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An SCL 3′ enhancer targets developing endothelium together with embryonic and adult haematopoietic progenitors

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SUMMARY

The *SCL* gene encodes a basic helix-loop-helix transcription factor which is expressed in early haematopoietic progenitors throughout ontogeny and is essential for the normal development of blood and blood vessels. Transgenic studies have characterised spatially distinct 5′ enhancers which direct *lacZ* expression to subdomains of the normal *SCL* expression pattern, but the same elements failed to produce appropriate haematopoietic expression. We now describe an *SCL* 3′ enhancer with unique properties. It directed *lacZ* expression in transgenic mice to extra-embryonic mesoderm and subsequently to both endothelial cells and to a subset of blood cells at multiple sites of embryonic haematopoiesis including the yolk sac, para-aortic splanchnopleura and AGM region. The 3′ enhancer also targeted expression to haematopoietic progenitors in both foetal liver and adult bone marrow. Purified *lacZ*$^+$ cells were highly enriched for clonogenic myeloid and erythroid progenitors as well as day-12 spleen colony forming units (CFU-S). Within the total gated population from bone marrow, 95% of the myeloid and 90% of the erythroid colony-forming cells were contained in the *lacZ*$^+$ fraction, as were 98% of the CFU-S. Activation of the enhancer did not require SCL protein. On the contrary, transgene expression in yolk sacs was markedly increased in an *SCL*$^{-/-}$ background, suggesting that *SCL* is subject to negative autoregulation. Alternatively the *SCL*$^{-/-}$ environment may alter differentiation of extra-embryonic mesoderm and result in an increased number of cells capable of expressing high levels of the transgene. Our data represents the first description of an enhancer that integrates information necessary for expression in developing endothelium and early haematopoietic progenitors at distinct times and sites throughout ontogeny. This enhancer provides a potent tool for the manipulation of haematopoiesis and vasculogenesis in vivo.

Key words: SCL, Haematopoiesis, Vasculogenesis, Endothelium, Mouse

INTRODUCTION

Blood formation begins early during embryogenesis in vertebrates and continues throughout the life of the animal to generate multiple lineages of mature cells. Haematopoiesis occurs in both extra-embryonic and intra-embryonic sites during mouse and avian development (Dieterlen-Lievre, 1975; Dzierzak and Medvinsky, 1995; Godin et al., 1995, 1993; Medvinsky et al., 1993). Murine multipotent progenitors and multipotential repopulating cells are also detected for the first time during development at day 8-9 of gestation in the yolk sac as well as within the embryo proper in the para-aortic splachnopleura (Cumano et al., 1996; Yoder et al., 1997). Haematopoietic stem cells (HSC), as defined by long term reconstitution (LTR) assays of irradiated adult recipient mice are detected from day 10 of gestation in the aorta, gonads, mesonephros (AGM) region (Medvinsky and Dzierzak, 1996; Muller et al., 1994). HSC are subsequently found in the in foetal liver and in the adult bone marrow (Spangrude et al., 1988). The lineal relationship between HSC arising in the yolk sac, p-sp and AGM region remains unclear. An additional layer of complexity is introduced by controversy over whether during development HSC in different locations arise directly from undifferentiated mesoderm, from the blood/endothelial common progenitor, the haemangioblasts, or from differentiated endothelial cells (Jaffredo et al., 1998; Nishikawa et al., 1998).

Identification of an enhancer that targets expression to multipotent haematopoietic progenitors would provide a powerful tool for probing the molecular regulation of early haematopoiesis. Expression of exogenous genes under the control of such an element is likely to produce important insights into the biology of the HSCs and may also have therapeutic applications in the context of gene therapy strategies. In addition, biochemical analysis of such an enhancer would provide important insights into the transcriptional programmes of early haematopoietic progenitors. Genes expressed in haematopoietic stem cells provide a source of candidate enhancers but elements that target this compartment have proved elusive. Regulatory

elements at the CD34 (He et al., 1994; May and Enver, 1995) and c-kit loci (Yamamoto et al., 1993; Yasuda et al., 1993) have been studied in vitro but enhancers from these genes have not been reported to target haematopoietic progenitors in vivo. Experiments by Dzierzak and colleagues have shown that a 14 kb fragment from the Ly-6E.1 locus will direct *lacZ* expression to adult HSC but not to those from foetal liver or yolk sac (Miles et al., 1997). However, analysis of Ly-6E.1 regulatory elements is complicated by the fact that Ly-6E.1 is a member of a large highly related family of genes (Kamiura et al., 1992) which exhibit complex and allele-specific expression in haematopoietic cells (Codias et al., 1989; Spangrude et al., 1988).

We have therefore elected to study regulatory elements of the *SCL* gene which encodes a bHLH transcription factor expressed in multipotent haematopoietic progenitors and is essential for the development of haematopoiesis. Mice lacking SCL protein die at embryonic day 9 with a complete absence of yolk sac haematopoiesis (Robb et al., 1995; Shivdasani et al., 1995). Moreover, SCL also plays an essential and cell autonomous role in the development of adult definitive haematopoiesis (Porcher et al., 1996; Robb et al., 1996). Several other lines of evidence suggest that SCL is also important for endothelial development. SCL null animals exhibit a defect in the development of yolk sac blood vessels (Robb et al., 1995; Visvader et al., 1998) and in the zebrafish *cloche* mutant both the endothelial and haematopoietic defects are partially rescued by ectopic expression of SCL (Liao et al., 1998). Furthermore, ectopic expression of SCL during zebrafish development has shown that SCL is capable of specifying haemangioblast formation from early mesoderm (Gering et al., 1998). The role of SCL in haematopoiesis and vasculogenesis is therefore very similar to the role of other bHLH proteins in myogenesis and neurogenesis (Lee et al., 1995; Weintraub et al., 1991).

SCL expression is highly conserved in the development of blood, endothelium and specific regions of the CNS (Drake et al., 1997; Green et al., 1992; Hwang et al., 1993; Kallianpur et al., 1994; Sinclair et al., 1999). During zebrafish development SCL is coexpressed with Flk-1 in presumptive haemangioblasts within the early posterolateral mesoderm (Gering et al., 1998). In the mouse, SCL is expressed at all sites of blood development, including embryonic and extra-embryonic mesoderm at 7.5 days postcoitum (dpc), and subsequently in the p-sp, yolk sac blood islands, the AGM region and foetal liver (Kallianpur et al., 1994; Silver and Palis, 1997). Within the murine and human adult haematopoietic system SCL is expressed in multipotent progenitor cells as well as in the erythroid, megakaryocyte and mast cell lineages (Cross et al., 1994; Green et al., 1991, 1992; Mouthon et al., 1993; Pulford et al., 1995; Visvader et al., 1991).

Two promoters on alternative 5′ exons have been identified in both murine and human *SCL* genes (Aplan et al., 1990; Begley et al., 1994; Bockamp et al., 1995, 1998, 1997; Lecointe et al., 1994), together with a third promoter within the body of the gene, the normal function of which remains unclear (Bernard et al., 1992). A systematic survey of chromatin structure surrounding the SCL locus (Göttgens et al., 1997; Leroy-Viard et al., 1994) has resulted in identification of a number of enhancers active in both transient and stable transfection assays (Göttgens et al., 1997). More recently transgenic analysis has identified three spatially distinct enhancers within the 5′ region of the murine *SCL* locus. These enhancers direct expression to three different regions (endothelium, midbrain and hindbrain/spinal cord) within the normal *SCL* expression domain (Sinclair et al., 1999). However, elements necessary for appropriate haematopoietic expression were not identified.

We now demonstrate that a region downstream of the murine *SCL* gene functions as a haematopoietic and endothelial enhancer in vivo. This 3′ enhancer directs *lacZ* expression to blood and endothelial cells at multiple sites of embryonic and adult haematopoiesis and targets the vast majority of CFU-S as well as most erythroid and myeloid progenitors.

MATERIALS AND METHODS

Transgenic reporter constructs

The *lacZ* gene including the SV40 polyadenylation signal was subcloned from plasmid pSDK *lacZ* pA (gift from Dr J. Rossant) into the pGL-2 basic plasmid (Promega). The SCL genomic DNA fragments were obtained from a Balb/c genomic library (Begley et al., 1994) and subcloned in the pGL-2 vector as previously described (Göttgens et al., 1997). The transgenic *lacZ* reporter constructs contained the following fragments: –0.9E3/*lacZ* contained a 3.8 kb *Bam*HI/*Xmn*I fragment starting at 0.9 kb upstream of the transcription start site at exon 1a with the *lacZ* gene fused in exon 3; +6E5/*lacZ* contained a 2.4 kb *Sau*3AI/*Nde*I fragment starting 6 kb downstream of promoter 1a with the *lacZ* gene fused in exon 5; SV40/*lacZ*, contained the minimal promoter from pGL-2. The 3′ enhancer is a 5.5 kb *Bgl*II/*Bgl*II fragment starting 14 kb downstream of promoter 1a. The 5.5 kb fragment was subcloned downstream of the *lacZ* gene to generate the –0.9E3/*lacZ*/3′En, +6E5/*lacZ*/3′En and the SV40/*lacZ*/3′En constructs (Fig. 1).

