Expression of the Ly-6E.1 (Sca-1) transgene in adult hematopoietic stem cells and the developing mouse embryo

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SUMMARY

The mouse hematopoietic marker Sca-1, encoded by the Ly-6E.1 and Ly-6A.2 genes, has been instrumental in the enrichment and characterization of the stem cell for the adult blood system. In the studies reported here, we use Ly-6E.1 genomic fragments to direct expression of a lacZ marker transgene in vivo to study Ly-6E.1 specific regulatory elements in the hematopoietic stem cell and to localize these cells in the developing mouse embryo. We demonstrate that a region approximately 9 kb downstream from the transcriptional start site is required for the distinct, restricted expression pattern of the Ly-6E.1-lacZ transgene within adult hematopoietic stem cells and embryos. We also demonstrate that viable and functional lacZ-expressing hematopoietic stem cells can be enriched by FDG staining and flow cytometric sorting. The Ly-6E.1-lacZ-mediated enrichment of hematopoietic stem cells from adult transgenic bone marrow in combination with the temporal expression pattern of the transgene in the pro/mesonephros suggest an intraembryonic site of development for these cells in the mouse.

Key words: mouse, hematopoietic cell, stem cell, Sca-1, Ly-6E.1, transgene

INTRODUCTION

In mammals, hematopoietic stem cells (HSC) are responsible for the daily production of millions of mature cells of all blood lineages throughout adult life. Our current knowledge of mammalian HSCs in vivo is derived primarily from experiments utilizing mouse radiation chimeras (Michlem et al., 1966). Transplantation studies in which marked cells from one animal are transferred into lethally irradiated syngeneic recipients indicate that HSCs are a rare population of self-renewing pluripotent cells in adult bone marrow (Abramson et al., 1977), fetal liver (Capel et al., 1989), aorta- gonad-mesonephros (AGM) region (Müller et al., 1994) and the yolk sac (Moore and Metcalf, 1970). The enrichment and further characterization of HSCs has relied on flow cytometry using antibodies detecting specific cell-surface markers (Spangrude et al., 1988b; Jordan et al., 1990), staining for mitochondrial activity (Ploemacher and Brons, 1989) and density gradient fractionation (Visser et al., 1984). Another approach for enrichment, additionally advantageous for the characterization of HSC-specific gene regulatory elements and the localization of the first HSCs within the developing embryo, would be to utilize a HSC-specific lacZ transgenic marker and flow cytometric sorting of cells staining positive with the fluorescein di-β-D-galactopyranoside (FDG) substrate. The FDG sorting procedure has previously been successful for enrichment of functional cells from Drosophila (Krasnow et al., 1991) and mammalian hematopoietic cell lines retrovirally transduced with an SV40-lacZ gene (Nolan et al., 1988). To date, no enrichment procedures for functional hematopoietic stem cells or other cells in the mouse have relied upon in vivo expression of a lacZ transgene marker.

Numerous candidate genes and gene regulatory elements for HSC-directed transgene marking studies are suggested from antibody enrichment and cell sorting experiments. HSCs of the adult mouse bone marrow and fetal liver express Sca-1, Thy-1, CD34 and c-kit (Spangrude et al., 1988b; Ikuta et al., 1990; Müller-Sieburg et al., 1986; Krause et al., 1994; Ikuta and Weissman, 1992), although none of these is exclusively a marker of the HSC. Previously, the Thy-1 gene has been used in transgenic experiments, but even a 12 kb genomic fragment appears to be unable to direct transgene expression in HSCs (Dzierzak et al., 1993 and unpublished results). The c-kit gene is spread over 70 kb of genomic DNA with 21 exons (Gokkel et al., 1992), thus making it difficult to manipulate for transgenic experiments. Furthermore, mouse CD34 gene expression has only recently been characterized in vitro (May and Enver, 1995). However, extensive analysis of Sca-1 protein expression patterns in vivo and gene regulatory elements in vitro suggest that it is a most suitable candidate for transgenic marking, gene regulation studies and HSC manipulations.

