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A dominant control region from the human β-globin locus conferring integration site-independent gene expression — Source link

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Apart from the inhibition of κ rearrangement, the transgenic mice also exhibit a decreased amount of rearrangement at the IgH loci. Of a total of 25 random λ^+-hybrids examined from the transgenic mice, 13 (52%) contained a germline J_H allele as detected using a probe for the region located between D-Q52 and J_H1 (one of the blots is shown in Fig. 3*b*). This is a considerably higher figure than would be expected from an analysis of IgH rearrangements both in primary B cells[18] as well as in random hybrids generated by fusing spleen cells from (C57BL/6×SJL) mice with X63.Ag8.653 (ref. 10). We cannot formally exclude the possibility that this inhibition is due to the secreted form of the human α2 heavy-chain polypeptide. A similar inhibition of IgH rearrangement, however, has recently been described in mice transgenic for a κ-light-chain gene where 22% of the hybrids were found to contain a germline J_H allele[19]. By analogy with the proposal of Manz *et al.*[19], we suspect that the increased frequency of germline J_H loci in the hybridomas from the IgA2,λ1 transgenic mice is probably caused by a combination of transgenic λ_1 chain with the endogenous μ_m-polypeptide leading to an early switch off of recombinase activity in pre-B-cells that have just rearranged their endogeneous IgH locus. This could then lead to a diminished amount of D–J_H rearrangement on the excluded IgH allele. The fact that we see a higher proportion of germline J_H alleles in the IgA2,λ_1 transgenic mice than has been described in the κ-transgenics could be due to that fact that expression of the λ_1 transgene is potentiated by the IgH enhancer; it may therefore be switched on earlier than the κ transgene. Because of this inhibition of both heavy and light chain rearrangement, it is not surprising that the transgene causes an overall depression of B-cell development as the number of spleen cells in the transgenic mice was ~70% that of their non-transgenic siblings.

The analysis of immunoglobulin light-chain gene expression in mouse plasmacytomas and in hybridomas from mice transgenic for a κ-light chain[7,8,12] supports a model in which V_κ–J_κ joining is switched off following a signal delivered by a complete IgM,κ antibody molecule. Recent data obtained using both transgenic mice and pre-B-cell lines were consistent with the membrane form of the IgM antibody mediating this feedback regulation[9,20]. From the work described here it is clear that a transgenic λ-light chain (presumably in association with the endogeneous μ_m-polypeptide) causes an inhibition of κ-locus rearrangement. It is probable that, following the production of intracellular μ_m polypeptide chains, any light-chain rearrangement be it λ or κ that leads to IgM on the cell surface results in a cessation of all V–J joining. It is attractive to speculate that whereas the presence of μ_m-polypeptide in the membrane of the endoplasmic reticulum signals a stop to heavy-chain rearrangement and a start to light-chain rearrangement, productive light-chain rearrangement causes the μ_m-polypeptide to be translocated to the plasma membrane where it signals to stop all V-gene rearrangement. □

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A dominant control region from the human β-globin locus conferring integration site-independent gene expression

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THE regulatory elements that determine the expression pattern of a number of eukaryotic genes expressed specifically in certain tissues have been defined and studied in detail. In general, however, the expression conferred by these elements on genes reintroduced into the genomes of cell lines and transgenic animals has turned out to be at a low level relative to that of endogenous genes, and influenced by the chromosomal site of insertion of the exogenous construct. We have previously shown that if regions flanking the human β-globin locus are introduced into the mouse genome along with the human β-globin gene, a level of expression comparable to that of endogenous genes can be achieved that is also independent of integration site[1,2]. We have now defined a dominant control region with these properties consisting of 6.5 kilobases of DNA encompassing erythroid cell-specific DNase I hypersensitive sites. The identification of such dominant control regions could have important applications in somatic gene therapy.

The human β-globin 'minilocus' construct contained 21 kilobases (kb) of DNA from the region 5 to the ε-globin gene encompassing four erythroid cell-specific DNaseI hypersensitive sites and 12 kb of DNA 3′ to the human β-globin locus[1]. To investigate the properties of these dominant control region (DCR) sequences, the 33 kb 5′ and 3′ sequences were replaced by a 6.5 kb DNA fragment containing only the upstream hypersensitive sites[1,3,4] (Fig. 1). Construct 1359 contains the four hypersensitive sites in the same orientation relative to the β-globin gene (and the *tk-neo*^{r} gene; tk, thymidine kinase; neo^r, neomycin-resistance) as that found on chromosome 11. Construct 1400 has the DNaseI hypersensitive sites in the opposite orientation and 1401 and 1357 have the four DNaseI hypersensitive sites placed 3′ to the β-globin gene (Fig. 1).