Transgenic mice

Transgenic fragments were separated from the plasmid vector by electrophoresis of *Xho*I/*Sal*I digested plasmid and purified using Quiax II following the manufacturer's instructions (Quiagen, UK). The fragments were dissolved in injection buffer (10 mM Tris-HCl, pH 7.5 and 0.1 mM EDTA) at a concentration of 4 μg/ml. DNA was then injected into the pronucleus of (C57Bl/6 × CBA) F_1 fertilised mouse embryos. In some experiments +6E5/*lacZ*/3′En DNA fragment was coinjected with +6E5/GFP/3′En including the *GFP* reporter gene instead of *lacZ*. The *Hin*dIII/*Hpa*I GFP fragment was subcloned from pCMX vector (Zernika-Goetz et al., 1996) into pGL-2+6E5 promoter vector. The 3′En fragment was then subcloned downstream of *GFP*. Lines 2262, 2257 and 2269 contained both constructs. The expression of *GFP* was not detected by fluorescence confocal microscopy or FACS (data not shown). The injected eggs were transferred into the oviducts of C57/Bl6 or CD1 pseudopregnant foster females as described by Miles et al. (1997).

For transient transgenic assays, foster females were killed 8 or 11-12 days after egg transfer. For stable lines, transgenic founders were bred to non transgenic (C57Bl/6 × CBA) F_1 mice. To analyse foetal tissue, timed matings of (C57Bl/6 × CBA) F_1 females with founder or F_1 transgenic males were performed. The day on which the plug was found was designated as day 0 of gestation. Genotype was performed by PCR using oligos for *lacZ* and *myogenin* (Miles et al., 1997).

+6E5/*lacZ*/3′En transgenic mice (line 2137) and –7E3/*lacZ* (line 453) (Sinclair et al., 1999) were bred with $SCL^{+/-}$ heterozygous mutant mice kindly provided by L. Robb and C. G. Begley (Robb et al., 1995) to obtain transgene positive $SCL^{+/-}$ mice. These were crossed with $SCL^{+/-}$ mice and embryos analyzed at 8.5 and 9.5 dpc.

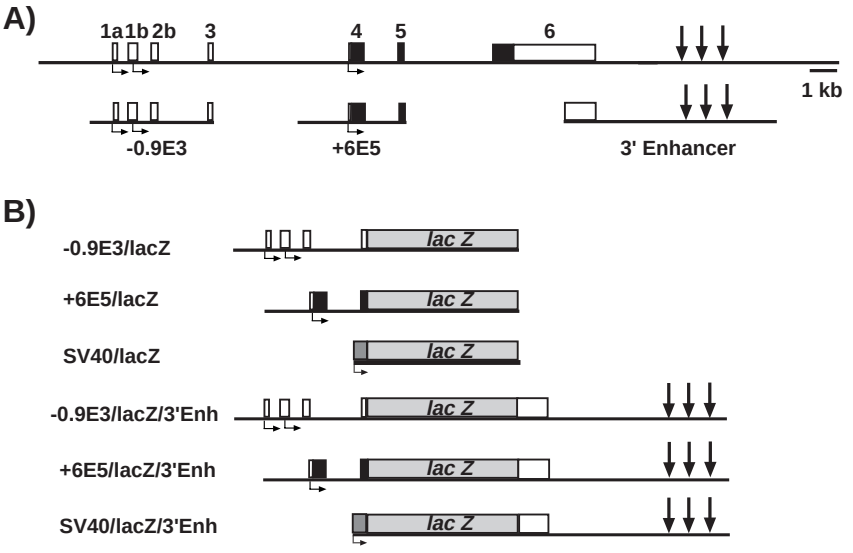

Fig. 1. SCL *lacZ* transgenic constructs. (A) Diagram of the SCL locus with the three *SCL* DNA fragments used in transgenic constructs indicated below. White and black boxes represent untranslated and translated exons respectively. Vertical arrows indicate DNAse I hypersensitive sites within the 3′ enhancer. (B) Transgenic constructs. *lacZ* transcription is driven by one of three promoter cassettes containing the *SCL* 5′ promoter region (-0.9E3), the exon 4 promoter (+6E5) or the SV40 minimal promoter. For each promoter, a reporter construct was generated with or without the *SCL* 3′ enhancer.

The embryo head was removed to obtain DNA for PCR to determine the *SCL* and *lacZ* genotype (Miles et al., 1997; Robb et al., 1995). The rest of the embryo was used for X-gal staining, paraffin embedding and sectioning.

β-galactosidase detection in whole embryos

X-gal staining was performed as previously described (Miles et al., 1997). Founder embryos were incubated overnight in staining solution. β-galactosidase expression was visualised in less that 1 hour in highly expressing transgenic lines and between 6-24 hours in low expressing lines. Prior to sectioning the embryos were dehydrated with increasing concentrations of ethanol and mounted in paraffin wax. 6-10 μm sections were counter stained with neutral red.

Fluorescence-activated cell sorting (FACS) analysis of β-galactosidase activity and surface marker expression

Individual foetal livers were removed from 11 dpc embryos derived from crossing heterozygous transgenic males with F_1 non transgenic females. Adult bone marrow, spleen and thymus were obtained from 3- to 7-month-old heterozygous transgenic mice. β-galactosidase activity in cell suspension was determined as described by Miles et al. (1997) using FDG (fluorescein di-β-D-galactopyranoside) (Sigma) subtrate. The fluorescence generated by FDG was detected on FACSsort (Becton & Dickinson) on the FITC analysis channel. Propidium iodide (PI) was added at 1 μg/ml to exclude dead cells. Non transgenic controls were analysed simultaneously.

For simultaneous FACS analysis of β-galactosidase and surface markers, cells were incubated with anti-Fcγ receptor antibody (2.4G2) to reduce the non-specific binding followed by incubation with PE or Biotin-conjugated antibodies and Streptavidin-PE (Pharmingen, San Diego) or Streptavidin-RED 670 (Gibco BRL, UK) as the secondary reagent. Antibody incubations were performed at 4ºC for 30 minutes. After washing in PBS the FDG reaction was performed. All monoclonal antibodies were from Pharmingen, San Diego and included anti-B220 (RA3-6B2), anti-c-kit (2B8), anti-CD4 (H129.19), anti-CD8 (53-6.7), anti-CD34 (RAM34), anti-Sca-1 (Ly-6A/E), anti-Ter119 (TER-119), anti-Mac-1 (M1/70), anti-Gr-1 (RB6-8C5), anti-IgM (R6-60.2) and anti-CD61 (2C9.G2). Rat PE-conjugated IgG2a and IgG2b isotype controls were used. Dead cells were excluded by propidum idodide (PI) staining.

For flow cytometric sorting bone marrow and foetal liver cells were incubated with FDG substrate and resuspended in cold PBS 5% FCS. Cells were sorted at 4ºC on a dual laser Coulter Epics Elite cell sorter and collected in PBS 5% FCS. Then cells were Trypan blue stained and counted under the microscope to adjust the cell concentration. In most experiments analysis of purity of sorted cell populations was performed; bone marrow purity was 85-90% for the FDG^+ fraction and >95 % for the FDG^-. In two experiments using foetal liver the purity was 90% and 82% in the FDG^+ fraction and >95% in the negative fraction. Dead cells were excluded by PI staining. Bone marrow erythroid cells with low to intermediate side (SSC) and low forward (FSC) light-scattering properties were also excluded.

Southern analysis

Genomic DNA (10 μg) for Southern blot analysis was digested with *Hin*dIII and electrophoresed through 1% agarose gels followed by transfer to nylon membrane. Probes used for hybridisation to Southern filters were labelled with [^{32}P]CTP using the Ready prime kit (Amersham, UK). The probe used was a 0.6 kb *Sac*I/*Sca*I 3′ enhancer fragment from plasmid pGL2-SV40/*lacZ*/3′En. This probe hybridises with the endogenous *SCL* gene giving a 2.8 kb band used as endogenous control and with the +6E5/*lacZ*/3′En transgene giving a 6.5 kb band. After hybridisation filters were exposed to X-ray films for 5 days and signals quantified using the VIH immage software.

In vitro colony assays for progenitors

FDG^+ and FDG^- fractionated and unfractionated cells from adult bone marrow and 11 dpc foetal liver were cultured in 0.3% agar in Iscoves MDM (Gibco, UK) supplemented with 25% FCS for myeloid colony formation (Metcalf, 1984) or in cytokine supplemented Methocult GF-M3434 (StemCell Technologies Inc., Vancouver, Canada) for erythroid and myeloid colony formation. Agar cultures were supplemented with conditioned medium from the Bhk cell line (a gift from Dr S. Tsai) as a source of stem cell factor and theWEHI 3B cell line (Metcalf, 1984) as source of IL-3, or with 2 ng/ml of recombinant IL-3 (R&D Sytems, UK). Duplicate (agar) or triplicate (methocult) cultures were incubated at 37ºC in a humidified atmosphere of 5% CO_2 and colonies scored 7 days after culture. To confirm haemoglobin-erythroid colonies, 2,7-diaminofluorene (Sigma) staining was performed at day 9 of culture (Kaiho and Mizuno, 1985). Controls with unsorted and sorted total bone marrow were performed and no significant differences in colony numbers were observed. The cell number plated were pre-established to obtain between 20-100 colonies in a 35-mm Petri dish. The cell number plated were from bone marrow: 5×10^4 unfractionated, $3\text{-}6\times10^3$ FDG^+ and $5\text{-}10\times10^4$ FDG^-; from foetal liver: 10^4 unfractionated, 10^3 FDG^+ and 10^4 FDG^- foetal liver cells.

The distributions (D^+ and D^-) of colony forming cells in the FDG^+

and FDG$^-$ fractions respectively were calculated for each experiment according to the following equations:

$$D^+ = \frac{(a \times b)}{(a \times b) + (c \times d)} \qquad D^- = \frac{(c \times d)}{(a \times b) + (c \times d)}$$

where a = % of sorted cells in FDG$^+$ fraction
b = mean colony frequency/5×10^4 cells in FDG$^+$ fraction
c = % of sorted cells in FDG$^-$ fraction
d = mean colony frequency/5×10^4 in FDG$^-$ fraction

In bone marrow the percentages of sorted cells in the FDG$^+$ and FDG$^-$ fractions were 7-16% and 75-85% respectively. Using foetal liver the corresponding values were 5-10% and 90-95%.

