The EMBO Journal vol.14 no.3 pp.564–574, 1995

Targeting gene expression to haemopoietic stem cells: a chromatin-dependent upstream element mediates cell type-specific expression of the stem cell antigen CD34

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Communicated by M.Greaves

The ability to target heterologous gene expression to haemopoietic stem cells will allow biological manipulation of this compartment and facilitate gene therapy of blood disorders. To identify regulatory elements with this potential, we have analysed the transcriptional regulation of the murine stem cell antigen CD34. Within haemopoiesis CD34 is expressed in stem and early progenitor cells, but it is also expressed in certain non-haemopoietic cell types, including fibroblasts. Comparison of CD34 chromatin in haemopoietic progenitor cells (416B and M1) and fibroblasts (Swiss 3T6) revealed several DNase I hypersensitive regions, one of which, a cluster of sites centred 3 kb upstream of exon 1, was specific to haemopoietic progenitors. This element stimulated expression by approximately two orders of magnitude in $CD34^+$ haemopoietic progenitors, but not in $CD34^+$ fibroblasts or in $CD34^-$ haemopoietic cells (18.8). Enhancer function was dependent upon chromosomal integration, although position-independent expression was not obtained. However, largely position-independent expression was conferred by addition of a downstream element which was hypersensitive in all the cell types analysed. We conclude that the murine CD34 gene is regulated by a chromatin-dependent, upstream enhancer element which acts in conjunction with a downstream domain-controlling element to confer high level gene expression in haemopoietic progenitor cells.

Key words: CD34/chromatin/LCR/stem cells/gene therapy

Introduction

The pluripotent haemopoietic stem cell is characterized by (i) its capacity for self-renewal and (ii) its ability to differentiate down one of at least eight possible lineage pathways (reviewed in Cross and Dexter, 1991). Constitutive dysregulation of the balance between self-renewal and differentiation is an essential feature of leukaemogenesis. While the mechanisms controlling these processes have not been defined, they are likely to involve complex interactions of stromal components and/or growth factors with cognate cellular receptors, ultimately resulting in a change in the functional balance of transcription factors within the stem cell. In contrast to the situation in terminally differentiated haemopoietic cells, where much is already known about both the patterns of gene expression and the *cis*- and *trans*-acting regulatory elements which are involved in controlling this expression, little is known about the transcriptional programmes of stem and early progenitor cells. Progress in this field has been hampered for two main reasons; first, stem cells are relatively rare *in vivo* and thus difficult to obtain in the numbers generally required for transcriptional analysis and, second, there are few obvious target genes to study. Genes whose expression appears to be associated with the stem cell progenitor phenotype include c-*kit* (Geissler *et al.*, 1988), *sca-1* (see Sinclair and Dzierzak, 1993, and references therein), *flk2* (Matthews *et al.*, 1991) and, most notably, the gene encoding the stem cell antigen CD34. CD34 antibodies are widely used to identify and purify human stem cells for both experimental and clinical purposes (Civin *et al.*, 1989); the successful engraftment of both lethally irradiated baboons and marrow-ablated humans with $CD34^+$ bone marrow cells supports the notion that CD34 expression defines a population containing haemopoietic stem cells (Berenson *et al.*, 1988, 1991). The function of CD34, however, remains unclear. The amino acid sequence predicts that CD34 is a type 1 transmembrane protein with a protein backbone molecular weight of 39 kDa and with no close homology to any other protein described to date. It is extensively glycosylated with both *N*- and *O*-linked carbohydrate, including sialic acid residues. These biochemical characteristics suggest that CD34 exists at the cell surface as an extended rod-like molecule with a net negative charge (Greaves *et al.*, 1992). It therefore seems likely that expression of CD34 at the cell surface would be able to modulate cellular interactions and adhesion and so play an important role in the interaction of stem cells with the stromal microenvironment. A recent report showing that L-selectin is able to bind to CD34 supports this view (Baumheuter *et al.*, 1993).

Against this backdrop, we elected to use the CD34 gene as a model system to investigate the transcriptional programmes of stem cells. Previous attempts to identify DNA elements regulating the human CD34 gene have demonstrated promoter activity spread over 3–4 kb of upstream sequences (Burn *et al.*, 1992; He *et al.*, 1994) and a weak enhancer (yielding 3- to 6-fold stimulation) downstream of the gene (Burn *et al.*, 1994). Because of the wider possibilities for analysis and manipulation of haemopoiesis in mice, we have focused on the murine CD34 gene. Murine CD34 cDNA and genomic clones have previously been isolated in this laboratory (Brown *et al.*, 1991) and it has recently been confirmed that the human and mouse CD34 genes have a similar overall structure (Satterthwaite *et al.*, 1992). The existence of CD34 as a stem cell marker in the mouse has been confirmed by the detection of CD34 transcripts in $Thy\text{-}1^{low}Lin^-$ haemopoietic progenitor cells (Salles *et al.*,

1992) and by the demonstration that 200 murine CD34$^+$ bone marrow cells are capable of haemopoietic reconstitution of lethally irradiated mice (Krause *et al.*, 1994). The expression of CD34 in murine haemopoietic progenitors suggests that CD34 function in haemopoiesis is conserved between mouse and human. However, it should be emphasized that CD34 is also expressed outside the haemopoietic system, with murine CD34 mRNA being present in embryonic fibroblast cell lines, brain and testis (Brown *et al.*, 1991). By comparing the regulation of CD34 in haemopoietic and fibroblast cells, we have examined whether the expression of the CD34 gene in haemopoietic progenitors is under the control of cell type-specific *cis*-acting regulatory elements. Such elements would provide a tool for further analysis of the transcriptional programme associated with haemopoietic stem cells and progenitors and allow the development of stem cell-targeting vectors, thus facilitating the production of murine models of human leukaemia and having the potential to be used in a gene therapy setting.

Results

Cellular models for CD34

In order to identify appropriate cellular models for analysis of the regulation of murine CD34, we performed Northern blotting on a panel of haemopoietic and fibroblastic cell lines. Within the haemopoietic cells tested (Figure 1, left panel), expression of CD34 mRNA was restricted to 416B (Dexter *et al.*, 1979) and FDCP-mix A4 (Spooncer *et al.*, 1984), with ~10-fold higher expression in the former. In fibroblasts, both Swiss 3T6 and NIH 3T3 fibroblasts were CD34$^+$, as is Swiss 3T3 (Brown *et al.*, 1991); only CMT 64/61, a lung fibroblast-like epithelial cell line, was negative for CD34 mRNA (Figure 1, right panel). As a result of this analysis we chose the multi-myeloid progenitor cell line 416B and Swiss 3T6 fibroblasts, the cell lines with the highest level of CD34 mRNA in each lineage, as models to test the hypothesis that expression of the CD34 gene in different cell types is under the control of different regulatory elements.

