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Expression of the *Ly-6A* (Sca-1) *lacZ* transgene in mouse haematopoietic stem cells and embryos

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Summary. The Sca-1 surface glycoprotein is used routinely as a marker for haematopoietic stem cell enrichment. Two allelic genes, *Ly-6A* and *Ly-6E*, encode this marker and appear to be differentially regulated in haematopoietic cells and haematopoietic stem cells. The Sca-1 protein has been shown to be expressed at a greater frequency in these cells from *Ly-6A* strains of mice. To study the specific expression pattern and haematopoietic regulation of the *Ly-6A* gene, we constructed a 14 kb cassette from a genomic *Ly-6A* fragment, inserted a *lacZ* reporter gene and created transgenic mice. We found that the *Ly-6A lacZ* transgene was expressed in the haematopoietic tissues and predominantly in the T-lymphoid lineage. Some expression was also found in the B-lymphoid and myeloid lineages. We demonstrated functional haematopoietic stem cell enrichment by sorting for β-galactosidase-expressing cells from the bone marrow. In addition, we found an interesting embryonic expression pattern in the AGM region, the site of the first haematopoietic stem cell generation. Surprisingly, when compared with data from *Ly-6E lacZ* transgenic mice, our results suggest that the *Ly-6A* cassette does not improve *lacZ* marker gene expression in haematopoietic cells.

Keywords: *Ly-6A/E*, Sca-1, haematopoietic stem cells, transgene, embryo.

Enrichment and characterization of the stem cells at the foundation of the haematopoietic hierarchy has relied on the Sca-1 phosphatidylinositol-linked cell surface glycoprotein marker (Spangrude *et al*, 1988). Through fluorescence-activated cell sorting using a monoclonal antibody specific for Sca-1, haematopoietic stem cells (HSC) can be enriched approximately 100-fold from adult bone marrow and, together with antibodies specific for other cell surface markers (i.e. Thy-1^{lo}, c-kit or depletion for cells with mature lineage markers), a greater than 1000-fold enrichment can be obtained (Spangrude *et al*, 1988; Okada *et al*, 1992).

The Sca-1 protein is encoded by the strain-specific allelic genes, *Ly-6E* and *Ly-6A* (van de Rijn *et al*, 1989; Khan *et al*, 1990; Stanford *et al*, 1992; Sinclair & Dzierzak, 1993), which are members of the multigenic *Ly-6* family (LeClair *et al*, 1986; Kamiura *et al*, 1992). The family consists of at least 18 highly homologous cross-hybridizing genes with diverse and overlapping patterns of expression (Kimura *et al*, 1984). Owing to the homologies of the *Ly-6* family of genes and proteins, and the suspected overlapping roles in cell adhesion played by members of this family, functional studies have been difficult (Bamezai & Rock, 1995; Stanford *et al*, 1997). Sca-1 protein expression is complex within *Ly-6A* and *Ly-6E* strains of mice (Kimura *et al*, 1984; van de Rijn *et al*, 1989; Spangrude & Brooks, 1993). The *Ly-6A* and *Ly-6E* genes differ only by three nucleotides in the coding sequence, resulting in two amino acid changes (LeClair *et al*, 1986; Reiser *et al*, 1988). Both gene products express the Sca-1 epitope recognized by the antibody E13–161–7 (LeClair *et al*, 1986; Palfree & Hammerling, 1986; Rock *et al*, 1986; Palfree *et al*, 1987; Reiser *et al*, 1988). Both genes are interferon inducible, but the *Ly-6A* allele appears to be more widely and highly expressed (Kimura *et al*, 1984; Rock *et al*, 1986; Spangrude & Brooks, 1993). Strains of mice with the *Ly-6A* gene express Sca-1 on 10–20% of adult thymocytes and 50–70% of peripheral T lymphocytes, while strains with the *Ly-6E* gene express Sca-1 on 5–10% of adult thymocytes and 10–15% of peripheral T lymphocytes. Similarly, *Ly-6A* strains of mice express Sca-1 on virtually all (99%) marrow repopulating cells, while *Ly-6E* strains express Sca-1 on only 25% of these cells (Spangrude & Brooks, 1993). Nonetheless, Sca-1 remains an important marker of HSCs and its gene

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regulatory elements are of current research interest to direct expression to HSCs for potential therapeutic applications.

