A transgenic mouse model of sickle cell disorder

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A SINGLE base-pair mutation (β^s) in codon 6 of the human β-globin gene, causing a single amino-acid substitution, is the cause of sickle cell anaemia[1]. The mutant haemoglobin molecule, HbS, polymerizes when deoxygenated and causes deformation of the erythrocytes to a characteristic 'sickled' shape. Sickling of cells in small vessels causes painful crises and other life-threatening complications[2,3]. Although the molecular basis for sickle cell anaemia has been known for 30 years, no definitive treatment is available[4]. An animal model of sickle cell anaemia would not only allow a detailed analysis of the factors that initiate erythrocyte sickling *in vivo* and of the pathophysiology of the disease, but would also permit the development of novel approaches to the treatment of the disease. By using the dominant control region sequences from the human β-globin locus, together with human α- and β^s-globin genes, we have obtained three transgenic mice with HbS levels ranging from 10 to 80% of total haemoglobin in their red cells. As observed in homozygous and heterozygous Hb^s patients, the erythrocytes of this mouse sickle readily on deoxygenation. Irreversibly sickled cells[2,3], which are characteristic of sickle-cell patients homozygous for β^s, are also observed in the peripheral blood of the mouse with high levels of HbS.

The dominant control region (DCR) sequences, which flank the human β-globin locus, direct high-level copy-number-dependent expression of the human β-globin gene in erythroid cells and in transgenic mice[5,9]. When these β-globin DCR sequences are linked to the human α-globin gene in transgenic mice, high-level expression is obtained[7-10]. It was therefore possible to obtain transgenic mouse lines expressing more human haemoglobin (HbA) than endogenous mouse haemoglobin by using a construct containing the β-globin DCR, the human β- and the α_1-globin gene[8]. These mice had a normal mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC)[8], but showed an imbalance in the synthesis of α- and β-globin, as only one α-gene, rather than the normal two α-genes, was used[8]. Our initial constructs for expressing HbS in transgenic mice included two human α-globin genes and the human β^s-globin gene (Fig. 1).

We obtained five transgenic animals and determined the transgene copy number by Southern blot analysis of tail-biopsy DNA (Fig. 2*a*, and data not shown). These mice fall into the usual two categories—mice that express at low levels because of deletions or mosaicism, and mice that are fully transgenic and express at high levels[5,7,8]. Mouse 41 had one copy of the transgene but had deletions in the β-globin gene (Fig. 2*a*) and the DCR (data not shown). The other transgenic mice did not have any detectable rearrangements and had the copy numbers shown in Table 1. We analysed RNA prepared from peripheral blood samples by S1 nuclease protection to determine the level of expression of the transgenic globin genes. Mouse 41 has a low level of correctly initiated β^s-globin messenger RNA (5% of mouse β-globin mRNA) caused by the deletion in the transgene. Two of the mice are mosaic and have low (mouse 75) or undetectable (mouse 64) levels of β^s-globin mRNA. Two mice have high levels of human α- and β^s-globin mRNA in peripheral blood and show, after correction for specific activities of the probes, a near-balanced synthesis of human α- and β^s-globin mRNA (Fig. 2*c* and Table 1). We analysed peripheral blood haemolysates by isoelectric focusing under conditions that resolve human haemoglobin (HbA), sickle cell haemoglobin (HbS), and mouse haemoglobin (MHb). In good agreement with the mRNA analysis, mice 29 and 54 have a large haemoglobin component that focuses at the same isoelectric point (pI) as HbS from a patient with sickle cell anaemia, whereas mouse 75 (mosaic) shows a minor component (Fig. 2*d*). Under these conditions we cannot distinguish mouse $\alpha\beta^s$-dimers from human $\alpha\beta^s$ dimers.