The presence of active *cis*-acting regulatory elements in DNA is usually associated with disruption of the chromatin structure, resulting in hypersensitivity of the DNA to nucleases. We therefore analysed the chromatin structure of the CD34 locus in 416B and Swiss 3T6 cells for the presence of nuclease-accessible regions. The results of DNase I hypersensitive site (HS) mapping 5′ and 3′ of the CD34 gene are presented in Figure 2A and B and summarized schematically in Figure 2C. A complex array of HSs was seen 5′ of the gene in 416B (Figure 2A, left panel). Within the first 15 kb of DNA 5′ of the gene we detected two major DNase I hypersensitive regions. The first region, 5′HS1, (band 1 in Figure 2A) maps close to the translational start and is coincident with the expected location of the promoter. The second more distal region, 5′HS2–4 (bands 2–4 in Figure 2A), comprises several discrete HSs located −2.6, −3.0 and −3.5 kb upstream of the ATG translational start. Four weak HSs were sometimes detected between the proximal and distal sites; these are not apparent in Figure 2A. Comparison with the Swiss 3T6 fibroblast line revealed the presence of the same proximal site (5′HS1; Figure 2A, right panel), but

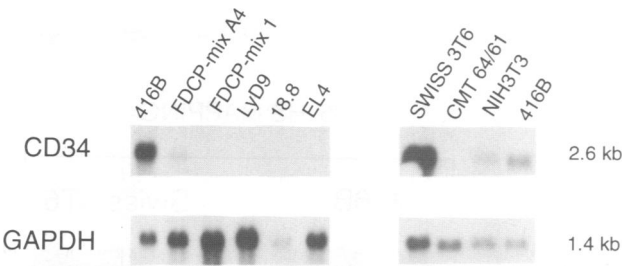


Fig. 1. CD34 expression in murine haemopoietic cells and fibroblasts. Total RNA was prepared from a number of murine haemopoietic and fibroblast cell lines and 12 μg analysed by Northern blotting. In the two upper panels, hybridization was with a full-length murine CD34 cDNA probe; the lower panels show re-hybridization of the membranes with a glyceraldehyde phosphate dehydrogenase (GAPDH) probe to control for differences in the amount of RNA loaded. Left panels, haemopoietic cell lines: 416B (multi-myeloid progenitor); FDCP-mix A4 (IL-3-dependent multipotential progenitor); FDCP-mix 1 (IL-3-dependent multipotential progenitor); LyD9 (lymphomyeloid progenitor); 18.8 (pre-B); EL4 (pre-T). Right panels, fibroblast lines: Swiss 3T6 (embryonic fibroblast); CMT 64/61 (C57 lung fibroblast-like epithelial); NIH 3T3 (embryonic fibroblast); 416B was included in the fibroblast experiment (far right lane) to allow direct comparison of CD34 expression levels between the haemopoietic and fibroblast lines. The sizes of the transcripts detected are shown to the right of the figure.

no distal hypersensitive cluster, suggesting that the distal sites 5′HS2–4 are involved in regulating the expression of CD34 specifically in haemopoietic cells. As might be expected, no HSs were seen in CMT 64/61 or EL4 (pre-T lymphoblast) cells, both of which were negative for CD34 mRNA by Northern blot analysis (data not shown).

Since *cis*-acting regulatory regions are found both upstream and downstream of genes, we also analysed the chromatin structure 3′ of the CD34 gene. Three HSs (3′HS1–3) were detected in 416B, located 2.4, 3.0 and 4.1 kb downstream of the polyadenylation signal (bands 1–3 in Figure 2B, left panel). However, at least two of these sites, and probably all three, were also present in Swiss 3T6 cells (Figure 2B, right panel) and so were not considered good candidates for cell type-specific regulatory elements. We decided, therefore, to focus on the haemopoietic-specific 5′ distal cluster of HSs (5′HS2–4) and to investigate their ability to stimulate transcription in haemopoietic and/or fibroblast cells.

Functional analysis of cis-acting elements

The role of the proximal and distal HSs in the regulation of CD34 transcription was tested by transient transfection analysis in 416B and Swiss 3T6 cells using a luciferase reporter gene. Cells were co-electroporated with various CD34−luciferase constructs and a β-galactosidase-expressing control plasmid. After 20 h, the cells were harvested and lysed to produce a cellular extract. Comparison of the luciferase and β-galactosidase activities present in each extract allowed the luciferase value to be corrected for differences in electroporation efficiency. The proximal HS region was found to have good promoter activity in 416B cells, increasing the level of luciferase activity ~40-fold compared with a promoterless control plasmid (data not shown). When the regulatory element in the plasmid was extended 5′ to include the distal HS2–4 [pCD34(4.4) in Figure 3A], luciferase activity was increased 1.25- to 2.5-fold over the CD34 promoter