The insert of construct 1359 was injected into fertilized mouse eggs and 56 embyos were collected after 13.5 days of gestation. Thirteen transgenics were obtained in two groups as defined by S1 nuclease protection (Fig. 2*a*) and Southern blot analysis to measure the expression levels and copy number of the human β-globin gene in the fetal liver (Table 1). Members of the first group expressed the insert at low levels because they were mosaics (three mice, for example lane 2), or carried a deletion in the insert (two mice, for example lane 3). The remainder express the gene at high levels in a copy-number dependent way: five of these looked normal (for example lane 4) and three looked anaemic (for example lane 1). These results are therefore similar to those obtained with the minilocus[1] and show that full erythroid-specific activation is obtained by the small reconstructed DCR, sometimes leading to a thalassaemia-like anaemia.

To study the different β-globin constructs and avoid mosaicism, three independent, stably transformed, MEL cell popula-

tions were generated for each construct[1]. RNA was prepared before (−) or after (+) differentiation[5] and analysed on northern blots with probes of similar specific activity. Figure 2b shows that human β-globin mRNA is present at levels as high as those of endogenous mouse globin mRNA after induction. For comparison, Fig. 2*b* shows equal amounts of RNA from two populations using a construct (1273) without the DCR. Human β-globin expression is increased at least 100-fold by the addition of the four DNaseI hypersensitive sites and this high level of expression is observed in all orientations and relative positions (1359, 1400, 1401 and 1357). Moreover, this level of expression seems to be higher than that seen previously with the β-globin minilocus[2] (1016, Fig. 2*b*). We used S1 nuclease analysis to quantitate the levels of mRNA using probes for the human β, mouse α and mouse β^{maj} globin genes (Fig. 3*a* and Table 1). Hybridization signals from Southern blots to probes for *tk-neo*^{r} and human β-globin genes were compared to those from the endogenous single-copy mouse Thy-1 gene and average β-globin gene copy numbers were calculated (Fig. 3*b*, Table 1). The expression per gene copy ratio is 120% per human β-globin gene compared to the endogenous mouse β^{maj}-globin gene (see Table 1 legend). As observed in the embryos, the expression levels seem to be directly proportional to the gene copy number as was observed for the human β-globin minilocus[1,2], although this argument is weakened by the fact that all the MEL populations generated have a similar average gene copy number (Fig. 3*b*, Table 1).

Figure 2*b* also shows that the 6.5 kb DNA fragment activates the heterologous herpes simplex virus (HSV) *tk* promoter in a similar way (compare 1359 to 1273). Unlike β-globin expression however, *neo*^{r} transcripts are present at about the same level as with the β-minilocus cosmid (ref. 2 and 1016), possibly because the *tk-neo*^{r} in the minilocus may already be transcribed maximally. Another difference between the minilocus and plasmid constructs is the higher level of β-globin and the *tk-neo*^{r} transcripts before erythroid induction (Fig. 2*b* and ref. 2). This may be due to the loss of specific sequences or to the greatly reduced size of the constructs. Therefore, the reconstructed DCR does not isolate the β-globin gene from the effects of flanking sequences before differentiation.

To investigate further the activation of the heterologous HSV *tk* promoter, constructs were prepared which carried the 6.5 kb DCR without any human β-globin gene sequence (1417) or carrying the previously described enhancers, either from within (1139) or from the 3′ flanking region of (1138) the β-globin gene[6,7,8,9]. Activation and erythroid inducibility of the HSV *tk* promoter is not dependent on the promoter or enhancers of the β-globin gene (Fig. 2*c*).

Interestingly the human β-globin gene expression per gene copy is increased, when compared to the β-globin minilocus (1016, Fig. 2b), but the *tk-neo*^{r} expression remains the same. The transcription machinery of the *tk* promoter may have been