Day 12 colony forming unit assay

Six- to eight-week old (C57Bl/6 × CBA) F_1 female mice were exposed to 950 rads of total irradiation from an X-ray irradiation source at a dose rate of 56 rads per minute. Unfractionated and fractionated FDG$^+$ and FDG$^-$ bone marrow cells were sorted as indicated, counted and resuspended in PBS. Cells were then injected, via the tail vein, in 0.5 ml PBS. 10^2, 10^3 and 10^4 cells of FDG$^+$ and FDG$^-$ were injected together with 10^4 total bone marrow cells. 5-6 mice were injected per cell dose in two different experiments. Twelve days after the injection, recipients were killed, their spleen fixed in glacial acetic acid/ethanol and macroscopic colonies were counted under a dissection microscope. One colony was found in 20 control irradiated mice that did not receive any injected cells.

RESULTS

An SCL 3′ enhancer directs *lacZ* expression to haematopoietic sites in mid gestation embryos

We have previously performed a systematic analysis of the chromatin structure of a 45 kb region spanning the murine *SCL* locus and thereby identified several transcriptional regulatory elements active in vitro (Göttgens et al., 1997). Three distinct 5′ enhancers were subsequently shown to direct *lacZ* expression to the developing neural system and endothelial cells in transgenic mice, but these 5′ constructs did not give rise to significant haematopoietic expression (Sinclair et al., 1999). In addition to the 5′ elements our previous studies had also identified a 3′ enhancer active in different haematopoietic cell lines (Göttgens et al., 1997) and we therefore proceeded to assess the activity of this enhancer in transgenic mice.

The 3′ enhancer exhibited promoter specificity in transfection assays (Göttgens et al., 1997) and so we elected to study the function of the 3′ enhancer in the context of all three known *SCL* promoters as well as in the context of a heterologous (SV40) promoter. A 5.5 kb fragment containing the 3′ enhancer was inserted downstream of a *lacZ* reporter gene (Fig. 1). Transcription of *lacZ* was driven by one of three promoter cassettes. The first of these (-0.9E3/*lacZ*) contained *SCL* promoter 1a and 1b. The second cassette (+6E5/*lacZ*) contained the SCL exon 4 promoter (Bernard et al., 1992). The third promoter cassette consisted of the 230 bp SV40 minimal promoter. For each promoter, a reporter construct was generated with or without the 3′ enhancer.

Transgene expression was assessed in mid gestation embryos by whole mount X-gal staining. Embryos were collected at 11-12 days of gestation. At this developmental stage both CFU-S and HSC are present in foetal liver, the AGM region and the yolk sac (Medvinsky et al., 1993; Muller et al., 1994) and erythropoiesis predominates in the foetal liver. In the absence of the 3′ enhancer, none of the promoter cassettes consistently directed *lacZ* expression to the foetal liver, AGM region, or yolk sac (Table 1), with the sole exception of urogenital ridge staining produced by the +6E5/*lacZ* construct in 3/6 embryos. All embryos transgenic for the –0.9E3/*lacZ* construct exhibited midbrain, hindbrain and spinal cord staining, consistent with our previous characterisation of midbrain and hindbrain/spinal cord enhancers (Sinclair et al., 1999).

In sharp contrast, *lacZ* expression was observed in foetal liver, AGM region, yolk sac and blood vessels of transgenic embryos generated with constructs containing the 3′ enhancer (Table 1). Interestingly, all three promoter cassettes were capable of supporting some degree of haematopoietic/vascular expression, but to varying extents. The –0.9E3/*lacZ*/3′En construct produced weak X-gal staining in the foetal liver of three embryos (Fig. 2A), no detectable signal in two embryos and strong staining in one additional embryo. Following dissection, *lacZ* expression was observed in the AGM region in 2/6 embryos. In both cases expression was localised to the dorsal aorta and was absent from the peri-aortic urogenital ridges. In 4/6 embryos weak staining was also evident in other blood vessels and in the heart, but no *lacZ* expression was found in the yolk sac.

Table 1. *lacZ* expression in 11-12 dpc transgenic embryos

Construct	Number of founder transgenic embryos	Number of embryos expressing *lacZ*						
		Yolk sac	AGM region		Liver	Blood vessels and heart	Brain/ spinal cord	Other locations
			DA	UR				
–0.9E3/*lacZ*	8/8	0	0	0	0	0	8	4[a]
+6E5/*lacZ*	5/6	0	0	3	0	0	0	5[b]
SV40/*lacZ*	3/15	0	0	0	0	0	0	3[c]
–0.9E3/*lacZ*/3′En	6/6	0	2	0	4	4	5	1[d]
+6E5/*lacZ*/3′En	15/15	11	12	12	12	13	0	3[e]
SV40/*lacZ*/3′En	56/6	4	4	4	4	4	0	2[f]

Founder transgenic embryos included transient transgenic embryos and embryos from established transgenic lines. Transgenic embryos were identified by *lacZ*-PCR and β-galactosidase activity by X-gal staining of whole embryos. AGM, aorta, gonads and mesonephros; DA, dorsal aorta; UR, urogenital ridges. Last column indicates variable and inconsistent expression in the following areas: (a) rostral areas and somites; (b) rostral areas, mesencephalon, spine, myelencephalon and limbs; (c) head and somites; (d) telecephalon; (e) myelencephalon, spinal cord, telencephalon, somites and limbs; (f) limbs and somites.

Staining was performed overnight. The number of established transgenic lines was as follows: 1 line for constructs +6E5/*lacZ*, –0.9E3/*lacZ*/3′En and SV40/*lacZ*/3′En; 7 lines for construct +6E5/*lacZ*/3′En. The number of transgenic embryos analysed from each line was at least 5. They were derived from a minimum of two litters. Within each line, the staining pattern was consistent in different embryos.

The +6E5/*lacZ*/3′En construct gave rise to higher and more consistent levels of *lacZ* expression in haematopoietic areas and blood vessels. Staining was observed in yolk sac vasculature, in the AGM region (both peri-aortic urogenital ridges and dorsal aorta) as well as in foetal liver (Fig. 2A,B; Table 1). Cephalic, intersomitic and limb blood vessels also expressed *lacZ* as did the heart (Fig. 2A,B; Table 1). This expression pattern was present in 6/8 transient transgenic embryos and in 6/7 transgenic lines after overnight incubation with X-gal. *lacZ* expression was relatively weak in two of the transgenic lines (lines 220 and 2137) and in 2 transient transgenic embryos. In the rest of the *lacZ*-positive embryos, *lacZ* expression was strong and could be observed after 1-3 hours incubation with X-gal.

The SV40/*lacZ*/3′En construct behaved similarly to the +6E5/*lacZ*/3′En construct (Fig. 2A; Table 1). Staining was observed in yolk sac, AGM region, foetal liver and blood vessels in 4 out of 6 embryos analysed.

Taken together these data demonstrate that in mid gestation embryos the *SCL* 3′ enhancer directed *lacZ* expression specifically to blood vessels and to regions associated with haematopoiesis. In view of its robust expression pattern subsequent detailed studies focused on mice transgenic for the +6E5/*lacZ*/3′En construct.

Characterization of haematopoietic cells targeted by the +6E5/*lacZ*/3′En construct in 11 dpc embryos

Dissection of the AGM region revealed prominent X-gal staining within the dorsal aorta and peri-aortic urogenital ridges (Fig. 2B). Staining was particularly intense in the mid-anterior part of the urogenital ridges, a region which has been shown to be especially rich in HSC activity (Medvinsky and Dzierzak, 1996). Histological sections showed strong staining in the endothelium of the dorsal aorta (Fig. 3A and B). In addition X-gal-positive cells with the appearance of blood cells were present in the lumen of the aorta and some large blast-like cells were attached to the inner surface of the endothelium (Fig. 3B). Cells with a similar undifferentiated appearance have been reported previously to form tight junctions with endothelial cells (Garcia-Porrero et al., 1995; Medvinsky et al., 1993). It is not clear whether these cells arise directly from the endothelium or whether they derive from the peri-aortic region and subsequently migrate across the wall of the aorta. Interestingly, clusters of large β-gal-expressing haematopoietic cells were also observed surrounded by endothelium within the peri-aortic urogenital ridges (Fig. 3C).

Histological sections indicated that only a proportion of foetal liver cells were X-gal$^+$. Unlike the AGM region, cell suspension of foetal liver cells can be easily obtained by mechanical dispersion, allowing β-galactosidase detection by flow cytometry using the fluorescent β-galactosidase substrate, FDG (Miles et al., 1997). Dual staining flow cytometry was therefore used to ascertain the phenotype of *lacZ*$^+$ cells. Foetal liver cells were co-stained with FDG and with antibodies to cell surface antigens. The mean proportion of FDG$^+$ foetal liver cells varied from 2.3 to 16.6% among the six +6E5/*lacZ*/3′En transgenic lines that exhibited haematopoietic vascular *lacZ* expression (Table 2). This variation correlated with the intensity of *lacZ* expression seen in whole mount X-gal stains, but not with the transgene copy number (Table 2). The antigen profile of the FDG$^+$ cells was very similar in lines with a high (line 2262) or low (line 220) proportion of FDG$^+$ foetal liver cells.