The Sca-1 hematopoietic stem cell marker is encoded by the...
allelic Ly-6E.1 and Ly-6A.2 genes (van de Rijn et al., 1989), which are members of the Ly-6 family consisting of at least 18 highly related genes (Kamiura et al., 1992). The Ly-6E.1 and Ly-6A.2 genes contain four exons that encode 876 and 830 base transcripts, respectively, and a 10-12 kDa GPI-linked cell surface glycoprotein (LeClair et al., 1986; McGrew and Rock, 1991; Palfree and Hammerling, 1986; Rock et al., 1986; Su and Bothwell 1989). The proteins are identical in sequence except for two amino acid differences resulting from three nucleotide differences (LeClair et al., 1986; Reiser et al., 1988). Within the hematopoietic system their expression patterns are complex (HSCs, progenitors and some subsets of T lymphocytes), interferon-inducible and allele-specific in inbred strains of mice (Kimura et al., 1984; Codias et al., 1989; Dumont and Boltz, 1987; Spangrude et al., 1988a,b), particularly in HSCs (Kimura et al., 1984; Reiser et al., 1988). The proteins are identical in sequence except for two amino acid differences resulting from three nucleotide differences (LeClair et al., 1986; Reiser et al., 1988). Within the hematopoietic system their expression patterns are complex (HSCs, progenitors and some subsets of T lymphocytes), interferon-inducible and allele-specific in inbred strains of mice (Kimura et al., 1984; Codias et al., 1989; Dumont and Boltz, 1987; Spangrude et al., 1988a,b), particularly in HSCs (Spangrude and Brooks, 1993). For example, Ly-6A.2 strains of mice express Sca-1 on 99% of stem cells with hematopoietic repopulating activity, while Ly-6E.1 strains express Sca-1 on only 25% of these cells. In addition, expression is found on epithelial cells, the kidney and the brain (Reiser et al., 1988; van de Rijn et al., 1989; Cray et al., 1990). While much is known about the protein expression patterns of Ly-6E.1 and Ly-6A.2 in the adult, little is known about Ly-6E.1/A.2 expression during mouse embryonic development. Only recently have yolk sac cells from embryos 11 days post coitum (dpc) been shown to be negative for Sca-1 expression, as measured by flow cytometry (Huang and Auerbach, 1993). RT-PCR analysis of RNA from 10 dpc embryos has confirmed the yolk sac as negative, and additionally, the intra-embryonic AGM region has been demonstrated to express Ly-6A.2/E.1 (Dzierzak et al., 1995).

Putative regulatory elements necessary for basal, interferon-inducible and allele-specific expression patterns of the Ly-6E.1/A.2 genes have been identified by deletion analysis (Khan et al., 1990; Sinclair and Dzierzak, 1993) and DNase I hypersensitive site (HSS) mapping (Sinclair and Dzierzak, 1993). Deletion analysis of HSS containing 5' and 3' flanking regions of these genes in transfected cells have revealed cis-regulatory elements at –1.2 and –0.11 kb upstream and +8.7 and +8.9 kb downstream of the transcriptional start site. The 3' sequences are required in vitro for high level, γ-IFN induced expression of the Ly-6E.1 gene in hematopoietic cells (Sinclair et al., 1996).

In order to direct high levels of lacZ expression to HSCs for flow cytometric sorting and enrichment, for characterizing gene regulatory elements of the Ly-6E.1 gene and for identifying sites of Ly-6E.1 expression in the mouse embryo, we have generated transgenic mouse lines that contain either a 14 kb or 9.4 kb Ly-6E.1-lacZ construct. We demonstrate the enrichment of functional HSCs by FDCG sorting of Ly-6E.1-lacZ-expressing bone marrow cells and show that the 3'-most region of the Ly-6E.1 gene is necessary for tissue-specific expression in adults and embryos. Our developmental studies demonstrate highly restricted transgene expression in the pro/mesonephros, hindgut, endoderm and mesoderm of the tail in early to mid-gestation embryos and suggest possible sites for the development of the first adult HSCs.

MATERIALS AND METHODS

Constructs and transgenic mice

The 14 kb Ly-6E.1 cassette, pL6Clu, was constructed by cloning the 3.6 kb Spiht-EcoRI fragment from pLR1Clu (upstream region and the first untranslated exon of Ly-6E.1 containing an inserted Cclu site) into a 12.3 kb Spiht-EcoRI partial fragment isolated from pAB14 (Sinclair and Dzierzak, 1993; Sinclair et al., 1996). This fragment contains the remaining 3' part of the Ly-6E.1 gene and the polyA site. The lacZ gene in p610ZA (gift of D. Meijer) was modified, converting a 3' Smal site to an NruI site using oligonucleotide adaptors. The 3.6 kb lacZ NruI fragment was cloned into p610Clu to generate pLALZ.

Fertilized (CBAxC57Bl/10)F1 oocytes were microinjected with Ly-6E.1-lacZ fragments (Fig. 1) (Grosveld et al., 1987). A 17.6 NotI fragment containing the 14 kb Ly-6E.1 genomic sequence with the inserted lacZ gene was obtained from pL6LZ and designated BL (for BamHI-lacZ). The truncated Ly-6E.1-lacZ fragment was obtained by Xbal and NotI digestion of pL6LZ and designated XN (for Xbal/NotI). Both fragments were gel-purified for removal of all vector sequences. Positive founder animals were bred with (CBAxC57Bl/10)F1 mice and lines were maintained as heterozygotes. Southern blot analysis of tail DNA was used to identify transgenic mice within a litter, to determine copy number and to assess the integration patterns of transgenes.