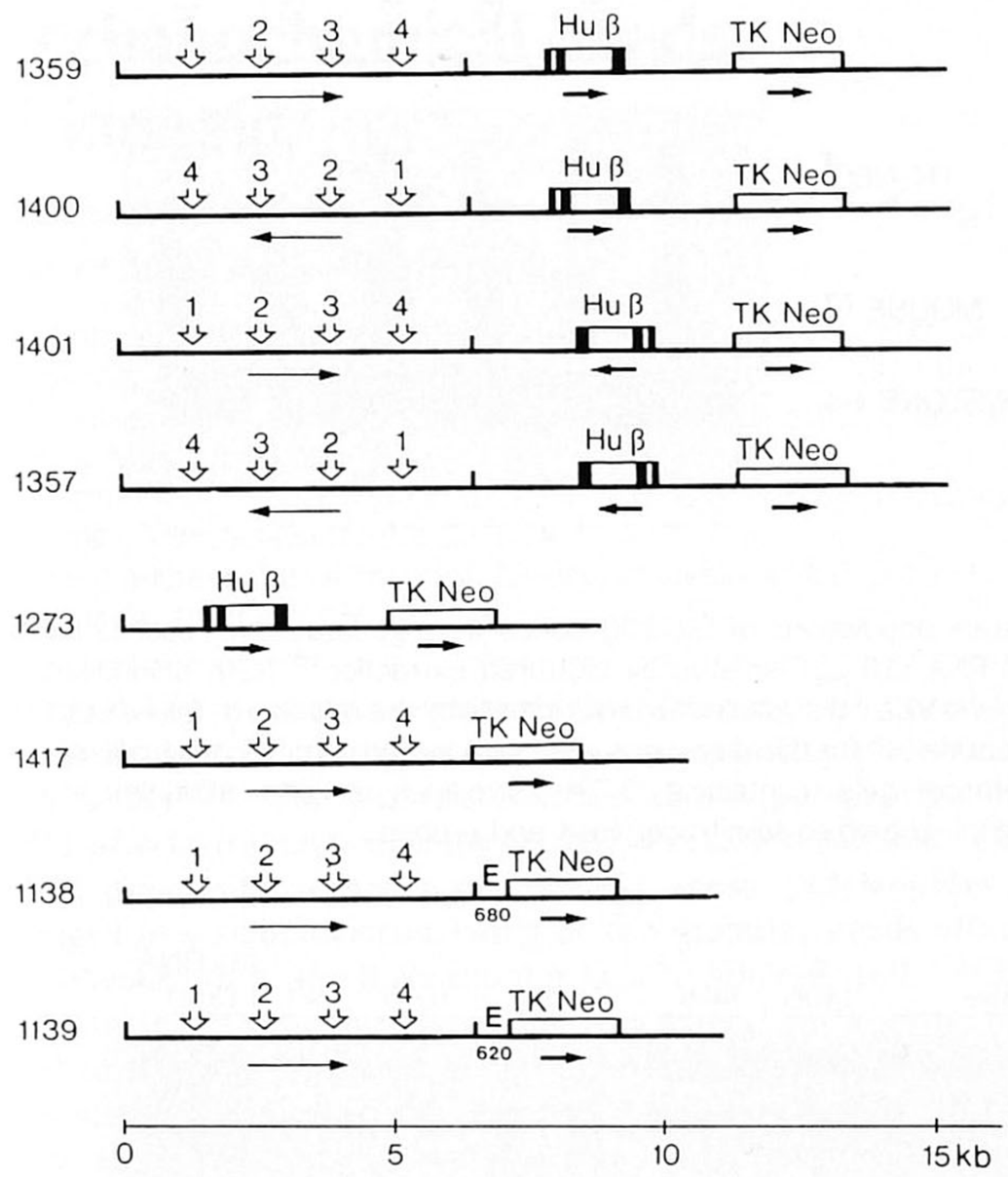


FIG. 1 Construction of the human β-globin plasmid locus.
METHODS. Plasmids 1359, 1400, 1401 and 1357 were constructed using restriction endonuclease fragments encompassing the 5′ DNaseI hypersensitive sites ligated into a pPoly-III-I vector (a gift from A. J. Clark, Edinburgh) in the same orientation as in the normal β-globin locus: 2.1 kb *Bam*HI–*Xba*I fragment containing hypersensitive site 1, a 1.9 kb *Hin*dIII fragment containing hypersensitive site 2, a 1.5 kb *Asp*718–*Bgl*II fragment containing hypersensitive site 3 and a 1.0 kb partial *Sst*I–*Hin*dIII fragment containing hypersensitive site 4. A 6.5 kb *Not*I fragment containing all of the restriction fragments of the hypersensitive sites was then cloned into a Bluescript vector (1273) containing the 4.8 kb *Bgl*II fragment of the human β-globin gene[1] and a 2.7 kb partial *Eco*RI *tk-neo*^{r} gene fragment from the cosmid pTCF (ref. 15). Vertical arrows show arrangement of the hypersensitive sites in the dominant control regions but do not show their actual positions within these constructs. Construct 1417 contains the 6.5 kb *Not*I fragment containing the four 5′ hypersensitive sites cloned into a specially designed polylinker in a pUC18 vector containing the 2.0 kb partial *Nar*I–*tk-neo*^{r} gene fragment. Construct 1138 contains the 680 bp *Dra*I–*Acc*I fragment of the 3′ human β-globin enhancer cloned between the *Kpn*I and *Cla*I sites of 1417. Construct 1139 contains the 620 bp *Dra*I fragment of the internal enhancer of the human β-globin gene[9]. Construct 1016 is the β-globin minilocus cosmid[1,2].