Previously, the *Ly-6E* transcriptional elements have been examined. Upstream *cis*-acting elements involved in regulating *in vitro* expression of *Ly-6E* have been identified (Khan *et al*, 1990, 1993) and sequence comparisons suggest that similar 5′ control elements are present in the *Ly-6A* promoter (McGrew & Rock, 1991). DNaseI hypersensitive site (HSS) mapping of both the *Ly-6E* and *Ly-6A* alleles show almost identical patterns (Sinclair & Dzierzak, 1993). Deletion studies using the *Ly-6E* and *Ly-6A* genes reveal that the region containing the two most distal 3′ HSS are responsible for high level, γ interferon-induced expression *in vitro* (Sinclair *et al*, 1996; Ma *et al*, 2001). Furthermore, this 3′ region is necessary in the context of a 14 kb *Ly-6E* expression cassette for high-level tissue-specific expression of a *lacZ* marker gene in transgenic mice (Miles *et al*, 1997). However, in such transgenic mice, it was found that some but not all HSCs can be sorted based on lacZ expression, suggesting that the *Ly-6E* expression cassette is not optimal for HSC expression *in vivo*. As subtle differences exist in HSS between the *Ly-6E* and *Ly-6A* alleles (Sinclair *et al*, 1996) and the *Ly-6A* gene product has been shown to be expressed in 100% of marrow repopulating cells, it was therefore of great interest to examine the *Ly-6A* sequences as a source for HSC-specific regulatory elements.

Thus, we cloned a *lacZ* reporter gene into a 14 kb *Ly-6A* gene cassette and generated transgenic mice. Here we present data from studies examining the differences in expression patterns and levels of *Ly-6A lacZ* transgene expression with that from a previously described *Ly-6E lacZ* transgene. In general, we found that the *Ly-6A lacZ* transgene is predominantly expressed in the cells of the T-lymphoid lineage. Moreover, we have shown that the *Ly-6A lacZ* transgene facilitates a > 100-fold enrichment of bone marrow HSCs. Surprisingly, while the *Ly-6A lacZ* transgene expression pattern in embryos was slightly more widespread than that of the *Ly-6E lacZ* transgene, the expected allele-specific differences in haematopoietic cell expression were not observed.

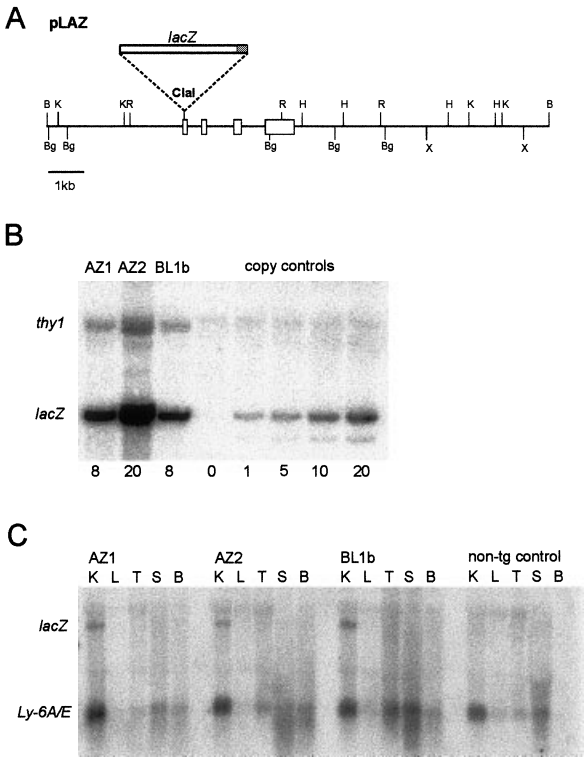

Fig 1. Generation and characterization of transgenic mouse lines. (A) Restriction map of the *Ly-6A lacZ* transgene in pLAZ. The *lacZ* marker gene (white rectangle) with SV40 poly A sequence (black rectangle) was cloned into the *Cla*1 site of the 14 kb *Ly-6A* cassette and this fragment was used to generate the AZ1 and AZ2 transgenic mouse lines. B = *Bam*H1, Bg = *Bgl*II, K = *Kpn*, R = *Eco*R1, H = *Hin*dIII, X = *Xba*. (B) Southern blot of transgenic mouse DNA. Hybridization with the *Thy1* probe (DNA normalization control) and *lacZ* probe (transgene) was performed and the signal detected by phosphorimaging was compared with plasmid copy controls for the determination of transgene copy number (indicated below each lane) in AZ1 and AZ2 transgenic lines. (C) Northern blot of total RNA from tissues of *Ly-6A lacZ* transgenic lines. Hybridization was performed with a *lacZ* probe for transgene expression and *Ly-6A/E*-specific probe for endogenous gene expression. Tissues of a BL1b and a non-transgenic control are also shown. K = kidney, L = lymph node, T = thymus, S = spleen, B = bone marrow.

MATERIALS AND METHODS

Constructs and transgenic mice. The 14 kb Ly-6A cassette (Ly-6A14) was constructed as described previously (Ma *et al*, 2001). The *lacZ* gene in p610ZA (gift of D. Meijer) was modified, converting a 3′ *Sma*1 site to a *Nar*1 site using oligonucleotide adaptors. The 3·6 kb *lacZ* Nar1 fragment was cloned into Ly-6A14 to generate pLAZ.

Fertilized (C57BL/10 × CBA)F1 oocytes were microinjected with a 17·6-kb Not1 fragment containing the *Ly-6A lacZ* gene from pLAZ (Fig 1A). This fragment was gel purified for removal of all vector sequences. Positive founder animals were bred with (C57BL/10 × CBA)F1 mice and lines were maintained as heterozygotes. The C57BL/10 strain contains an endogenous *Ly-6A* allele and the CBA strain contains an endogenous *Ly-6E* allele. (C57BL/10 × CBA)F1 mice co-dominantly express both alleles. Southern blot analysis of tail DNA was used to identify founder transgenic mice.