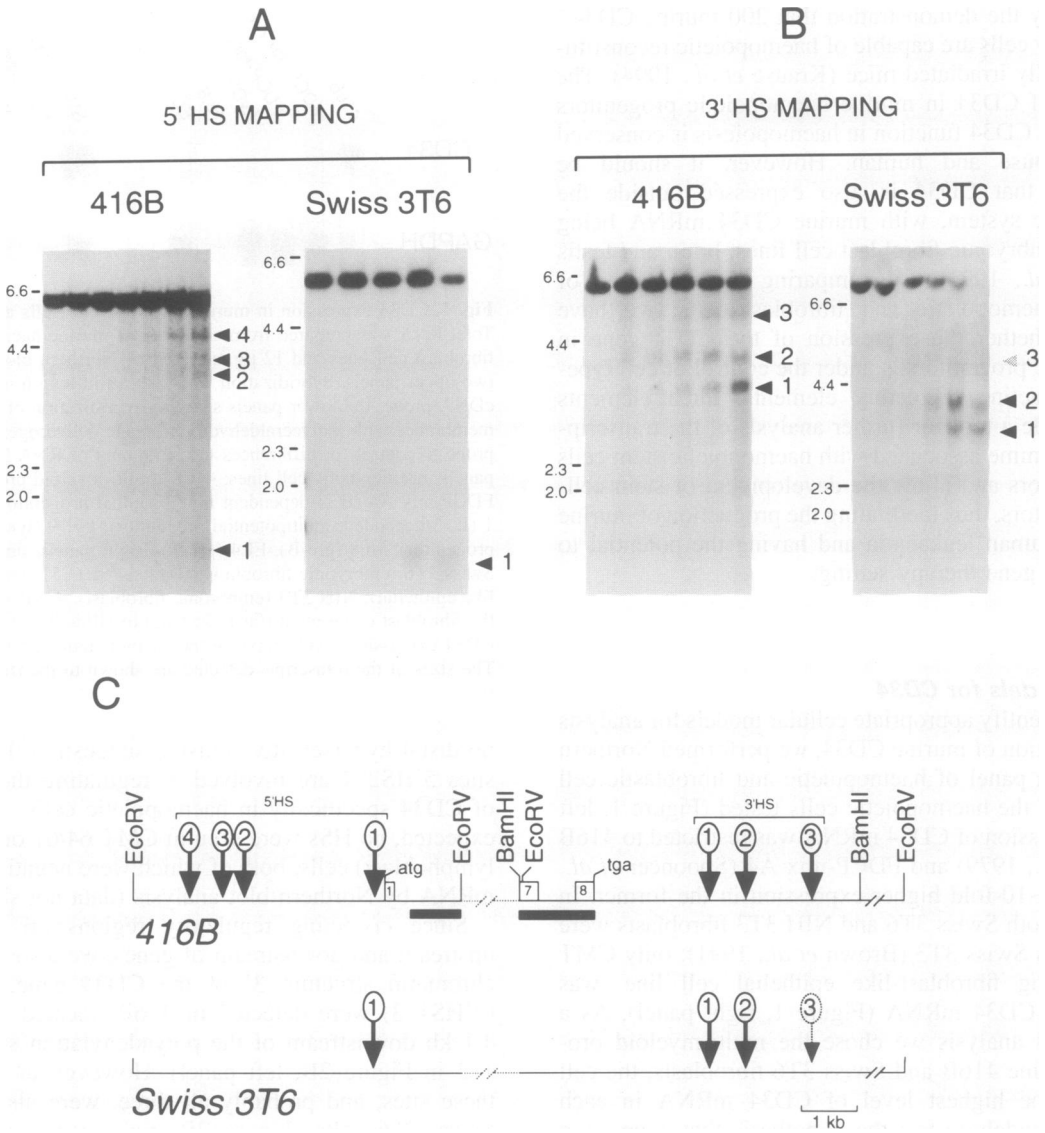


Fig. 2. Analysis of CD34 chromatin in murine haemopoietic progenitor (416B) and fibroblast (Swiss 3T6) cell lines. Nuclei were prepared and treated with a range of DNase I concentrations (0–7.5 μg/ml, increasing left to right) and analysed as described in Materials and methods. The cell type used is indicated above each blot, and the sizes in kilobases of radiolabelled lambda/*Hin*dIII markers are shown to the left. DNase I HSs are indicated by arrowheads and termed 5′HS1–4 and 3′HS1–3, with site 1 in each case lying closest to the gene coding sequences. (**A**) Analysis of the 5′ flanking region of the CD34 gene. DNase I-treated DNA was digested with *Eco*RV and hybridized with a 900 bp *Eco*RV–*Eco*RI probe (filled box in Figure 2C) derived from the first intron. Note that 5′HS2–4 are unique to 416B. (**B**) Analysis of the 3′ flanking region of the CD34 gene. DNase I-treated DNA was digested with either *Bam*HI (416B) or *Eco*RV (Swiss 3T6) and hybridized with a 1.1 kb probe which includes exons 7 and 8 (filled box in Figure 2C). 3′HS1 and -2 were clearly present in both cell lines; 3′HS3 was partially obscured by background in Swiss 3T6 cells (stippled arrowhead). (**C**) Schematic representation of the CD34 5′ and 3′ regions showing the location of the HSs (numbered as above) and the restriction sites and DNA probes (filled boxes) used in the analysis. The thin horizontal lines represent murine CD34 genomic sequences, with open boxes depicting exons 1, 7 and 8.

alone [pCD34(1.4)] following transfection into 416B cells (Figure 3A, middle panel). Although the magnitude of this effect was small, this level of stimulation was reproducibly observed in 10 independent experiments. Furthermore, the stimulation of expression was cell type-specific; in Swiss 3T6 cells, pCD34(4.4) consistently produced slightly less luciferase activity than pCD34(1.4) (Figure 3A, right panel). To confirm that this activity could be attributed to the distal HS cluster, we investigated its ability to influence expression from two heterologous promoters. When placed upstream of either the SV40 or HSV TK viral promoters (plasmids p3.0-SV and p3.0-TK), the distal HS element again consistently increased luciferase activity relative to the promoter alone in 416B cells (2- to 3-fold), but decreased luciferase activity in Swiss 3T6 cells (0.7-fold) (Figure 3B and C). Preliminary experiments using constructs in which the HSs from the 3′ flanking region of the CD34 gene were placed downstream of the luciferase gene indicated that they had little ability to affect expression in transient transfection (data not shown).

The distal 5′ HSs function in the context of chromatin

Since the putative regulatory elements under investigation were originally identified as nuclease HSs in chromatin, we speculated that their full activity might only be apparent

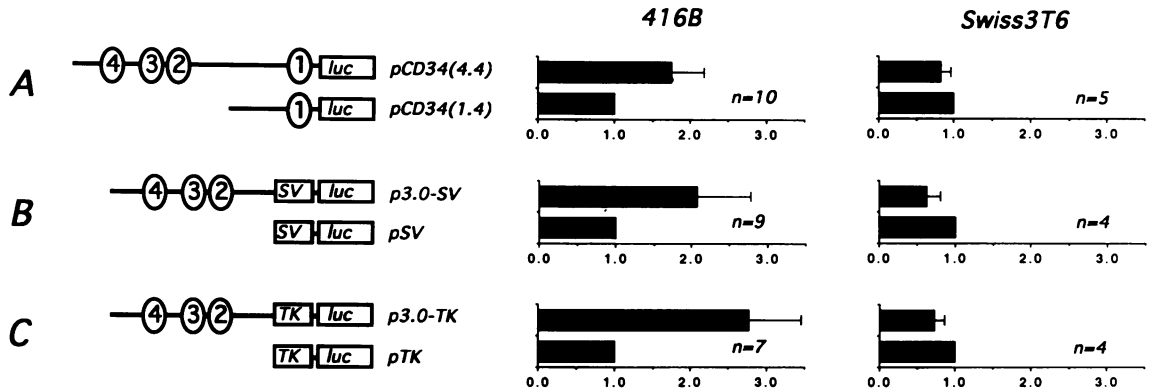


Fig. 3. Functional analysis of the CD34 5′ HSs by transient transfection. This figure summarizes the ability of the progenitor-specific HSs (numbered 2–4 here and in Figure 2A) to enhance transcription from the native CD34 promoter (**A**) or heterologous SV40 (**B**) and HSV TK (**C**) promoters. 416B and Swiss 3T6 cells were transiently transfected with the luciferase (*luc*) reporter constructs shown schematically alongside each graph. The locations of the HSs detected in the endogenous gene (see Figure 2) are indicated on the construct maps by open circles. pCD34(4.4) contains 4.4 kb of CD34 5′ flanking sequences and includes 5′HS1–4; pCD34(1.4) contains 1.4 kb of CD34 5′ flanking sequences which includes only 5′HS1. Vector promoter and gene sequences are shown as open boxes. Luciferase activities were corrected for differences in construct size and transfection efficiency and the final luciferase value for each construct containing 5′HS2–4 was expressed relative to the value obtained for the corresponding promoter construct. The graph therefore shows mean fold enhancement; *n* indicates the number of experiments performed and the bars show the standard deviation of the mean.

when integrated into host cell chromatin. We therefore determined the ability of the 5′ distal HS element (5′HS2–4) to stimulate luciferase expression following stable transfection into 416B and Swiss 3T6 cells. This experiment, which was intended to identify the extent to which the presence of the distal HSs would increase the level of expression over that obtained from the CD34 promoter alone, was performed using pools of clones. Cells were co-electroporated with one of the CD34−luciferase constructs previously tested by transient transfection and a TKneo plasmid to provide resistance to the drug G418. Six independent pools (numbered 1–6) were derived from each electroporation; each pool was estimated to contain 30–50 individual clones. After selection in G418 for 2–3 weeks, the luciferase activity of the pools was measured and the average gene copy number determined by Southern blotting. In contrast to the results obtained in transient assay, the pCD34(4.4) construct was found to produce 75-fold greater mean luciferase activity than the smaller pCD34(1.4) construct in stably transfected 416B cells, after correcting for differences in gene copy number (Figure 4). We also tested the activity of the 3′ HSs 3′HS1–3 in the same type of experiment, using a construct in which a 6 kb *Bam*HI fragment containing exons 7 and 8 of the CD34 gene and 3′HS1–3 (see Figure 2C) was inserted into pCD34(4.4) downstream of the luciferase gene [construct pCD34(4.4)+3′(6.0)]. The presence of the 3′ HSs did not further increase the luciferase activity over that obtained from the 5′ element alone. When the experiment was repeated in Swiss 3T6 cells, the difference in activity of the two 5′ constructs, pCD34(1.4) and pCD34(4.4), was only 1.3-fold (Figure 4). Interestingly, the activity of pCD34(1.4) was considerably higher in Swiss 3T6 than in 416B cells, although some caution should be exercised in comparing absolute levels of expression between different cell lines in transfection studies. Addition of the 3′ element [pCD34(4.4)+3′(6.0)] gave a further 2.1-fold increase in luciferase activity over pCD34(4.4), yielding a 2.7-fold increase in total. In summary, the distal 5′ element enhances expression from the CD34 promoter 75-fold in 416B, but is inactive in Swiss 3T6 cells. The ability of this element to influence transcription in 416B and Swiss 3T6 cells therefore correlates with the presence or absence of its constituent HSs (5′HS2–4) in the endogenous CD34 gene. Furthermore, we conclude that this element functions predominantly at the level of chromatin, since its activity in 416B is apparent in the stable but not the transient assay. Unexpectedly, the 3′ element, whose HSs are present in both 416B and Swiss 3T6, had little effect in either cell type in this assay system.

