EXPRESSION OF THE Ly-6E.1 (Sca-1) GENE IN TRANSGENIC MICE FOR THE STUDY OF HAEMATOPOIETIC STEM CELLS.

DURING DEVELOPMENT.

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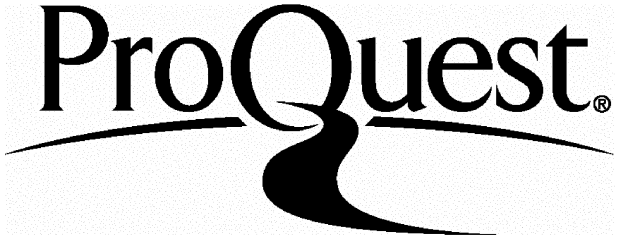

ABSTRACT

The haematopoietic stem cell is the progenitor of all mature blood cells. The processes involved in the maintenance and differentiation of haematopoietic stem cells throughout adult life are poorly understood and a greater understanding of these processes would be of clinical benefit for many haematological disorders.

The Sca-1 antigen is a marker of murine haematopoietic stem cells and has been used in haematopoietic stem cell enrichment procedures. This thesis describes the production and analysis of transgenic mice containing DNA constructs based upon the Ly-6E.1 (Sca-1) gene.

In the first series of experiments, the genetic regulatory elements of the Ly-6E.1 gene were studied by deletion analysis *in vivo* using Ly-6E.1/*lacZ* transgenes. The Ly-6E.1 transgene was shown to be functional *in vivo* and to possess a genetic element with some characteristics of a chromatin opening domain. More importantly, the Ly-6E.1 transgene was shown to be capable of directing heterologous gene expression to haematopoietic stem cells. Analysis of the expression pattern of Ly-6E.1/*lacZ* transgenes during development supports the notion of an intraembryonic origin for definitive haematopoiesis and suggests Ly-6E.1 may be a marker of haematopoietic stem cells throughout ontogeny.

In the second series of experiments, transgenic mice were produced which expressed either the *c-myc* or the *bcl-2* proto-oncogenes under the transcriptional control of the Ly-6E.1 gene. All transgenic mice containing proto-oncogenes displayed perturbation of multiple haematopoietic lineages and the expression of these

genes has facilitated the derivation of a novel cell line with a cell surface phenotype resembling that of a haematopoietic stem cell.

These data demonstrate that the cloned 14Kb Ly-6E.1 gene is a useful tool for the study *in vivo* of the regulatory elements of a gene which is expressed in haematopoietic stem cells. Studies such as these could serve as a model leading to an understanding of the transcriptional programmes active in self-renewing, pluripotent haematopoietic stem cells. In addition, the results presented in this thesis provide evidence for the feasibility of using this genetic tool for the expression of heterologous genes in transgenic mice to study haematopoiesis *in vivo* and for the isolation of cell lines. Ultimately, such studies will enable the mechanisms of haematopoietic stem cell self-renewal and pluripotence to be understood.

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INTRODUCTION

1. Haematopoietic stem cells.

Mature cells of the blood system have a finite lifespan, hence there is a need for sustained production of haematopoietic cells throughout an animal's life. Mature haematopoietic cells are continuously generated from haematopoietic stem cells that reside in the adult bone marrow (Spangrude, 1992). The continual, lifelong production of haematopoietic cells from stem cells in the bone marrow has been utilised clinically, providing the basis for therapies directed against various haematological disorders. Allogenic bone marrow transplantation can be used to treat disorders such as immunodeficiencies, whilst autologous bone marrow transplantation is commonly used in the treatment of leukaemia (Fisher *et al.*, 1990; Kersey *et al.*, 1987; Thomas, 1991). It is hoped that with recent advances in molecular biology, genetic disorders of the blood such as thallassaemias and immunodeficiencies, may prove suitable candidates for gene therapy by making use of the pluripotent, regenerable properties of bone marrow haematopoioetic stem cells as targets for therapeutic genes.

1.1 Characterisation of haematopoietic stem cells.

Initial studies by Till and McCulloch demonstrated the existence of cells capable of migrating and responding to the haematopoietic environment of the irradiated mouse spleen by producing haematopoietic cells of erythroid, myeloid and megakaryocytic lineages. Bone marrow cells, when injected intravenously into irradiated mice formed macroscopic colonies on the spleen, referred to as CFU-S, colony forming unit - spleen (Till *et al.*, 1961; Wu *et al.*, 1967). Further studies demonstrated that the cells giving rise to these spleen colonies could self-renew, producing cells capable of giving rise to spleen colonies in secondary transplant recipient irradiated mice (Siminovitch *et al.*, 1963; Siminovitch *et al.*, 1964). Thus we have the beginning of a working definition of the haematopoietic stem cell, self-renewal and multilineage differentiation, that has formed the base for all following studies and continues to be used at present.

The potential of spleen colonies to give rise to secondary colonies was found to vary with the time of their harvest. Colonies taken 14 days post injection of bone marrow had a greater potential to form secondary colonies than those taken 8 days post injection, suggesting some heterogeneity exists within the CFU-S cells of the bone marrow (Magli *et al.*, 1982; Worton *et al.*, 1969). Upon detailed analysis of radiation chimaeras, it became evident that the CFU-S cells were not responsible for reseeding the bone marrow and were representative of more mature cells, commited progenitors, with CFU-S_{14} being more immature than CFU-S_8. Neither class of CFU-S was capable of consistently providing long term reconstitution (Jones *et al.*, 1990; van der Loo *et al.*, 1994), though the CFU-S assay remains a useful tool providing

an indication of haematopoietic activity for myeloid, erythroid and megakaryocytic lineages (Medvinsky *et al.*, 1993; Moore & Metcalf, 1970).

In addition to the CFU-S assay, numerous *in vitro* culture based assays for haematopoietic potential have been devised. In general, these rely on the growth of haematopoietic cells in the presence and under the influence of growth factors and/or stromal cells and score progenitors on the ability to give rise to differentiated haematopoietic progeny (Metcalf, 1984). A widely used assay is the *in vitro* CFU-C (colony forming unit - culture) in which test cells are seeded in semisolid media, often methylcellulose, containing specific, well defined growth factors. Colonies can be scored and typed from 7 to 14 days in culture and, if desired, can be replated to encourage growth of more primitive cells. In general, this technique is only useful for detecting cells of the myeloid and erythroid lineages. To detect lymphoid cells, a less well defined approach has been employed, utilising stromal or organ culture. B lymphopoiesis is detected using stromal cells and growth factor supplements in a 14 day culture system (Ogawa *et al.*, 1988), whereas T lymphopoiesis requires T cell depleted foetal thymic explants as a stroma to provide a source of the growth factors/cellular signals necessary for T cell production (Liu & Auerbach, 1991). Recently, several multistep culture systems have been reported, for example, the SMA (Godin *et al.*, 1995), single cell multipotential assay, in which cells are first seeded in multiwell plates at single cell dilution onto stromal cells in the presence of interleukin 7 (IL-7), interleukin 3 (IL-3) and c-kit ligand (KL) for 10 to 15 days prior to transferring each clonally derived culture into myeloid, B and T cell cultures to assay for multipotency; the LTC-IC (long term culture initiating cell) and the CAFC (cobblestone area forming cell) assays (Dexter *et al.*, 1977; Lemieux *et al.*, 1995)

rely upon the establishment of a healthy bone marrow stroma, the addition of complex cocktails of growth factors and highly specialised culture conditions. Unlike the SMA, the LTC-IC and CAFC assays provide only an indirect assessment of haematopoietic stem cells and do not test the potential of single cells.

The most stringent assay for a haematopoietic stem cell is the ability of that cell to give rise to long term multilineage reconstitution of an irradiated mouse (Abramson *et al.*, 1977; reviewed in Orlic & Bodine, 1994). As is explained in detail in section 1.3, *in vivo* analyses not only define haematopoietic stem cells, they are the only way to accurately assess the frequency and pluripotency of haematopoietic stem and progenitor cells. For this reason the majority of our current understanding about haematopoietic stem cells has come from these types of study.

As the bone marrow consists of a heterogenous mix of haematopoietic cells at all stages of maturity from stem cells to terminally differentiated cells, it was of interest to determine the frequency of the haematopoietic stem cell. Harrison, 1980, generated chimaeric mice from congenic C57BL/6 donors and recipients that differed at their β-globin and glucose phosphate isomerase loci (Harrison, 1980), employing competitive repopulation between different mice and detecting donor cell engraftment on the basis of electrophoretic differences. Such competitive repopulation experiments, coupled with limiting dilution assays have estimated the frequency of the long-term haematopoietic stem cell in bone marrow to be approximately one in 50,000 nucleated cells (Boggs *et al.*, 1982; Boggs *et al.*, 1984; Russel, 1979).

As the haematopoietic stem cell is capable of multilineage reconstitution, it was of interest to investigate the clonality of haematopoietic reconstitution, ie, was an individual haematopoietic stem cell capable of repopulating all mature

haematopoietic lineages? The first experiments to address this involved the induction of cytological damage, with radiation, to donor bone marrow cells prior to transplantation. Such radiation induced chromosomal markers allowed the fate of these donor cells to be followed after transplantation into lethally irradiated recipient mice. Progeny of the donor marked cells were detected in all haematopoietic lineages of the recipient mice thus demonstrating clonal repopulation of the adult haematopoietic system by a single haematopoietic stem cell (Abramson *et al.*, 1977; Wu *et al.*, 1968).

The generation of replication defective recombinant retroviruses harbouring marker genes enabled cell lineage and precursor/progeny studies to be conducted in a reliable and detailed fashion, without using potentially damaging radiation induced chromosomal translocations to mark cells (Lemishka *et al.*, 1986; Price *et al.*, 1987; Sanes *et al.*, 1986). Bone marrow (or any test cell population) is infected with the replication defective retrovirus, which will integrate into the DNA of each infected cell at random. Given the size of the genome, it is likely that no two cells of those originally infected will have the same viral integration site. As cells from the original preparation divide, each specific integration pattern will be inherited, thereby clonally marking the progeny of each starting cell. Analysis of radiation chimaeras generated from retrovirally marked bone marrow proved that a single cell could differentiate into all haematopoietic lineages and provide long term reconstitution (Dick *et al.*, 1985; Keller *et al.*, 1985; Lemishka *et al.*, 1986).

In addition to providing the most stringent assay for haematopoietic stem cells, the analysis of retrovirally marked radiation chimaeras over a period of time has yielded interesting information regarding the dynamic aspects of bone marrow

transplantation. Upon analysis of the radiation chimaeras, it became apparent that reconstitution occurred in waves, emanating from the most immature haematopoietic stem cells as well as from more commited progenitor cells. The retroviral integration patterns fluctuated over a six month period, demonstrating a highly dynamic situation in which short lived clones expand and atrophy, becoming sequentially activated. Some clones could be activated by transplantation into secondary recipients, indicating that after replicating *in vitro* (a prerequisite for retroviral infection) they could return to a quiescent state for many months. Thus, these experiments demonstrate that although generally quiescent, haematopoietic stem cells can be retrovirally marked and contribute to high level, long term multilineage reconstitution and that only very few haematopoietic stem cells generate the complete adult haematopoietic system at any one time (Jordan & Lemishka, 1990; Keller, 1992; Keller & Snodgrass, 1990). The existence of short lived clones, contributing to single lineages and the initial clonal fluctuations observed are likely to result from the retroviral infection of committed progenitor cells. Detailed analysis of this type of radiation chimaera and, in particular the short lived clones, could increase our understanding of the branchpoints involved in haematopoietic development.

1.2 Enrichment and purification of haematopoietic stem cells.

The basis of the regenerable and sustained haematopoiesis emanating from the bone marrow is the haematopoietic stem cell. The haematopoietic stem cell is present in murine bone marrow at only between one in 10^4 and 10^5 nucleated cells. To

characterise haematopoietic stem cells it would be desirable to work with as many and as pure a population of stem cells as possible which necessitates the development of enrichment proceedures (reviewed in Spangrude, 1989). Haematopoietic stem cell enrichment protocols would also be of clinical use as the purification of haematopoietic stem cells away from malignant cells, in the case of leukaemia (Gribben *et al.*, 1991), or from alloreactive T cells which could potentially initiate graft-versus-host disease (Ferrara & Deeg, 1991) is vital to the success of autologous or allogeneic bone marrow transplants respectively.