DNA and RNA analysis. Genomic DNA (5–10 μg) for Southern blot analysis (Miles *et al*, 1997) was digested using BamH1 and electrophoresed through 1% agarose/Tris, acetate, EDTA gels prior to transfer to Hybond-N membranes. Transgene copy number controls were generated by addition of appropriate amounts of pLAZ to non-transgenic genomic DNA. Filters were probed with lacZ and *Thy-1* gene fragments. Normalization for DNA content of each lane was performed after phosphorimage analysis of *Thy-1* signal. Copy number was determined subsequently by comparing the *lacZ* signal obtained from the transgenic mice with that of the plasmid controls on the linear portion of the standard curve.

Total cellular RNA for Northern blot analysis was prepared using the lithium chloride/urea method and 5–15 μg was fractionated on 1% agarose/formaldehyde gels (Fraser *et al*, 1990) prior to transfer to Hybond-N membranes. Filters were probed with *lacZ* and *Ly-6E* cDNA fragments.

Probes used for hybidization to Southern or Northern filters were labelled using the random oligonucleotide priming procedure incorporating [^{32}P]-ATP. The fragments used were as follows: 1·1 kb *Bam*H1–*Eco*RV *lacZ* containing fragment from p610ZA; 1·2 kb *Xba*1–*Nru*1 *Thy-1* gene fragment from pD7 (Spanopoulou *et al*, 1988); 761 bp *Eco*R1 *Ly-6E* cDNA fragment from pLy6·1–2R (LeClair *et al*, 1986). After hybridization, filters were washed to a stringency of 0·2 × saline sodium citrate (SSC)/0·1% sodium dodecyl sulphate (SDS) and exposed to a phosphorimager screen for quantification using IMAGEQUANT software.

Genomic DNA (200 ng) from the peripheral blood of transplant recipients was analysed using polymerase chain reaction (PCR) with oligonucleotide primers for myogenin-specific sequences: (myo1) 5′-TTACGTCCATCGTGGACAGC-3′ and (myo2) 5′-TGGGCTGGGTGTTAGTCTTA-3′; and for *lacZ*-specific sequences: (lacZ1) 5′-GCGACTTCCAGTTCAACATC-3′ and (lacZ2) 5′-GATGAGTTTGGACAAACCAC-3′.

DNA was subjected to an initial 5 min denaturation at 94°C followed by 30 cycles of denaturation (5 s at 94°C), annealing (30 s at 60°C), elongation (30 s at 72°C). Serial dilutions of blood DNA from a transgenic animal were used as a control to evaluate the levels of donor cell reconstitution in transplanted mice.

β-galactosidase and antibody staining. For analysis of β-galactosidase expression in transgenic bone marrow, thymus, spleen and lymph node, 10^6 cells were suspended in 100 μl of prewarmed phosphate-buffered saline (PBS) with 5% fetal calf serum (FCS) prior to loading with 100 μl of 2 mmol/l fluorescein di-(β-D-galactopyranoside) (FDG) in H_2O. Cells were incubated at 37°C for 60 s. The uptake was stopped by the addition of 2 ml of ice-cold PBS with 5% FCS and the reaction was allowed to proceed for 1–3 h on ice in the dark. Propidium iodide (PI, 1 μg/ml) or 7-amino-actinomycin D (7AAD, 2·5 μg/ml; Pharmingen, Alphen a/d Rijn, The Netherlands) was used to exclude dead cells. A FACSCAN and FACSVANTAGE SE (Becton-Dickinson, Alphen a/d Rijn, The Netherlands) were used for analysis and sorting.

Sca-1, CD4, CD8, B220 and Mac-1 antibodies were direct phycoerythrin (PE) conjugates (Pharmingen). Briefly, after 1–2 h of FDG staining, 10^6 cells were stained with antibody, incubated on ice for 30 min and washed three times in cold PBS with 5% FCS.

Whole embryos were isolated into ice-cold PBS, fixed in 1 ml of X-gal fix (1% formaldehyde, 0·2% gluteraldehyde) at 4°C for 1 h and stained overnight at room temperature in 1 mg/ml X-gal (Sigma, Zwijndrecht, The Netherlands). After staining, embryos were dehydrated through increasing concentrations of ethanol in ice-cold PBS and mounted in paraffin wax. Sections (6–10 μm) were cut onto APES (3-aminopropyltriethoxysilane, Sigma, Zwijndrecht, The Netherlands)-coated microscope slides and dried overnight at room temperature. Slides were dewaxed in Histoclear and rehydrated through decreasing concentrations of ethanol before standard counterstaining with haematoxylin-eosin and mounting.