As shown in Fig. 3, all FDG$^+$ foetal liver cells were c-kit$^+$. Furthermore 50% and 20% of the FDG$^+$ foetal liver cells expressed CD34 and Mac-1 antigens respectively. HSC from foetal liver have been shown to be c-kit$^+$ CD34$^+$ Mac-1$^+$ (Sanchez et al., 1996). No expression of the erythroid antigen Ter119 was detected in FDG$^+$ foetal liver cells, despite the fact that foetal liver is predominantly erythroid at this developmental stage and *SCL* is expressed in erythroid cells. This observation suggests the existence of additional *SCL* haematopoietic enhancers. The alternative possibility that *SCL* and *lacZ* are differentially regulated at a post-transcriptional level in erythroid cells, is excluded by the observation that *lacZ* 'knocked into' the *SCL* locus is expressed in erythroid cells (Elefanty et al., 1998).

In order to investigate whether the +6E5 promoter casette was necessary for the pattern of transgene expression described above, the phenotype of 11 dpc foetal liver cells was also analysed in one –0.9E3/*lacZ*/3′En and in one SV40/*lacZ*/3′En transgenic line (2175 and 895 respectively). A mean of 5 and 2% respectively of the liver cells expressed *lacZ*. In both transgenic lines the FDG$^+$ cells were all c-kit$^+$ and approximately 50% expressed CD34 (data not shown).

Taken together our data therefore demonstrate that (1) the +6E5/*LacZ*/3′En transgene directs expression to part of the AGM region known to be rich in HSC; (2) this transgene targets foetal liver cells expressing antigens found on HSC; (3) the 3′ enhancer is primarily responsible for this pattern of expression.

The precise location of *lacZ*$^+$ cells within the AGM region and the phenotype of *lacZ*$^+$ cells in foetal liver strongly suggest

Table 2. FACS analysis of *lacZ* expression in haematopoietic tissues

Transgenic lines	Transgene copy number	% FDG-positive cells: 11 dpc foetal liver	Bone marrow	Spleen	Thymus
+6E5/*lacZ*/3′En					
2262	2	16.6±7.2	5±1.4	5.4±1.4	59±13
2269	7.6	14±3.6	2.1±0.9	1.4±0.6	5.8±5
2257	4	5±3.4	3.3±0.7	1.6±0.5	5.3±0.9
2270	2.8	14±1.6	1.2±0.5	0.6±0.2	2.8±2.2
2137	14.2	3±0.2	0.4±0.5	<0.1	<0.1
220	4.6	2.3±0.7	0.6*	0.6*	<0.1*
2266	20	<0.1	<0.1*	<0.1*	0.5*
–0.9E3/*lacZ*/3′En					
2175	ND	5.2±1.7	2.2±1	4±3	15±11
LC-12*	ND	<0.1	<0.1	<0.1	<0.1
SV40/*lacZ*/3′En					
895	ND	2±0.2	<0.1	<0.1	<0.1
+6E5/*lacZ*					
219	ND	ND	<0.1	<0.1	<0.1

11 dpc foetal liver and adult bone marrow, spleen and thymus cells from different transgenic lines were stained with FDG and analysed by flow cytometry. Data represent the mean of results obtained from at least three mice per line except where indicated with an asterisk where only one mouse was analysed. Transgene copy number was determined by Southern blot analysis. Adult mice were 2-6 months of age.

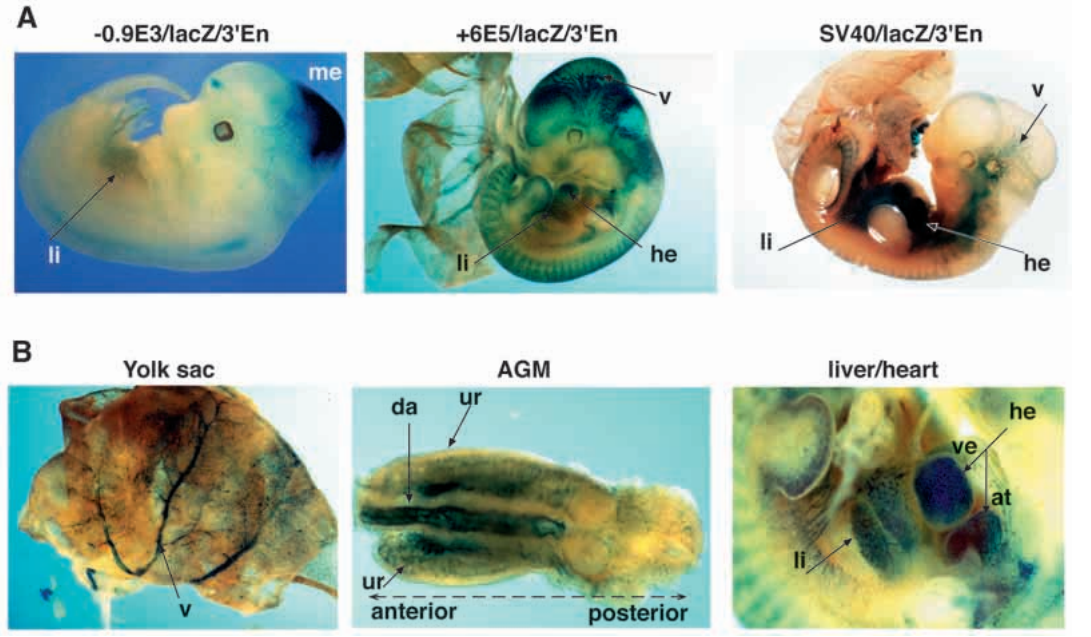

Fig. 2. Expression of transgenes containing different promoter regions and the *SCL* 3′ enhancer in 11-12 dpc embryos. Whole embryos were stained with X-gal for detection of *lacZ* expression. (A) Transgenic embryos expressing the indicated transgenes. li, liver; me, mesencephalon; he, heart; v, blood vessels. The –0.9E3/*lacZ*/3′En and SV40/*lacZ*/3′En panels show 12 dpc and 11 dpc founder embryos respectively that were stained overnight with X-gal. The +6E5/*lacZ*/3′En panel shows an 11 dpc transgenic embryo from line 2262 after 2 hours incubation in X-gal. (B) Individual haematopoietic organs from an 11 dpc embryo carrying the +6E5/*lacZ*/3′En transgene (line 2269). The panels show a dissected yolk sac, a dissected AGM region and a high power view of the foetal liver and heart. da, dorsal aorta; ur, urogenital ridges; ve, ventricle; at, atrium; li, liver; he, heart.

that the +6E5/*lacZ*/3′En construct directs expression to early haematopoietic progenitors at the 11 dpc stage of development.

The SCL +6E5/*lacZ*/3′En transgene targets haematopoietic and endothelial progenitors in early embryonic development

Expression of the +6E5/*lacZ*/3′En transgene was assessed in early stages of mouse development, starting with 7.5 dpc embryos, the time point at which haematopoietic cells first become apparent. At this stage *lacZ* expression was observed in the extra-embryonic region which will form the yolk sac (Fig. 4A). This pattern was consistent in all six transgenic lines expressing *lacZ* at 11 dpc. Four of the transgenic lines exhibited high levels of expression as shown in Fig. 4. Two additional transgenic lines (220 and 2137) displayed punctuated expression in the same region and line 2266 did not express *lacZ*. Serial sections of embryos from three independent high expressing lines demonstrated X-gal staining in cells within the extra embryonic mesoderm (Fig. 4B,C), whereas only occasional mesodermal cells within the embryo proper expressed *lacZ*. Staining was also observed in a few cells in the allantoic bud (Fig. 4B), which is derived from extra

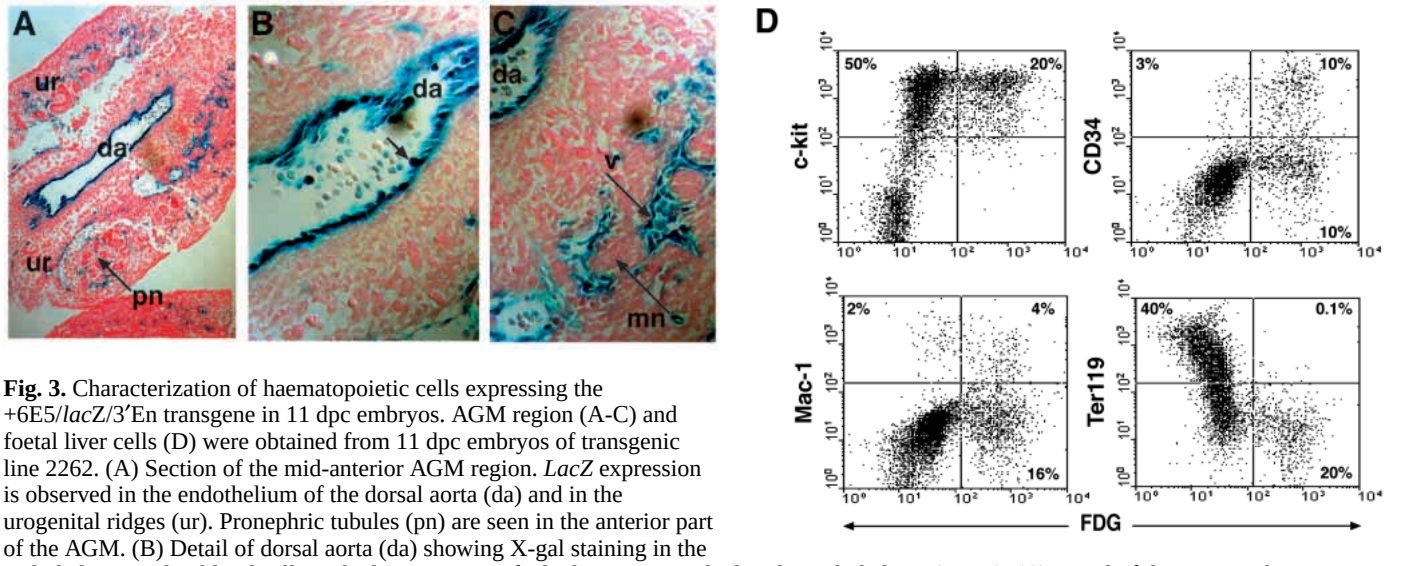

Fig. 3. Characterization of haematopoietic cells expressing the +6E5/*lacZ*/3′En transgene in 11 dpc embryos. AGM region (A-C) and foetal liver cells (D) were obtained from 11 dpc embryos of transgenic line 2262. (A) Section of the mid-anterior AGM region. *LacZ* expression is observed in the endothelium of the dorsal aorta (da) and in the urogenital ridges (ur). Pronephric tubules (pn) are seen in the anterior part of the AGM. (B) Detail of dorsal aorta (da) showing X-gal staining in the endothelium, and in blood cells in the lumen, some of which appear attached to the endothelium (arrow). (C) Detail of the mesonephros region of the urogenital ridges showing X-gal staining in vascular structure (v) adjacent to the mesonephric tubules (mn). *lacZ* expression is seen in the endothelium and in presumed haematopoietic cells in the lumen. (D) FACS analysis of 11 dpc foetal liver cells. Foetal liver cells from transgenic and non-transgenic embryos were stained with antibodies against the indicated cell surface molecules followed by incubation with the β-galactosidase substrate FDG. Quadrants were established according to values in non transgenic embryos and isotype controls.