DNA and RNA analysis

Genomic DNA (5-10 μg) for Southern blot analysis (Sambrook et al., 1989) was digested with BamHI and electrophoresed through 1% agarose/TAE gels prior to transfer to Nytran nylon membranes. Transgene copy number controls were generated by the addition of appropriate amounts of pL6LZ to non-transgenic genomic DNA.

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 prior to transfer to Hybond-N membranes (Southern et al., 1980). Northern blots were probed with a GAPDH probe for quantitation of RNA in each lane. After normalization the intensity of lacZ hybridizing signal was compared with that of Ly-6E.1 on a phosphorimager and expressed as a percentage of endogenous Ly-6E.1 expression. The specific activities of the lacZ and Ly-6E.1 probes were equivalent. The Ly-6.1-2R probe (see below) used to detect Ly-6E.1 RNA cross-hybridizes to transcripts from closely homologous Ly-6 genes and therefore the percentage lacZ expression is an underestimate.

Genomic DNA (200 ng) from the peripheral blood of transplanted mice was analyzed by PCR using the following oligonucleotide primers: for myogenin-specific sequences TTACGTCCA TCGTG-CAGCTGCTGATGCAGC (Myo1) and TGGGCTGGGTGTTAGTCTTA (Myo2); and for lacZ-specific sequences GCCGACTTCCAGTTCAACA TC (lacZ1) and GATGAGTTTGGACAAACCAC (lacZ2). DNA was subjected to agarose/TAE gels prior to transfer to Nytran nylon membranes. Transgene copy number controls were generated by the addition of appropriate amounts of pL6LZ to non-transgenic genomic DNA.

For RT-PCR analysis of sorted cell populations, RNA was prepared using the lithium chloride/urea method (Fraser et al., 1990). RNA from 4.5×10⁶ FDCG, Sca-1 double-positive, 8×10⁶ FDCG single-positive and 1×10⁶ unsorted bone marrow cells was primed with oligo dT and reverse-transcribed using AMV reverse transcriptase (HT Biotechnology, Cambridge UK) under the manufacturers’ recommended con-
ditions. PCR was performed on 5-10% of each RT product with the same cycling parameters as described above, using the following primer pairs: lacZ1 and lacZ2 (described above); GAPDH primers gapdh-s, CTCACCCACCGGGAAGGG and gapdh-a, CCA-CCCTGTTGCTTGAGGC (cDNA product = 670 bp); for Ly-6E.1 primers PE5, ACTGTGCCTGCAACCTTGAGA and Ex4+2, GTCCAGGTTGCTGCGTCACT (cDNA product = 425 bp).

**Lymphocyte activation**

The medium used in all primary cultures of thymus, spleen or lymph node cells consisted of αMEM supplemented with 10% FCS, 10 μg/ml penicillin, 10 μg/ml streptomycin, 2 mM L-glutamine and 50 μM β-mercaptoethanol. Cells were seeded at 2×10^5/ml and activated for 2-3 days in the presence of either 5 ng/ml ConA (Sigma) or 5 ng/ml PMA (Sigma) and 500 ng/ml ionomycin (Sigma).

**β-galactosidase assays and antibody staining**

For analysis of β-galactosidase activity in viable transgenic bone marrow, thymus, spleen or lymph node cells, 10^6 single cells were resuspended in 20 μl of PBS with 5% FCS prior to loading with 20 μl of 2 mM fluorescein di-(β-D-galactopyranoside), FDG (Sigma) in dH2O and followed by incubation at 37°C for 75 seconds. The uptake was stopped by the addition of 500 μl of ice-cold PBS with 5% FCS and the reaction was allowed to proceed for 1-3 hours on ice in the dark. Before flow cytometric analysis and sorting, propidium iodide (Sigma) was added to a final concentration of 1 μg/ml to allow the exclusion of dead cells. The fluorescence generated by β-galactosidase was detected on the FACScan or FACStar (Becton-Dickinson) on a FITC analysis channel.

**RESULTS**

The 3′ flanking sequences of the Ly-6E.1 gene are required for tissue-specific expression in adult transgenic mice

Previously, in transfected γ-IFN-induced murine erythroleukemia (MEL) cells, a 14 kb genomic Ly-6.1 DNA has been shown to express surface protein to the same levels as the endogenous MEL cell Ly-6A.2 gene (Sinclair and Dzierzak, 1993). Deletional analysis of Ly-6E.1 constructs in transfected MEL cells has demonstrated that a region approximately 9 kb downstream from the transcriptional start site is required for high-level γ-IFN-induced expression (Sinclair et al., 1996). In this region, which contains two DNase I hypersensitive sites at +8.7 and +8.9, an IFN-stimulated response element (ISRE) and numerous other transcription factor consensus sites were found. Based on this in vitro information, we set out to test whether this region would direct high-level and/or tissue-specific expression in vivo.