To determine the haematological consequences of the presence of human HbS in mouse red cells, we measured the haemoglobin levels and red cell indices (Table 1). It is clear that the transgenic mice do not have anaemia and the only haematological abnormality, apart from morphological changes (see below) is a slightly lower MCV in mouse 29 (Table 1). We then performed a conventional 'sickling test'[13]. We incubated peripheral blood samples from transgenic and control animals under a sealed cover slip with the reducing agent sodium metabisulphite, and followed the changes in red cell morphology by microscopy. In mouse 29, nearly 100% of red cells sickled (Fig. 3*c*), like those of a human HbAS heterozygote (Fig. 3*a*), whereas cells from a control mouse (Fig. 3*b*) or mouse 54 did not sickle. It is of interest that 3–4% of the cells from the mosaic mouse 75 also sickled (data not shown); we therefore conclude that its transgenic red cells, like the red cells of mouse 29, have a sufficiently high ratio of human β^s-globin to mouse β-globin (>80%) to allow sickling. By contrast, mouse 54 still expresses sufficient levels of mouse globins to prevent sickling[14].

To determine if erythrocyte sickling was occurring *in vivo*, we examined peripheral blood films. No sickle cells were found in nontransgenic animals (Fig. 3*d*) or in mouse 41. By contrast, sickle cells were present in mouse 29 at levels of about 1 in every 1,000 cells (Fig. 3*e*, *f*). Because we prepared the films after thorough oxygenation of the blood samples, these cells are by definition irreversibly sickled cells (ISCs)[15]. This is confirmed by the finding of fibrillar structures in the red cells of mouse 29, characteristic of HbS polymers[16] (Fig. 4*a*, *b*). ISCs are believed to have suffered dehydration and membrane damage resulting from repeated cycles of sickling and unsickling[17]. Given the short half-life of mouse erythrocytes relative to that of human

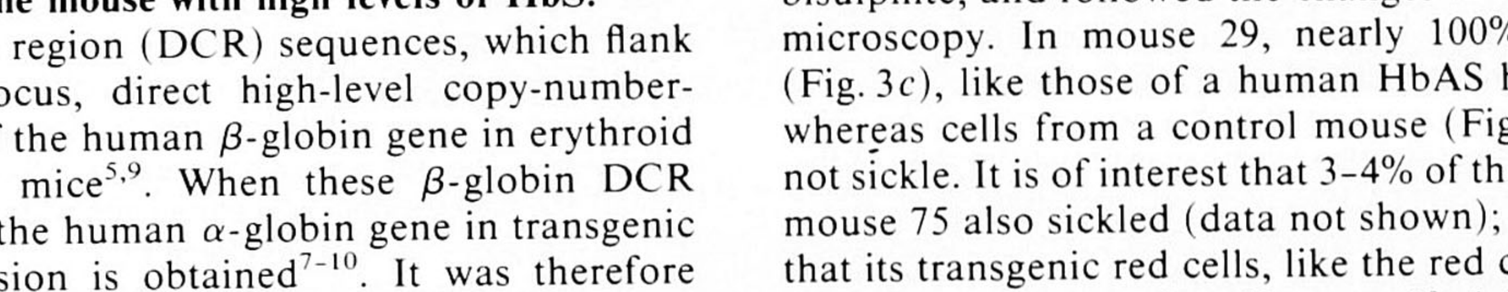

FIG. 1 Dominant control region $\alpha\alpha\beta^s$-gene construct 1665. Plasmid vector sequences are not shown; the orientation of the genes is denoted by horizontal arrows, the vertical arrows represent the erythroid-specific DNaseI hypersensitive sites. A, *Asp*718 site; C, *Cla*I site; S, *Sst*II site.
METHODS. A 3.0-kilobase (kb) *Cla*I–*Xba*I fragment of the human α_1-globin gene from construct 1254 (ref. 8) was cloned in the vector KS$^+$ (Stratagene) and subsequently cloned between *Cla*I and *Asp*718 sites of the microlocus vector 1417[7,12]. An *Nco*I–*Xba*I fragment of a β^s-globin allele was cloned into an Asp718–linkered 4.8 kb *Bgl*II fragment of the human β^A-globin gene. The presence of the sickle mutation was confirmed by *Sau*I digestion and the *Asp*718 fragment cloned into the microlocus α-construct (construct 1642). The 3.0-kb *Cla*I-*Asp*718 human α_1-globin gene fragment was ligated to *Cla*I linkers and cloned into the unique *Cla*I site of construct 1642 to give construct 1665. The final construct was digested with *Sst*II and a 17.5-kb fragment purified for microinjection into fertilized mouse eggs. Abbreviation: *tk–Neo*^{r}, thymidine kinase gene promoter linked to neomycin-resistance gene.