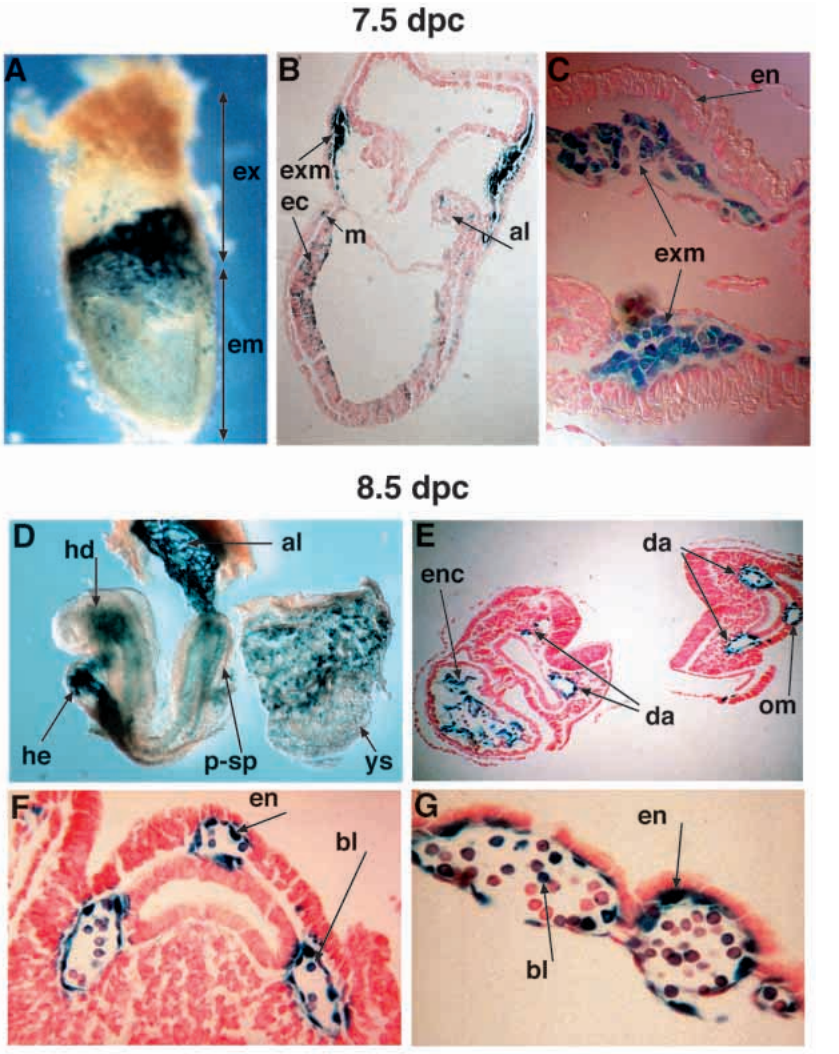

Fig. 4. Expression of +6E5/*lacZ*/3′En transgene in 7.5 and 8.5 dpc mouse embryos. X-gal staining of 7.5 dpc embryos (A-C) and 8.5 dpc embryos (D-G). Embryos are from transgenic line 2262 in A and C and line 2269 in B and D to G. (A) Whole-mount embryo: ex, extraembryonic tissue; em, embryo proper. (B) Sagittal section of 7.5 dpc embryo showing X-gal staining in extraembryonic mesoderm (exm), allantois (al), embryonic ectoderm (ec) and in a few embryonic mesodermal cells (m). (C) Transverse section of 7.5 dpc embryo showing X-gal staining in extra embryonic mesodermal cells (exm) surrounded by endoderm (en). (D) Whole-mount 8.5 dpc embryo and disected yolk sac (ys). Whole-mount embryos showed X-gal staining in the allantois (al), head (hd), heart (he), and para-aortic splachnopleural region (p-sp). (E) Transverse section through the heart and caudal region showing staining in the endocardium (enc), dorsal aorta (da) and omphalomesentheric vein (om). (F) Transverse section of the caudal region of the para-aortic splanchnopleura. X-gal staining is present in vessel endothelium (en) and in some blood cells (bl) in the lumen of these vessels. (G) Section of yolk sac showing two blood islands. Staining in endothelial (en) and blood cells (bl) is indicated.

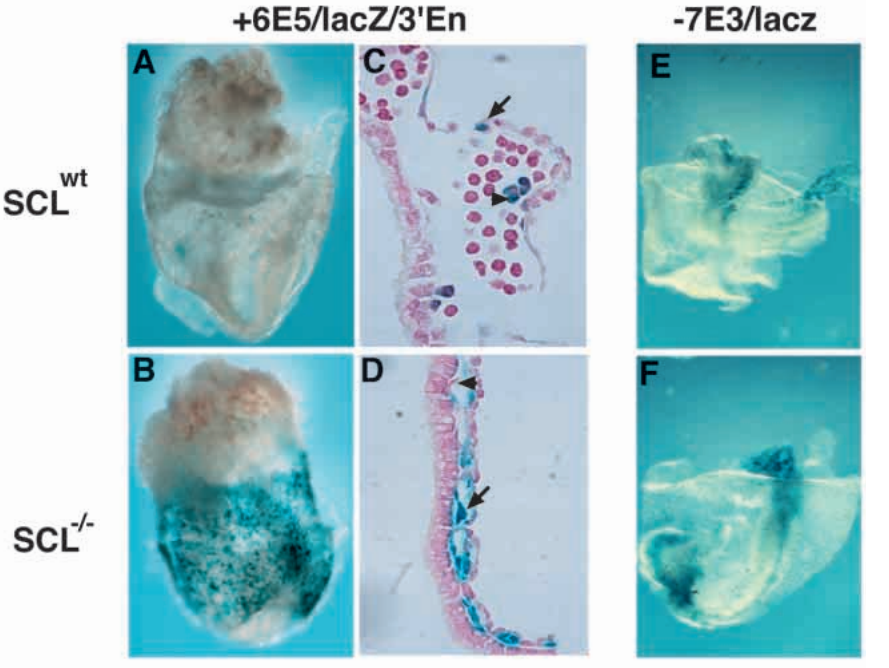

Fig. 5. SCL is not required for activation of the *SCL* 3′ enhancer. (A,B) X-gal staining of 8.5 dpc embryos carrying a +6E5/*lacZ*/3′En transgene (line 2137) in an SCL^{wt} or $SCL^{-/-}$ genetic background. (C) Histological section of a yolk sac blood island from an SCL^{wt} embryo showing occassional X-gal staining in endothelium (arrow) and haematopoietic cells (arrowhead). (D) Histological section of yolk sac from an $SCL^{-/-}$ embryo showing prominent *lacZ* staining in cells resembling undifferentiated mesoderm cells (arrow) and others resembling endothelial cells (arrowhead). (E and F) X-gal staining of embryos carrying a −7E3/*lacZ* transgene in an SCL^{wt} or an $SCL^{-/-}$ genetic background.

embryonic mesoderm and has been shown to contain haematopoietic and endothelial precursors in birds (Caprioli et al., 1998). In two transgenic lines (2257 and 2269) X-gal staining was also present in a subset of presumptive ectodermal cells within the embryo proper. The significance of this staining is unclear and may represent a position effect.

In 8.5 dpc embryos prominent X-gal staining was consistently observed in all +6E5/*lacZ*/3′En lines in the blood vessels, heart and yolk sac (Fig. 4D). *lacZ* expression was also seen in the cephalic region and allantois in 4/7 lines (not detectable in 220 and 2137). Histological sections of the embryo proper showed X-gal staining in the endocardium (Fig. 4E and F) and in angioblast-like cells within the cephalic mesenchyme (not shown). Within the para-aortic splachnopleura, X-gal staining was evident in the endothelium of the omphalomesenteric vein and the paired dorsal aortae (Fig. 4E,F), as well as in a subset of circulating haematopoietic cells (Fig. 4F). Yolk sac sections showed *lacZ* expression within blood islands, in both haematopoietic cells and endothelium (Fig. 4G). It should be noted that staining variations among transgenic lines were observed in the proportion of X-gal$^+$ blood and endothelial cells in the yolk sac blood islands and p-sp. These differences may reflect, at least in part, differences in the level of *lacZ* expression since transgenic lines which exhibited strong *lacZ* expression had a higher proportion of X-gal$^+$ cells. It has previously been reported that residual β-galactosidase protein may persist within differentiated haematopoietic cells even after transcriptional down-regulation of the transgene (Lawrence et al., 1987; Tewari et al., 1996).