Bone marrow transplantation. Donor transgenic bone marrow cells for transplantations were FDG, Sca-1 and Hoechst 33258 stained *ex vivo* in PBS with 5% FCS. FACS-sorted cells were counted, diluted and suspended in a final volume of 500 μl of PBS for intravenous injection into the tail vein of male (C57BL/10 × CBA)F1 mice. On the day of transfer, the recipients were exposed to a split dose (3 h interval) of 900 rad irradiation from a ^{137}Cs source. Adult (C57BL/10 × CBA)F1 spleen cells (2×10^5) were co-injected with the donor cells to promote short-term survival. All recipients were housed in filter-top isolators and received 1·6 g/l neomycin in drinking water for at least 1 month. Peripheral blood was taken at 1 and 4 months post transplantation for analysis.

RESULTS

The Ly-6A lacZ transgene is expressed in adult mice

The *Ly-6A* gene was previously cloned and analysed for *in vitro* expression in haematopoietic cells. A genomic expression cassette containing a distal 3′ fragment with strong DNaseI hypersensitive sites (Sinclair & Dzierzak, 1993) was found to yield high level, γ interferon-induced expression (Ma *et al*, 2001). To determine if this cassette could be used to express exogenous genes in haematopoietic stem cells *in vivo*, we inserted a *lacZ* marker gene into an engineered *Cla*1 site in the first untranslated exon of the *Ly-6A* gene (Fig 1A; Ma *et al*, 2001). Two transgenic mouse lines were produced with the *Ly-6A lacZ* construct: AZ1 and AZ2. Southern blotting of DNA from these established mouse lines was compared with DNA from a previously generated *Ly-6E lacZ* transgenic line (BL1b) which, in the homozygous state, contains eight copies of this allelic transgene. Figure 1B shows that AZ1 contains eight copies and AZ2 contains > 20 copies of the *Ly-6A lacZ* transgene in the hemizygous state. Northern blot analysis was performed on RNA derived from various haematopoietic and non-haematopoietic tissues of these transgenic lines (Fig 1C). High level *Ly-6A lacZ* transgene expression was found in the kidney of both the AZ1 and AZ2 transgenic lines and was similar to that observed in the BL1b transgenic line. Other tissues, such as the bone marrow, spleen and thymus, show little or undetectable expression. No expression was found in a non-transgenic littermate control. In general, the tissue-specific expression pattern followed closely the transcription of the endogenous *Ly-6A/E* gene. Interestingly, the higher copy AZ2 line showed equivalent levels of expression to the AZ1 and BL1b lines using this analysis. Thus, the specific expression pattern of the *Ly-6A lacZ* transgene was similar in both AZ1 and AZ2 adult tissues and was consistent with the general pattern in several lines of *Ly-6E lacZ* transgenic mice (Miles *et al*, 1997) including BL1b.

Ly-6A lacZ transgene is expressed in haematopoietic cells

Although Northern blot analysis showed little expression in haematopoietic tissues, a more sensitive method,

FDG-FACS, was performed on cells from thymus, spleen, lymph node and bone marrow of transgenic mice to detect β-galactosidase expression. To determine if allelic-specific differences in transgene expression could be observed, the two *Ly-6A lacZ* transgenic lines were analysed and compared with the *Ly-6E lacZ* BL1b transgenic line. As shown in the representative FACS histograms in Fig 2, no FDG-positive cells were found in the tissues of a non-transgenic control mouse, while both AZ1 and AZ2 mouse lines expressed the *Ly-6A lacZ* transgene in all four haematopoietic tissues. When the FACS-FDG profiles of the *Ly-6A lacZ* tissues were then compared with those of the *Ly-6E lacZ* transgenic line BL1b, similar percentages of FDG-positive cells were observed in all four tissues.

To determine in which adult haematopoietic lineages the *Ly-6A lacZ* transgene expresses, we performed FDG-FACS analysis together with antibodies specific for T-lymphoid, B-lymphoid and myeloid cells. Table I shows the percentages of CD4-, CD8-, B220- and Mac-1-positive cells in the FDG^+ fraction of bone marrow, spleen, thymus and lymph node cells. As expected, predominant transgene expression was found in the T-lymphoid lineage, with some expression in the B-lymphoid and myeloid lineages. In addition, the percentages of FDG^+ cells of the different lineages found in the bone marrow, spleen and thymus of *Ly-6E lacZ* and *Ly-6A lacZ* transgenic adults were similar. Slight differences were found in the bone marrow CD4 and Mac-1 subsets, probably the result of low sample numbers. Taken together, these results strongly suggest that the lineage distribution of *lacZ* marker expression is not different for the *Ly-6E* and *Ly-6A* allelic transgene cassettes.