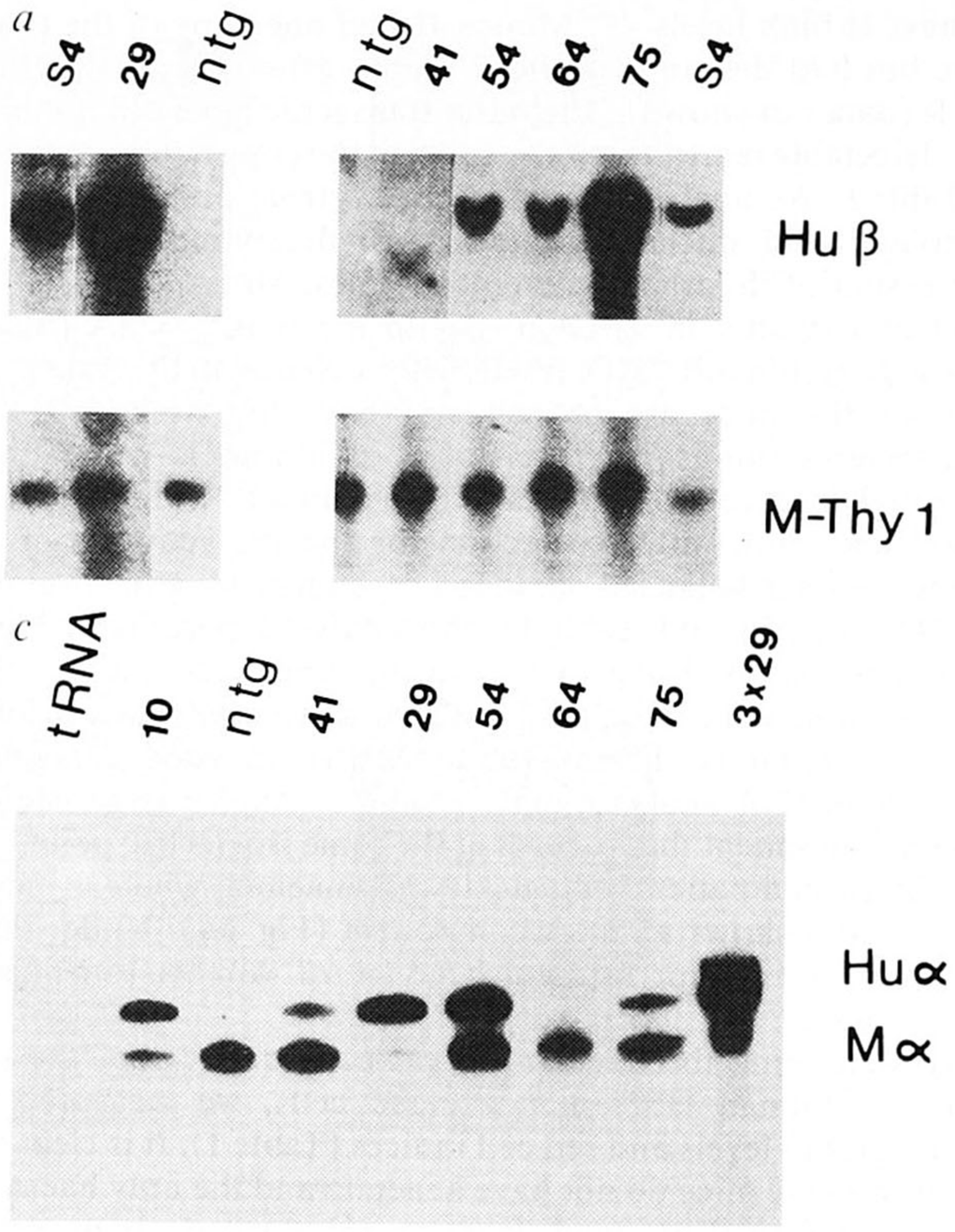

FIG. 2 Copy number, RNA and haemoglobin analysis of transgenic mice. H, human; M, mouse; ntg, nontransgenic. *a*, Tail biopsy DNAs of transgenic mice were digested with *Pst*1, fractionated by electrophoresis, transferred to nitrocellulose and hybridized to human β-globin and mouse Thy-1-specific probes. Placental DNA of a transgenic mouse (S4) with four copies of a human β-globin construct was used as a copy-number standard. *b*, Peripheral blood RNA samples (1 μg) were analysed using a 5′ human β-globin *Acc*I fragment (525 base pairs) and a mouse β-globin *Nco*I–*Hind*III probe (700 base pairs) in an S1 nuclease protection assay; specific activity ratio of the probes, 1:2 (human:mouse). Spleen RNA of a five-copy transgenic $\beta^A\alpha\theta$ transgenic mouse line (10; ref. 8) was used as positive control; nontransgenic blood RNA (ntg) and 10 μg transfer RNA (tRNA) as negative controls. To show that the assay was performed in conditions of probe excess, 1 and 3 μg of mouse 29 RNA were analysed (3×29). *c*, Peripheral blood RNA (1 μg) was analysed with the same control RNAs as in *b*, using a 3′ human α-globin *Bst*EII probe (700 base pairs) and a mouse α-globin *Bam*HI probe (300 base pairs). Specific activity ratio of the probes, 2:1 (human:mouse). *d*, Haemolysates of $\alpha\alpha\beta^S$-transgenic mice were analysed on an LKB Multiphor IEF horizontal slab gel. Sickle cell patient (SS), normal human blood (AA) and normal mouse blood (ntg) were run as markers. HbS, HbA and MHb indicate the position of sickle cell Hb, normal human adult Hb and mouse adult Hb, respectively. The gel was photographed without staining. Gel slices were excised, the haemoglobin was eluted into water and quantitated by absorption spectroscopy at 415 nm (Table 1).
METHODS. Southern blots, S1 analyses and IEF were performed as described previously[8].

erythrocytes[18,19], the presence of even a few ISCs proves that repeated sickling must have occurred *in vivo*.