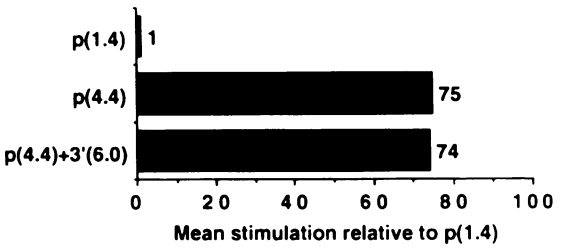
We next confirmed our observations made in 416B cells by analysing an additional myeloid progenitor cell line, namely M1. Like 416B, M1 expresses CD34 at both the mRNA (Figure 5A) and protein levels (data not shown). Analyis of M1 nuclear chromatin (Figure 5B) reveals the presence of four discrete HSs at the CD34 distal 5′ element. Three of these coincide with the major distal 5′ HSs (5′HS2–4) consistently observed in 416B cells; the fourth corresponds to an HS whose appearance in 416B cells is variable. The results obtained from stable transfections of M1 cells using CD34–luciferase constructs are presented in tabular form in Figure 5C and summarized diagrammatically in Figure 5D. Results obtained in similar experiments conducted in 18.8 cells, a pre-B lymphocyte line that does not express CD34, are presented in Figure 6. In M1 cells, the CD34 promoter alone had extremely low activity; addition of the CD34 5′ and 3′ regulatory elements yielded a 249-fold mean stimulation over this basal activity, compared with the 74-fold stimulation seen in 416B cells. Unexpectedly, the CD34 promoter was active in 18.8 cells, but, in marked contrast to M1 and 416B, inclusion of additional CD34 sequences provided no stimulation.

Is the distal 5′ HS element a locus control region?

Locus control regions (LCRs) have been operationally defined on the basis of their ability to confer position-independent expression on a linked gene in transgenic animals; in some studies, copy number-dependent expres-

416B

Pool	Luciferase activity/gene copy in cpm		
	p(1.4)	p(4.4)	p(4.4)+3'(6.0)
#1	507	524616	284931
#2	225	164119	89927
#3	7412	71281	67730
#4	2585	265027	156125
#5	3599	54616	222372
#6	3007		455582
Mean	2889	215932	212778



Swiss 3T6

Pool	Luciferase activity/gene copy in cpm		
	p(1.4)	p(4.4)	p(4.4)+3'(6.0)
#1	33319	47800	89664
#2	36935	37910	122898
#3	39573	21355	119568
#4	25284	78384	50211
#5	28352	37301	102271
#6	40616		67761
Mean	34013	44550	92062

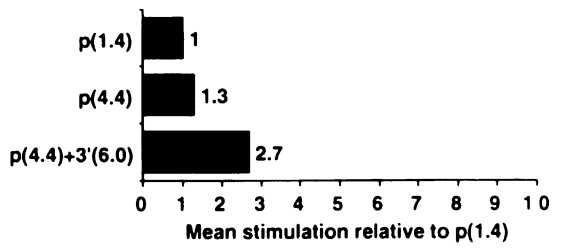


Fig. 4. Analysis of CD34 regulation by stable transfection of $CD34^+$ haemopoietic progenitor (416B) and fibroblast (Swiss 3T6) cell lines. 416B: stably transfected 416B cells were obtained by co-electroporation of CD34−luciferase constructs with a selectable TKneo plasmid encoding resistance to the drug G418. p(1.4), p(4.4) and p(4.4)+3′(6.0) are abbreviations for plasmids pCD34(1.4), pCD34(4.4) and pCD34(4.4)+3′(6.0) respectively; the latter contains the same 5′ region as p(4.4), but 6.0 kb of CD34 3′ flanking sequences (including 3′HS1–3) have been inserted downstream of the luciferase gene (see Figure 3 and Materials and methods). Six independent pools of cells were derived from each electroporation and randomly numbered 1–6; one of the pools carrying p(4.4) was lost during G418 selection through contamination. The luciferase activity per gene copy (determined by Southern blot) of each pool and the mean of the values obtained from pools transfected with the same construct are shown in the table. The graph shows the mean stimulation of transcription obtained with these constructs in 416B cells, expressed relative to the activity of p(1.4); for p(4.4) these values range from 18.9 to 181.6, with a mean of 74.7, and for p(4.4)+3′(6.0) the values range from 23.4 to 157.7, with a mean of 73.6. Swiss 3T6: pools of stably transfected Swiss 3T6 cells were obtained and analysed as described for 416B cells; the results are presented in tabular form on the left and the graph on the rights shows the mean stimulation obtained relative to p(1.4); for p(4.4) these values range from 0.63 to 2.30, with a mean of 1.3, and for p(4.4)+3′(6.0) the values range from 1.48 to 3.61, with a mean of 2.71.

sion of the transgene has also been observed (reviewed in Epner *et al.*, 1992; Dillon and Grosveld, 1993). Whilst the most extensively characterized LCR is that of the human β-globin gene cluster, LCRs or LCR-like elements have also been identified in a number of other loci (detailed in Talbot *et al.*, 1994). The analysis of pools of stably transfected cells described above was able to demonstrate the chromatin dependence of the enhancer activity, but could not address the question of LCR-like activity, because positive and negative position effects due to the chromatin environment at the site of integration should balance out in a pool and thus not significantly affect the overall expression level. The ability of the CD34 5′ regulatory element described above to confer position-independent expression in 416B cells was therefore assessed in individual clones. Single clones carrying the pCD34(4.4) construct were expanded and analysed for luciferase expression and copy number. Only three of the five clones obtained expressed the luciferase reporter gene; the other two clones failed to express, despite containing multiple intact copies of the luciferase gene, i.e. expression was not position-independent (data not shown). These results indicate that the upstream element does not have the ability to overcome chromatin effects at the site of integration and therefore does not in itself constitute an LCR.