We inserted a lacZ reporter gene in the first exon of the Ly-6E.1 14 kb genomic fragment (Fig. 1A). Four transgenic lines were produced with this construct: BL1a, BL1b, BL7 and BL19. Fig. 1B shows a Southern blot used for determination of transgene copy number in each of these transgenic lines. All contain 4-15 copies of the intact sequences, as indicated. To test whether the 3′ region containing the +8.7 and +8.9 DNase I HS sites was required for Ly-6E.1-lacZ expression, we deleted 4.6 kb of Ly-6E.1 downstream genomic sequences. With this NX construct (Fig. 1A), six transgenic lines XN23, XN37, XN224, XN225, XN229 and XN231 and one founder animal XN25 (>90% chimeric for the transgene) were produced. Southern blot analysis (Fig. 1B) of tail DNA demonstrated that between one and 25 copies of the transgene were present in these animals. The non-transgenic XN28 mouse was used as a control.

Transgene expression analysis was performed by northern blotting of kidney RNA from BL and XN mice, since it is known that kidney expresses high levels of endogenous Ly-6E.1/A.2. When transgenic kidney RNA was examined (Fig. 1C), lacZ-specific signal was found in all four BL lines. However, only one out of eight XN transgenics showed kidney expression of lacZ. The percent level of lacZ expression per transgene copy, as compared to endogenous Ly-6E.1/A.2 gene expression, was found to range from 2.0% to 4.7% for the BL transgenics. Only one XN transgenic line, XN23, expressed lacZ to a level of 1.4% per copy. The tissue-specific expression patterns of the Ly-6E.1-lacZ transgenes were examined in liver,
muscle, bone marrow, spleen, lymph node and thymus RNA (Fig. 1D) in high copy number BL19 (15 copies) and XN229 (25 copies) mice. lacZ transgene expression was found in all BL19 tissues positive for endogenous Ly-6E.1/A.2 expression. The XN229 transgenic showed low level lacZ signal only in the lymph node RNA. Tissue-specific expression of lacZ was found in all BL transgenic mice (data not shown), while no XN transgenics were found to express lacZ in a tissue-specific manner. In a few XN lines, spurious lacZ expression was found in some tissues (see legend to Table 2), possibly indicating integration site-dependent expression. These data strongly suggest that the 3' flanking region of the Ly-6E.1 gene containing the +8.7 and +8.9 DNase I hypersensitive sites is required for in vivo tissue-specific expression.

**Specific Ly-6E.1 lacZ expression in hematolymphoid cells can be detected only in BL transgenic mice**

Ly-6E.1 is known to be expressed on a high percentage of activated T lymphoid cells of the adult mouse (Kimura et al., 1984). As a first test for β-galactosidase activity in the hematopoietic and non-hematopoietic tissue RNAs from BL19 and XN229 transgenic mice. Hybridization with lacZ and Ly-6E.1 probes shows transgene and endogenous gene expression. Ethidium bromide-stained gel shows 18S ribosomal RNA as a quantitation control. K, kidney; L, liver; M, muscle; B, bone marrow; S, spleen; LN, lymph node; T, thymus.
and bone marrow cells from control non-transgenic and high copy number BL19 and XN229 transgenic mice was performed. As shown in Fig. 3, a distinct population of FDG-positive cells is found in all four hematopoietic tissues of the BL19 mouse, but no positive cells are found in the XN229 tissues. These data were confirmed by staining other BL and XN transgenic lines (not shown). Also, the direct comparison of X-gal and FDG staining of BL19 transgenic cells showed complete correspondence, with 9.9, 39.3, 45.8 and 10.3% X-gal-positive cells in thymus, spleen, lymph node and bone marrow, respectively. In combination these data demonstrate that, within the sensitivity of both substrates, only the 14 kb BL transgene expresses in the hematopoietic lineage in vivo.

Examination of the BL transgene expression pattern within various hematopoietic lineages was performed by double staining of bone marrow, spleen and thymus cells with FDG and Thy.1, CD4, CD8, B220, Mac-1 and Gr-1 specific antibodies (Table 1). As expected, predominant transgene expression was found in the T lymphoid lineage, with some expression in the B lymphoid and myeloid lineages. Double staining of bone marrow cells with FDG and the Sca-1 antibody (Fig. 4A) shows 1.7% of these cells co-express the transgene and Sca-1. However, all Sca-1-positive cells (4.2% of total bone marrow) are not FDG-positive and a small fraction of the total bone marrow cells are single-positive for FDG (5.2%). To determine whether both the endogenous gene and the transgene were transcriptionally active in the same cells, we performed RT-PCR analysis on sorted FDG, Sca-1 double-positive and FDG single-positive bone marrow cells (Fig. 4B). Ly-6E.1-lacZ transgene and Ly-6E.1/A.2 endogenous gene transcripts are found in both populations, strongly suggesting that translational or post-translational differences account for the single-positive population.