The Ly-6A lacZ transgene marks functional haematopoietic stem cells in adult bone marrow

As the Ly-6A (Sca-1) protein is used extensively for the enrichment of HSCs from the bone marrow of adult mice and the *Ly-6A lacZ* transgene is expressed in 5–6% of adult bone marrow cells, we determined, using limiting dilution transplantation analysis, whether HSC activity was enriched in the FDG^+ population. To begin these studies, we first examined what percentage of bone marrow cells were positive for transgene and endogenous Sca-1 expression. The FACS plots in Fig 3A show the distribution and percentages of negative, double-positive and single-positive cells found in representative *Ly-6A lacZ* AZ1 and AZ2 transgenic bone marrow. The percentage of cells within each of the four quadrants was similar between AZ1 and AZ2 as well as BL1b (not shown). While some cells expressed both markers, not all FDG^+ cells were Sca-1^+ and vice versa. Thus, regulation of transgene expression overlapped but did not completely recapitulate endogenous *Ly-6A/E* gene regulation.

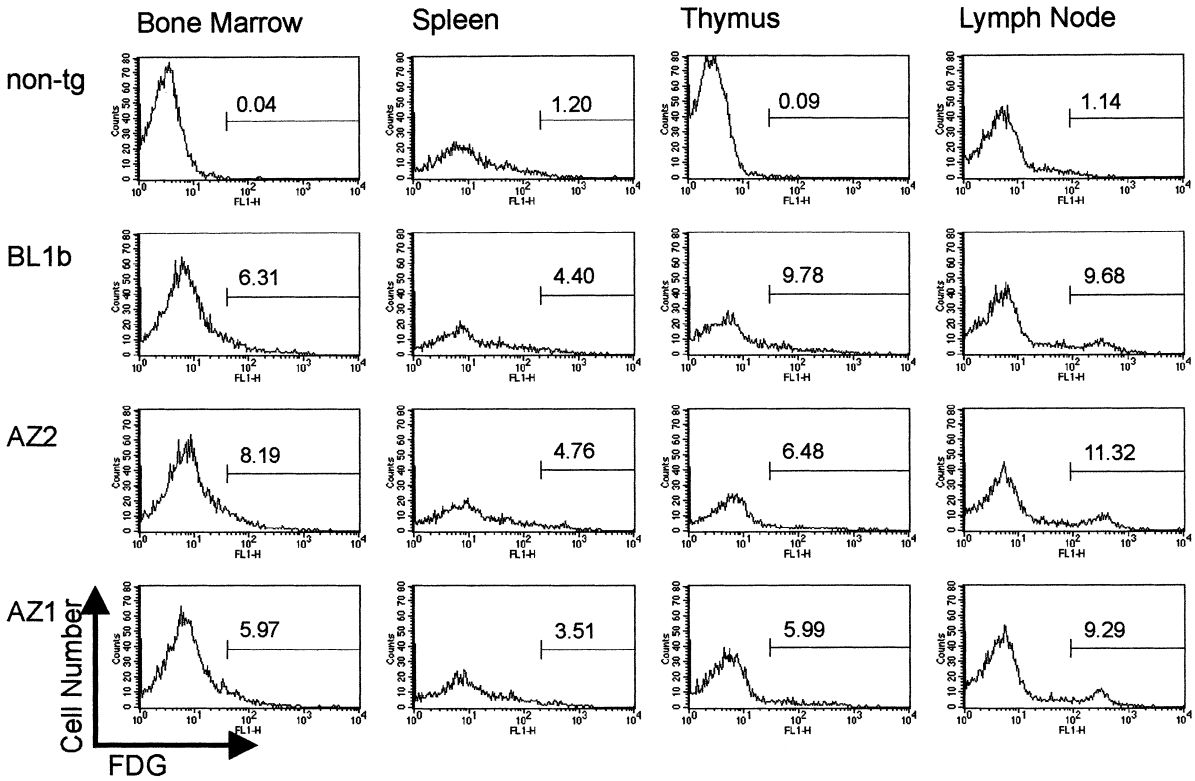

Fig 2. Representative fluorescein di-(β-D-galactopyranoside) fluorescence-activated cell sorting (FDG-FACS) analysis of *lacZ* transgene expression in haematopoietic tissues. Bone marrow, spleen, thymus and lymph node cells from control non-transgenic, BL1b, AZ1 and AZ2 age-matched male transgenic mice were stained with the FDG substrate and analysed using flow cytometry. Histograms show levels of fluorescence intensity on a logarithmic scale (abscissa) and number of cells (ordinate). Percentages of FDG-positive cells are indicated.

Table I. Subsets of haemato/lymphoid cells found in the FDG$^+$ fractions of *Ly-6E lacZ and Ly-6A lacZ* transgenic mice.

		Mean percentage of subset in total FDG$^+$ population (SD)			
Tissue	Transgene	CD4	CD8	B220	Mac-1
Bone marrow	Ly-6E lacZ	28·2*	14·2	20·8	33·1
	Ly-6A lacZ	46·5	19·4	22·8	12·5*
Spleen	Ly-6E lacZ	42·9	29·0	16·5	5·8
	Ly-6A lacZ	49·2 (17·4)	38·7 (13·9)	12·5 (5·7)	6·7 (3·7)
Thymus	Ly-6E lacZ	68·7	37·8	ND	ND
	Ly-6A lacZ	64·9 (13·3)	38·8 (18·1)	ND	ND
Lymph node	Ly-6E lacZ	55*	35·3*	3·5*	ND
	Ly-6A lacZ	58·3 (15·5)	39·2 (12·9)	3·6 (3·4)	ND

*Only one experiment performed.