We next tested whether the combination of CD34 upstream and downstream elements might possess LCR activity by analysing single clones stably transfected with pCD34(4.4)+3′(6.0). In contrast to the pCD34(4.4) clones, all six of the pCD34(4.4)+3′(6.0) clones were active, with the lowest luciferase activity obtained being 100-fold higher than the background value from untransfected 416B cells. This observation suggested that the pCD34(4.4)+3′(6.0) construct might be able to express regardless of its position of integration. This was confirmed by analysing a further six pCD34(4.4)+3′(6.0) clones, all of which also expressed the luciferase reporter gene. The

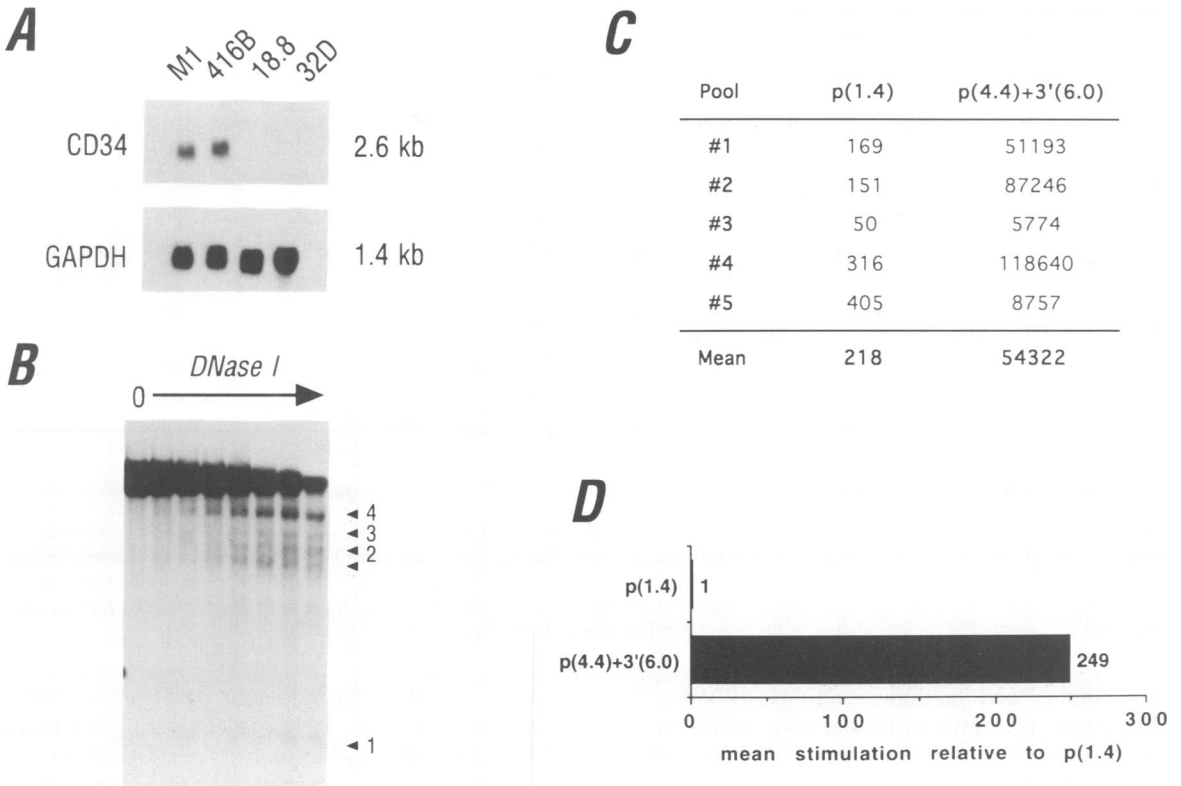


Fig. 5. Analysis of CD34 regulation in the CD34$^+$, myeloid-progenitor cell line M1. (**A**) Northern blot analysis of CD34 expression in the murine myeloid progenitor cell lines M1 and 32D; 416B and 18.8 (pre-B) cells are included as positive and negative controls respectively. The upper panel shows hybridization with a full-length murine CD34 cDNA probe and the lower panel shows re-hybridization of the membranes with a GAPDH probe to control for any differences in the amount of RNA loaded; note that M1 and 416B cells express similar levels of CD34 mRNA. The sizes of the transcripts are indicated in kb. (**B**) DNase I HS mapping. DNase I-treated DNA was restricted with *Eco*RV and hybridized with a 900 bp *Eco*RV–*Eco*RI probe derived from the first intron (see legend to Figure 1 and Materials and methods for details). 5′HS1–4, which were previously observed in 416B cells, are indicated by arrowheads. Also indicated is an additional HS which corresponds to an HS whose appearance in 416B cells is variable. DNase I concentrations increase left to right from 0 to 7.5 μg/ml. (**C** and **D**) Analysis of stably transfected M1 pools. Pools of M1 cells, stably transfected with the constructs indicated, were obtained and analysed in a manner similar to that described for 416B cells. The luciferase activity per gene copy is shown in tabular form in (C) and the diagram in (D) shows the mean stimulation obtained, relative to p(1.4), by inclusion of the CD34 5′ and 3′ flanking regions; the values ranged from 26.5 to 544, with a mean of 249.

18.8

luciferase activity/gene copy in cpm

Pool	p(1.4)	p(4.4)+3'(6.0)
#1	35174	10592
#2	14565	46335
#3	72933	19917
#4	30484	26614
#5	116356	12447
#6	13981	20124
Mean	**47249**	**22672**

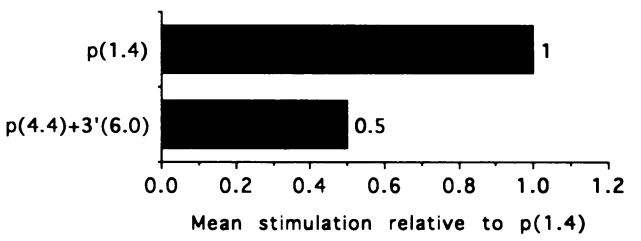


Fig. 6. Analysis of CD34 regulation in the CD34$^-$, pre-B cell line 18.8. Stably transfected 18.8 cells were obtained by co-electroporation of the CD34–luciferase constructs p(1.4) and p(4.4)+3′(6.0) with a selectable TKneo plasmid encoding resistance to the drug G418. The luciferase activity per gene copy (determined by Southern blot) of each pool and the mean of the values obtained from pools transfected with the same construct are shown in the table. The graph shows the mean stimulation of transcription obtained by inclusion of the CD34 5′ and 3′ flanking regions expressed relative to the activity of p(1.4); the values ranged from 0.22 to 1.02, with a mean of 0.48.

clone	copies	luciferase
#1	8	665416
#2	253	8451816
#3	25	1334952
#4	89	644920
#5	57	316360
#6	19	3399944
➡ #7	47	17097144
#8	136	1284192
#9	19	900152
#10	14	12912
#11	242	1914496
#12	42	3529848

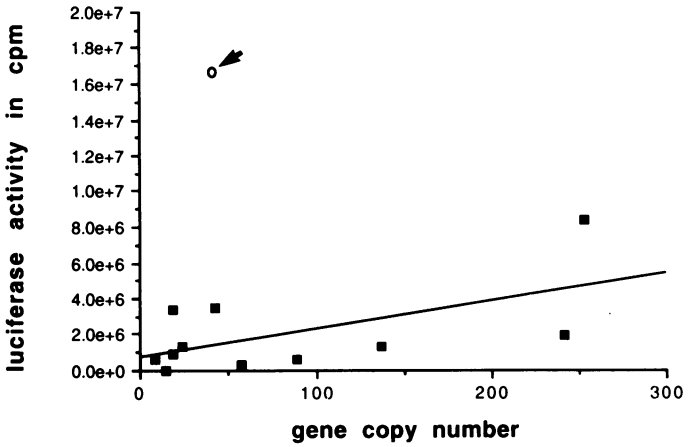


Fig. 7. Analysis of individual haemopoietic progenitor clones stably transfected with pCD34(4.4)+3′(6.0). Twelve individual clones of 416B cells stably transfected with pCD34(4.4)+3′(6.0) were analysed for transgene copy number and expression. The gene copy number (determined by Southern blotting and corrected to the nearest integer) and the luciferase expression (in c.p.m.) for each clone is presented in the table. A scatter plot of luciferase activity versus gene copy for these clones is shown on the right. Clone 7 (arrowed in both the table and the graph) appears to be an outlying point and was omitted in the calculation of the regression line plotted in the figure (see text for details).