TABLE 1 Copy number and expression levels of the human β-globin gene in transgenic mice and transfected cells

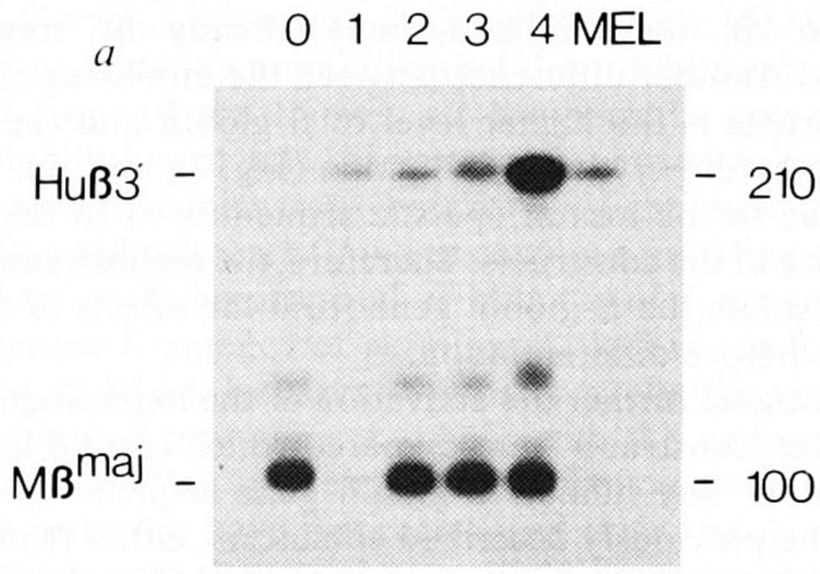
		c.p.m. Hu β	Copy number Hu β-globin	Percentage expression Hu β/ Mα	Hu β/ $M\beta^{maj}$	Mα/ $M\beta^{maj}$
Mouse	1	315*	4.0	ND	160	ND
	2	449	mosaic	ND	—	ND
	3	654	deletion	ND	—	ND
	4	5,385	3.0	ND	160	ND
1357	a	14,738	7.0	70	130	190
	b	10,859	4.3	90	130	140
	c	10,692	2.6	110	210	200
1359	a	12,692	7.7	70	110	170
	b	18,855	7.0	100	140	150
	c	17,533	7.0	80	150	200
1400	a	19,670	5.8	90	100	100
	b	20,924	5.8	90	90	100
	c	18,695	6.2	90	90	100
1401	a	14,643	3.4	120	120	100
	b	17,835	4.5	100	110	110
	c	13,262	4.9	70	70	100
		Average expression/gene copy		90	120	140
		Standard deviation		16	36	42
1359	a	39,709	7.7	60	120	180
3x	b	55,012	7.0	90	150	160
	c	56,059	7.0	80	160	210
1016	a	2,684	5.0	18	50	270
	h	1,289	7.0	11	30	280

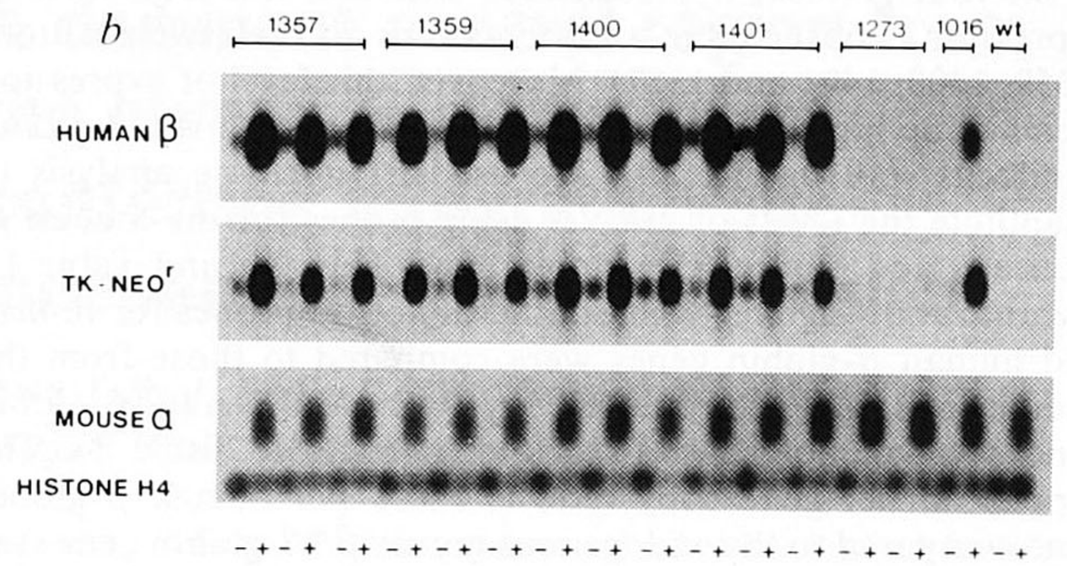
Copy numbers of human β-globin (Hu β) were determined by Southern blotting and laser densitometry as described. Background-corrected Cerenkov counts for human β-globin, mouse β-globin major and mouse α-globin protected fragments were expressed as ratios of the exogenous human β-globin message (Hu β) to the endogenous mouse α and β^{maj} signals (Mα and $M\beta^{maj}$), correcting for relative specific activities. This expression ratio was then reduced to an expression per gene copy ratio by dividing it by the ratio of the number of copies of human β-globin to the number of endogenous gene copies (two for mouse β-globin major and four for mouse α-globin). Clones a and h seem to have threefold lower human β-globin expression than the average minilocus MEL population due to the loss of endogenous mouse β-globin genes[2]. ND, not determined, * Anaemic.