Early enrichment studies relied upon the spleen colony forming assay and *in vitro* colony forming assays to detect haematopoietic activity in populations of cells sorted using physical parameters, such as density gradient centrifugation (Jones *et al.*, 1990; Worton *et al.*, 1969; Worton *et al.*, 1969b). In order to reach the levels of sophistication employed today in the search for purity in haematopoietic stem cells, two advances were necessary. With the advent of antisera specific to cell surface molecules and fluorescence activated cell sorting, a powerful new technology was employed in the purification of haematopoietic stem cells (Herzenberg & Sweet, 1976). To date, there is no single, definitive antigenic determinant that marks haematopoietic stem cells exclusively. However, through the combined use of numerous antibodies to cell surface markers, it is possible to achieve significant enrichments in haematopoietic stem cell activity from murine bone marrow (Spangrude, 1989).

The first step towards haematopoietic stem cell purification was the finding that monoclonal antibodies directed towards the Thy-1 glycoprotein marked haematopoietic stem cells (in addition to other cells). To further enrich for stem

cells, bone marrow was depleted of cells which expressed markers of mature B cells (B220), T cells (CD4 and CD8), granulocytes (Gr-1) and myelomonocytic cells (Mac-1). Bone marrow cells expressing low levels of Thy-1 in the absence of mature lineage markers (Thy-1^{lo}, Lin^-) were 50-200 fold enriched in haematopoietic progenitor activity (Müller-Seiberg *et al.*, 1986). This Thy-1^{lo}, Lin^- population has been further enriched using a monoclonal antibody E13 161-7 (Aihara *et al.*, 1986) which reacts with an antigen referred to as Sca-1 (stem cell antigen 1). The Thy-1^{lo}, Lin^-, Sca-1^+ fraction of bone marrow from C57BL/Thy-1.1 mice represents approximately 0.05% of bone marrow cells and is approximately 2,000 fold enriched for haematopoietic stem cell activity in radioprotection assays, *in vitro* assays and, more importantly, long term multilineage reconstitution assays (Li & Johnson, 1992; Smith *et al.*, 1991; Spangrude *et al.*, 1988). In addition, no other bone marrow cell type possesses haematopoietic stem cell activity (Uchida & Weissman, 1992). The precise designation of the mouse strain used is of considerable importance as both Thy-1 and Sca-1 exhibit strain specific polymorphisms, resulting in differing antibody staining profiles in bone marrow of mice of different backgrounds. These polymorphisms cast doubt on the use of Thy-1 in haematopoietic stem cell enrichment procedures, as not only does the low cell surface level of expression make cell sort windows difficult to determine, it has been shown that haematopoietic stem cells in Thy-1.2 strains of mice are both Thy-1^{lo} and Thy-1^- (Spangrude, 1994). Similarly, Sca-1 exhibits strain specific differences in bone marrow expression and is thought not to be expressed on all haematopoietic stem cells in certain mouse strains (Sangrude and Brooks, 1993). However high level Sca-1 expressing long term reconstituting haematopoietic stem cells can be found in bone marrow of mice from

all backgrounds tested (Jurecic *et al.*, 1993; Okada *et al.*, 1992; Spangrude & Brooks, 1993).

In order to further enrich for haematopoietic stem cells from the Thy-1^{lo}, Lin^-, Sca-1^+ bone marrow fraction, antibodies directed towards the c-kit receptor were employed. Separation of Thy-1^{lo}, Lin^-, Sca-1^+ bone marrow cells into c-kit expressing and non-expressing subsets demonstrated that only c-kit^+ cells possessed CFU-S activity and were enriched in long-term repopulating activity (Ikuta & Weissman, 1992). Detailed analysis demonstrated that Sca-1 expression is associated with more immature bone marrow haematopoietic stem cells than is c-kit. As haematopoietic stem cells mature and differentiate, they rapidly lose Sca-1 antigen from their cell surface, whilst the c-kit marker continues to be expressed into the committed progenitor stage. When used in combination, both Sca-1 and c-kit provide a powerful system for haematopoietic stem cell enrichment (Ikuta & Weissman, 1992; Okada *et al.*, 1991; Okada *et al.*, 1992).

Haematopoietic stem cells are resistant to treatment with 5FU and other cell cycle active agents (Hodgson & Bradley, 1979), suggesting they exist in the bone marrow in a quiescent state. This observation has been utilised for *in vivo* methods of haematopoietic stem cell enrichment. Haematopoietic stem cell quiescence has led to the development of other parameters for the enrichment of the most primitive haematopoietic stem cells. Low metabolic activity results in haematopoietic stem cells staining only weakly with the mitochondrial dye rhodamine 123 (Bertoncello *et al.*, 1985) and, as haematopoietic stem cells are not in cell cycle, they exhibit low staining with the DNA dye Hoechst 33342 (Wolf *et al.*, 1993). By combining the use of dyes such as these and cell surface markers, it is possible to recover cell

populations capable of long-term multilineage reconstitution after transplantation of fewer than 50 cells (Li & Johnson, 1992; Wolf *et al.*, 1993).

1.3 Heterogeneity of haematopoietic stem cells.

To accurately study the biochemistry and molecular biology of the haematopoietic stem cell in detail, it is desirable to isolate haematopoietic stem cells from the bone marrow in as purified form as possible. In addition, haematopoietic stem cell purification would have great clinical significance, for example, eliminating the problem of bone marrow transplants containing leukaemic or immunocompetent cells and providing a pure target cell population for gene therapy directed at haematological disorders.

Despite the advances in haematopoietic stem cell enrichment procedures outlined in the previous section, the haematopoietic stem cell has not been purified to homogeneity, a point illustrated by the study of the kinetics of haematopoietic reconstitution in irradiated mice. Following the fate of individual stem cell clones using retrovirally marked bone marrow, it became evident that not all multilineage contributing clones were equivalent and that some were short lived whilst others were long lived (Ikuta & Weissman, 1992; Jordan & Lemishka, 1990; Keller, 1992). Similar functional heterogeneity has been observed for Thy-1^{lo}, Lin^{-}, Sca-1^{+} bone marrow cells (Smith *et al.*, 1991; Uchida *et al.*, 1994). Despite displaying the same cell surface phenotype and giving rise clonogenically to both myeloid and lymphoid

lineages in limiting dilution transplantation assays, these cells had productive lifespans which varied between several weeks and two years (Smith *et al.*, 1991).

These examples of heterogeneity within the Thy-1^{lo}, Lin$^-$, Sca-1$^+$ bone marrow subset, however do not constitute mature cells or single lineage commited progenitors (Spangrude *et al.*, 1991; Uchida *et al.*, 1994). They appear to represent heterogeneity within the stem cell compartment. It may be that the "haematopoietic stem cell" consists of a dynamic cell population, rather than a specific individual quiescent cell.

In addition to functional heterogeneity, more detailed analysis has revealed a surprising degree of phenotypic heterogeneity within the haematopoietic stem cell compartment. Thy-1^{lo}, Lin$^-$, Sca-1$^+$ "haematopoietic stem cells" display heterogeneity in terms of their cell cycle status (Fleming *et al.*, 1993), rhodamine 123 staining (Li & Johnson, 1995; Spangrude & Johnson, 1990) and low level expression of certain lineage markers, such as CD4 (Onishi *et al.*, 1993; Wineman *et al.*, 1992) and mac-1 (Morrison & Weissman, 1995).

By collating observations from several independent laboratories, it is possible to reconcile some of this heterogeneity and suggest that a heirarchical organisation exists for early haematopoietic differentiation. Thy-1^{lo}, Lin$^-$, Sca-1$^+$, Rh123^{lo} cells are the most primitive long-term reconstituting haematopoietic stem cells (Li & Johnson, 1992); Thy-1^{lo}, Lin$^-$, Sca-1$^+$, Rh123^{hi} are short term multipotent stem cells (Spangrude & Johnson, 1990), presumably, the quiescent Rh123^{lo} cell has become activated and these cells are likely to be equivalent to the Lin$^-$, Sca-1$^+$, c-kit$^+$, Rh123^{hi} short term haematopoietic repopulating cell (Li & Johnson, 1995). The Lin$^-$, Sca-1$^-$, c-kit$^+$, Rh123^{hi} cell also with short term repopulating activity could be an

intermediate stage before the c-kit$^+$, Sca-1$^-$ committed progenitor which is defined by ability to respond to single cytokines *in vitro* in the absence of growth factor synergy (Li & Johnson, 1995). At some point prior to this commitment stage, a subset of haematopoietic stem cells may express low levels of lineage markers, possibly signalling their intent to become fully activated along the pathway of haematopoietic differentiation.

Caution must be exercised when interpreting such FACSorting experiments that artefacts are not inadvertently introduced. For example, the monoclonal antibody AA4.1 has been used to purify haematopoietic stem cells from foetal liver (Jordan *et al.*, 1990). However, analysis of adult bone marrow stem cells has revealed that they only express the AA4.1 antigen during recovery from 5FU treatment (Spangrude, 1994). A recent study by Spangrude *et al.* (Spangrude *et al.*, 1995) demonstrated that cell surface phenotype alone can be an unreliable criterion for defining haematopoietic stem cells. Upon sorting bone marrow from radiation chimaeras generated with Thy-1^{lo}, Lin$^-$, Sca-1$^+$ bone marrow cells, an expansion in the number of Thy-1^{lo}, Lin$^-$, Sca-1$^+$ cells of up to 1000-fold above input was observed. However, secondary transplantation of Thy-1^{lo}, Lin$^-$, Sca-1$^+$ cells showed they were not functionally equivalent to the original haematopoietic stem cells, generally lacking haematopoietic reconstitution activity. The answers to such questions will require a combination of haematopoietic stem cell enrichment strategies and retroviral marking of individual cells to follow the precise fate of individual cells.

1.4 Haematopoiesis during ontogeny.

Prior to adult bone marrow haematopoiesis, where do haematopoietic stem cells originate? Morphologically, the first embryonic tissue to demonstrate evidence of haematopoiesis, in the form of erythroid blood islands, is the yolk sac at day 7.5 *post coitum* (p.c.). In addition to erythropoiesis, precursors for granulocytes and macrophages (Morris *et al.*, 1991; Sonada *et al.*, 1983) have been identified as well as other, *in vitro* colony forming cells such as BFU-E and CFU-mix (Moore & Metcalf, 1970; Wong *et al.*, 1986) within the yolk sac between days 7.5 and 9 p.c. Lymphoid precursors have been demonstrated in yolk sac between days 8 and 9 p.c. by their ability to seed foetal thymic organ culture (Liu & Auerbach, 1991). The T cells produced in this culture system expressed T cell markers such as CD4, CD8, CD3 and were responsive to concanavalin A and anti-CD3 stimulation. However, they were unlike adult type T cells in that they were of a subtype Vγ3.

Despite a lack of retroviral marking and haematopoietic stem cell purification experiments using yolk sac, the detection of haematopoietic progenitors in yolk sac at early, preliver stages of development has led to the theory that the yolk sac is the origin of the haematopoietic stem cell (Moore & Metcalf, 1970). At present little is known about the cell surface phenotype of yolk sac haematopoietic cells due to technical difficulties with flow cytometry and high background staining with fluorescent antibodies (Ikuta & Weissman, 1993). The work of Huang and Auerbach has suggested that haematopoietic progenitor cells in yolk sac are AA4.1$^+$ and Sca-1$^-$, although this study failed to examine yolk sac cells from the time of the first observation of blood islands (Huang & Auerbach, 1993).