gene copy number and luciferase activity of these 12 individual clones are shown in tabular form in Figure 7. The expression of all 12 clones contrasts with the results obtained with the 5′ distal element alone and suggests that the combination of 5′ and 3′ elements displays one of the characteristics of an LCR, namely the ability to confer expression regardless of the site of integration; this has been termed position-independent expression. We next asked whether the level of expression obtained was directly related to the number of copies of the construct present. This analysis is presented in Figure 7 as a scatter plot. The correspondence between gene copy number and luciferase expression was reasonably good in 11 of the 12 clones. However, relative to these, clone 7 (arrowed in the figure) displayed very high expression for its gene copy number. Linear regression analysis of all 12 clones did not indicate a significant correlation between expression level and gene copy number (not shown). Since linear regression is sensitive to outlying data points, we repeated the analysis omitting clone 7. The regression line produced is shown in Figure 7 and has a correlation coefficient of 0.586, which is statistically significant at the 95% confidence level. The lack of strict copy number dependence in these clones may indicate that the integrated constructs are only partially protected from the influence of flanking chromosomal sequences; this is considered in more detail in the Discussion.

Discussion

The aim of this study was to identify regulatory elements which function in haemopoietic stem cells, using the murine CD34 gene as a model system. Regulatory elements of a number of other genes whose expression is associated with the haemopoietic stem cell phenotype have recently been identified; like CD34, the expression of these genes is not completely restricted to haemopoietic stem and progenitor cells. For example, the stem cell factor receptor gene, c-*kit*, is expressed in early erythroid cells, mast cells, melanocytes, germ cells and the central nervous system as well as haemopoietic progenitors (Nocka *et al.*, 1989; Keshet *et al.*, 1991). In comparing our results with those obtained from the analysis of c-*kit* and human CD34 gene regulation, it is important to emphasize two key differences in the experimental strategies employed. (i) Since our goal was to identify a progenitor-specific component of murine CD34 regulation, we initially compared the transcriptional regulation of the murine CD34 gene in two different tissue types (haemopoietic progenitors and fibroblasts), both of which express high levels of CD34. In contrast, studies of c-*kit* and human CD34 regulatory elements have compared reporter gene expression in c-*kit*/CD34$^+$ versus c-*kit*/CD34$^-$ cell types. (ii) In addition to transient reporter gene assays, we have also analysed reporter gene activity in stably transfected cells; stable transfection places reporter constructs in an environment which is considered to better reflect the chromatin-imposed constraints under which regulatory elements must normally act (reviewed by Felsenfeld, 1992). The ability of a regulatory element to function within chromatin is also critical if it is to be useful in constructing vectors capable of directing long-term expression of heterologous gene products in the haemopoietic compartment.

Transcriptional programmes of haemopoietic stem cells

Our results show that the murine CD34 gene is flanked both upstream and downstream by DNase I HSs, with the more distal cluster of upstream sites (5′HS2–4) being restricted to haemopoietic cells. The proximal upstream hypersensitive region (5′HS1) has promoter activity in both haemopoietic progenitor and fibroblast cells in transient transfection; neither the distal upstream sites nor the downstream sites significantly stimulate expression in a transient assay system. However, the upstream sites enhance transcription 75-fold in stably transfected CD34$^+$ haemopoietic progenitor cells; the same sites do not stimulate transcription in CD34$^+$ fibroblasts or in CD34$^-$ haemopoietic cells. Murine CD34 expression is therefore regulated in haemopoietic progenitor cells by an upstream

enhancer which displays both tissue- and stage-specificity and which functions in a chromatin-dependent fashion. Our failure to detect significant activity of this element in transient assays may provide an explanation for the results obtained by Burn *et al.* (1992) in attempting to identify regulatory elements upstream of the human CD34 gene by deletion and transient transfection. In that study, deletion of the region between −4.5 and −2 kb upstream of the ATG gave a 2- to 3-fold decrease in the activity of a reporter construct following transient transfection into a $CD34^+$ human T lymphoblastic cell line. These results are similar to those reported here comparing pCD34(4.4) and pCD34(1.4) in transient assays. Unfortunately, the various human CD34 constructs were not tested for activity in stably transfected cells and so further comparison with our data is difficult. In the analysis of c-*kit* gene regulation, sequences from the mouse c-*kit* 5′ flanking region have been shown to have promoter activity, with sequences within 200 bp of the transcription initiation site giving 36-fold greater stimulation in mast cells (c-kit^+) than in myelomonocytic cells (c-kit^-) in transient assays (Yasuda *et al.*, 1993). Similar results have been obtained for the human c-*kit* gene (Yamamoto *et al.*, 1993). However, neither the mouse nor the human c-*kit* promoter elements have been tested for activity in non-haemopoietic or more primitive haemopoietic cells which normally express c-*kit*. We found that the murine CD34 promoter region was active in both progenitors and fibroblasts in transient assays, with greater activity in fibroblasts than in $CD34^+$ progenitors when stably transfected. If the mechanism of regulation of the c-*kit* gene is similar to that of the murine CD34 gene, then c-*kit* promoter sequences may also prove to be active in several or all c-*kit*-expressing tissues, with differential tissue regulation being conferred by additional DNA elements. Surprisingly, the murine CD34 promoter alone was more active in stably transfected $CD34^-$ haemopoietic cells than $CD34^+$ cells, raising the possibility that part of the function of the more distal regulatory elements is to alleviate repression of the promoter in progenitor cells.

The gene encoding a second stem cell-associated protein Ly-6E.1 has recently been cloned and its regulation investigated (Khan *et al.*, 1990; Sinclair and Dzierzak, 1993). Ly-6E.1 is a mouse strain-specific allele encoding one of the murine antigens recognized by the Sca-1 antibody, which has been used to purify and characterize mouse stem cells (Spangrude *et al.*, 1988). Sca-1 is expressed in haemopoietic stem cells, progenitors and T cells and other non-haemopoietic tissues, such as kidney and brain (van de Rijn *et al.*, 1989; Cray *et al.*, 1990). Sinclair and Dzierzak (1993) detected multiple DNase I HSs both upstream and downstream of the gene; stable transfection of a 14 kb gene fragment showed that these HSs were sufficient to confer Ly-6E.1 expression at levels equivalent to that of the endogenous *sca-1* gene in erythroid MEL cells induced to express *sca-1*. However, the ability of individual sites or groups of sites to stimulate transcription of heterologous genes in different cell types has not yet been determined. A third gene whose expression is associated with haemopoietic stem cells is the tyrosine kinase *flk2* gene (Matthews *et al.*, 1991). Again, the gene is also expressed outside haemopoiesis, in tissues such as placenta, brain and gonads (Rosnet *et al.*, 1991). Transcriptional elements regulating this gene have not yet, to our knowledge, been identified. Thus, our demonstration of a CD34 regulatory element which is capable of enhancing transcription 75-fold specifically in haemopoietic cells represents a significant advance in the search for stem cell-specific regulatory elements. Characterization of the transcription factors whose binding presumably underlies the hypersensitivity of the CD34 distal element should allow analysis of the transcription factor programme associated with the self-renewal phenotype.

