stained with cresyl violet, and the nucleoli of facial motor neurons were counted in every fifth section on both sides as previously described[4]. Counts were not corrected for split nucleoli[4,15]. The mean of the counts on both sides was used for each animal. Phrenic nerves were prepared after perfusion of the animals with 4% formalin. Nerves were postfixed in 4% formalin, dehydrated, then 5-μm transverse sections made and stained according to ref. 16. Myelinated axons were counted from photographs taken from nerve sections under the light microscope. Data shown are means ± s.e.m. for each group. ND, not determined.

* Statistical significance was tested by Student's t-test, $P < 0.0005$.