Our data therefore demonstrate that the +6E5/*lacZ*/3′En construct directed *lacZ* expression to presumptive haematopoietic and endothelial progenitors at 7.5 dpc as well as to both endothelium and haematopoietic cells at 8.5 dpc. This early pattern of *lacZ* expression was primarily dependent on the *SCL* 3′ enhancer, since a similar pattern was observed in yolk sac and p-sp from whole mount 8.5 dpc embryos carrying the SV40/*lacZ*/3′En and −0.9E3/*lacZ*/3′En transgenes (2/2 embryos and 1/1 embryo analysed respectively. The precise pattern of expression was very similar to that displayed by endogenous SCL protein and mRNA (Kallianpur et al., 1994; Silver and Palis, 1997). In the light of recent evidence that *SCL* is expressed in the haemangioblast (Gering et al., 1998) our data raise the possibility that the *SCL* 3′ enhancer directs expression to mesodermal cells as they are activating the transcriptional programmes necessary for haematopoietic and endothelial differentiation.

Activation of the 3′ enhancer in yolk sac endothelium is independent of SCL protein

Auto regulation is a strategy utilised by a number of key developmental regulators (Cheng et al., 1995; McDevitt et al., 1997). In order to investigate whether SCL protein is necessary for activity of the 3′ enhancer, the +6E5/*lacZ*/3′ En transgene (line 2137) was bred into an *SCL*$^{-/-}$ background. In a wild-type background, line 2137 expresses relatively low *lacZ* levels in yolk sac, however the number of X-gal$^+$ cells observed in 8.5 dpc or 9.5 dpc yolk sacs was markedly increased in *SCL*$^{-/-}$ relative to *SCL*$^{+/-}$ or *SCL*$^{+/+}$ embryos (compare Fig. 5A with B). This dramatic increase in *lacZ* expression was observed in 4/4 *SCL*$^{-/-}$ embryos compared to 0/4 *SCL*$^{+/+}$ and 0/14 *SCL*$^{+/-}$ embryos. In sections of 8.5 dpc *SCL*$^{+/+}$ yolk sacs *lacZ* expression was detectable in occasional endothelial and haematopoietic cells (Fig. 5C). By contrast, a large number of X-gal$^+$ cells was evident between the endoderm and mesothelium in *SCL*$^{-/-}$ yolk sacs (Fig. 5D). Most of the X-gal$^+$ cells had the morphological appearance of endothelial cells with a minority resembling undifferentiated mesoderm. These data demonstrate that the 3′ enhancer does not require SCL protein for its activation in yolk sac endothelial and/or mesodermal cells.

We considered the possibility that the increased number of X-gal$^+$ endothelial cells might represent a physiological response to the absence of blood in *SCL*$^{-/-}$ embryos. In order to address this issue we performed similar studies using a different endothelial enhancer. A −7E3*lacZ* transgene, containing an endothelial enhancer from the 5′ region of the *SCL* gene (Sinclair et al., 1999), was therefore bred into the *SCL*$^{-/-}$ background. In *SCL*$^{+/+}$ yolk sacs *lacZ* expression was again observed in a minority of endothelial cells but with this construct only a minimal increase of *lacZ* expression was seen in *SCL*$^{-/-}$ yolk sacs (compare Fig. 5E with Fig. F). These results argue that the dramatic increase in the number of *lacZ*$^+$ cells produced by the +6E5/*lacZ*/3′En construct in *SCL*$^{-/-}$ yolk sacs did not represent a non-specific response of endothelial cells to the lack of blood. Instead, activity of the +6E5/*lacZ*/3′En transgene may be negatively regulated by SCL protein. Alternatively it is possible that the *SCL*$^{-/-}$ environment alters mesodermal and/or endothelial differentiation resulting in an increased number of cells capable of expressing high levels of the +6E5/*lacZ*/3′En transgene. Our data do not stringently distinguish between these two possibilities and indeed they are not mutually exclusive.

Analysis of transgene expression in adult haematopoietic cells

In adult mice *SCL* is expressed in erythroid, mast and megakaryocytic cells, together with multipotent progenitors. In order to determine the specificity of the +6E5/*lacZ*/3′En construct for different populations of haematopoietic cells in adult mice, we performed flow cytometric analysis of bone marrow, spleen and thymus from transgenic lines carrying the +6E5/*LacZ*/3′En transgene.

In thymus, 3/6 lines exhibited *lacZ* expression in 2.8-5.8% of cells and one additional line showed expression in 59% of thymocytes (Table 2). *lacZ* expression was also seen in 0.5% of thymocytes from line 2266. The proportion of thymocytes expressing detectable *lacZ* varied significantly between individuals of the same age within a given transgenic line. In addition, transgene expression was generally higher in newborn mice (e.g. a mean of 78% FDG$^+$ cells for line 2257 and 17% in line 2269) relative to adult mice (Table 2).

In bone marrow and spleen the percentage of FDG$^+$ cells also varied among *lacZ*-expressing transgenic lines with a range of 0.4%-5.0% in bone marrow and 0.6%-5.4% in spleen (Table 2). Dual parameter FACS analysis was then performed on spleen and bone marrow from three lines (2257, 2262 and 2269) to assess coexpression of *lacZ* and a variety of lineage-associated antigens. The data from line 2262 are shown in Fig. 6 and similar results were obtained using the other 2 lines. Half of the FDG$^+$ cells in spleen were CD4$^+$ T cells whereas

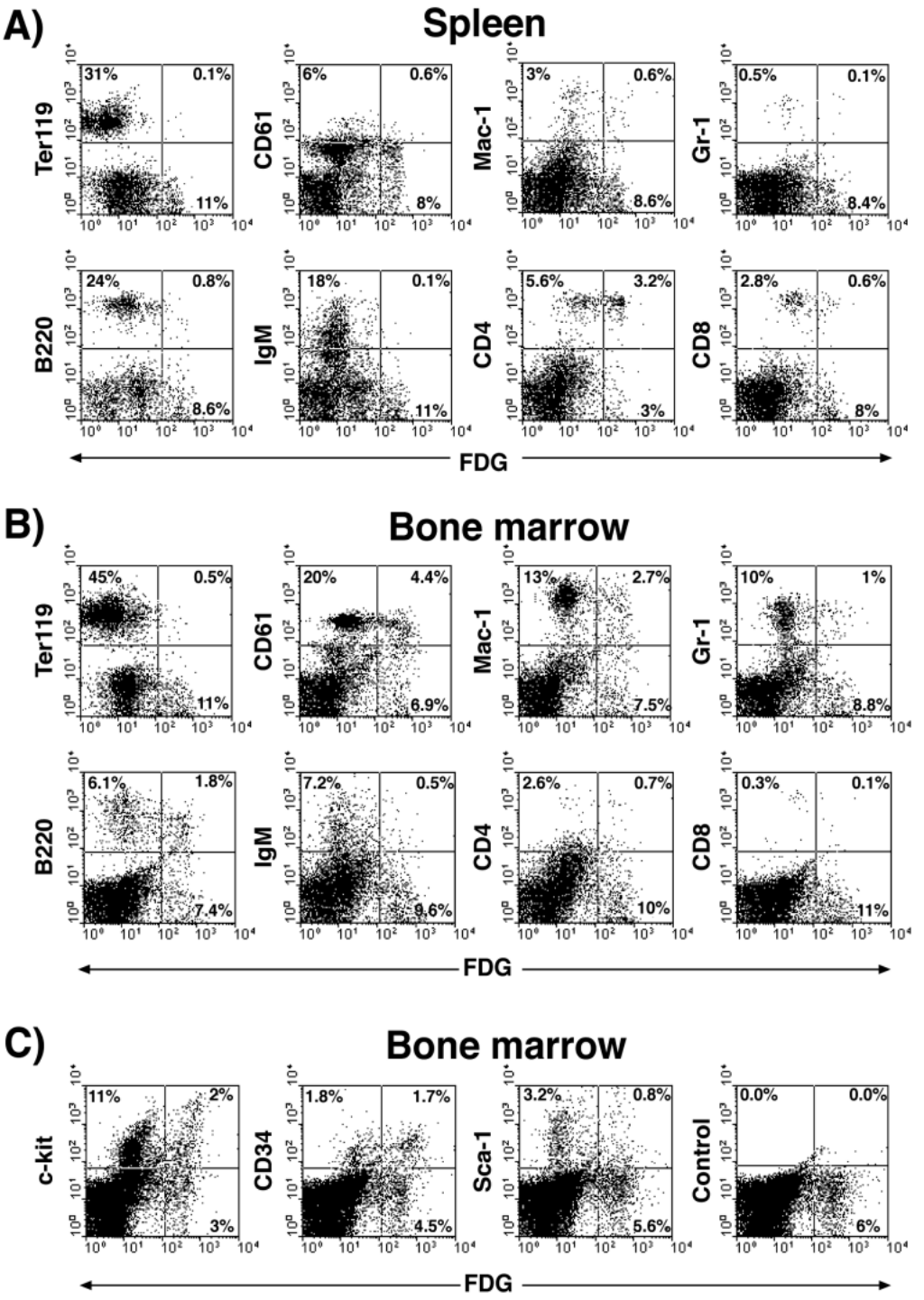

Fig. 6. FACS analysis of spleen and bone marrow from adult +6E5/*lacZ*/3′En transgenic mice. Cells were obtained from six-month-old transgenic mice (line 2262) stained with PE-conjugated antibodies against the indicated cell surface antigens and subsequently incubated with the *lacZ* substrate, FDG. Quadrants were established according to values obtained using cells from non transgenic mice and isotype controls in the same experiment. Dead cells were excluded by propidium iodide staining.

all other lineage markers were each present on <10% of the FDG^+ cells. In bone marrow CD61, Mac-1 and Gr-1 were present on 39%, 26%, 20% and 10% respectively of the FDG^+ population whereas all other lineage markers were present on <10% of the FDG^+ cells (Fig. 6B). It is worth noting that neither bone marrow nor spleen FDG^+ cells expressed Ter119, a finding consistent with our observations using foetal liver (see above).