Cell suspensions were stained with the FDG substrate and specific antibodies against the indicated cell lineage markers. At least 2×10^4 cells were examined. For the *Ly-6E lacZ* results, BL1b and BL19 transgenic adult mice were examined (see Miles *et al*, 1997). For the *Ly-6A lacZ* results, AZ1 and AZ2 transgenic adult mice were used. Numbers in brackets (SD) are the standard deviation (three experiments performed). ND = not done.

To test for the presence of HSCs in each of the phenotypically described populations, AZ1 and AZ2 bone marrow cells were sorted based on FDG and Sca-1 staining and injected in varying doses into irradiated adult recipients. At 4 months post transplantation, the recipient mice were tested for donor cell haematopoietic engraftment. As shown in Fig 3B, the combined results of two independent experiments show the highest enrichment of HSCs in the sorted Sca-1$^+$FDG$^-$ and Sca$^+$FDG$^+$ cells (as few as 100 sorted cells yield repopulation). Some enrichment was also observed in the sorted Sca-1$^-$FDG$^+$ cells (2×10^4 cells yield repopulation). In contrast, the Sca-1$^-$FDG$^-$ population of bone marrow was greatly decreased in HSC activity, requiring greater than $1–5 \times 10^5$ cells for repopulation. Unsorted control bone marrow was found to be at least five times more efficient than the Sca-1$^-$FDG$^-$ sorted bone marrow. When these transplantation data were compared with equivalent sorting and transplantation data from *Ly-6E lacZ* transgenic mice (Miles *et al*, 1997; and data not shown), no clear quantitative difference was found between *Ly-6A lacZ* and *Ly-6E lacZ* transgenics in bone marrow HSC activity enriched by FDG sorting.

Embryonic expression of the Ly-6A lacZ transgene in the AGM region is similar to that of the Ly-6E lacZ transgene

The expression of the *Ly-6A lacZ* transgene in the haematopoietic lineages and the HSCs of the adult mouse led us to examine the specific expression pattern of this transgene during development. Localization of β-galactosidase expression by X-gal staining could indicate the first site(s) of HSC appearance within the embryo. At E11, the expression pattern of *Ly-6A lacZ* was limited to the embryo body, with no expression in the yolk sac. The most striking X-gal staining was in the caudal tail region and the limb buds of AZ1 and AZ2 embryos (Fig 4A). The caudal expression pattern along the dorso-ventral axis in the AZ1 and AZ2 lines was slightly more widespread than in BL1b embryos. However, the antero-posterior limit of expression in all three lines was confined to the posterior area containing the hindgut. The high-level limb bud expression was specific to the AZ1 and AZ2 lines and was not observed in the BL1b line. Furthermore, limb bud expression was not observed in other *Ly-6E lacZ* transgenic lines (Miles *et al*, 1997). Thus, in mid-gestational mouse embryos the *Ly-6A lacZ* transgene was differentially expressed compared with the *Ly-6E lacZ* transgene.

Histological sectioning and staining was performed to determine in which embryonic tissues the *Ly-6A lacZ* transgene was expressed. In transverse sections from the truncal region of E11 AZ1, AZ2 and BL1b transgenic embryos, intense blue staining was observed in the epithelial cells lining the tubules of the pronephros and mesonephros (Fig 4B). The staining pattern was identical between all three lines. As the dorsal aorta and the surrounding mesenchyme have been found to be the only area with the AGM region containing functional HSCs (de Bruijn *et al*, 2000), we carefully examined the transverse sections for β-galactosidase activity at the site. No X-gal staining was found in the dorsal aorta or surrounding mesenchyme in any of the E11 sections examined from AZ1, AZ2 or BL1b transgenic lines. As the counterstaining may obscure the weak β-galactosidase signal from this area, we also examined transverse sections stained only with X-gal. While the pro/mesonephros showed high level β-galactosidase expression, not even weak X-gal staining was observed in the dorsal aorta or surrounding mesenchyme (data not shown). More sensitive FACS analysis verified this result, strongly suggesting that AGM HSCs are negative or beneath the limits of detection for *Ly-6A lacZ* transgene expression.