The fact that mouse 29, with 83% Hb^S, had a normal somatic development, no obvious manifestations of disease, and no evidence of haemolytic anaemia is interesting and constitutes a difference from the homozygous sickle cell disease. We are not sure about the reason for this discrepancy, but one explanation might be that, because of its physico-chemical properties[14], 17% mouse Hb is still sufficient to protect the animal to some extent. It is remarkably reminiscent of the well-characterized human condition of heterozygosity for β^S and HPFH[2,20] which results in HbS levels ranging from 65 to 90%, no anaemia, normal red cell indices and mostly normal reticulocyte counts; just as mouse 29. It appears that the 17% mouse Hb does not prevent HbS polymer formation in mouse 29 (or 75, Fig. 4), but may protect it against haemolytic anaemia, just as similar levels of fetal haemoglobin protects the human β^S/HPFH heterozygotes. However, joint pains and infarctive phenomena have been reported in these subjects[21,22], indicating that sickling does take place in them *in vivo*, just as in mouse 29. The proportion of HbS could be increased further by crossing these transgenic mice with thalassaemic mice[23,24]. Alternatively, transgenic lines could

TABLE 1 Globin gene expression and haematological analysis of $\alpha\alpha\beta^S$-transgenic mice

Mouse no.	copy no. $\alpha\alpha\beta^S$-gene	HUβ^S/Mβ mRNA	Huα/Mα mRNA	HbS (% of total Hb)	Age (weeks)	Hb (g dl^{-1})	PCV (l l^{-1})	MCV (fl)	MCH (pg)	MCHC (g dl^{-1})	Reticulocytes (%)	Sickling test (%)	ISC
41	1*	0.11	0.17	0	6			58	15.7				
29 (Sample I)	8–10	2.40	2.68	83%	15	14.4	0.49	47	13.8	29.4	ND	>95.0	+
29 (Sample II)		ND	ND	ND	30	13.5	0.41	45	14.6	32.7	4.4	>95.0	+
54	5	0.50	0.58	35%	4	12.5	0.46	53	14.5	27.4	8.1	0	—
64	Mosaic	0.00	0.00	0	4	14.4	0.50	58	16.9	28.8	9.0	0	—
75	Mosaic	0.17	0.24	10%	4	11.5	0.40	66	19.0	28.9	12.0	3.3	—
NtC						13.4 (±2.2)	0.46 (±0.07)	53 (±3)	15.9 (±1.0)	29.8 (±1.5)	5.3 (±1.7)	0	—

NtC, nontrangenic controls (n=16; 4–15 weeks; number in parentheses, s.d.); ND, not determined; Hb, haemoglobin concentration; PCV, packed cell volume; MCH, mean corpuscular haemoglobin; Hu, human; M, mouse
* Deletion in the β-globin gene (see text).