To determine what lineages of cells are represented in the Sca-1, FDG double-positive bone marrow population, we performed triple staining with antibodies against the lymphoid- and myeloid-lineage specific markers (Table 1).

**Table 1. Percentage of lymphoid and myeloid cells with the FDG+ population**

<table>
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<tr>
<th>Cell Markers</th>
<th>Thy 1.2</th>
<th>CD4</th>
<th>CD8</th>
<th>B220</th>
<th>Mac-1</th>
<th>Gr-1</th>
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<td>Bone marrow</td>
<td>24%</td>
<td>7%</td>
<td>12%</td>
<td>22%</td>
<td>29%</td>
<td>20%</td>
</tr>
<tr>
<td>Spleen</td>
<td>72%</td>
<td>34%</td>
<td>22%</td>
<td>23%</td>
<td>3%</td>
<td>1%</td>
</tr>
<tr>
<td>Thymus</td>
<td>93%</td>
<td>65%</td>
<td>30%</td>
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</tr>
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</table>

Single cell suspensions of bone marrow, spleen and thymus cells were stained with the FDG substrate and antibodies against the above cell markers. 10^6 cells were examined on a Becton-Dickinson FACStar for percentage myeloid and lymphoid lineage cells within the β-galactosidase-positive population. ND = not done.

**Fig. 3.** Expression analysis of the Ly-6E.1-lacZ transgene in the hematopoietic tissues of BL19 and XN229 mice with the FDG substrate. Thymus, spleen, lymph node and bone marrow cells from control non-transgenic and BL and XN transgenic mice were stained with the FDG substrate and analyzed by 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.
specific markers Thy-1 and B220. Within the gated double-positive population, 26% of the cells are Thy-1+ and 44% are B220+ (Fig. 4C). Thus, approximately 70% of the double-positive cells are lymphocytes. Interestingly, 33% of the double-positive cells (<1% of total bone marrow) are Thy1lo, a phenotype characteristic of HSCs and progenitors.

**Functional hematopoietic stem cells can be enriched by FDG sorting of Ly-6E.1-lacZ transgenic bone marrow cells**

As Ly-6E.1 gene product has been used for the enrichment of HSCs from the bone marrow and we have detected Ly-6E.1-directed lacZ expression in BL transgenic bone marrow, it was of great interest to determine whether the FDG-positive population was enriched in HSC activity. Thus, we tested the ability of FDG-positive BL transgenic bone marrow cells to contribute to long-term, multilineage hematopoiesis by transplantation of sorted cells into lethally irradiated adult recipients. In two separate experiments, bone marrow cells were obtained from male homozygous BL1a (eight copies) or heterozygous BL19 (15 copies) transgenics. Limiting numbers of sorted transgenic FDG-positive and FDG-negative cells were injected into lethally irradiated female mice along with 2.5x10^5 non-transgenic female splenocytes to provide for short term hematopoiesis. Peripheral blood DNA was examined for evidence of engraftment by a lacZ transgene-specific PCR assay at 4 months post-transplantation. As shown in Fig. 5, as few as 10^3 FDG-positive donor bone marrow cells resulted in 100% engraftment of one out of three recipients, while 1000-fold more (10^6) FDG-negative cells were required for high-level engraftment of five out of six recipients. Considering that transplantation of total bone marrow requires at least 10^8 cells for high-level engraftment, FDG-positive bone marrow cells are approximately 100-fold enriched for HSC activity. Engraftment with FDG-positive cells was determined to be multilineage by Southern blot analysis of hematopoietic tissues and lineages (not shown). These results demonstrate, in two independent transgenic mouse lines, Ly-6E.1-lacZ expression in HSCs and the ability to enrich for functional HSC activity by FDG sorting of transgenic bone marrow.

**A distinct and restricted urogenital expression pattern of Ly-6E.1-lacZ in the developing embryo**

The fidelity of tissue-specific expression, particularly in HSCs, of the BL transgene in adult mice led us to examine the specific expression pattern of Ly-6E.1-lacZ during development. Embryonic localization of β-galactosidase staining could indicate specific sites of HSC development within the AGM region, which was previously shown to express Ly-6A.2/E.1 by