From day 10 p.c. erythropoiesis can be macroscopically discerned in the foetal liver (Russel & Bernstein, 1966). In contrast to yolk sac derived erythrocytes, those produced in the foetal liver from day 10 p.c. are four to five times smaller and enucleated, resembling bone marrow derived erythrocytes (Russel & Bernstein, 1966). This suggests that there is a degree of heterogeneity associated with the development of haematopoietic stem cells. Between days 10 and 12 p.c. granulocytes, macrophages, B lymphocytes (Velardi & Cooper, 1984) and even bipotential precursors of B cells and macrophages (Cumano *et al.*, 1992) have been identified in the liver. The analysis of the surface phenotype of foetal liver haematopoietic cells is, like that of the yolk sac, less advanced than that of the bone marrow for techinical reasons. Erythropoiesis dominates the foetal liver and only recently with the development, and inclusion in the Lin cocktail, of the monoclonal antibody Ter119, has it been possible to exclude mature erythroid lineage cells from foetal liver preparations (Ikuta *et al.*, 1990). By inclusion of the Ter119 antibody in the Lin cocktail, foetal liver cells with the surface phenotype Thy-1^{lo}, Lin$^-$, Sca-1$^+$ were shown to have similar properties to analogous cells isolated from the bone marrow (Ikuta & Weissman, 1992). In addition, detailed analysis of the foetal liver has been conducted by Lemishka (reviewed in Lemishka, 1993) using a different enrichment protocol. The selection of Sca-1$^+$, Lin$^-$ cells of 1.066 - 1.070 qcm^{-3} density which did not adhere to fibronectin and stained positive with monoclonal antibody AA4.1 demonstrated that haematopoietic stem cell activity was associated with the Sca-1$^+$ and not the Sca-1$^-$, Lin$^-$ fraction. Lemishka demonstrated at the level of the single cell with retrovirally marked AA4.1$^+$ foetal liver cells in competitive repopulation of irradiated mice that foetal liver haematopoietic stem cells were functionally equivalent

to those in adult bone marrow. Long term reconstitution was either mono or oligoclonal after an initial period of fluctuation (Jordan & Lemishka, 1990; Jordan *et al.*, 1990).

Despite phenotyopic similarities between foetal liver and adult bone marrow haematopoietic stem cells, an elegant experiment by Ikuta and Weissman demonstrated that, at the functional level, they exhibit clear lineage potential differences in the foetal thymic organ culture system (reviewed in Ikuta & Weissman, 1993). Foetal liver haematopoietic stem cells generated T cells predominantly of the Vγ3 and Vγ4 type whereas adult bone marrow haematopoietic stem cells do not give rise to Vγ3 and Vγ4 cells but instead generate $\alpha\beta$ T cells. In addition, when these stem cells were tested in the adult environment (by intrathymic injection of either foetal or adult haematopoietic stem cells) neither gave rise to Vγ3 T cells (Ikuta *et al.*, 1990). Given these observations and the fact that foetal haematopoietic stem cells can give rise to both foetal and adult erythroid cells (Fantoni *et al.*, 1967), Ikuta & Weissman speculate that haematopoietic stem cells may lose some developmental potential during ontogeny, ie, that the haematopoietic stem cell itself is undergoing a process of commitment during development (Ikuta & Weissman, 1993). This also questions part of the definition of a haematopoietic stem cell. If the self renewal criterion were strictly adhered to, the daughter cells should possess the same potential and not be more commited. In the face of such examples of heterogeneity, the precise definition of haematopoietic stem cells at any given time in development is still unclear.

1.5 Haematopoietic development in non-mammalian vertebrates.

Despite such elegant experiments as enrichment of murine haematopoietic stem cells, retroviral marker clonal analysis and long term repopulation studies, these approaches have left several key questions unanswered concerning the pre-liver origin and development of the adult haematopoietic stem cell. The study of the origins of the adult haematopoietic system in non-mammalian vertebrates has provided an insight into the ontogeny of the mammalian haematopoietic system. Specifically, the study of non-mammalian vertebrates allows for the use of a range of grafting techniques not possible in mammals due to the *in utero* development of the mammalian embryo. In particular the generation of interspecific embryo chimaeras, in which cells from each donor can be readily distinguished and their fate followed in the context of the whole animal, has proven invaluable in determining the developmental origin of particular cell lineages.

This approach has been most extensively employed in the study of avian haematopoiesis and makes use of the difference between chick and quail cells. The nuclei of quail cells possess a nucleolar marker, a mass of heterochromatin which is not found in chick cells. Quail embryo bodies were engrafted onto intact chick yolk sacs to generate chimaeras in which the contribution of chick/quail cells to particular haematopoietic lineages was determined by DNA staining for the nucleolar marker (LeDourain, 1973). Analysis of interspecific chick/quail chimaeras raised to adult stages clearly demonstrated that the adult haematopoietic system was generated from the quail embryo body and not from the chick yolk sac (Dieterlen-Lievre, 1975). Due to the asynchronous development of the haematopoietic system in chick/quail

chimaeras, homospecific chimaeras, differing by sex chromosomes/MHC antigens/immunoglobulin allotypes, were generated and confirmed the result observed in the chick/quail chimaeras (Lassila *et al.*, 1982). Thus, in birds, the yolk sac provides only transient embryonic haematopoiesis whilst definitive, long term, adult haematopoiesis originates within the embryo body (Dieterlen-Lievre & LeDourain, 1993).

Whilst embryo grafting experiments localised the origin of the definitive haematopoietic system to the embryo body, the first visible indication of haematopioetic cells within a specific region of the avian embryo body is the appearance of large foci within the dorsal aorta (Dieterlen-Lievre & Martin, 1981). Although very few antibodies exist to avian haematopoietic cells, *in situ* analysis has localised the expression of some haematopoietic specific markers to cells within the wall of the aorta (Dieterlen-Lievre, 1984). In addition, when cells from the para-aotic region were cultured in semisolid media with haematopoietic growth factors, monocytic, myeloid and erythroid colonies were produced whilst cultures of the remainder of the embryo produced no colonies (Cormier & Dieterlen-Lievre, 1988). Thus these experiments suggest the definitive haematopoietic system in birds originates in the para-aortic intraembryonic region.

In similar embryo grafting experiments, amphibians also show transient embryonic and stable adult haematopoietic activities. Homospecific grafting experiments using diploid and triploid embryos demonstrated that the ventral blood island (analogous to the yolk sac) produces primitive haematopoietic cells and that definitive, adult haematopoiesis originates from the dorsal lateral plate, in the region of the pro/mesonephros (Turpen *et al.*, 1981). Recently, the results of *in situ*

hybridisation experiments have demonstrated the expression of members of the *GATA* family of haematopoietic transcription factors in the dorsal lateral plate of *Xenopus* embryos (Kelley *et al.*, 1994) and in the region of the pronephric duct of zebrafish embryos (Detrich *et al.*, 1995; Neave *et al.*, 1995). Thus, amphibian and fish definitive haematopoiesis originates within the embryo and is most likely generated in the pro/mesonephric region.

Unlike birds, however, some haematopoietic cells derived from the ventral blood island of *Xenopus* persist in the adult and contribute to both erythroid and lymphoid systems. This phenomenon is not restricted to frogs alone, there is evidence in mice of a population of B cells which predominate during foetal development, found in the pleuroperitoneal cavities, that are not generated from adult bone marrow in radiation chimaeras (Herzenberg *et al.*, 1986). Maybe these cells originate from a primitive, embryonic haematopoietic stem cell. Alternatively, they may develop *in situ* and their microenvironment is merely destroyed by irradiation.

1.6 The AGM region and primitive *vs* definitive haematopoiesis in mammals.

To investigate whether the principles concerning the origins of embryonic and definitive haematopoiesis in amphibians and birds may be similar for mammals (mice), Medvinsky and co-workers tested for intraembryonic haematopoiesis in the developing mouse using the CFU-S assay in radiation chimaeric mice (Medvinsky *et al.*, 1993). The results of their studies clearly demonstrate the presence of potent and abundant CFU-S haematopoietic progenitors in a region comprising dorsal Aorta, Gonads and Mesonephros (AGM). From 9 days p.c., CFU-S progenitors are found

at far greater frequency in the AGM region than in the yolk sac and before such activity is detectable in the liver. The frequency of CFU-S progenitors obtained from the AGM region was equivalent to that obtained from adult bone marrow (Medvinsky, 1993). In contrast, the liver contained a low frequency of CFU-S which increased on day 11 p.c. and was coincident with a decrease in CFU-S frequency in the AGM region. As seen previously in chick/quail chimaeras, this may represent colonisation of secondary haematopoietic tissues by cells which originated within the AGM region of the embryo body (Dieterlen-Lievre & LeDourain, 1993).

These *in vivo* haematopoietic progenitor studies were extended to assay for haematopoietic stem cells in various embryonic tissues using long term reconstitution of irradiated mice. At late day 10 p.c., cells from the AGM region were the only cells from the embryo capable of reconstitution (Müller *et al.*, 1994). More significantly, in this most stringent of haematopoietic assays, complete multilineage reconstitution was demonstrated and secondary/tertiary transplantation highlighted the self-renewal capacity of these cells. The fulfilment of these criteria indicate that the day 10p.c. mouse AGM region does contain definitive adult haematopoietic stem cells. These experiments also provide indirect evidence of expansion of haematopoietic stem cells if one assumes that a frequency of 3% reconstitution corresponds to single cell dilution. This may be in accord with the hypothesis that haematopoietic stem cells lose potential and become commited during development (Ikuta & Weissman, 1993) and that only the most primitive can expand their numbers.

Evidence for the existence of an intraembryonic source of haematopoietic cells has been been obtained independently by Godin and co-workers, whilst investigating

the origin of the unusual $CD5^+$ B cell population (Godin *et al.*, 1993). Transplantation of the whole para-aortic splanchnopleura region, P-Sp (the ebryonic rudiment of the AGM region) under the kidney capsule of SCID mice resulted in generation of several B cell subsets, including B1a. *In vitro* analysis of cells from this region showed the potential for production of T cells (foetal thymic organ culture), B cells, macrophages, megakaryocytes, myeloblasts, basophils and erythroblasts - though the evidence for one cell being a common precursor is not conclusive (Godin *et al.*, 1995). In these studies, in contrast to the findings of Müller *et al.*, the yolk sac shows a parallel increase in haematopoietic potential to the P-Sp, although 10-fold less. This discrepancy may reflect inherent differences between the *in vitro* culture system and the more stringent *in vivo* long term multilineage reconstitution employed to detect definitive haematopoietic stem cells.

The functional differences between yolk sac and intraembryonic haematopoiesis recently documented by two independent groups of investigators, in addition to those previously known, strongly argues for the development of primitive and definitive haematopoiesis in two separate sites in the mouse embryo. However, in the absence of grafting/chimaera technology, proving this remains difficult. Although haematopoietic activity can be detected simultaneously in yolk sac (low) and AGM (high), CFU-S generated from AGM morphologically resemble those generated from the liver *post* day 11 p.c. and adult bone marrow, ie, organs of definitive haematopoiesis (Medvinsky *et al.*, 1996). Additionally, culture experiments comparing embryonic tissue explants suggest that day 10 p.c. AGM can sustain the expansion of both CFU-S and long term reconstituting activity in isolation from all other embryonic tissues whilst yolk sac cannot (Medvinsky, pers. comm.). Long

term reconstitution of irradiated mice by 2 day cultured day 10 p.c. AGM reached a frequency of 90% compared to 3% by fresh day 10 p.c. AGM. Day 10 p.c. yolk sac showed no reconstituting ability either when fresh or after culture. However, both day 10 p.c. and day 12 p.c. yolk sac maintained their original (low) frequency of CFU-S activity indicating that culture conditions were not inhibitory to CFU-S formation. AGM derived haematopoietic stem cells appear, in two separate assays, to have the ability to expand their numbers, possibly due to their gaining competency and maturing within the AGM region. The results of the organ culture experiments go further and suggest that haematopoietic stem cells may be produced *de novo* in the AGM region. The AGM associated haematopoietic stem cells seem sufficiently different from yolk sac associated haematopoietic activity in terms of function that they may well represent definitive and primitive haematopoiesis. It is also a fact that transplantable, long-term reconstituting haematopoietic stem cells have not been detected in yolk sac before embryonic day 11 in the mouse (Müller *et al.*, 1994; Sonada *et al.*, 1983, Huang & Auerbach, 1993) despite readily detectable, overt haematopoiesis there in the form of CFU-C and CFU-S activity.