Taken together, these data demonstrate that in adult mice the +6E5/*LacZ*/3′En transgene directed expression to a sub-population of haematopoietic cells which included cells expressing different lineage-associated antigens. Some of these lineages are not thought to normally express *SCL*. Thus only occasional normal thymocytes stain positively for SCL protein (Kallianpur et al., 1994) and the vast majority of thymocytes do not express SCL mRNA (Aplan et al., 1997). There are several possible explanations for the apparent discrepancy between transgene expression and endogenous *SCL* expression, including: (1) The +6E5/*lacZ*/3′En construct may lack one or more silencers present at the *SCL* locus, (2) *SCL*

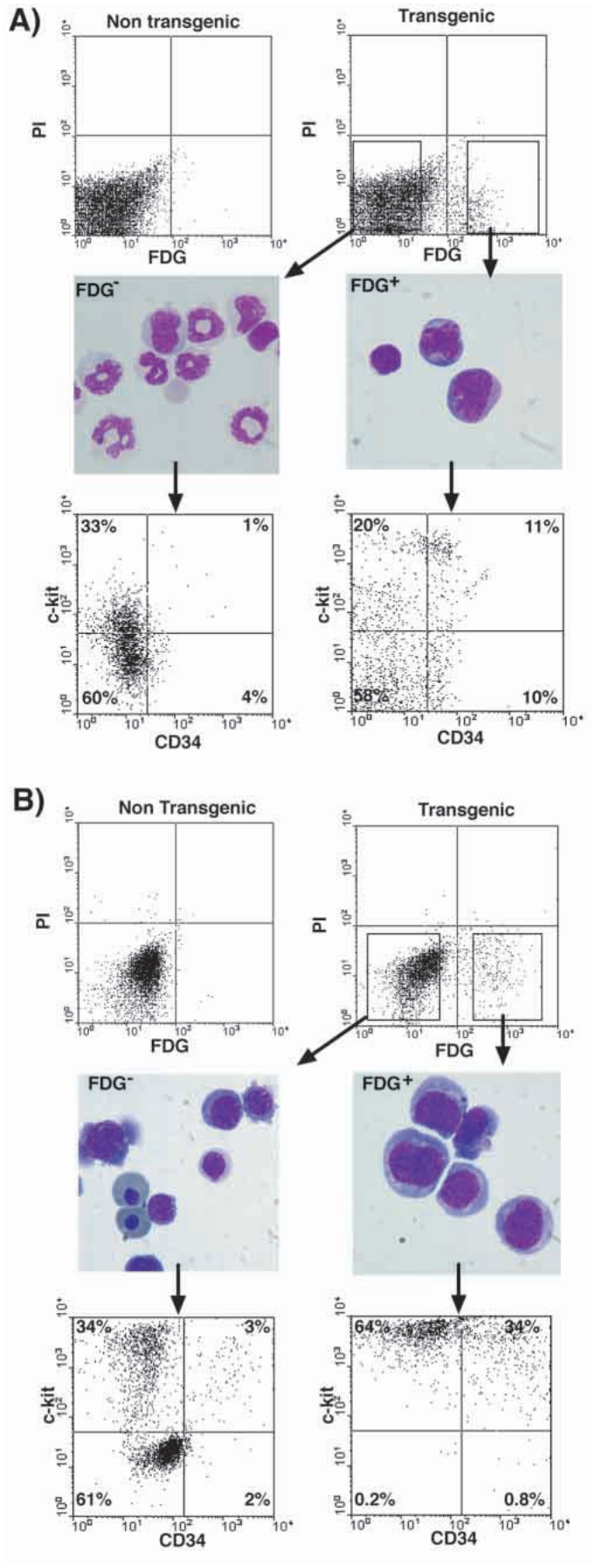

Fig. 7. The +6E5/*lacZ*/3′En transgene targets undifferentiated blasts. Adult bone marrow (A) and 11 dpc foetal liver cells (B) from non-transgenic and transgenic mice were incubated with the β-galactosidase substrate, FDG. Sort windows for FDG^+ and FDG^- cells are represented after excluding dead cells by propidium iodide (PI) staining and bone marrow red blood cells by forward and side scatter. Purity of the sorted cells varied from 85-95% as assessed by FACS reanalysis. Cytospin preparations of the different sorted fractions were stained with May-Grunwald-Giemsa (400× magnification). In a representative experiment sorted cells were also stained with PE-c-kit and Biotin-CD34 followed by incubation with Streptavidin-Red 670. Quadrants were established according to values with isotype controls and secondary reagent.

and *lacZ* mRNA may undergo differential post-transcriptional regulation in normal thymocytes. The former possibility is more likely in view of the observation that *lacZ* 'knocked in' the *SCL* locus is not expressed in thymocytes (Elefanty et al., 1998).

In view of the possibility that the +6E5/*lacZ*/3′En construct was expressed in early progenitors we assessed coexpression of *lacZ* with c-kit, CD34 or Sca-1, all known to be expressed on bone marrow haematopoietic stem cells (Morel et al., 1996; Okada et al., 1991; Spangrude et al., 1988). *lacZ* was expressed in 50%, 20% and 15% of the CD34-, Sca-1- and c-kit-positive cells respectively (Fig. 6C). Moreover, the FDG^+ cells contained the majority of the c-kit bright cells, which have previously been shown to contain most of the CFU-S activity (Okada et al., 1991). These data are therefore consistent with expression of the +6E5/*lacZ*/3′En transgene in early multipotent progenitors from bone marrow.

The +6E5/*lacZ*/3′En transgene targets erythroid, myeloid and multipotent progenitors

In order to directly assess the biological function of haematopoietic progenitors targeted by the +6E5/*lacZ*/3′En construct, colony assays were performed on FDG^+ and FDG^- fractions of bone marrow and foetal liver.

In bone marrow undifferentiated blasts were greatly enriched in the FDG^+ relative to the FDG^- fraction (65% compared to 3% respectively) and most of he $c\text{-}kit^+CD34^+$ progenitor cells were detected in the FDG^+ population (Fig. 7A). The frequency of erythroid and myeloid colonies was over 50- and over 100-fold higher, respectively, in FDG^+ relative to FDG^- fractions (Table 3). Within the total gated population, 90-95% of the colony forming cells were in the FDG^+ fraction.

In foetal liver most of the cells were erythroid in both FDG^+ and FDG^- fractions as assessed by the morphology of cytospin preparations. However, in the FDG^+ population the erythroid cells were left-shifted with a conspicuous absence of late normoblasts, whereas erythroid precursors at all stages of differentiation were evident in the FDG^- fraction (Fig. 7B). In additon $c\text{-}kit^+CD34^+$ cells were enriched in the FDG^+ population. The frequency of erythroid and total colonies was approximately 40- and 30-fold higher in the FDG^+ relative to the FDG^- fractions respectively (Table 3). Within the total gated population 76% of total colony forming cells were in the FDG^+ fraction.

These data demonstrate that the +6E5/*lacZ*/3′En construct directed *lacZ* expression to erythroid and myeloid progenitors. In order to establish whether the transgene was active in more primitive multipotent cells, we assessed CFU-S activity in bone

Table 3. Frequency of colony forming cells in FDG⁻ and FDG⁺ haematopoietic cells

Assay	Stimulus	Colony type	Frequency per 5×10^4 cells			Colony distribution (%)	
			Unseparated	FDG⁻	FDG⁺	FDG⁻	FDG⁺
ADULT BONE MARROW							
Agar	Bhk/W3B	d7 myeloid	72±21	3±2	493±164	4±1	95±2
(*n*=4)	IL-3	d7 myeloid	50±23	2±2	301±110	4±3	95±3
Methylcellulose	Methocult	d7 total	83±24	7±4	648±157	7±5	92±3
(*n*=3)	Methocult	d7 erythroid	28±9	3±1	197±59	3±1	90±5
FOETAL LIVER							
Methylcellulose	Methocult	d7 total	395±40	90±35	2965±1125	26±6	76±10
(*n*=2)	Methocult	d7 erythroid	290±40	50±10	1950±575	19±7	64±11

Myeloid or erythroid colonies were enumerated after culture in agar or methyl cellulose as indicated. In each experiment, colonies were counted in duplicate or triplicate. Cells from transgenic lines 2262 and 2257 were used with similar results. Colony distribution represents the mean of the individual colony distributions calculated for each experiment (see Materials and Methods). *n*, number of independent experiments; Bhk, conditioned medium from Baby Hamster Kidney cell line expressing stem cell factor; W3B, conditioned medium from WEHI 3B cell line.

marrow FDG⁺ and FDG⁻ fractions (Table 4). Within the total gated population, a striking 98% of CFU-S were found in the FDG⁺ fraction. These results are consistent with our immunophenotype data and directly demonstrate that the +6E5/*lacZ*/3′En construct targeted *lacZ* expression to the vast majority of primitive multipotent progenitors.

DISCUSSION

In this paper we have identified an *SCL* 3′ enhancer which directs expression to blood and endothelium throughout development, and which targets early haemopoietic progenitors.