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Fig. 4. Sca-1 and FDG double-stained subsets of BL transgenic bone marrow. (A) FACS dot plots of Sca-1 antibody (PE), isotype control antibody (PE) and FDG (FITC) substrate double staining are shown for bone marrow cells from control non-transgenic and BL19 transgenic mice. Percentages of cells in each quadrant are indicated. Analysis was performed on 10^4 cells. (B) RT-PCR analysis of sorted Ly-6E.1-lacZ bone marrow subsets from a BL19 transgenic mouse. Specific RNA transcripts for transgene lacZ, endogenous Ly-6E.1 and normalization control GAPDH were examined. Lane 1, no cells; lane 2, FDG single-positive sorted BL19 bone marrow; lane 3, FDG and Sca-1 double-positive sorted BL19 bone marrow; lane 4, BL19 transgenic unsorted bone marrow; lane 5, non-transgenic unsorted bone marrow. RT-PCR products were run on a 1.2% agarose gel and stained with ethium bromide. (C) Analysis of Sca-1/FDG double-positive bone marrow cells from BL19 transgenic bone marrow with Thy 1.2- and B220-specific antibodies. Percentages of Thy 1.2 and B220-positive cells within the Sca-1/FDG double-positive population are indicated. Note that Thy 1.2 expression levels vary from bright to dim.
Expression patterns were examined in numerous embryos of each line, giving cells of +++ , ++, + and +/- intensity of X-gal staining. Staining was consistent within each line of mice. Ectopic expression was seen in embryos at the following sites: BL7, diencephalon and liver; BL19, otic vesicle; XN23, mesenchyme of limb buds, midline of telenchephalon, region of the first branchial arch and otic vesicle; XN37, hindbrain, telenchephalon and liver; XN225, dorsal root ganglia; XN225, otic vesicle; XN229, otic vesicle. The following ectopic expression patterns were observed in adults of the following lines: XN37, activated lymphocytes; XN231, otic vesicle. The following ectopic expression patterns were consistent between all four BL transgenic lines and correlates with endogenous Ly-6A.2/E.1 gene expression, as determined by RT-PCR analysis (not shown). In the 11.5 dpc embryo (Fig. 6C), another site of intense staining was found by removing a portion of the body wall in the region of the aorta, mesonephros and gonads (AGM). To localize the expression within the AGM region, embryos were dissected after X-gal staining. Only the anterior portion of the pro/mesonephros is positive for β-galactosidase activity (Fig. 6D). The AGM region is known to harbor HSC activity, beginning late 10 dpc, and at some low frequency we have been able to find staining in the pro/mesonephros of transgenic 10.5 dpc embryos (not shown). In addition, hematopoietic cell-containing yolk sacs from various stages were stained and examined. As shown in Fig. 6E, no blue-staining cells are found in 11 dpc yolk sac. This is consistent with the lack of Sca-1 antibody staining of 11 dpc yolk sac (Huang and Auerbach, 1993) and lack of Ly-6E/A-specific RNA at 10 dpc (Dzierzak et al., 1995).

Cross sectioning of 11 and 11.5 dpc embryos in the mid- and caudal regions yielded further localization of β-galactosidase-positive cells. Within the pro/mesonephros, intense X-gal staining is restricted to the epithelial-like cells of the tubules (Fig. 7A,B). Some lower level staining is seen in the surrounding mesenchymal cells. This low-level staining is probably not due to diffusion since the hindgut exhibited strong staining only in the gut wall, with no positive surrounding cells (Fig. 7C). In the tail region, endodermal cells of the hindgut are strongly positive for X-gal staining and surrounding mesodermal cells show various levels of staining (Fig. 7D). At day 8 pc, the diverticulum of the hindgut is well defined and extends almost to the posterior end of the tail. Ly-6E.1-lacZ expression is seen from the tail to the anterior end of the hindgut in 8.5 dpc embryos onwards. At later stages after fusion of the hind and foreguts, staining stops abruptly at this border (not shown).

Late in gestation at 13.5 dpc, staining of tubules is widespread in the degenerating mesonephros and surrounding cells (Fig. 8A,B). Faint staining is observed in some cells of the Mullerian duct in females (Fig. 8B). In males at 15.5 dpc, intense staining is found in the epididymus (Fig. 8C), which is thought to be derived from the mesonephric tubules (Kaufman, 1992). Finally, expression in the developing kidney (metanephros) appears to be restricted to the cortical tubules at 18 dpc (Fig. 8D) and is consistent with the adult kidney expression pattern of the Ly-6E.1/A.2 (van de Rijn et al., 1989).

The 3' flanking sequences of the Ly-6E.1 gene appear to be responsible for tissue-specific transgene expression during embryonic development

The spatial expression patterns of the 14 kb and 9.4 kb Ly-6E.1-lacZ transgenes in embryos of the BL and XN lines were examined to determine if the 3' sequences of the Ly-6E.1 gene are necessary for tissue-specific embryonic expression. Data for 11 dpc embryos is summarized in Table 2. Mesonephros and tail expression was consistently high in all four BL transgenic lines. However, only one XN line, XN23, expressed the transgene highly in the mesonephros, but it did not express the transgene in the tail region. The XN229 line expressed the transgene reliably in the tail but at a lower level as compared to the tail expression in the BL lines. Furthermore, while ectopic expression of the BL transgene occurred rarely, ectopic

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Table 2. Embryonic expression patterns of the Ly-6E.1-lacZ transgenes
expression was found very frequently in the XN lines in regions such as the limb buds, liver, brain, etc. Thus, the high level tissue-specific embryonic expression pattern of the Ly-6E.1-lacZ transgene appears to require the presence of the 3′-most gene flanking sequences.