Further evidence to suggest that primitive and definitive haematopoiesis develops in two separate embryonic sites is provided by analysis of the cell surface antigens expressed on haematopoietic stem cells during development. Both foetal liver and bone marrow haematopoietic stem cells are Thy-1^{lo}, Lin^-, Sca-1^+, c-kit^+ (Ikuta & Weissman, 1992; Li & Johnson, 1992; Spangrude *et al.*, 1988). In addition, foetal liver cells are AA4.1^+ (Lemishka, 1993) and, recently, have been associated with low levels of Mac-1 staining (Morrison & Weissman, 1995). Intruiguingly, AA4.1 positive selection appears to enrich for the haematopoietic activity associated

with the P-Sp (Godin *et al.*, 1995). Stronger evidence is provided by recent observations that AGM derived cells capable of long term multilineage reconstitution reside wholly within a c-kit$^+$ population and that some haematopoietic stem cells in day 11p.c. AGM region are Mac-1$^+$ (Sanchez, pers. comm.) as are foetal liver haematopoietic stem cells. In contrast to definitive haematopoietic stem cells in foetal liver and bone marrow, primitive yolk sac progenitor cells do not express Sca-1 (Huang & Auerbach, 1993). Thus it seems that markers of mature haematopoietic stem cells such as c-kit and Sca-1 also mark definitive embryonic haematopoietic stem cells.

Recently described mouse mutants, generated by gene targeting, support the hypothesis that primitive, yolk sac haematopoiesis is independent from definitive, intraembryonic haematopoiesis. For example, mice lacking the gene for the transcription factor GATA-3 exhibit severely abnormal foetal liver haematopoiesis (Pandolfi *et al.*, 1995) but not yolk sac haematopoiesis, likewise, mice deficient in Myb exhibit a proliferative defect in definitive haematopoietic progenitors only (Mucenski *et al.*, 1991). These observations suggest that there are fundamental molecular and genetic differences between primitive and definitive haematopoiesis.

Considering evidence from embryologic studies of the mouse and examples provided by other species, it seems likely that the AGM is the origin of definitive haematopoiesis in the mouse. The enrichment and characterisation of AGM haematopoietic stem cells based on their surface antigen expression, in particular Ly-6E.1 based enrichment procedures for definitive haematopoietic stem cells, should enable the ontogeny of haematopoietic stem cells to be studied from the earliest stages of the mouse development.

2. Ly-6E.1

After depletion of cells bearing surface markers specific for mature haematopoietic lineages, positive selection with the Sca-1 antibody is the principal element in haematopoietic stem cell enrichment procedures (Li & Johnson, 1992; Smith *et al.*, 1991; Spangrude *et al.*, 1988; Uchida & Weissman, 1992). The Sca-1 antibody, E13 161-7 (Aihara *et al.*, 1986), detects a glycosylphosphatidylinositol (GPI) linked cell surface protein that is encoded by the allelic Ly-6E.1 and Ly-6A.2 genes (van der Rijn *et al.*, 1989). The coding regions of Ly-6E.1 and Ly-6A.2 differ at three nucleotide point mutations which result in two amino acid differences between the proteins (LeClair *et al.*, 1986; Palfree *et al.*, 1987; Reiser *et al.*, 1988). When enriching for bone marrow haematopoietic stem cells, monoclonal antibody D7 which detects a different epitope of Ly-6E/A can be substituted for Sca-1 without affecting the efficiency of the procedure (Li & Johnson, 1995; Spangrude *et al.*, 1995). As both D7 and Sca-1 antibodies recognise separate epitopes found on both Ly-6E.1 and Ly-6A.2 proteins, it is likely that Ly-6E.1 and Ly-6A.2 are allelic genes expressed in haematopoietic stem cells. Immunoprecipitation analysis has demonstrated directly that Ly-6E/A specific monoclonal antibodies react with the Sca-1 antigen and furthermore, that Ly-6E/A mRNA can be detected in lin^-, $Sca\text{-}1^+$ cells and not in lin^-, $Sca\text{-}1^-$ cells (van der Rijn *et al.*, 1989). The Ly-6E.1 and Ly-6A.2 genes exhibit strain specific differences in expression pattern within the bone marrow (Spangrude & Brooks, 1993). It has been shown, however that the Sca-1 antigen marks long term haematopoietic reconstituting stem cells from either Ly-6.1 or Ly-6.2 strains of mice (Jurecic *et al.*, 1993; Okada *et al.*, 1992; Spangrude & Brooks,

1993). The genes encoding both the Ly-6E.1 (Sinclair & Dzierzak, 1993) and Ly-6A.2 (McGrew & Rock, 1991; Stanford *et al.*, 1992) forms of Sca-1 have been cloned. Thus, a novel, molecular genetic approach may be applied to the study of haematopoietic stem cells, namely the use of these genetic elements to target heterologous gene expression to haematopoietic stem cells. This thesis will focus on the Ly-6E.1 gene for the following reasons: a larger genomic fragment has been isolated for the Ly-6E.1 gene (Sinclair & Dzierzak, 1993), whilst the smaller Ly-6A.2 genomic clone possesses a 5' bias (Stanford *et al.*, 1992) which may result in the absence of certain genetic regulatory elements from the Ly-6A.2 sequence and the regulatory elements of the cloned Ly-6E.1 gene have been more extensively studied than those of Ly-6A.2 (Sinclair & Dzierzak, 1993; Sinclair *et al.*, 1996).

2.1 Ly-6E.1 and the Ly-6 superfamily

Ly-6E.1 is a 10-12 kDa GPI-linked murine protein (Rock *et al.*, 1989). On the basis of amino acid sequence homology, Ly-6E.1 has been assigned to the Ly-6 superfamily of proteins (Barclay *et al.*, 1993) which includes, in addition to many murine proteins; human CD59 (Davies *et al.*, 1989; Stefanova *et al.*, 1989), squid neuronal protein gp2 (Williams *et al.*, 1988), human urokinase plasminogen activator (Palfree *et al.*, 1991), snake venom neurotoxins (Fleming *et al.*, 1993) and E48, a putative human desmosomal protein (Brakenhoff *et al.*, 1995). Homology searches have indicated that these sequences have been conserved through evolution and are distantly related to the extracellular domains of some growth factor receptors

(MacNeil *et al.*, 1993; Palfree *et al.*, 1991). Members of the Ly-6 superfamily are all GPI-anchored to the cell surface and are characterised by 10 cystein residues, which are wholly conserved between family members (Barclay *et al.*, 1993; Gumley *et al.*, 1992). These 10 conserved cystein residues are equally spaced in the amino acid sequence of every member of the Ly-6 superfamily and are predicted to form disulphide bridges resulting in a complex protein structure consisting of a tight, globular core with finger-like projections (Fleming *et al.*, 1993). Such a predicted structure could be involved in ligand binding or cell-cell interactions.

Within the haematopoietic system of the mouse, antisera initially defined at least six Ly-6 related antigens; A.2, B.2, C.2, D.2, E.1 & ThB, each exhibiting a distinct expression pattern (Hogarth *et al.*, 1987; Kimura *et al.*, 1980; Kimura *et al.*, 1984; Yutoku *et al.*, 1974). Genetic linkage studies localised the Ly-6 encoding genes to a particular region of chromosome 15 (LeClair *et al.*, 1987), closely linked to *IL-2Rβ* , *myc*, *Pdgf* and *sis* genes (Huppie *et al.*, 1988; MacNeil *et al.*, 1993; Malek *et al.*, 1993; Meruelo *et al.*, 1987). However, it remained unclear whether some, or all, of the Ly-6 antigens were derived from differentially processed forms of the same gene or whether the locus contained multiple, related genes. Cloning of the Ly-6E.1 cDNA and its subsequent use as a probe in genomic Southern blot analysis identified numerous hybridising bands suggestive of the presence of a family of genes with considerable sequence homology (LeClair *et al.*, 1986). Utilising the homology between Ly-6 coding regions, Ly-6C.2 (Palfree *et al.*, 1988), Ly-6A.2 (Palfree *et al.*, 1987; Reiser *et al.*, 1988), ThB (Gumley *et al.*, 1992), TSA-1 (MacNeil *et al.*, 1993), Ly-6F.1 and Ly-6G.1 (Fleming *et al.*, 1993) have also been cloned, either as cDNA or genomic clones. Long range mapping techniques have

demonstrated that the Ly-6 locus exceeds 1600Kb and contains at least 18 highly homologous genes arranged in three clusters (Kamiura *et al.*, 1992), suggesting that the Ly-6 family has evolved as a result of gene duplication events. In view of the complexity within the Ly-6 locus, it is difficult to determine how many of the Ly-6 related genes are functional and how many are merely pseudogenes. The non-murine members of the Ly-6 superfamily characterised to date show such DNA sequence diversity that they are thought either not to be homologues or to have diverged early in evolution (Fleming *et al.*, 1993). However, given the degree of conservation of certain aspects of gene structure and amino acid sequence observed across such species as mice, human, snakes and squid it is likely that the Ly-6 superfamily represents an ancient family of genes (Barclay *et al.*, 1993).

Recent work by Brakenhoff and co-workers is suggestive of the existence of a human Ly-6 family analogous to mouse, with a novel and exciting function (Brakenhoff *et al.*, 1995). The monoclonal antibody E48 has been developed to assist the treatment of squamous cell carcinoma and seems to detect an antigen associated with desmosomal cell to cell junctions - consistent with the presumed importance of desmosomes in the development of transformed cells into metastatic, invasive tumour cells. Using the E48 antibody to purify protein for sequencing and to screen expression libraries, Brakenhoff *et al* cloned the cDNA encoding the E48 antigen and demonstrated that E48 possessed the 10 conserved cystein residues that place it within the Ly-6 superfamily. Unlike any other non-murine member of the Ly-6 superfamily, the human E48 antigen showed a high degree (>70%) of Ly-6 amino acid sequence homology across its entire sequence. E48 is homologous to murine ThB, GPI-anchored and the gene encoding it maps to human chromosome 8 in the q24 region,

syntenic with chromosome 15 of the mouse which is the location of the Ly-6 locus. Analysis of mouse tissues showed that, in addition to lymphocytes, both mThB and mLy-6A.2 are expressed on keratinocytes (Hogen-Esch *et al.*, 1993). Additionally, the overexpression of both Ly-6A.2 (Bamezai & Rock, 1995) and the E48 antigen (Brakenhoff *et al.*, 1995) can induce cell aggregation in transfected cell lines. Thus, these striking similarities between mouse and human Ly-6 proteins suggest that the Ly-6 superfamily may have an additional role in metastasis and cell migration/infiltration.

2.2 The expression pattern of Ly-6E.1 and the Ly-6 antigens.

The Ly-6 proteins exhibit complex, often overlapping expression profiles throughout many murine tissues. Studies have tended to focus on expression in cells of the haematopoietic system, for example, Ly-6E/A was initially described as a T cell activation marker (Horton & Sachs, 1979). Ly-6E.1 and Ly-6A.1 are also expressed in the central nervous system (Cray *et al.*, 1990), some keratinocytes and dendritic cells (Hogen-Esch *et al.*, 1993), osteoblasts (Horrowitz *et al.*, 1994), kidney (Blake *et al.*, 1993) and on many transformed cell lines (Lollini *et al.*, 1992). Ly-6E.1 and Ly-6A.2 are dramatically upregulated upon T cell activation, with the result that all activated T cells are Ly-6E/A$^+$ (Kimura *et al.*, 1984). Also, expression is often upregulated in other tissues as a result of cytokine stimulation. All three interferons, α, β and γ upregulate Ly-6E/A expression suggesting that common control mechanisms operate in a diverse range of tissues (Blake *et al.*, 1993;

Horrowitz *et al.*, 1994). Due to the high degree of homology that exists between members of the Ly-6 family at both the nucleotide sequence level and in terms of the predicted protein structure, detailed expression studies are inherently difficult to interpret as both *in situ* hybridisation probes and monoclonal antibodies/antisera have a tendency to cross react. The remainder of this discussion will focus on haematopoietic specific expression of the Ly-6 family.