A common origin for blood and endothelium

A close developmental link between blood and endothelium has long been recognised. It is particularly evident in the yolk sac blood islands in which blood and endothelial cells arise at the same time (Haar and Ackerman, 1971; Murray, 1932; Sabin, 1920). Within the embryo proper, haematopoiesis also occurs in close association with endothelium in chicken (Dieterlen-Lievre and Martin, 1981), mouse (Garcia-Porrero et al., 1995; Wood et al., 1997) and man (Tavian et al., 1996). Several additional lines of evidence support the concept of blood and endothelium arising from a common progenitor, the haemangioblast. A large number of genes are expressed in both blood and endothelial cells, including *SCL, MB 1/QH 1* and *MEP 21* in birds (Drake et al., 1997; McNagny et al., 1997; Pardanaud et al., 1987) and *SCL, CD34, CD31* and the erythropoietin receptor in mammals (Anagnostou et al., 1994; Baldwin et al., 1994; Kallianpur et al., 1994; Young et al., 1995). Moreover, *Flk-1* is required for normal vasculogenesis and haematopoiesis (Shalaby et al., 1997) and *Flk-1*⁺ cells from chick embryos (Pardanaud et al., 1996) or ES cells (Choi et al., 1998) can give rise to both haematopoietic and endothelial cells.

The simple haemangioblast model is complicated by two recent observations. Firstly, experiments using chicken/quail chimeras have demonstrated the existence of two endothelial lineages within the embryo proper (Pardanaud et al., 1996). One arises from splanchnopleural mesoderm, is associated with haematopoiesis and contributes endothelial cells to the ventral floor of the aorta and to visceral organs. The other lineage arises from paraxial mesoderm and forms the vasculature of the body wall and kidney. Thus only a subset of endothelial progenitors are associated with haematopoiesis. Secondly, two groups have reported that mature endothelial cells, as assessed by morphology, immunophenotype and functional assays, can give rise to haematopoietic cells (Jaffredo et al., 1998; Nishikawa et al., 1998). These latter papers suggest that there may be unexpected plasticity in the relationship between differentiated endothelium and blood cells.

It has recently become clear that SCL plays a key role in the development of endothelium as well as blood. *SCL* is expressed in normal endothelium (Drake et al., 1997; Hwang et al., 1993) and *SCL*$^{-/-}$ embryos exhibited defects in the formation of yolk sac blood vessels (Robb et al., 1995; Visvader et al., 1998) which reflect a requirement for SCL in yolk sac angiogenesis (Visvader et al., 1998). Direct evidence that SCL also plays a role in specification of endothelial progenitors has come from zebrafish studies (Gering et al., 1998). The idea that SCL specifies haemangioblast development is also consistent with the observation that ectopic *SCL* expression partially rescues both haematopoietic and vascular defects in the zebrafish *cloche* mutant (Liao et al., 1997).

In this paper we have identified an *SCL* 3′ enhancer which targets blood and endothelium. Several features are of particular note. Firstly, this enhancer was already active in extra-embryonic

Table 4. Frequency of day 12 CFU-S in transgenic bone marrow

Bone marrow fractions	Cells injected per recipient	Spleen colonies
Uninjected		0
Unfractionated	10^4	6±3
FDG⁻	10^2	0
	10^3	0
	10^4	0.5±0.5
FDG⁺	10^2	0.6±0.8
	10^3	3.4±3
	10^4	23±16

Bone marrow cells from transgenic mice were injected into irradiated recipients. The number of animals used in two independent experiments are: uninjected, 11 animals; unfractionated, 5 animals, FDG fractions, 6 animals per group. Spleen were harvested after 12 days, fixed and macroscopic colonies counted. Values represent the mean of the two experiments ± s.d.

mesoderm at 7.5 dpc prior to the development of morphologically identifiable endothelial or blood cells and at a time when *SCL* and *Flk-1* are both normally expressed (Shalaby et al., 1995; Silver and Palis, 1997). These data therefore suggest that the *SCL* 3′ enhancer is active in murine haemangioblasts, a possibility that is consistent with expression of *SCL* in zebrafish (Gering et al., 1998). Secondly the 3′ enhancer subsequently targets blood and endothelium at multiple embryonic sites associated with haematopoiesis including yolk sac, para-aortic splanchopleura and AGM region, as well as directing expression to endothelial cells participating in both angiogenesis and vasculogenesis. Thirdly the 3′ enhancer was active in both primitive and definitive haematopoietic cells.

SCL protein is not required for activity of the 3′ enhancer

Transcriptional autoregulation is a feature of several transcription factors with important roles in both vertebrate and invertebrate development (Bienz and Tremml, 1988; McDevitt et al., 1997; Rhodes et al., 1993). Autoregulation of key regulators may contribute to the stability of differentiated phenotypes and may also form part of molecular switches that define the spatial or temporal limits of a transcription programme. Ectopic *SCL* expression in zebrafish embryos resulted in an increased number of cells expressing the endogenous *SCL* gene (Gering et al., 1998), thus providing circumstantial evidence that SCL may regulate its own transcription either directly or indirectly. We have performed a genetic test of this model by breeding the +6E5/*lacZ*/3′En transgene into an $SCL^{-/-}$ background. Our results demonstrate that activity of the 3′ enhancer was not dependent on the presence of SCL protein. This observation is similar to previous data which demonstrate that GATA-1 protein was not required for activation of GATA-1 expression (McDevitt et al., 1997), despite previous evidence for autoregulation of GATA-1 (Hannon et al., 1991; Nicolis et al., 1991; Tsai et al., 1991). However, in contrast to the GATA-1 results, the number of cells expressing the +6E5/*lacZ*/3′En transgene was substantially increased in a $SCL^{-/-}$ background. This observation suggests that SCL may negatively regulate its own expression in yolk sac mesoderm and/or endothelium. However our data do not exclude the possibility that mesoderm or endothelial differentiation may be altered in $SCL^{-/-}$ yolk sacs with a consequent expansion of cells in which the transgene is particularly active.

The SCL 3′ enhancer targets early haematopoietic progenitors

Very little is known about the transcriptional programs of multipotent haematopoietic progenitors. Our data represent the first description of an enhancer which targets haematopoietic progenitors throughout ontogeny and which is capable of integrating the information needed to direct expression to such progenitors at different sites and at distinct stages of development.

The 3′ enhancer directed *lacZ* expression to a subset of haematopoietic cells in the yolk sac, splanchnopleura and AGM region. In adult bone marrow and foetal liver, *lacZ* was coexpressed with c-kit and CD34, both markers of early progenitors. In both tissues the $lacZ^+$ fraction was greatly enriched for committed myeloid and erythroid progenitors and in bone marrow the $lacZ^+$ fraction also contained the vast majority of multipotent CFU-S. However $Ter119^+$ erythroid cells did not express *lacZ*. Elefanty and colleagues (Elefanty et al., 1998) have reported the haematopoietic expression of *lacZ* ‘knocked into’ the SCL locus. They found that the $lacZ^+$ fraction in bone marrow was enriched for day-12 CFU-S and also contained >90% of myeloid and >99% of erythroid colony-forming cells. Importantly, differentiated erythroid cells expressed *lacZ*. Comparison with our results therefore suggests a model in which the 3′ enhancer is responsible for expression in early haematopoietic progenitors, but that additional regulatory elements are needed for expression in differentiating erythroid cells. Taken together with the phenotype of $SCL^{-/-}$ mice (Porcher et al., 1996; Robb et al., 1996), and the previously reported pattern of *SCL* expression (Cross et al., 1994; Elefanty et al., 1998; Kallianpur et al., 1994; Mouthon et al., 1993), our results also suggest that the 3′ enhancer is likely to be active in haematopoietic stem cells. Long-term reconstitution experiments will be needed to confirm this prediction. In view of our observation that Ter119-positive fetal liver cells and bone marrow cells did not express *lacZ*, it is interesting to note that *lacZ* was expressed in cells within the lumen of blood vessels in the AGM region and yolk sac blood islands at 11 dpc and 8.5 dpc (Fig. 3B,C and Fig. 4F,G). By contrast 99% of 11 dpc peripheral blood cells (obtained after removal of the placenta and leaving the embryos in medium for 10-20 minutes) were Ter119+ and did not express *lacZ*, as determined by FDG staining (data not shown). This does not reflect a technical problem as Graubert and colleagues (Graubert et al., 1998) have shown that there is no difference in the proportion of red cells stained with FDG and X-gal. Instead our data suggest that there may be a differential distribution of cell types at different sites. The AGM and yolk sac are both sites of active haematopoiesis and we postulate there may be an increased numbers of progenitors in these locations.

Although there is considerable interest in identifying enhancers that target early haematopoietic progenitors, very few candidate elements have been described. Two separate enhancers flanking the CD34 gene have been reported to confer high-level expression in haematopoietic cell lines (May and Enver, 1995). However, it is not known whether these elements are sufficient to target haematopoietic progenitors in vivo. The only expression cassette currently known to target multipotent progenitors in vivo is a 14 kb genomic cassette containing the *Ly-6E.1* gene (Miles et al., 1997).This cassette targets haematopoietic stem cells from adult bone marrow. However, unlike the *SCL* 3′ enhancer, the *Ly-6E.1* cassette is not expressed in foetal liver or yolk sac progenitors. Furthermore, no individual enhancer within the *Ly-6E.1* genomic construct has been shown to be sufficient for targeting haematopoietic progenitors.

The *SCL* 3′ enhancer therefore exhibits unique properties. It targets blood and endothelial progenitors and probably the bipotent ancestors of both lineages. Moreover, within the haematopoietic compartment it targets expression to committed and multipotent haematopoietic progenitors. Preliminary data suggests that full enhancer activity resides within a 2.5 kb fragment (B. G., unpublished data). Detailed characterisation of this element is likely to identify the transcriptional programmes responsible for the formation of haematopoietic and endothelial progenitors, as well as providing a potent tool for their manipulation.

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