Chromatin-dependent elements

In recent years the discovery of chromatin-dependent transcriptional elements, together with evidence from chromatin reconstitution experiments aimed at investigating competition between histones and *trans*-acting factors for binding to DNA, has reinforced the view that chromatin structure is of paramount importance in determining gene expression (reviewed by Felsenfeld, 1992). The archetypal chromatin-dependent regulatory element is the LCR of the human β-globin gene cluster, first reported as an erythroid-specific, developmentally stable cluster of HSs located 6–18 kb 5′ of the ε-globin gene (Tuan *et al.*, 1985; Forrester *et al.*, 1986). Direct evidence for LCR function was first provided by Grosveld *et al.* (1987), who showed that linkage of the LCR to the human β-globin gene resulted in high level expression which was independent of the position of integration and directly proportional to the number of copies of the transgene. Results from chromosome-mediated gene transfer experiments have shown that the β-globin LCR affects the chromatin structure and replication of the β-globin domain over more than 200 kb of DNA (Forrester *et al.*, 1987, 1990). The notion that the LCR functions at the level of chromatin is further supported by the observation that two of its constituent HSs (5′HS3 and 4) potentiate transcription only after integration into chromatin. The regulatory element that we have identified 3 kb upstream of the murine CD34 gene is also clearly dependent upon integration into chromatin for its activity, the stimulation it produces being comparable to that from the β-globin LCR when tested in stably transfected cell lines (75-fold versus 10- to 100-fold; Blom van Assendelft *et al.*, 1989; Forrester *et al.*, 1989; Talbot *et al.*, 1989). However, our analysis of single clones did not indicate an ability to confer position-independent expression, because not all of the clones expressed the reporter gene, and thus this element does not appear to be an LCR. It may more closely resemble the recently described *MyoD* enhancer, which is tissue-specific and functions only when integrated into chromatin, but does not appear to confer completely position-independent expression in that only ~50% of individual clones are active (Tapscott *et al.*, 1992). Since the CD34 gene, unlike globin, is expressed at high levels in a number of different tissues, its domain-controlling element would presumably be expected to function in all $CD34^+$ tissues. The CD34 5′ distal HS element does not fulfill this expectation, in that it does not stimulate transcription in stably transfected fibroblasts and, more importantly, is not DNase I hypersensitive in fibroblast chromatin. From this perspective, a better candidate for a CD34 domain-controlling element is provided by the downstream cluster of HSs, which are strongly detected in both haemopoietic progenitor and fibroblast chromatin. All clonal lines stably

transfected with a construct containing this downstream element in addition to the 5′ element expressed the linked reporter gene at high levels, indicating that expression could now occur independent of the site of integration. Whilst the level of expression in 11 out of 12 of the clones was statistically correlated with gene copy number, considerable variability was observed between individual clones. This variability may be inherent in the stable transfection approach, in which transfectants are isolated on the basis of expression of a linked or co-transfected drug resistance gene (for a discussion, see Epner *et al.*, 1992). An alternative explanation is that the CD34 elements are not completely dominant over the pre-existing chromatin regime at the site of transgene integration. We postulate that chromatin-dependent elements with different degrees of penetrance exist, depending on the strength of the positive and negative chromatin effects that they encounter in their native chromosomal location.

Implications for gene therapy and gene transfer

Gene therapy by gene addition offers a potential therapy for a number of blood disorders, including thalassaemia. For such an approach to succeed, two main problems have to be solved. Firstly, transcriptional regulatory elements capable of directing both appropriate levels and cell type-specificity of expression of the added gene need to be defined. Secondly, since blood cells are constantly being turned over and rederived from a self-renewing population of pluripotent stem cells, the stable propagation of the novel genetic information requires its delivery to the stem cell compartment. Delivering genes to the stem cell compartment has proved difficult, because stem cells are rare and difficult to purify. A solution to this second problem could, in principle, be provided by the inclusion in gene addition vectors of a dominant selectable marker under the transcriptional control of a stem cell-specific regulatory element. This would allow gene transfer into unpurified cell populations followed by drug selection; only stem cells harbouring the novel genetic information would survive, since the drug resistance gene would only be transcribed in stem cells. To date, no such element has been described, largely because studies of transcriptional regulation have focused on genes transcribed in terminally differentiated cells. Our analysis of the transcriptional control of the gene encoding the stem cell antigen CD34 has revealed a potent upstream regulatory element which appears to be a strong candidate for a stem cell-specific enhancer; the ability of this element to direct expression in stem and progenitor cells *in vivo* is currently being tested by murine transgenesis, both with and without the downstream CD34 element. Ultimately, if this element is to be used therapeutically in a targeting construct, further attention will also have to be paid to the CD34 promoter itself, which appears to function in both $CD34^+$ and $CD34^-$ cells. In addition to its potential use in a gene therapy setting, the CD34 5′ distal element provides a unique tool for biological analysis of haemopoiesis, since it should permit targeting of a range of regulatory molecules (oncogenes, receptors, transcription factors, etc.) to the haemopoietic stem cell compartment of transgenic mice. This would directly test the role played by these molecules in self-renewal versus differentiation decisions, as well as facilitate the production of murine models of human leukaemias.

Materials and methods

Cell culture

416B cells (a kind gift from T.M.Dexter, Paterson Institute, Manchester, UK) were maintained in Fischer's medium supplemented with 20% horse serum and 4 mM L-glutamine; Swiss 3T6 (ECACC 88031146) and M1 (ATCC TIB192) cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum and 4 mM L-glutamine. Other cell lines used for the preparation of RNA were obtained and cultured as described (Ford *et al.*, 1992).

Plasmids used for transfection

The plasmids described here are also shown schematically in Figure 3. pCD34(4.4) was prepared by ligating a 4.4 kb *NciI*–*Eco*RV fragment from the mouse CD34 5′ region to the promoterless pGL2-basic luciferase reporter plasmid (Promega) linearized with *Sma*I. The *Nci*I site lies 47 bp upstream of the CD34 ATG, within the region of published sequence (Brown *et al.*, 1991); the location of the *Eco*RV site used was determined by restriction analysis. pCD34(1.4) was prepared by digesting pCD34(4.4) with *Asp*718 to release a 1.4 kb fragment running from the *Asp*718 site in the vector multiple cloning site to an *Asp*718 site upstream of the CD34 promoter region. This 1.4 kb fragment containing the putative CD34 promoter was ligated to pGL2-basic previously linearized with *Asp*718; in pCD34(1.4), the promoter is in the sense orientation with respect to the luciferase gene. p3.0(SV) was prepared by transferring the distal portion of the pCD34(4.4) insert containing 5′HS2–4 as a 3 kb *Sma*I–*Asp*718 fragment into the pGL2-P vector (Promega) such that it lies in the sense orientation upstream of the SV40 promoter (see Figure 3B). p3.0-TK contained the same 3 kb element inserted into pTK109 (Nordeen, 1988), which contains 109 bp of the herpes simplex virus thymidine kinase promoter (see Figure 3C). pCD34(4.4)+3′(6.0) was created by inserting a 6 kb *Bam*HI fragment, containing CD34 exons 7 and 8 plus ~5 kb of 3′ flanking region (see Figure 2C), into pCD34(4.4) linearized with *Bam*HI just downstream of the vector polyadenylation signal. In pCD34(4.4)+3′(6.0), the 3′ *Bam*HI insert is in the sense orientation relative to the luciferase gene. The pSV-β-galactosidase vector (Promega) was used to control for transfection efficiency in transient experiments. DNA for transfections was prepared using Qiagen columns (Qiagen Inc.), followed by phenol extraction and ethanol precipitation. pTKneo contains a neomycin (G418) resistance cassette under the control of the herpes simplex virus thymidine kinase promoter (Enver *et al.*, 1991). DNA concentration was determined using a Hoefer TKO 100 fluorometer; the concentration and integrity of the DNA samples used for electroporation were verified by running linearized and undigested samples on an agarose gel and staining with ethidium bromide.