The expression pattern of the Ly-6 family of proteins in the haematopoietic system of the mouse is complex (see Table 1) and although allelic forms can account, in part, for the serological complexity seen, it is clear that these genes have intricate tissue specific, developmentally regulated patterns of expression that often overlap. The functional consequences of their complex expression pattern is not known but may reflect an important role for Ly-6 proteins in the subtle modulation of the haematopoietic system. Although Ly-6E.1 and Ly-6A.2 are strain specific alleles of the same gene, table 1 shows that they have different expression patterns within the haematopoietic system. Ly-6A.2 is expressed on a greater number of lymphocytes within thymus, lymph nodes and spleen than Ly-6E.1 (Codias *et al.*, 1989; Kimura *et al.*, 1984; Rock *et al.*, 1986; Yeh *et al.*, 1986). However caution must be exercised in the interpretation of such comparisons between individual animals. Although Ly-6E.1 and Ly-6A.2 are expressed on 100% of T cells activated *in vitro*, individual animals used in these studies may be in different states of health and possess different numbers of activated lymphocytes *in vivo*. Consequently, differences in health may distort the result of *in vivo* studies of Ly-6E.1 and Ly-6A.2 expression. In addition, although the expression of the Ly-6E.1 and Ly-6A.2 genes

Table 1. Expression analysis of Ly-6 antigens within the haematopoietic system.

Surface antigen expression within specific tissues is represented as follows: ++++ = 75-100%; +++ = 50-75%; ++ = 25-50%; + = 5-25%; - = less than 5%; n.d. = not done. Data compiled from: Kimura *et al.*, 1984; Houlden *et al.*, 1986; Tucek *et al.*, 1992; Fleming *et al.*, 1993.

Antigen	Thymus	Lymph Node	Spleen	Bone Marrow	Activated Lymphocytes
Ly-6A.2	+	++	++	+	++++
Ly-6B.2	-	+	+	++	-
Ly-6C.2	-	++	++	++	++
Ly-6D.2	+++	+++	++	+	++++
Ly-6E.1	+	+	+	+	+++
Ly-6G.1	-	-	-	+++	-
TSA-1	++++	-	-	+++	n.d.
ThB	++++	++	++	++	n.d.

is thought to be codominant (Codias *et al.*, 1989), that both are expressed normally in F1 heterozygotes, detailed studies on the strain specific nature of Ly-6 expression patterns remain to be conducted.

The general observation that Ly-6A.2 is expressed on a greater number of haematopoietic cells than Ly-6E.1 may be indicative of a more widespread phenomenon common to all Ly-6 related genes on chromosome 15. Both ThB (Brakenhoff *et al.*, 1995; Gumley *et al.*, 1992) and Ly-6C (McCormack *et al.*, 1993) demonstrate variable levels of expression between mice of the Ly-6.1 and Ly-6.2 haplotypes with both being expressed at higher levels on cells derived from Ly-6.2 mice. In the case of ThB, the strain specific high and low expression levels are observed for lymphoid expression but not expression on keratinocytes. Keratinocytes express high levels of ThB in both Ly-6.1 and Ly-6.2 strains of mice (Brakenhoff *et al.*, 1995; Gumley *et al.*, 1992) possibly reflecting different cell specific regulatory mechanisms. The differences in expression occur at the level of mRNA, as detected by Northern blot analysis (Brakenhoff *et al.*, 1995), suggesting transcriptional regulation is important. It will be interesting to determine whether the whole locus exhibits differential expression depending upon mouse strain and how tissue specificity of expression is regulated.

2.3 What is the function of Ly-6 proteins?

Binding of monoclonal antibodies to Ly-6E.1 and Ly-6A.2 on lymphocytes induces the production of IL-2 and results in mature T and B cell activation (Malek *et al.*, 1986; Rock *et al.*, 1986; Codias and Malek, 1990). Although antibody cross linkage of other GPI-linked proteins, for example Thy-1 and Qa-2, can result in T cell activation (Kroezek *et al.*, 1986; Steinberg *et al.*, 1987) the specific importance of Ly-6A.2 has been demonstrated by the study of mutant cell lines lacking Ly-6A.2 or cell lines depleted of Ly-6A.2 using antisense oligonucleotides (Yeh *et al.*, 1988; Flood *et al.*, 1990). In these experiments, Ly-6A.2 was shown to be necessary for antigen mediated activation through the T cell receptor (TCR). Further investigations involving antibodies to other Ly-6E.1/Ly-6A.2 epitopes have suggested that, depending upon the types of costimulatory molecules utilised, for example the presence of specific CD3 chains, the response to mitogenic stimuli may be repressed (Codias *et al.*, 1990, 1992).

Lu *et al.*, (1989) also presented evidence that the Ly-6E.1 and Ly-6A.2 molecules play a role in modulating the immune system. In this study, monoclonal antibodies to Ly-6E.1 and Ly-6A.2 were able to surpress sarcoma, leukemia and melanoma growth *in vivo*, inducing tumour regression in mice. This effect was dependent upon the Ly-6 haplotype of the host and not the tumour, suggesting that the antibodies were able to upregulate host T cell and natural killer (NK) cell antitumour responses. Further, detailed analysis *in vitro* showed that anti-Ly-6E.1 antibodies stimulated T cells to produce TNFα which increased the IL-2 dependent antitumour activity of NK cells (Lu *et al.*, 1989; Lu *et al.*, 1994).

Although the precise role of the Ly-6E.1/Ly-6A.2 proteins is unclear, several lines of evidence point towards their involvement in the transduction of signals across the cell membrane in response to extrinsic stimuli. GPI linked proteins have no intracellular domain, however, an unidentified cytoplasmic tyrosine kinase activity (other than that of p56*lck*) has recently been copurified with Ly-6E.1/Ly-6A.2 in immunoprecipitation experiments (Stefanova *et al.*, 1991; Bohuslav *et al.*, 1993). Association with tyrosine kinases may be the way in which antibodies to Ly-6E.1 and Ly-6A.2 can bring about such changes in cellular physiology as T and B cell activation.

Loss of function experiments conducted by Lee *et al.* (1994) suggests an identity for the unknown tyrosine kinase and provides direct molecular evidence for the importance of Ly-6E.1 in normal T cell function. In these studies, a $CD4^+$, $CD45^+$, Ly-6A.2^+ mature T cell clone was depleted of Ly-6A.2 by generating transfectants expressing antisense Ly-6A.2. In Ly-6A.2^- and Ly-6A.2^{lo} cells, T cell activation was inhibited, TCR expression was reduced and p59*fyn in vitro* kinase activity was impaired. Rescuing TCR expression with a TCRβ transgene only partially restored T cell activation potential and had no effect on p59*fyn* kinase activity. Rescuing the Ly-6A.2 defect with an Ly-6E.1 transgene fully restored activation potential and p59*fyn* kinase activity (Lee *et al.*, 1994). This work provides the most conclusive evidence of a role for Ly-6A.2/Ly-6E.1 in signal transduction and also demonstrates that Ly-6E.1 and Ly-6A.2 are functionally equivalent, at least in this T cell clone. This apparent functional equivalence raises the question of why Ly-6E.1 and Ly-6A.1 are expressed at different levels and on different cells within the haematolymphoid system of Ly-6.1 and Ly-6.2 mice.

In a separate study, Bamezai *et al* generated transgenic mice constitutively over expressing Ly-6A.2 on T cells throughout their development in the thymus (Bamezai *et al.*, 1995), under control of the human CD2 3' enhancer element. In contrast to transgenic mice over expressing Ly-6E.1 under complete control of the human CD2 promoter and LCR (Appendix, this report), Ly-6A.2/CD2 transgenic mice display a marked impairment of T cell development within the thymus (Bamezai *et al.*, 1995) and transgenic thymocytes exhibit homotypic adhesion in culture (Bamezai & Rock, 1995). Bamezai and Rock concluded that Ly-6A.2 binds a cell surface ligand which is expressed on all thymocytes. Intriguingly, over expression of the recently cloned E48 protein, a putative human desmosomal protein, in cell lines also results in homotypic aggregation (Brakenhoff *et al.*, 1995). Ly-6A.2/CD2 transgenic thymocytes should provide a useful screening tool for the identification of an Ly-6A.2 ligand. The fact that CD2/Ly-6E.1 transgenic thymocytes do not display altered adhesion properties suggests that the allelic Ly-6E.1 and Ly-6A.2 molecules have different ligand specificities. Either Ly-6E.1 and Ly-6A.2 are not functionally equivalent in the thymus, though both exhibit a dramatic downregulation in cell surface expression at the double positive $CD4^+$ $CD8^+$ stage of development (Altemeyer *et al.*, 1991; Codias *et al.*, 1989), or differences between the design of the two experiments may account for the different phenotypes. It is possible that the background strain of transgenic mice used, either BALB/c or (CBA/Ca x $C57/Bl10)_{outbred}$ or the cell surface protein expression levels could affect both T cell development and homotypic adhesion in the different transgenic experiments. In addition, the Ly-6A.2/CD2 transgene used the Ly-6A.2 proximal promoter, whereas the CD2/Ly-6E.1 transgene used the human CD2 5' promoter, so there may be

subtle, yet significant, differences in the cell type over expressing either Ly-6E.1 or Ly-6A.2. The resolution of these questions is vital for the interpretation of these experiments and more lines of transgenic mice may need to be analysed to enable conclusions to be drawn.

In summary, our understanding of the function of the Ly-6 family of proteins in particular, Ly-6E.1/Ly-6A.2 remains unclear. Evidence obtained to date suggests a role for Ly-6E.1/Ly-6A.2 in both signal transduction resulting in subtle modulation of the haematopoietic system and intercellular interactions/adhesion.

2.4 The regulation of Ly-6E.1 gene expression.

The pattern of Ly-6E.1/Ly-6A.2 protein expression has been extensively studied and shown to be complex (see Table 1). Recent evidence points to an emerging concept that, despite their often overlapping expression profiles, members of the Ly-6 superfamily define functionally distinct subsets of cells within the haematopoietic system (Codias and Malek, 1993). Not only is this of interest physiologically, it also raises the question of how these diverse, yet coordinated, expression patterns are regulated and, in particular, what is the nature of the mechanisms controlling gene expression within the haematopoietic stem cell.

The expression of Ly-6E.1 and Ly-6A.2 has been shown to be influenced by certain cytokines. Interferons (IFNs) can dramatically upregulate Ly-6E.1 and Ly-6A.2 expression on T and B cells (Dumont and Boltz, 1987; Snapper *et al.*, 1991b) as can tumour necrosis factor (TNF) α and interleukin 1 (IL-1) (Altmeyer *et al.*,

1991). IFNs and TNF-α can synergise in their enhancement of Ly-6E.1/Ly-6A.2 expression on thymocytes and bone marrow cells (Malek *et al.*, 1989). This synergy only occurs in mature T cells in strains of mice of the Ly-6.1 haplotype, adding a further level of compexity to the regulation of Ly-6E.1 gene expression and suggesting that the regulation is at the level of the Ly-6 genetic locus itself. Successful ectopic expression of both Ly-6E.1 and Ly-6A.2 cDNAs also provides evidence that, in the thymus at least, the regulation of Ly-6E.1 expression probably occurs at the level of transcription.

The genetic elements of the Ly-6 genes that direct their complex expression profiles have yet to be fully analysed. The Ly-6E.1 and Ly-6A.2 genes are comprised of four exons, characteristic of the mouse Ly-6 family and a trait conserved throughout Ly-6 superfamily members in all species (Bothwell *et al.*, 1988; Fleming *et al.*, 1993). Analysis of cDNAs from the two alleles show that Ly-6E.1 contains an extra 46bp of untranslated sequence (LeClair *et al.*, 1986; McGrew and Rock, 1991) which may play a role in the regulation of Ly-6E.1/Ly-6A.2 expression.

Khan *et al.* (1990), isolated overlapping genomic phage clones corresponding to portions of the Ly-6E.1 and Ly-6A.2 genes and undertook the first analysis of their genetic regulatory elements. By linking a panel of 5' deletion constructs to a chloramphenicol acetyltransferase (CAT) reporter gene and analysing transfectants of the fibroblast L cell line, several elements of the promoter were discovered. Of particular interest were a γ-interferon activation site (GAS) located between 0.9Kb and 1.76Kb upstream of the transcription start site as well as Y-box and ISRE motifs within the proximal promoter, these showing homology to regions of the MHC class II promoters (Khan *et al.*, 1990). Both Ly-6E.1 and Ly-6A.2 promoters are also

known to lack a TATA box but to contain five CCAAT boxes, a purine rich element at -110bp to the start site which is necessary for constitutive expression in L cells and a B1 repeat at -300bp (Khan *et al.*, 1990; Stanford *et al.*, 1992). These elements have also been located within the Ly-6C.1 gene (McGrew and Rock, 1991) illustrating the sequence similarity between members of the Ly-6 superfamily.