DISCUSSION

The results of our transgenic mouse studies with the genetic elements encoding the HSC marker Sca-1 have demonstrated that a 14 kb Ly-6E.1 genomic fragment can direct high level, tissue-specific expression of the lacZ marker gene in adult mice. We have shown here through the use of the XN deletion transgene construct the importance of distal 3′ sequences for in vivo expression. While previous data have indicated that 5′ Ly-6E.1 sequences were insufficient to direct reporter gene expression in transgenic mice (Dr A. Bothwell, personal communication), we have been able to reproducibly detect lacZ RNA- and β-galactosidase-positive cells in all BL transgenic mice carrying 11 kb of 3′ Ly-6E.1 sequence. Transgene expression levels are high and average at 3.2% per Ly-6E.1 lacZ copy, as compared to endogenous gene transcription. However, this value is most likely an underestimate, since the probe used to detect endogenous Ly-6E.1/I A.2 expression cross-hybridizes with transcripts from other Ly-6 family members. This is further supported by the high levels of β-galactosidase activity found in the BL transgenic mice (efficient X-gal and FDG staining are seen within 1 hour). Also, the levels of lacZ RNA in the four BL transgenic lines appear to closely correspond to transgene copy number. While we have not produced enough Ly-6E.1 lacZ transgenic lines to draw a strong conclusion, preliminary data from additional transgenic lines with the 14 kb Ly-6E.1 expression cassette containing other gene inserts (unpublished observations) support copy number dependency.

The Ly-6E.1 lacZ expression pattern observed in the kidney, thymus, spleen, lymph nodes, bone marrow and certain hematopoietic lineages of BL transgenic mice corresponds to the known in vivo expression pattern of endogenous Ly-6E.1/I A.2. We found only rare ectopic expression in the BL lines as compared to embryos and adults of all the XN lines. While the BL transgenics show a reproducible expression

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Fig. 6. Whole and specific organ X-gal staining of BL embryos. Whole embryos at (A) 8.5 dpc, (B) 10.5 dpc and (C) 11.5 dpc were stained with X-gal for visualization of Ly-6E.1-lacZ expression. Embryos were of the BL1b transgenic line. Arrow indicates intra-embryonic staining. (D) Dissected AGM region from 11.5-12 dpc BL1a transgenic embryo. The pro/mesonephros is the most lateral vertically oriented tissue, with the genital ridge positioned slightly over the pro/mesonephros. The dorsal aorta runs vertically along the midline between these tissues. As indicated by the arrow, X-gal-positive cells are located at the anterior tips of the mesonephros. (E) Yolk sac from an 11 dpc BL1b transgenic embryo. Blood vessels of the yolk sac appear yellow and connecting yolk sac tissue appears blue because of the background. No X-gal-positive cells can be observed in the circulating blood, blood vessel walls or other cells of the yolk sac.

Fig. 7. X-gal-stained transverse-sections from 11.5 dpc BL transgenic embryos. Sections through the caudal region of BL1b embryos stained with X-gal and counterstained with eosin. (A) Transverse section through the region containing the aorta, mesonephros and genital ridge. (B) Higher magnification of the mesonephros. (C) Transverse section through the hindgut region. (D) Transverse section through the posterior hindgut and tail region. da, dorsal aorta; hg, hindgut; l, liver; m, mesonephros; mt, mesonephric tubule; tm, tail mesoderm.
pattern, all the regulatory elements directing tissue specificity may not be located in the downstream fragment, since some of the XN transgenic lines express the transgene with partial specificity. For example, the XN37, XN23 and the XN229 lines express the transgene in T lymphocytes, the mesonephros and the tail, respectively. Thus, the 3′ Ly-6E.1 genomic sequences clearly play an important role in directing high-level expression and may confer integration site independence. In combination, the integration site-independent expression and the copy number-dependent expression suggest that the region 9 kb downstream of the Ly-6E.1 gene may contain a locus control region (LCR, Dillon and Grosveld, 1993).

This downstream region of the Ly-6E.1 gene has previously been shown to contain several transcription factor binding consensus sequences (Sinclair et al., 1996). Of potential importance is an ISRE consensus sequence (AGAACAGAAAC), which is located within the strong DNase I hypersensitive site at +8.7. The 1 kb region surrounding this consensus sequence is highly homologous (80%) to an upstream region (~2.3 to −1.6) of Ly-6E.1, which contains an incomplete ISRE (Khan et al., 1990). The 3′ ISRE, along with other transcription factor motifs, may function to increase expression in vivo, although all other ISREs previously described are upstream of gene sequences (Porter et al., 1988; Reid et al., 1989). The identification of the precise Ly-6E.1 gene regulatory elements that specify copy number-dependent and integration site-independent expression in the different tissues and during different developmental stages awaits more detailed deletion and mutation analysis.