Isolation of RNA and Northern blot hybridization

Total cellular RNA was isolated from cell lines (5×10^7 cells) using the guanidium isothiocyanate procedure of Chirgwin *et al.* (1979). RNA (10–12 μg) was prepared for electrophoresis by denaturation in 50% formamide, 2.2 M formaldehyde and 20 mM sodium phosphate, pH 7.6, and run through 1.1% agarose gels essentially as described (Pritchard and Holland, 1985), except that the concentration of formaldehyde in the running buffer was reduced to 0.66 M (Ford *et al.*, 1988). Transfer to nitrocellulose, hybridization and washing were essentially as described below for DNA.

DNase I HS mapping

Nuclei were isolated and treated with DNase I essentially as described elsewhere (Siebenlist *et al.*, 1984), except that the procedure was scaled down to accommodate the use of 5×10^7 cells. Briefly, cells were lysed by the addition of 0.2% Nonidet P-40 and nuclei purified by centrifugation through a sucrose cushion. Nuclei were resuspended at 4–5×10^7/ml in a buffer solution containing 0.3 M sucrose and 5% glycerol and digested with DNase I at concentrations ranging from 0 to 7.5 μg/ml. Purified DNA from each sample was digested with the appropriate restriction enzyme, electrophoresed through a 0.8% 0.5× TBE agarose gel and transferred in 20× SSC onto a nitrocellulose membrane. Membranes were hybridized with [α-^{32}P]dCTP-oligolabelled probes as described (Parslow and Granner, 1982), except that the hybridization buffer was supplemented with 0.2% PVP, 0.2% Ficoll, 0.1% SDS and 0.2 mg/ml sonicated salmon sperm DNA. After hybridization overnight at 65°C,

membranes were washed in 0.5× SSC, 0.5% SDS for 1 h and autoradiographed.

Transient transfection

416B cells in logarithmic growth (4–7×10^5 cells/ml) were harvested by spinning at 1200 r.p.m. in a benchtop centrifuge, washed with RPMI 1640 medium containing 25 mM HEPES and resuspended at 5×10^7 cells/ml in the same medium. Each transfection was of 1×10^7 cells (0.2 ml). Cells were mixed with 5 μg of the relevant luciferase reporter plasmid and 1 μg pSV-β-galactosidase control plasmid and incubated at room temperature for 10 min before electroporation at 270 V and 960 mF using 0.4 cm electrode gap cuvettes (Bio-Rad) and a Bio-Rad Gene Pulser. The electroporated cells were stored on ice for 10 min and then transferred into 20 ml normal growth medium for overnight culture. Transfection of Swiss 3T6 cells followed essentially the same procedure, except that cells were trypsinized before harvesting and the voltage for electroporation was reduced to 230 V.

Luciferase activity was determined using Promega's luciferase assay system. Cells were harvested 20 h post-electroporation, washed once with phosphate-buffered saline and resuspended in 100 μl cell lysis culture reagent. After incubation at room temperature for 15 min, cell debris was pelleted by centrifugation in a microfuge for 30 s and the luciferase activity of the lysate determined by mixing 20 μl lysate with 100 μl luciferase assay reagent; the latter contains the enzyme substrate luciferin and coenzyme A. The light produced was measured immediately after mixing using a scintillation counter with the coincidence circuit switched off. The β-galactosidase activity was determined by incubating 5 μl of the same cell lysate with 100 μl of a chemiluminescent substrate (Lumi-Gal 530; Lumigen Inc.) at 37°C for 50 min and measuring the light produced with a scintillation counter, as for luciferase. The luciferase activities obtained were corrected for transfection efficiency by comparison with the activity of the co-transfected β-galactosidase control and for the differences in the molar amount of plasmid used (by correcting for size differences).

Stable transfection

In order to establish stably transfected clones, cells were co-transfected with 5 μg pTKneo (to confer resistance to the drug G418) linearized at the *Sca*I site and a 3:1 molar excess of luciferase reporter plasmid, also linearized with either *Sca*I [pCD34(1.4) and pCD34(4.4)] or *Sal*I [pCD34(4.4)+3′(6.0)]. 416B and Swiss 3T6 cells were electroporated as described above; for M1 and 18.8 cells voltages of 270 and 230 V were used respectively. Twenty four hours post-electroporation the culture volume was increased from 20 to 50 ml and the medium supplemented with either 0.7 mg/ml (for 416B and M1) or 1 mg/ml (Swiss 3T6 and 18.8) G418 (Geneticin; Gibco-BRL) to select for G418 resistance. The cells from each electroporation were divided into six 6 ml pools and the remainder into 1 ml aliquots for the derivation of single clones. The pools were analysed as soon as the viable cell count exceeded 5×10^6 cells, in order to minimize the possible effects of uneven clonal expansion, usually 2–3 weeks after electroporation. The luciferase activity in each pool was determined as described for transient assays, except that the cell lysate was made from 1×10^6 viable cells, the lysate volume reduced to 40 μl and only 5 μl assayed by reaction with 100 μl luciferase assay reagent. The remainder of the cells were harvested on the same day and used to prepare genomic DNA for Southern blot analysis. Individual 416B clones were picked from the 1 ml cultures using a Gilson pipette once the colonies were sufficiently large to be readily visible to the naked eye. The individual clones were analysed exactly as described for the pools, but about 4–6 weeks after electroporation. Linear regression analysis was performed using Cricket Graph (Cricket Software Inc.).

Southern blotting of stably transfected cells

Cells were lysed in 100 mM Tris−HCl, pH 8.0, 50 mM NaCl, 12.5 mM EDTA, 0.2% SDS and digested overnight at 37°C with 100 μg/ml proteinase K. DNA was extracted with phenol, ethanol precipitated and resuspended in distilled H_2O. The DNA was limit-digested with *Asp*718 and *Eco*RV, separated on a 1% agarose 0.5× TBE gel and transferred in 0.4 M NaOH transfer buffer onto Hybond-N^+. The membrane was hybridized with the 1.4 kb CD34 promoter-containing insert from pCD34(1.4) labelled with [α-^{32}P]dCTP by random oligonucleotide priming, which detects an internal 1.4 kb *Asp*718 fragment from each copy of each construct and a 2.6 kb *Asp*718−*Eco*RV fragment derived from the endogenous CD34 gene. Membranes were washed with 0.1× SSC, 0.1% SDS for 20 min before quantitation using a PhosphorImager (Molecular Dynamics). Comparison of the intensities of the construct and genomic bands within each lane gave a direct estimate of construct copy number in the DNA samples. Membranes were then stripped of probe and rehybridized with an 800 bp *Eco*RI−*Eco*RV fragment derived from the luciferase gene of pGL2-basic (Promega). This probe detects a 1.4 kb *Asp*718−*Eco*RV fragment containing the luciferase gene (corresponding to nucleotides 8–1404 of the original pGL2-basic vector) from each construct. Detection of the correct sized band confirmed that no gross rearrangements of the luciferase gene had occurred.

Acknowledgements

We wish to thank Caroline Bennett for assistance with the Northern analysis, Professor Mel Greaves and Dr Leanne Wiedemann for discussion of the manuscript, and all our colleagues at the LRF Centre for their help and support. We are grateful to the Leukaemia Research Fund who supported this work.

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Received on April 6, 1994; revised on October 7, 1994