saturated, which also explains why the addition of human β-globin gene enhancers to the 6.5 kb DNA fragment does not result in any further increase in the number of *tk-neo*^{r} transcripts in MEL cell populations (Fig. 2*c*). We therefore suggest that a requirement for reproducible but low expression levels of biologically active molecules could be met by the combination of the DCR and inefficient or mutagenized promoters.

DNaseI fadeout analysis on nuclei from uninduced cells shows that the hypersensitive sites 1–4 are regenerated in uninduced MEL cells, but not in L-cells with the exception of site 3 (data not shown and ref. 2). Weaker DNaseI hypersensitive sites can be seen on the β-globin gene promoter, 3′ enhancer and *tk-neo*^{r} promoter. Therefore, at this level of resolution, the establishment of an 'active' chromatin configuration is separable from full β-globin gene transcription.

The human β-globin gene constructs in these experiments are





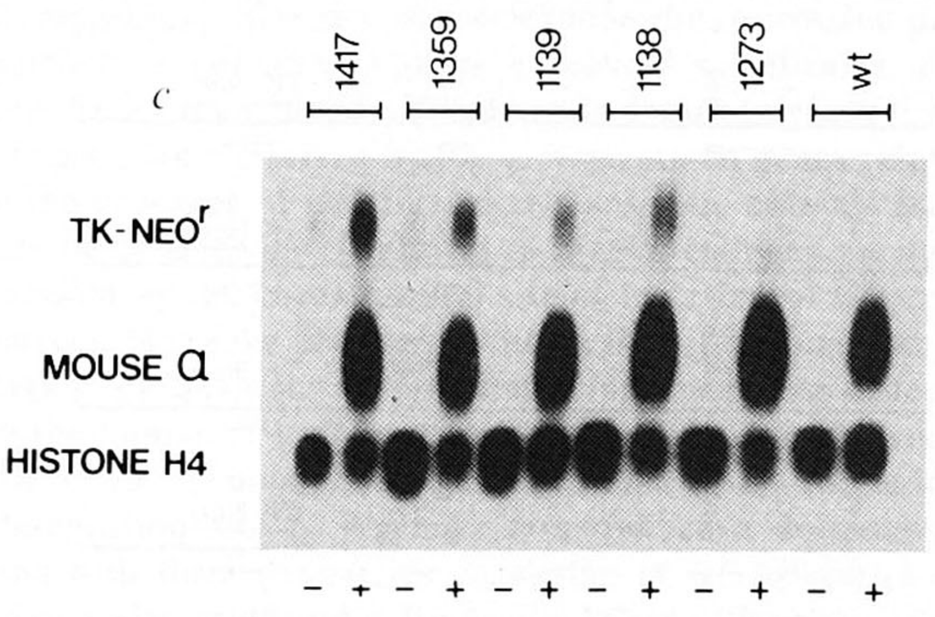
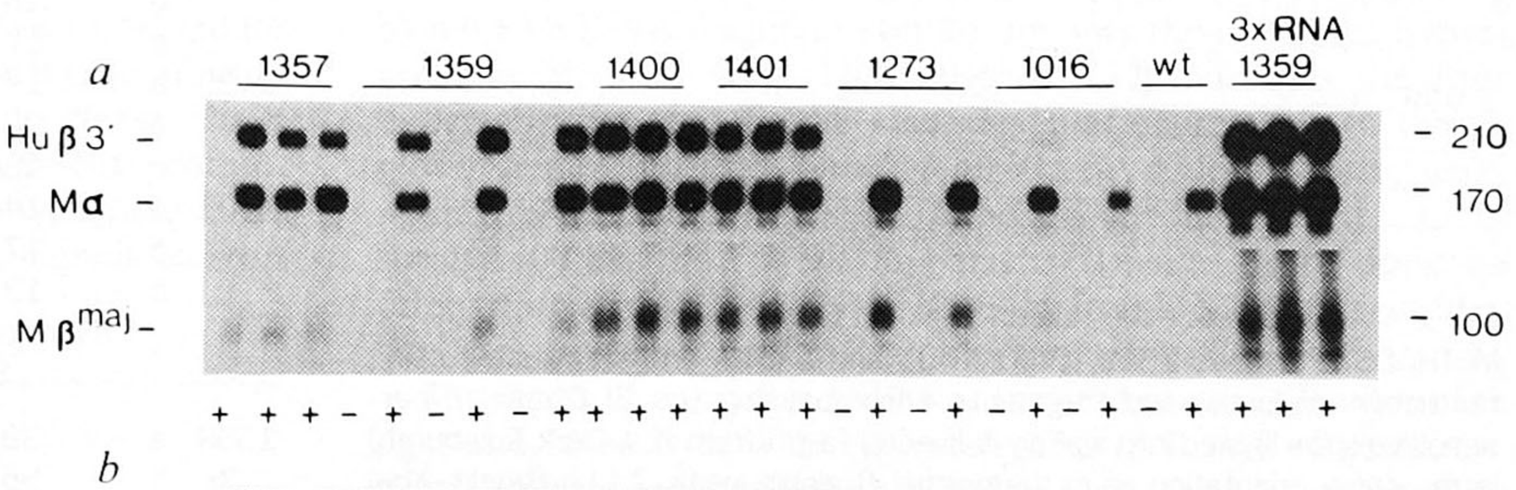