Of greater interest to our studies is the demonstration that the 14 kb Ly-6E.1 genomic sequence can direct heterologous gene expression in HSCs of the adult bone marrow. At present, the only method to clearly demonstrate transgene expression in HSCs is by FACS sorting and transplantation into lethally irradiated recipient mice for long-term, multilineage hematopoietic repopulation. For these purposes, the use of the lacZ gene has been problematic, as others have reported high levels of endogenous β-galactosidase activity in hematopoietic cells with the widely used substrates (Hendriks et al., 1994). However, in the context of the 14 kb Ly-6E.1 expression cassette, X-gal and FDG substrates provide the necessary sensitivity in hematopoietic cells, well above background staining, for the enrichment of functional lacZ-expressing HSCs from the bone marrow. The enrichment is approximately 100-fold and is the expected degree of enrichment, as found with the Sca-1 antibody. Thus, we have shown, for the first time, the expression of the lacZ gene in HSCs of transgenic mice, and that FDG-FACS can be performed on primary cells without compromising their in vivo function.

As revealed by FACS analysis, β-galactosidase is expressed in lymphoid, myeloid and hematopoietic progenitor/stem cells. Each of these lineages, particularly the T lymphoid lineage, is known to contain Sca-1-positive cells. However, double staining experiments reveal that there is an incomplete overlap in Sca-1 and FDG staining. This could be expected since the allele-specific pattern of the Ly-6A.2 allele is more widespread than Ly-6E.1, and we have used an Ly-6E.1 gene (from the Balb/c strain) in (CBA×C57Bl/6)F1 mice, which carry both alleles. These allelic differences are even more complex in F1 hybrid mice, where the expression has been found to be distinct from either parent (Codias et al., 1989; Spangrude and Brooks, 1993). Preliminary double staining experiments using allele-specific antibodies and the FDG substrate suggest that the Ly-6E.1 lacZ transgene expression pattern is complex. In addition, we have found a small percentage of Sca-1-negative cells with β-galactosidase activity. While β-galactosidase is a very stable cytoplasmic protein, Sca-1 is a GPI-linked cell-surface glycoprotein requiring complex post-translation modifications, transport and surface turnover. In Drosophila larva, such differences between β-galactosidase and endogenous protein expression have been exploited in the study of segmentation (Lawrence et al., 1987). In Ly-6E.1 lacZ mice, such differences may be useful for studies of hematopoietic cell differentiation and migration.

The reproducible, high-level caudal region expression pattern in the hindgut, tail and pro/mesonephros of BN embryos as compared to XN embryos strongly supports a role for the 3′ sequences of the Ly-6E.1 gene in development. The pro/mesonephric localization of the Ly-6E.1 lacZ expression is most interesting with respect to the developmental origins of

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**Fig. 8.** X-gal-stained late gestation BL embryo whole tissues and sections. (A) Sagittal section through 13.5 dpc BL1b embryo stained with X-gal and counterstained with eosin. (B) Transverse section through a 13.5 dpc BL1b embryo stained with X-gal and counterstained with eosin, showing the degenerating mesonephros and Mullerian duct. (C) Whole urogenital system of a 15.5 dpc BL1b male transgenic embryo stained with X-gal. Staining can be seen in the urogenital system of a 15.5 dpc BL1b male transgenic embryo. (D) Transverse section through a 13.5 dpc BL1b embryo stained with X-gal and counterstained with eosin, showing staining in the epididymus and faintly in the kidney. (D) Transverse section of the kidney from a 18 dpc BL1b transgenic embryo stained with X-gal. Staining of the cortical tubules can be seen. ct, cortical tubules; e, epididymus; g, gonad; k, kidney; m, mesonephros; md, Mullerian duct; mt, mesonephric tubule; s, stomach; t, testis.
the mammalian hematopoietic system (reviewed in Dzierzak and Medvinsky, 1995). In non-mammalian vertebrates it has been found that during development the pronephros serves as an intra-embryonic site of hematopoiesis. By embryo grafting experiments, the amphibian dorsal lateral plate, which gives rise to the pronephros, is the dominant source of the adult hematopoietic system rather than the ventral blood islands, which are analogous to the mammalian yolk sac (Turpen et al., 1981). Our previous findings of adult HSC activity in the AGM region beginning at day 10.5 pc (Medvinsky et al., 1993; Müller et al., 1994) and the X-gal staining of Ly-6E.1-lacZ transgenic embryos described here, also beginning at day 10.5, suggest that the pro/mesonephros may be the analogous developmental site for mammalian HSCs. Thus, the highly-specific and regulated expression pattern of the Ly-6E.1-lacZ transgene in adult HSCs, diverse hematopoietic cell lineages and the pro/mesonephros of the developing embryo poses many interesting questions for further study on the origins and manipulation of definitive HSCs.

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