The recent cosmid based cloning of a full length Ly-6E.1 gene (Sinclair and Dzierzak, 1993) has provided an opportunity to conduct a more extensive study of the regulatory elements of this gene. Initial studies, using nuclease sensitivity of chromatin (Gross *et al.*, 1988) to identify putative regulatory elements, have identified eight regions of DNaseI hypersensitivity associated with the Ly-6E.1 gene (Sinclair and Dzierzak, 1993). The appearance of some of the DNaseI hypersensitive sites (HSSs) correlate with the expression of the gene in haematopoietic cell lines. In particular, five of the DNaseI HSSs were induced in mature cells (the mature T cell BW5147 and the mouse erythroleukaemia (MEL) cell line) and an immature, multipotent cell line (FDCP-1), suggesting that they correspond to putative genetic regulatory elements (Sinclair and Dzierzak, 1993). As expected, if this were the case, hypersensitive sites were mapped upstream of the gene at -1.2Kb and -0.1Kb. The location of these sites corresponds to the interferon inducible element and the purine rich element previously identified in L cell transfectants (Khan *et al.*, 1990). It is interesting to note the presence of six regions of DNase 1 hypersensitivity in the 3' region of the Ly-6E.1 gene (Sinclair and Dzierzak, 1993), as far distal as 8.9Kb from the transcriptional start site. The appearance of the four most distal HSSs correlate precisely with expression of Ly-6E.1 in the panel of haematopoietic cell

lines. Thus Sca-1 expression on haematopoietic cells is associated with regions of DNaseI hypersensitivity 3' to the coding region of Ly-6E.1.

Recently more direct evidence has indicated that regions containing the DNaseI HSSs are required for high levels of Ly-6E.1 expression in transfected haematopoietic cells. In these studies, Ly-6E.1 constructs were linked to a human growth hormone reporter gene and expression levels determined in stably transfected MEL cell populations (Sinclair *et al.*,1996). The MEL cell line does not normally express its endogenous Ly-6A.2 gene and requires γ-IFN induction to stimulate expression. When various 5' and 3' deletion constructs were assessed for expression, the proximal Ly-6E.1 promoter up to 1.8Kb upstream yielded almost undetectable levels of growth hormone after γ-IFN induction, consistent with previous studies (Khan *et al.*, 1990; Stanford *et al.*, 1992). High levels of expression in transfected MEL cell populations required constructs containing the 3' hypersensitive sites at +8.7 and +8.9 (Sinclair *et al.*, 1996). Sequence analysis of the region encompassing DNaseI HSSs +8.7 and +8.9 showed the presence of an ISRE consensus sequence and potential binding sites for both Myb and AP-1 at +8.7 and for Ets-1 at +8.9 (Sinclair *et al.*, 1996). The dramatic increase in expression brought about by the addition of 3' sequences, in particular, the +8.7 and +8.9 region of Ly-6E.1 strongly suggests an important role for this region in the regulation of Ly-6E.1 expression *in vivo*.

Previously, analysis of the 5' part of the Ly-6E.1 gene, extending 6Kb upstream, has shown that this region is insufficient to direct expression of a linked reporter gene *in vivo* using transgenic mice (Weissman, pers. comm.). For this reasons and in view of the results of Ly-6E.1 deletion analysis in stable transfectants,

this thesis will examine the *in vivo* expression of the full length 14Kb subclone of the Ly-6E.1 gene, containing both 5' and 3' flanking regions, linked to heterologous genes in transgenic mice.

3. Project Aims.

As outlined in the previous sections, the precise identification and molecular characterisation of haematopoietic stem cells remains elusive. One property consistently associated with haematopoietic stem cells is expression of the Sca-1 antigen. The cloning of cDNAs and genomic regions corresponding to the Sca-1 encoding alleles Ly-6E.1 and Ly-6A.2 has facilitated the application of molecular biological techniques to the study of Sca-1 and haematopoietic stem cells.

The studies in this thesis attempt to further characterise the cloned Ly-6E.1 gene at the molecular level and to assess the feasibility of using this gene in molecular appoaches to study haematopoietic stem cells *in vivo*. In particular, the specific aims are: i) To determine whether the cloned Ly-6E.1 gene contains sufficient regulatory sequences for tissue specific expression *in vivo*, using transgenic mice; ii) Having described an Ly-6E.1 clone that is functional *in vivo*, perform deletion analysis to identify elements of the gene involved in regulating levels and tissue specificity of expression; iii) To investigate the suitability of cloned Ly-6E.1 sequences for the molecular targeting of haematopoietic stem cells by directly assessing Ly-6E.1 directed heterologous gene expression in haematopoietic stem cells *in vivo*; iv) To manipulate haematopoietic stem cells *in vivo* by expressing either *c-myc*, to induce

cell cycling, or *bcl-2*, to block apoptosis, in an attempt to disrupt the processes of haematopoietic stem cell quiescence and self-renewal; and v) To investigate the potential of Ly-6E.1/*myc*/*bcl-2* bone marrow to facilitate the derivation of early haematopoietic cell lines. The results of these investigations and their implications for haematopoietic stem cell biology will be the subject of this thesis.

MATERIALS AND METHODS

1. Nucleic acids - DNA.

1.1 Genomic DNA preparation.

Tissue samples (~100mg) were carefully homogenised with a glass mortar and eppendorf tube in 430μl of tail mix buffer. To this, 50μl of 10% SDS, and 20μl of proteinase-K (10mg/ml) were added with gentle mixing. The samples were incubated at 55°C overnight, followed by a 1hr incubation at 37°C with 10μl of 10mg/ml DNAse-free RNAse. The DNA was cleaned by 2x phenol/chloroform extractions, plus one chloroform extraction, and precipitated from the aqueous layer with 0.6 volumes of isopropanol. The DNA was hooked out using a sterile glass pasteur pipette, washed in 70% ethanol, and air-dried for 5 minutes. It was then resuspended in 50-200μl of TE pH 7.4, and stored at -20°C until needed.

DNA from tail cuts of mice was obtained as above, with the exception of the homogenisation step, while cells from culture were resuspended in tail mix, before proceeding as described.

1.2 DNA fragment purification.

All DNA fragments for either cloning or DNA probes were isolated using the GELase™ (Cambio, Cambridge) or QIAEX™ (QIAGEN) protocols.

QIAEX™ method: The DNA was run on an ethidium bromide (1μg/ml) stained, 1xTAE/agarose (0.8-1.5%) gel, and the desired band removed with a sterile scalpel blade. The gel slice was weighed in an eppendorf tube, and 300μl of QX1™ solution per 100mg of gel was added. To this, 10-20μl of QIAEX™ resin was added and the sample was incubated at 50°C for 10 min. After centrifugation at 14K rpm for 30 seconds the supernatant was removed. 2x washes with QX2™ solution and QX3™ solution followed, with centrifugation at 14K rpm after each wash. The QIAEX™ resin pellet was then air-dried, before the DNA was eluted in 20-50μl of TE pH 7.4.

GELase™ method: The DNA was run on an ethidium bromide (1μg/ml) stained, 1xTAE/low melting point agarose (0.8-1.5%) gel, and the desired band removed with a sterile scalpel blade. The gel slice was weighed in an eppendorf tube and 1μl of 50x GELase™ buffer added per 50mg of gel. The gel slice was then completely melted at 70°C (approx. 20 min). The sample was then equilibrated to 42°C for 10 min., before 1 unit of GELase™ enzyme was added per 300mg of 1% LMP agarose gel. Incubation was from 1hr to overnight. One volume of 5M ammonium acetate, followed by 4x the original volume of room temperature absolute ethanol was then added. The DNA was pelleted by centrifugation for 30 min. at 14k rpm, and the supernatant removed. After air-drying the DNA was resuspended in 10-50μl of TE pH7.4.

1.3 DNA quantitation.

For concentrations of DNA thought to be in excess of 300ng/μl, the sample was diluted 1:200 and the absorbance was read at 260/280nm on a spectrophotometer. For samples less than 300ng/μl, 1-2μl of the DNA was run on a 1% agarose/1xTAE ethidium bromide (1μg/μl) stained gel, alongside known amounts of standard DNA. The concentration of the sample was then estimated by comparing the intensity of its fluorescence to that of the standards, under a UV light.

1.4 DNA restriction digests.

DNA digestion with restriction endonucleases were performed using the optimal conditions as recommended by the manufacturer. Digests were performed using 1x buffer, DNA (10ng to 1μg/μl) and 0.5-5 units of enzyme per μg of DNA. The total voume of enzyme was always limited to no more than 10% of the reaction volume to avoid glycerol inhibition of digestion. Incubations were at 37°C, unless otherwise instructed, for periods from 1hr to overnight.

1.5 Slot blot analysis of DNA.

Slot blot analysis of DNA from tissue samples. 5μg of DNA was added to dH_2O, to a final volume of 180μl. To this 20μl of 4M NaOH was added, mixed and

left at room temperature for 5min. The slot blot manifold was prepared by laying 2 sheets of 2x SSC soaked Watman 3MM paper onto the lower half of the apparatus. On this was placed a pre-wet nitrocellulose filter (0.45-micron pore size) that had been soaked in 1M ammonium acetate for 10min. The top manifold was fitted and clamped in place. The vacuum line was attached and turned on. Under suction, each slot was filled with 1M ammonium acetate and allowed to empty. To the DNA samples 200μl of 2M ammonium acetate was added and mixed. The 400μl sample was then added to the slot and allowed to pass through onto the filter. Known amounts of plasmid DNA added to 5μg of normal mouse genomic DNA were used as copy number controls. After the last sample the filter was allowed to dry under suction for 5min. The vacuum line was then removed. The filter was air-dried then baked in an 80°C oven for 2hr. The blots were hybridised as for Southern bolt analysis.

1.6 Southern blot analysis of DNA.

Between 5-20μg of genomic DNA was digested overnight with the appropriate restriction enzyme(s). The sample was then mixed with 0.2 volumes of orange-G loading dye, and loaded onto an ethidium bromide (1μg/μl) stained agarose (0.8%-1.2%)/TAE (1x) gel. Bacteriophage Lambda DNA, digested with BstEII, was used as a DNA size marker, and plasmid DNA of known concentration was added to normal non-transgenic mouse DNA to generate copy number controls. The samples were run through the gel at between 1-5 V/cm, until the orange G front had reached

the end of the gel. The gel was photographed with ultraviolet light using a video camera and image processing system (Cybertech CS1) and the position of the Lambda size markers were noted. The gel was inverted and soaked in 0.25M HCl for 20 min at room temperature, with gentle agitation. The gel was then washed twice with 0.5M NaOH/1.5M NaCl for 20min., followed by 2x 20min. washes in 0.5M Tris pH 7.4/1.5M NaCl. The inverted gel was then placed onto a wick comprised of a piece of Watman 3MM paper dipped into a tray of 20x SSC. The following was placed on top of the gel ensuring that no air bubbles were trapped; 1 piece of nylon (Nytran) filter soaked initially in H_2O then in 20x SSC for 20 min., 2-4 20x SSC-soaked pieces of 3MM paper, 20-40 sheets of dry 3MM paper, a 5cm stack of dry paper towels, a glass plate and two 500ml bottles half filled with water (weight approx. 0.5kg). This blotting set-up was left for 12-16hr. The apparatus was dismantled and the position of the wells were marked on the filter with a water-proof pen. The filter was then baked at 80°C for 2hr. and hybridised as described below.