FIG. 2 Analysis of human β-globin expression. *a*, S1 nuclease protection analysis of RNA from the transgenics (sizes are shown on the right). *b*, Northern blot of RNA prepared before (−) or after (+) differentiation of the stably transformed MEL cell lines. Lanes marked 1016 contain RNA from a MEL cell clone stably transfected with the human β-globin minilocus[2]. Wt, RNA from untransfected MEL C88 cells. The probes were for human β-globin (760 bp *Eco*RI–*Msp*I) or the *tk-neo*^{r} gene (580 bp *Sph*I–*Bgl*II). The filter initially probed with human β-globin was reprobed with both the mouse histone H4 probe (460 bp *Eco*RI–*Tth*111I) and the mouse α-globin probe (300 bp *Bam*HI). *c*, Northern blot of total RNA (10 μg) from uninduced (−) and induced (+) transfected C88 cell populations, probed simultaneously with the *tk-neo*^{r} probe, the mouse α-globin probe and the mouse histone H4 probe.

METHODS. In (*a*), an 11.5 kb *Eco*RV fragment from construct 1359 was gel-purified and microinjected into fertilized mouse eggs[1]. The levels of human β-globin mRNA were determined by S1 nuclease analysis with a mixture of probes for the human β-globin and mouse β^{maj}-globin genes[1,2]. After autoradiography, each band was excised from the gel, placed at the bottom of an Eppendorf tube and quantitated by Cerenkov counting. A local background count was obtained by measuring a gel slice immediately above the band of interest and was subtracted from the actual count in subsequent calculations (see Table 1). For the Northern blots (*b*, *c*), plasmid DNA of indicated constructs (100 μg) was linearized by digestion with *Pvu*I and introduced into MEL C88 cells by electroporation[9]. Three independent G418-resistant populations of 50–100 clones were generated for each construct. Total RNA (10 μg) isolated by LiCl/urea extraction[16] from uninduced MEL cells, and MEL cells induced to differentiate by the addition of 2% v/v dimethyl sulphoxide (+) for five days, was electrophoresed on duplicate 1.5% agarose denaturing gels containing 0.7% formaldehyde and ethidium bromide (1 μg ml^{-1}) blotted to nitrocellulose and probed.



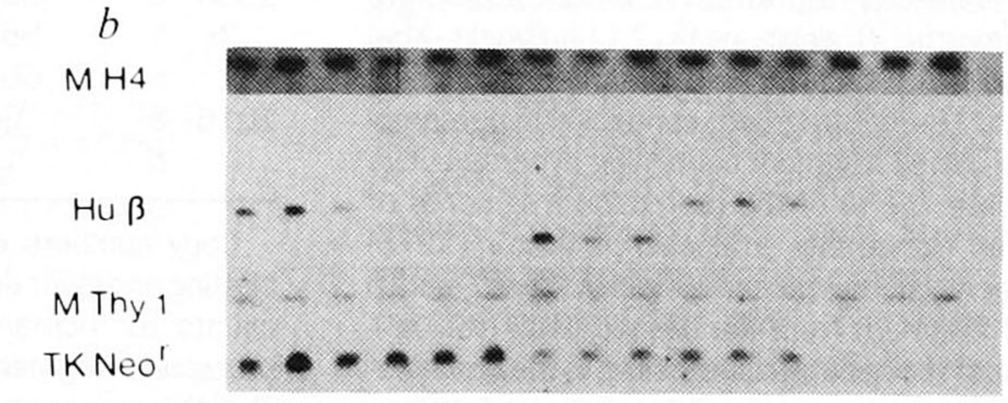


FIG. 3 S1 nuclease analysis and copy number determinations. *a*, S1 nuclease analysis of total RNA from uninduced (−) and induced (+) MEL cells. *b*, Southern blotting of genomic DNA from MEL populations.