DISCUSSION

The results of the studies presented here demonstrate that the *Ly-6A lacZ* transgene is transcribed consistently in a lineage-specific manner. This 14 kb cassette produced high

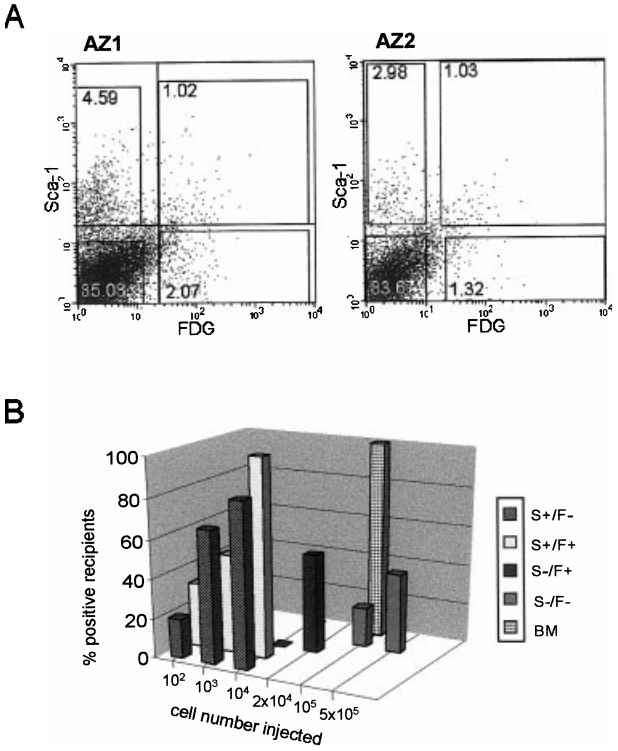

Fig 3. Repopulation of adult recipients using flow cytometric-sorted bone marrow cells from *Ly-6A lacZ* transgenic bone marrow. (A) Fluorescence-activated cell sorting (FACS) plots and sorting gates of bone marrow cells from AZ1 and AZ2 transgenic mice used for limiting dilution transplantation experiments. Adult bone marrow cells were stained with FDG and Sca-1 and sorted into four populations. Percentages of cells in each of the quadrants is indicated. (B) Limiting dilution repopulation frequency of sorted FDG and Sca-1 stained bone marrow from *Ly-6A lacZ* bone marrow. AZ1 and AZ2 bone marrow was sorted into double-negative, double-positive and single-positive populations and injected into irradiated adult recipients in limiting numbers. Engraftment by donor cells was tested at greater than 4 months post transplantation and mice found to be more than 10% repopulated in the peripheral blood (as determined by *lacZ* semiquantitative polymerase chain reaction) were considered positive. The percentage of positive recipients is plotted on the ordinate and the number of sorted cells transplanted is plotted on the abscissa. Coded vertical bars represent the various sorted cell populations injected into the recipient mice. S = Sca-1, F = FDG and BM = whole unsorted bone marrow.

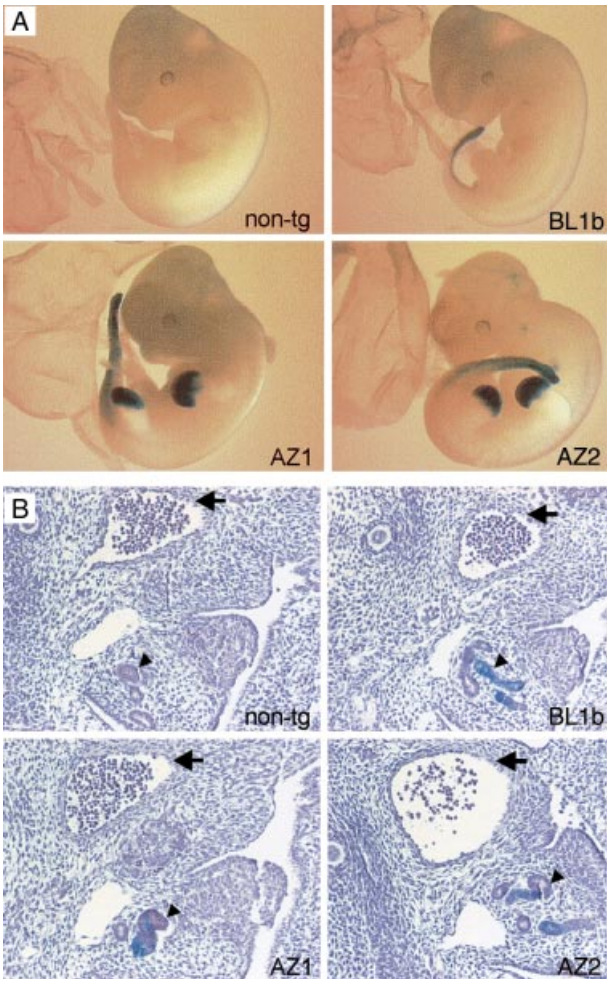

Fig 4. *Ly-6A lacZ* expression in E11 transgenic embryos. (A) Whole embryos. E11 non-transgenic, BL1b, AZ1 and AZ2 embryos were stained with the X-gal substrate to detect β-galactosidase expression. Staining is observed in the limb buds and caudal regions of *Ly-6A lacZ* embryos. No staining is observed in the yolk sac of transgenic embryos or the control. (B) AGM transverse sections. E11 embryos (as above) were sectioned after X-gal staining to reveal the β-galactosidase expression pattern. Counterstaining was performed after transverse sectioning. Expression is observed in the pro/mesonephric tubules (arrow head). The arrow indicates the ventral wall of the dorsal aorta which appears negative for β-galactosidase expression.

levels of *lacZ* transcripts in the kidney as previously observed in *Ly-6E lacZ* transgenic mice and recapitulated the endogenous *Ly-6A/E* gene transcription expression pattern in adults. Although we examined only two *Ly-6A lacZ* transgenic lines, the levels of transcription of the transgene appeared to be identical between the AZ1 line, carrying eight copies of the transgene, and the AZ2 line, which had 20 copies of the transgene. Furthermore, the transcriptional levels were similar to that of the BL1b *Ly-6E lacZ* transgenic line which carries eight transgene copies (in homozygous animals). Thus, in the context of the *lacZ* reporter gene, the *Ly-6A* cassette does not appear to direct copy number dependent expression.