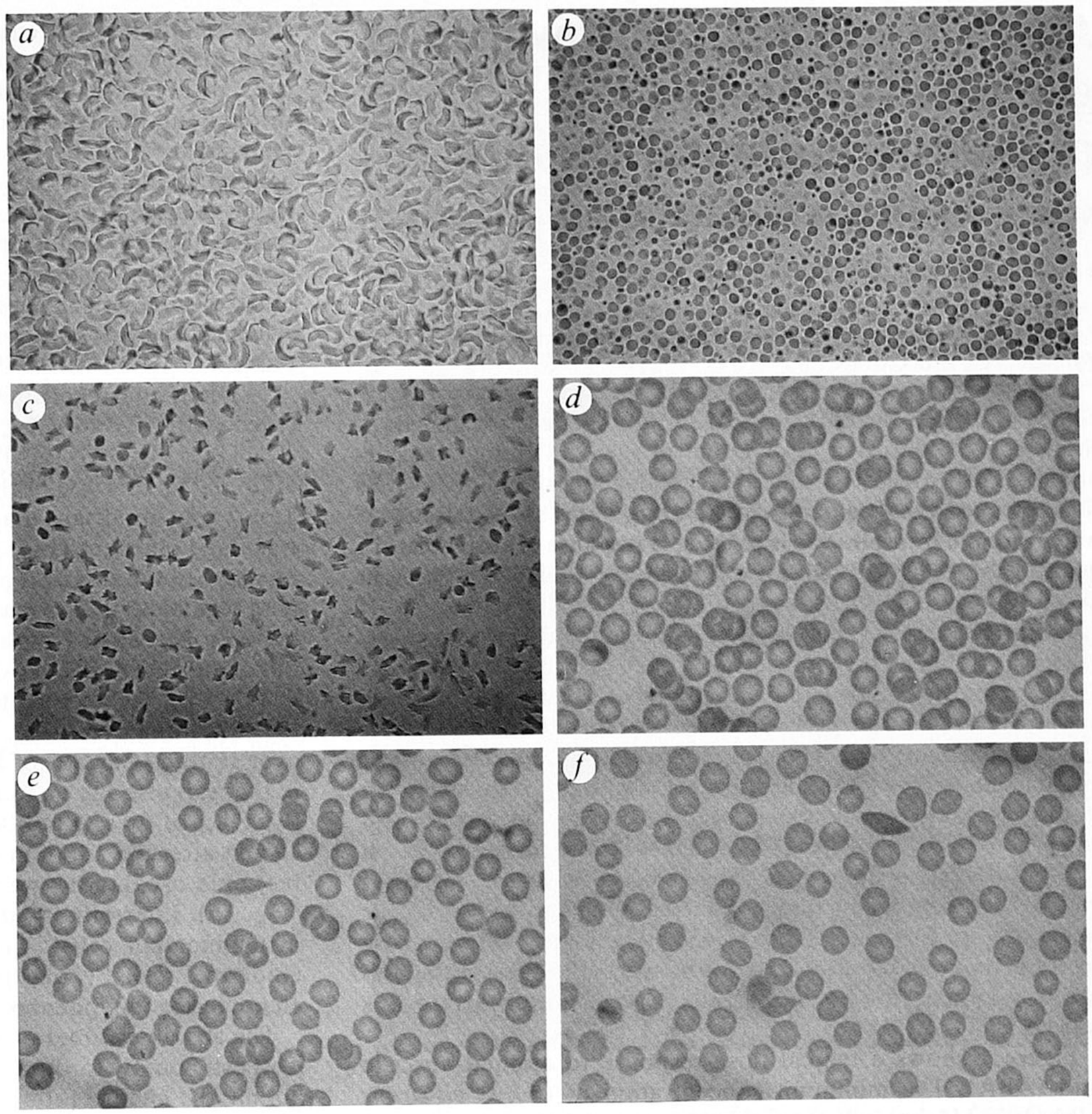

FIG. 3 Haematological analysis of transgenic mice. Sickle tests[13] were performed on blood of a sickle trait patient (*a*), peripheral blood of a non-transgenic mouse (*b*) and peripheral blood of $\alpha\alpha\beta^{s}$-transgenic mouse 29 (*c*). May-Grunwald Giemsa-stained blood films are shown for nontransgenic mouse blood (*d*) and for blood of transgenic mouse 29 (*e*, *f*).