1.7 Oligo-labelling of DNA probes (random priming).

DNA fragments from 100-5000bp were used as DNA probes. 100ng of DNA in 7μl of ddH_2O was denatured by boiling at 100°C for 5min, then placed on ice. To this, 12μl of 2xOLB mix, 1μl of 1mg/ml BSA, 3μl of ^{32}P α-dATP and 1μl of Klenow enzyme (5U/μl) were added. The mix was incubated at 37°C for 30min, with the reaction terminated by the addition of 10 μl of 0.25M EDTA. The labelled DNA was separated from unincorporated nucleotides by spinning the DNA through a 2x SSC-

equilibrated G50 sephadex column. Probes with a specific activity above $1x10^8$ cpm/μg of DNA were used.

1.8 End-labelling of DNA probes.

DNA probes were end-labelled using T4 polynucleotide kinase. The DNA fragment was prepared by leaving a 5' overhang at the end of the DNA that is to be labelled, by digestion with an appropriate restriction endonuclease. The phosphate was removed from this 5' end by incubation with 1μl of calf intestine alkaline phosphatase (1U/μl) for 30 min at 37°C. The DNA was cleaned using 1x phenol/chloroform plus 1x chloroform extraction, followed by an ethanol precipitation. The labelling reaction involved 100 ng of this prepared DNA in 10μl. To this 4μl of 5x kinase buffer, 5μl of ^{32}P γdATP, and 1μl of T4 polynucleotide kinase were added, and the mixture incubated at 37°C for 30 min. The reaction was terminated by adding 1μl of 0.5M EDTA and 1μl of 10% SDS and the labelled DNA separated from the unincorporated nucleotide by spinning it through a G50 sephadex column. Probes with a specific activity greater than $1x10^7$ cpm/μg were used.

1.9 Hybridisation of DNA filters.

DNA filters were pre-wet in 2x SSC then placed in Hybaid hybridisation bottles. The filters were pre-hybridised for 2hr at 68°C in 20ml of DNA pre-

hybridisation solution without the radio-labelled probe. The pre-hybridisation solution was removed. The probe was then heat denatured at 100°C for 5 mins, added to 20ml of DNA hybridsation solution and incubated overnight at 68°C. The filters were then washed twice for 20 min in 2x SSC/0.1% SDS and twice for 20 min in 0.2x SSC/0.1% SDS. The filters were then covered in Saran wrap and exposed to either Kodak XAR5 or Fuji RX100 film at -70°C with intensifying screens. Alternatively, the filters were exposed to a Molecular Dynamics Phosphorimager screen. The image was then scanned into the phosphorimager and analysed using the ImageQuant softwear package.

1.10 Polymerase chain reaction (PCR).

Reagents for PCR, including equipment and test samples, were kept physically isolated from potential sources of contamination - such as plasmids and amplified PCR products - by the use of separate laboratories/laminar flow hoods. 200ng genomic DNA prepared under "PCR clean" conditions using filtered Gilson tips was used in each assay. To this, a reaction mix containing PCR mix(2x) and 100ng of each oligonucleotide was added, overlayed with mineral oil and subjected to thermal cycling as detailed below. PCR products were electrophoresed through agarose/TAE gels of between 1.2-2% and visualised by ethidium bromide staining.

All genomic DNA PCRs were subject to an initial 5 min denaturation at 94°C followed by 30 cycles of: denaturation - 5 sec @ 94°C; annealing - 30 sec @ 60°C;

30 sec elongation @ 72°C. A final elongation step of 5 min at 72°C ensured all PCR products were full length.

Oligonucleotides used in PCR:

name	Sequence (5'-3')	Comment
PE4	GAA GTG TCC ATC CTC AGA GAA GGG	Ly-6E.1 exon 2
PE5	ACT CTG CCT GCA ACC TTG TCT GAG	Ly-6E.1 exon 1
CMEX2	GGC ATC GTC GTG GCT GCT TG	c-myc exon 2
BC2PE	GTG CAG CTG ACT GGA CAT CTC TGC	bcl-2 cDNA
YMT2	CAG TTA CCA ATC AAC ACA TCA C	Y chromosome
YMT1	CTG GAG CTC TAC AGT GAT GA	Y chromosome
MYO1	TTA CGT CCA TCG TGG ACA GC	myogenin cDNA
MYO2	TGG GCT GGG TGT TAG TCT TA	myogenin cDNA
LACZ1	GCG ACT TCC AGT TCA ACA TC	
LACZ2	GAT GAG TTT GGA CAA ACC AC	

2. Nucleic acids - RNA.

2.1 RNA preparation.

For the preparation of RNA, gloves, sterile plastic, and fresh DEPC-treated and autoclaved solutions were used, and the samples were kept at 4°C or below at all times. Tissue samples or cell pellets were placed directly into 3ml of 3M LiCl/6M urea on ice. The tissue samples were homogenised with the ultra-turrax on full power for 1 min. Samples were then sonicated for 1-2 min to shear the genomic DNA and then left at 4°C overnight. The solution was centrifuged at 14K rpm at 4°C for 30 min. The supernatant was removed and the pellet pipetted back and forth in 500μl of 3M LiCl/6M urea for 1-2 min. The sample was again centrifuged at 4°C for 30min and the supernatant was removed. The pellet was then resuspended in 300μl of 10mM Tris/0.5% SDS. Folllowing this, 2x phenol/chloroform, plus 1x chloroform extractions were performed. The RNA was precipitated with 30μl of NaOAc and 800μl of ethanol. The sample was left on dry ice for 30 min, before it was pelleted at 4°C, 14K rpm, for 30 min. The pellet was washed with 70% ethanol and resuspended in 20-100μl of DEPC-treated water. The samples were stored at -70°C until required.

2.2 Northern blot analysis of RNA.

The gel tank and apparatus was first washed well with 0.1M NaOH and rinsed in DEPC-treated H_2O. RNA samples (5-30μg) were made up to 4.5μl in DEPC-treated H_2O. To this, 2μl of 10x MOPS, 3.5μl of 37% formaldehyde, and 10μl of 100% formamide were added and mixed well. The samples were then incubated at 68°C for 15min before being removed to ice. Northern loading dye (0.2 vol.) was added, and the samples were loaded onto an agarose (0.8%-1.2%)/MOPS (1x)/formaldehyde (6%) gel. The samples were run through the gel at 1-5 V/cm until the furthest blue dye front had reached the end of the gel. The gel was then soaked in 50mM NaOH/100mM NaCl for 20min, 100mM Tris pH 7.6 for 20 min, and 20x SSC for 20 min. The gel was then blotted to 20x SSC soaked nitrocellulose of Hybond-N (Amersham) filters in the manner described for Southern blot transfer. After 12-16hr the filter was removed and baked in an 80°C oven for 2hr, and hybridised as described below.

2.3 Hybridisation of RNA filters.

RNA filters were pre-wet in 2x SSC then placed in Hybaid hybridisation bottles. The filters were pre-hybridised for 2hr at 42°C in 20ml of RNA hybridisation solution without the radio-labelled probe. The pre-hybridisation solution was removed. The probe was then added to 20ml of fresh RNA hybridisation solution and incubated overnight at 42°C. The filters were then washed twice for 20 min in 2x

SSC/0.1% SDS and twice for 20 min in 0.2x SSC/0.1% SDS. The filter was then covered in Saran wrap and exposed on film (4hr-1wk) or on the Molecular Dynamics Phosphorimager.

2.4 S1 nuclease protection analysis.

S1 nuclease protection analysis was used to detect specific mRNAs in mouse tissues. 5-30μg of RNA and 10ng of end-labelled probe were mixed, made up to 300μl with H_2O, and precipitated using 30μl of 2M NaOAc and 800μl of 96% ethanol (10 min dry ice). The nucleic acids were pelleted by centrifugation at 14k rpm, air-dried for 5min, and resuspended well in 15μl of S1 nuclease hybridisation buffer. The samples were then incubated at 90°C for 5 min to denature the RNA, and then swiftly transferred to a water bath at 50-55°C. The probe and RNA were allowed to anneal in these conditions for at least 16hr. To each sample 200μl of ice cold S1 digestion mix, containing 1x digestion buffer, 10μg of tRNA carrier, and 100 units of S1 nuclease, was added as the tubes were removed from the water bath. They were quickly sealed, vortexed and placed on ice. After each had been removed from the bath, all the samples were placed at 23°C for 2hr 15min. The digestion reaction was terminated by placing the samples on ice. The remaining nucleic acids were phenol/chloroform extracted and ethanol precipitated in the presence of 0.1 vol. of 2M NaOAC. After centrifugation the pellet was resuspended in 5μl of S1 loading buffer. The samples were then denatured for 5 min at 90°C, placed on ice, and loaded onto a 7% denaturing polyacrylamide gel, made with Accugel 40™ acrylamide (40%)

(National Diagnostics), 1xTBE, 6M urea, 0.1% ammonium persulphate and 0.1% TEMED. Gels were cast between glass plates, using 0.4mm spacers, and run in 1x TBE at a power of between 60-80 Watts. The gels were placed on Watman 3MM paper, covered with Saran wrap, and dried under vacuum at 80°C for 1 hr. The gel was exposed either to film or to a Phosphorimager screen as described above.

2.5 Reverse transcription.

Reverse transcription of RNA (and PCR amplification of the resulting cDNA fragments - see above) was used to analyse the presence of specific transcripts in various mouse tissues. 1-10μg of RNA in 5μl of H_2O was heat denatured for 5 min at 65°C then placed on ice. To this, 2μl of 10x RT buffer, and 10 units of Super RT (HT biotechnology) were added and made up to 20μl with H_2O. The mix was incubated at 42°C for 2hr. 30μl of 1mM Tris pH 7.5 was then added and the resulting cDNA solution stored at -20°C until required.

3. Cloning procedures.

3.1 Generation of constructs.

The 14Kb Ly-6E.1 cassette, pL6Cla, was constructed by cloning the 3.6Kb Sph1-EcoR1 fragment from pLR1Cla - consisting of the upstream region and the first exon containing a Cla1 site (gift A. Sinclair) - into a 12.3Kb Sph1-EcoR1(partial) fragment isolated from pAB14 (Sinclair & Dzierzak, 1993). This fragment contains the remaining 3' part of the Ly-6E.1 gene and pPolyIII. The lacZ gene in p610ZA (gift D. Meijer) was modified, converting the 3' Sma1 site to a Nar1 site using oligonucleotide adptors, thus enabling the isolation of lacZ as a 3.6Kb Nar1 fragment - which was cloned into pL6Cla. The orientation of the insert was verified by the characteristic restriction pattern caused by the asymmetrically located EcoR1 site in the lacZ fragment.

p1271 containing exons 2 and 3 of the murine c-myc gene was used to provide the myc sequences which were cloned into pL6Cla, in the same way as the lacZ gene. The Stu1 and BamH1 sites flanking c-myc in p1271 were converted into Nar1 sites using oligonucleotide adaptors and exons 2 and 3 were cloned into pL6Cla as a 4.8Kb Nar1 fragment. The orientation of this insert was verified by a diagnostic EcoRV digest - EcoRV being assymetrically located within the myc fragment.

The bcl-2 cDNA was cloned into pL6Cla in the same way. The Sma1 site in the bluescript polylinker of pBcl-2 was converted into a Nar1 site using an oligonucleotide adaptor and the bcl-2 cDNA was isolated as a 865bp Nar1(partial)-

ClaI fragment and cloned into pL6Cla. In this case assymmetrically located EcoR1 and HindIII sites allowed the orientation of the insert to be determined.

The 760bp Ly-6E.1 cDNA (LeClair *et al.*, 1986) was cloned as an EcoRI fragment into the human CD2 expression cassette p2629 (gift from D. Kioussis) and the orientation of the insert was determined by restriction analysis using NcoI and SstI digestion, both of which are assymetrically located within the Ly-6E.1 cDNA. The 3' LCR of the hCD2 gene from p2694 (gift from D. Kioussis) was added as a 4.5Kb BamHI-NotI fragment to the plasmid containing the Ly-6E.1 cDNA in a sense orientation with respect to the hCD2 cassette.

3.2 Ligations.

In the cloning process, 10-50ng of linearised plasmid and 100ng of the insert fragment were mixed together in 8μl of H_2O. To this, 1μl of 10x ligation buffer and 1μl of T4 DNA ligase (1U/μl) were added. The reaction was incubated overnight at 16°C. For transformation into *E. coli*, 2μl of these samples were used.