Both lines of *Ly-6A lacZ* transgenic adult mice express β-galactosidase similarly in haematopoietic cells. As expected from the previous results of flow cytometric analysis with the Sca-1 antibody, the *Ly-6A lacZ* transgene is expressed in all haematopoietic organs. Similar percentages of FDG-positive cells were found in the bone marrow, thymus, spleen and lymph nodes of the *Ly-6A lacZ* transgenic mice and corresponded to the percentages found in *Ly-6E lacZ* transgenic tissues. The predominant lineage

expressing the transgene is the CD4 subset of T cells. Also, cells of the CD8 subset, B and myeloid lineages were positive for transgene expression. Again, these data on the *Ly-6A lacZ* transgenic mice correspond well with the percentages of haematopoietic subsets positive for *Ly-6E lacZ* transgene expression.

Surprisingly, we did not observe the allele-specific differences noted by previous Sca-1 FACS analysis of the different allelic mouse strains and the percentages of FDG^+ haematopoietic cells were always slightly less than $Sca\text{-}1^+$ cell percentages.

Indeed, in the *Ly-6A lacZ* transgenic mice we found FDG expression in some but not all functional adult repopulating HSCs. Flow cytometric sorting of FDG and Sca-1 double-stained bone marrow showed that not all adult HSCs were in the FDG fraction. While almost all HSCs are in the Sca-1 fraction, equal numbers of HSCs are found in the FDG^+ and FDG^- fractions. We observed this same distribution in the *Ly-6E lacZ* transgenic mice. The incomplete overlap in FDG and Sca-1 staining in bone marrow may be owing to the following: (1) Ly-6A/E molecules are surface GPI-linked glycoproteins, while β-galactosidase is cytoplasmic. Thus, the kinetics of protein production as well as protein half-life could be vastly different. (2) Not all the appropriate transcriptional control elements are contained within the 14kb *Ly-6A/E* cassettes or, more likely, position effect variegation has occurred. (3) The cell permeability to FDG is inefficient. Either the entry of FDG into the cells is suboptimal or there is a loss through leakage, or both. (4) The *lacZ* gene is bacterial in origin and may be constrained in its expression in mice. For example, β-galactosidase production may reach a physiological threshold with higher levels being toxic and, thus, levels appear to be limited in the *Ly-6A lacZ* transgenic mice. At this time it is unclear which of these possibilities is responsible for suboptimal transgene expression. However, we have made several lines of transgenic mice in which mammalian genes such as the *tal-1* transcription factor (unpublished observations) and the *Bcl-2* antiapoptotic gene (unpublished observations) have been inserted into these cassettes. Both genes have been found to be expressed in haematopoietic cells.

Unlike the adult, differences in *Ly-6A lacZ* and *Ly-6E lacZ* expression were observed in transgenic embryos. The consistent expression in the limb buds and dorsal-caudal tail of the *Ly-6A lacZ* but not *Ly-6E lacZ* embryos strongly suggests that the proper regulatory elements are present, at least for these tissues. However, no functional HSCs have been isolated from either *Ly-6A lacZ* or *Ly-6E lacZ* AGMs. Sectioning and staining of this region (together with preliminary data with a *Ly-6A GFP* transgene) suggests that β-galactosidase expression is not high enough to yield an enrichment of HSCs from the AGM region.

Despite incomplete expression of the *Ly-6A lacZ* transgene in $Sca\text{-}1^+$ cells, this transgene cassette does lead to faithful expression in some HSCs. For manipulation of HSCs *in vivo* and *in vitro* and for localization of HSCs within the whole animal, the *Ly-6A* cassette appears at present to be the best transgene construct, outside of targeting a marker gene by homologous recombination in embryonic stem cells. The clear advantage in the use of *Ly-6A* sequences for regulated expression in HSCs is the relative size of this gene and, particularly important, the 3′ distal 1 kb regulatory sequence, compared with other genes encoding proteins expressed in HSCs. The genes encoding HSC marker proteins c-kit and AML-1 (Gokkel *et al*, 1992; Levanon *et al*, 2001) span over 100 kb of sequence and contain many exons and introns, thus making identification of regulatory elements difficult. Thus, the further dissection of the regulatory elements of the *Ly-6A* gene expression cassette should lead to the generation of retroviral vectors for efficient transduction of and expression in HSCs.

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