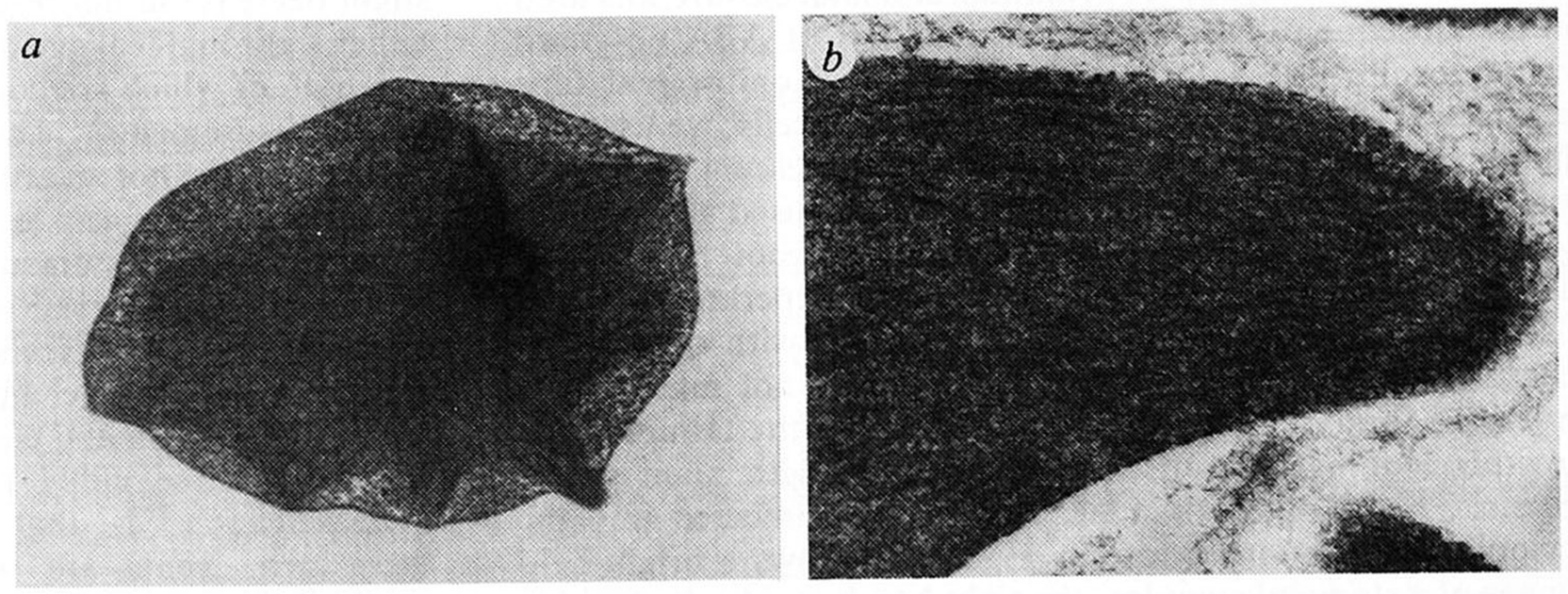

FIG. 4 *a*, Intracellular HbS polymers in mouse red cells. Fibres are seen in large portions of a cell which would not be classified as sickled by light microscopy (original magnification, ×10,000). *b*, Numerous parallel polymer fibres are seen in a knob protruding from a cell which appears to be in the process of sickling (original magnification, ×40,000).

be obtained with higher copy numbers. If either of these approaches will mimic not only sickling but also human sickle cell anaemia remains to be seen.

The present model should already make it possible to carry out an analysis of the factors which precipitate sickling *in vivo* and would also provide a means of screening for antisickling agents which might be clinically useful. After crossing with thalassaemic mice[23,24] the low copy number mice should provide an excellent animal model to develop somatic gene (addition) therapy by using the β-globin DCR. □

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