METHODS. (*a*) Total RNA (10 μg) from uninduced (−) and induced (+) populations of MEL cells were hybridized to a mixture of mouse β-globin major, mouse α-globin and human β-globin probes described previously[1,2]. Relative specific activities were 1, 10 and 8, respectively, for the above probes. Three controls containing 30 μg of RNA (3 × RNA) were performed to prove that the S1 probes were in excess. Quantitation was as described in Fig. 2*a*. 1016 RNAs are from two MEL cell clones containing the human β-globin minilocus[2]. Sizes are shown on the right. *b*, Genomic DNA (8 μg) of MEL populations was digested with *Eco*RI and electrophoresed on a 0.6% agarose gel. After Southern blotting, the filter was probed with human β-globin intervening sequence probe (900 bp *Bam*HI–*Eco*RI and subsequently with a *tk-neo*^{r} resistance probe (580 bp *Sph*I–*Bgl*II), a mouse Thy-1 probe (M Thy 1) (600 bp *Pst*I) and a histone H4 probe (M H4) (460 bp *Eco*RI–*Tth*111I). Laser densitometry using a range of autoradiographic exposures was used to quantitate human β-globin copy number. Mouse Thy-1 and histone H4 were used to correct for DNA loading. Human placental DNA was used to obtain an estimate of the actual β-globin copy number. The human β-globin copy number of the populations of cells containing 1359 were compared to the other constructs through the *tk-neo*^{r} gene signal as the *Eco*RI digestion gave a larger 9.3 kb human β-globin *Eco*RI fragment.

integrated at different sites into host cell DNA and have been replicated as chromatin. This contrasts with transient transfections using extra-chromosomal DNA templates not packaged into chromatin. Viral enhancers can stimulate β-globin gene expression several hundredfold in transient assays[10], but this is not observed after stable integration, and levels of expression are dependent upon the site of integration into host DNA.

The observation that only 6.5 kb of DNA from the 5′ boundary of the human β-globin locus allows high levels of expression of the human β-globin gene and the heterologous *tk-neo*^{r} gene in erythroid cells has implications for the prospects of somatic gene therapy. Currently, the most realistic approach for therapy of a β-globin gene disorder (thalassaemia or sickle-cell anaemia) is by *in vitro* retroviral infection of bone marrow and transplantation[11–14]. Retroviruses carrying the human β-globin gene give rise to low levels of expression that are dependent upon the site of integration. The observation that the DCR works equally efficiently in all orientations and positions should facilitate production of retrovirus stocks with higher titres. The size of the reconstituted DCR with a smaller human β-globin gene is within the range that can be packaged into retroviral particles. Such constructs have been made and are currently being tested. □

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Identification of globular mechanochemical heads of kinesin

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KINESIN is a mechanoenzyme which uses energy liberated from ATP hydrolysis to transport particles towards the 'plus ends' of microtubules[1–6]. The enzyme consists of two polypeptide heavy chains of relative molecular mass (M_r) ≈ 110,000–140,000 (110K–140K) plus copurifying light chains; these polypeptides are arranged in a structure consisting of two globular heads attached to a fibrous stalk which terminates in a 'feathered' tail[7–11]. Here we report that a function-disrupting monoclonal antikinesin, which binds to the 45K fragment of the kinesin heavy chain[12,13], recognizes an epitope located towards the N-terminal end of the heavy chain, and decorates the two globular heads lying at one end of the intact molecules (one antibody per head). The results show that the two heavy chains of native kinesin are arranged in parallel, and that the 45K fragments, which display nucleotide-sensitive interactions with microtubules[12,13], represent mechanochemical 'heads' located at the N-terminal regions of the heavy chains. Thus, it is likely that the kinesin heads are analogous to the subfragment-1 domains of myosin.

Electron microscopy[7,10,11] and DNA sequence analysis[15] indicate that kinesin heavy chains are organized into distinct domains. We have obtained evidence for such domains from the proteolytic cleavage of kinesin, which yields heavy-chain fragments of $M_r \approx 45K$ and 76K[12,13]. The 45K fragment is bound by three monoclonal antikinesins that inhibit kinesin-driven microtubule motility[12], one of which, SUK 4, was used in the present study. Interestingly, the proteolysis reaction that produces the SUK 4-reactive 45K fragments is nucleotide- and microtubule-sensitive (Fig. 1). Irrespective of the presence or absence of microtubules, the 130K kinesin heavy chain is resistant to cleavage in the absence of nucleotide whereas the formation of the 45K peptide is enhanced by magnesium ATP. In the absence of microtubules, MgATP also promotes the formation of a small quantity of a 25K subfragment of the 45K fragment (Fig. 1, lane 5). The presence of AMPPNP (an unhydrolysable ATP analogue) or ADP seems to favour subdigestion of the 45K domain in the absence of microtubules; consequently no SUK 4-reactive products are visible on immunoblots. The 45K fragment is, however, stabilized by the presence of microtubules in AMPPNP or ADP. It seems therefore that the proteolytic reactions that produce and subdigest the 45K fragment are affected by the conformational differences of kinesin–micro-

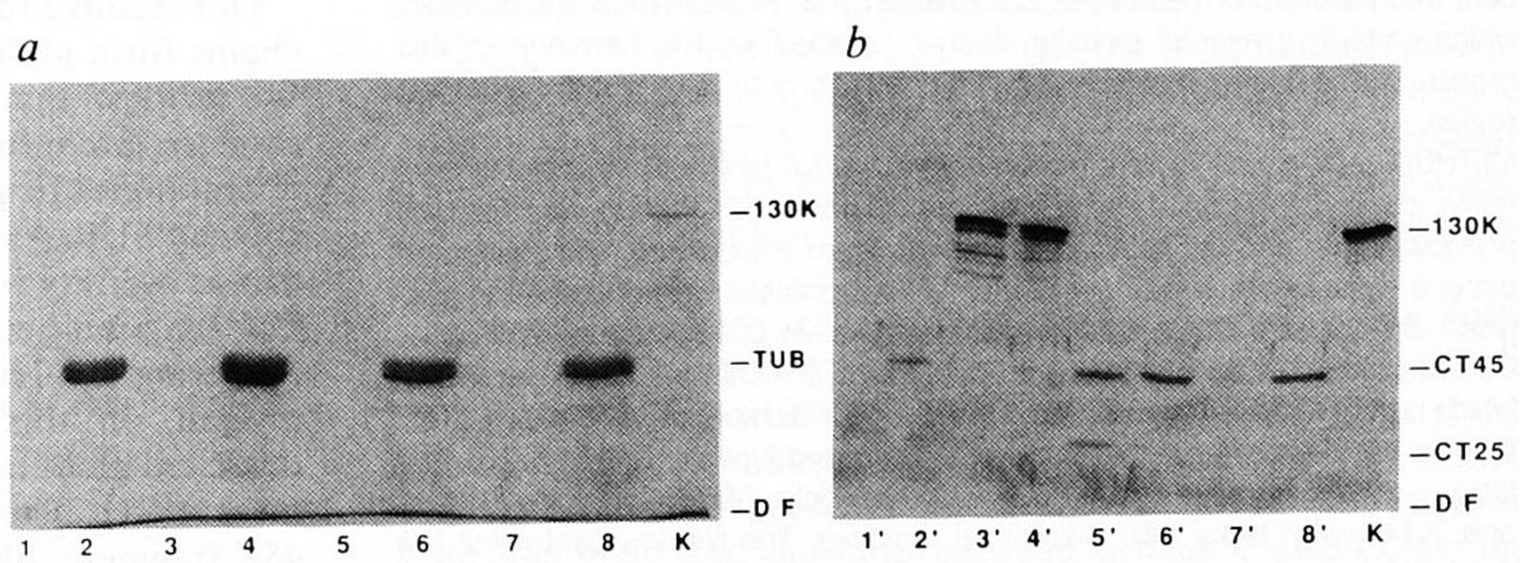

FIG. 1 Nucleotide and microtubule-sensitive proteolysis of sea-urchin kinesin 130K heavy chains. *a*, Coomassie-stained SDS polyacrylamide gel; *b*, corresponding immunoblot probed with the function-blocking monoclonal antikinesin SUK 4, which binds to the 45K peptide[12].
METHODS. Partially purified sea-urchin kinesin (lanes marked k) was obtained by AMPPNP-microtubule affinity binding, MgATP release, and Biogel A5M chromatography in the absence of MgATP (ref. 12). The kinesin in our standard PMEG buffer[5] was digested with α-chymotrypsin[12] in the presence (lanes 2, 4, 6, 8) or absence (lanes 1, 3, 5, 7) of microtubules (2 mg ml^{-1}) assembled using taxol from purified bovine brain tubulin. Lanes 3 and 4 were incubated with apyrase 50 μg ml^{-1} to remove residual ATP or ADP; lanes 5 and 6 show digests performed in MgATP (5 mM), whereas 7 and 8 were performed in the presence of Mg AMPPNP (5 mM). Lanes 1 and 2 show digests performed using kinesin from the Biogel column and which presumably contained bound MgADP (ref. 4) (identical results were obtained in parallel digestion reactions performed in the presence of additional MgATP (1 mM) although in the presence of NaCl (0.5 M) microtubules did not protect the 45K peptide). 130K, intact kinesin heavy chain; TUB, tubulin; CT45, the 45K chymotryptic fragment of the kinesin heavy chain; CT25, a minor 25K fragment; DF, dye front.