3.3 Competent bacteria.

For transformation, $CaCl_2$ competent *E. coli* (strains DH5α or DH10β), were prepared. A single colony was innoculated into a 20ml LB media culture and grown overnight at 37°C with vigorous shaking. 100μl of this culture was then added to a

fresh flask containing 250ml of pre-warmed LB media and incubated at 37°C. The OD_{600} was monitored at regular intervals until it reached 0.5. The culture was then cooled rapidly in an ice bath, followed by the centrifugation of the bacteria at 4k rpm for 10 min at 4°C. The cells were then resuspended in 62.5ml of ice cold sterile 0.1M $MgCl_2$, and re-pelleted by centrifugation at 4k rpm at 4°C for 10 min. The cell pellet was resuspended in 31.25ml ice cold sterile 0.1M $CaCl_2$ and left on ice for 20 min. After centrifugation at 4k rpm at 4°C for 10 min, the cells were resuspended in 26.5ml of ice cold 0.1M $CaCl_2$ and 3.5ml of glycerol. 200μl aliquots of this solution were then snap frozen in eppendorf tubes and stored at -70°C until required.

3.4 Bacterial transformations.

The DNA to be transformed was mixed with 100μl of rapidly thawed competent cells and left on ice for 30 min. The mixture was then transferred to a 42°C water bath for 90 seconds, then onto ice for 5 min. 1ml of LB media was then added and the culture allowed to incubate at 37°C for 1hr. The cells were pelleted by centrifugation for 10 sec and all the supernatant, with the exception of 50μl, removed. The cells were resuspended in this volume, then spread onto LB plates containing 100μg/ml ampicillin. After overnight incubation at 37°C, single colonies were picked and inoculated to 5ml LB/ampicillin (100μg/ml) cultures for miniprep purification of their plasmid DNA.

3.5 Plasmid miniprep purification.

Single plasmid-containing *E. coli* colonies were inoculated into 5ml of LB media with 100μg/ml ampicillin. The cultures were incubated overnight at 37°C. 1.5ml of the culture was transferred to an eppendorf and centrifuged at 14k for 30 sec, the supernatant being discarded. 270μl of TEN buffer was added, with the pellet resuspended by vortexing. To this, 30μl of 10% SDS was added, vortexed, followed by 150μl of 2M NaOAc pH 5.2. After mixing well 1x phenol/chloroform and 1x chloroform extractions were performed. To the final aqueous layer 0.9ml of 96% ethanol was then added, the sample placed on ice for 5 min, and centrifuged at 14k rpm for 10 min to pellet the DNA. The pellet was washed with 70% ethanol, air-dried and resuspended in 50μl of TE. RNAse treatment using 1μl of 10mg/ml RNAse A was then carried out for 30 min at 37°C, followed by a phenol/chloroform extraction, ethanol precipitation and resuspension of the final DNA pellet in 50μl of TE pH 7.4.

3.6 Plasmid maxiprep purification.

A single bacterial colony, grown up in 5ml cultures as described for miniprep purification, was used to inoculate a 1 litre culture of LB media with 100μg/ml ampicillin. This was incubated overnight at 37°C. The culture was then centrifuged at 4k rpm in a 1 litre bottle for 20 min, the supernatant being discarded. The bacterial pellet was then resuspended in 40ml of 1x glucomix by vortexing. To this, 80ml of

0.2M NaOH/1% SDS was added, mixing gently, and left to stand for 5 min. 40ml of ice-cold 5M KOAc pH 4.8 was then added, again mixing gently and leaving to stand for 5 min. The mixture was then centrifuged at 4k rpm for 20 min, then poured through 8 layers of cheesecloth to remove the debris. To the remaining supernatant, 0.6 vol. of isopropanol was added, with gentle mixing to precipitate the DNA. The DNA was then pelleted by 20 min centrifugation at 4k rpm, washed with 70% ethanol, air-dried for 30 min, and resuspended in 5ml TE pH 8.0. The solution of DNA was then transferred to a pre-weighed Falcon 50ml tube, and adjusted to 9g with TE pH8.0. To this, 10.2g of CsCl and 1ml of 5mg/ml ethidium bromide was added. The solution was then transferred to Quick-seal™ centrifuge tubes and sealed with a heat clamp. The samples were then centrifuged for 24hr at 56,000 rpm in a 70.1 Ti Beckman rotor. The lower supercoiled band of plasmid DNA was removed using a syringe and needle. This was transferred to a 15ml Falcon tube, made up to 4ml with H_2O, and then mixed with 8ml of 96% ethanol. The DNA was pelleted by centrifugation at 4k rpm for 10 min, followed by a wash in 70% ethanol and resuspension in 500μl of TE pH 8.0. To this, 10μl of 0.5M EDTA and 5μl of 10% SDS were added, followed by 2x phenol/chloroform extractions and a chloroform extraction. To the final aqueous phase, 50μl of 2M NaOAc pH 5.5 and 1ml of 96% ethanol were added to precipitate the DNA. After 5 min on dry ice, the sample was centrifuged for 10 min at 14k rpm. The DNA pellet was washed in 70% ethanol, and resuspended in 450μl TE pH 8.0, plus 50μl of 1M NaCl. To this 2μl of 10mg/ml RNAse A was added, and incubated at 37°C for 30 min. Then followed 2x phenol/chloroform extractions, 1x chloroform extractions, the addition of 50μl of 2M NaOAc pH 5.5 to the final aqueous phase, and the precipitation of the DNA by the

addition of 1ml of 96% ethanol. After pelleting the DNA by centrifugation at 14k rpm for 10 min, a 70% ethanol wash was followed by the resuspension of the DNA in 500μl of TE pH 8.0. The plasmid DNA was then stored at -20°C until needed.

4. Cell biology procedures.

4.1 FACS analysis.

FACS analysis was used to detect antigens on lymphocytes from transgenic mice. In each experiment, two transgenic animals and two normal littermates were analysed together and the experiments were repeated at least twice to verify the observations. Thymus, spleen and lymph nodes were removed and homogenised to single cell suspension in ice cold FACS media; bone marrow cells were obtained by flushing femurs with ice cold PBS prior to homogenisation in ice cold FACS media. Accurate cell counts were obtained. 10^6 cells were then washed in 5ml of FACS media, pelleted and the supernatant removed. Antibodies were added at a dilution of 1:200 in FACS media and incubated for 30 min on ice. Cells were washed once with 5ml of cold FACS media, washed once with 5ml of cold PBSa, fixed in 1% formaldehyde/PBS and filtered through nylon mesh. Stained cells were analysed with a Becton Dickinson FACSCAN cell sorter and the LYSIS II software package.

Antibodies used were a FITC-conjugated hamster monoclonal antibody against mouse CD3δ (Pharmingen, San Diego, CA); PE-conjugated rat monoclonal antibody against murine CD4; a FITC-conjugated rat monoclonal antibody against murine CD8 (both Becton Dickinson Immunocytometry Systems, San Jose, CA); and the following rat monoclonal antibodies all from Pharmingen:
CD8-PE, CD25-FITC, CD44-CY, mac1-PE, c-kit-FITC, B220-FITC, Ly-6A/E-PE, Ly-6A/E-FITC, HSA-PE, ter119-PE, Thy1.2-PE.

For the analysis of apoptosis, 10^6 cells were washed in PBS, and then incubated overnight at 4°C in 1.5ml of PI buffer. The cells were then run through the FACScan machine with events aquired on the FL-3 channel.

For the analysis of β-galactosidase activity, 10^6 single cells were placed in 20μl of ice cold PBS/5%FCS prior to loading with 20μl of 2mM fluorescein di-(β-D-galactopyranoside),FDG (Sigma) in dH_2O at 37°C for 75s. The uptake was stopped by the addition of 500μl of ice cold PBS/5%FCS and the reaction was allowed to proceed for 1-3 hours on ice, in the dark. Propidium iodide (Sigma) was added to a final concentration of 1μg/ml to allow the exclusion of dead cells. The fluorescence generated by β-galactosidase was detected on the FL-1 channel.

4.2 Cell sorting.

Cell sorting was performed to isolate populations of cells exhibiting β-galactosidase activity above endogenous levels. Cells were homogenised to single cell suspension in ice cold Leibowitz L-15 medium (Flow Labs) with 5%FCS and stained with FDG as described, with the exception that L-15 medium with 5%FCS instead of PBS/5%FCS and ten times all volumes was used throughout. Cells were passed through a Beckton Dickinson cell sorter, generally under sterile conditions, and the resulting populations collected into L-15/5%FCS. The fidelity of the sort was verified by reanalysing a small fraction of the sorted cells.

4.3 Cytological staining of haematopoietic cells.

$1x10^5$ cells in 150μl of PBS were centrifuged directly onto a glass slide for 5 minutes at 950rpm ("Cytospin", Shandon, Runcorn, UK) and allowed to air dry prior to staining. Slides were stained using the "Diff-Quick" system (Baxter Healthcare, Thetford, UK) comprising a 5s fixation with 0.002g/l fast green in methanol, 10s stain with eosin G in PBS pH6.6, 15s counterstain with thiazine dye in PBS pH6.6 and a 5s wash in dH_2O. After air drying, slides were mounted in DPX mountant (BDH Ltd, Poole, UK).

4.4 X-Gal staining of embryos.

Embryos were isolated into ice cold PBS and washed briefly. Depending upon the size of the embryos, they were fixed with 1ml ice cold X-gal fix at 4°C for between 30 mins (for e7.5 embryos) and 90 minutes (e12.5 embryos) prior to washing 4 times in embryo wash solution at room temperature (20 mins and 1ml each). Embryos were generally incubated in X-gal stain solution overnight at room temperature, in the dark. After staining, the embryos were washed in two changes of PBS prior to whole mount photography and/or processing.

Prior to sectioning, washed, stained embryos were dehydrated through increasing concentrations of ethanol in ice cold PBS for at least 30 mins at each step (70%, 85%, 95%, 100%). From absolute ethanol, the embryos were cleared using two 30 min washes in Histoclear and then washed through increasing concentrations

of paraffin wax in Histoclear before mounting in 100% wax. 6-10μm sections were cut on a Reichert-Jung microtome and dried onto APES coated microscope slides overnight at room temperature. The dried slides were dewaxed in Histoclear (5 mins) and rehydrated through decreasing concentrations of ethanol (100%, 95%, 70%, dH_2O) before counterstaining for 15s in 0.25% eosin. Counterstained slides were dehydrated through increasing ethanol concentrations, washed in Histoclear for 10 mins and mounted with DPX mountant.

4.5 X-gal staining of single cell suspensions

10^6 cells were generally used for X-gal staining in a modified embryo staining protocol. Cells were washed once with PBS, fixed for 10 mins on ice in 0.5X X-gal fix, washed with 5 volumes of PBS and resuspended in 1ml X-gal stain. Incubation was typically overnight at room temperature with constant agitation. After staining, cells were washed once with 10ml PBS and resuspended in 1ml PBS. 200μl of stained cells was centrifuged using the Cytospin system as described previously and the resulting slide counterstained by using the "Diff-Quick" solutions omitting the final thiazine dye stain.

4.6 Cell culture techniques.

NIH 3T3 murine fibroblasts were grown in DMEM media with 10% FCS, 10U/ml penicillin, 10μg/ml streptomycin, 2mM L-glutamine and maintained at between 10^6 and 10^7 cells per 10 cm Costar petri dish in a humid incubator at 37°C and 5%CO_2.

All other cell cultures were of primary haematopoietic cells. The base media used in all primary cultures consisted of αMEM supplemented with 10%FCS, 10u/ml penicillin, 10μg/ml streptomycin, 2mM L-glutamine and $5x10^{-5}$M β-mercaptoethanol. Cells were kept in standard tissue culture plasticwear (Costar) at 37°C with 5% CO_2.

For each individual methyl cellulose assay, between 10^4 and 10^5 cells were suspended in 200μl base media plus any additional growth factors (recombinant mouse erythropoietin (epo), Boehringer; Poke weed mitogen spleen cell conditioned medium (SCM), Stem Cell Technologies inc, Vancouver; leukaemia inhibitory factor (LIF), gift A. Stewart. To this, 800μl of Methocult media (Stem Cell Technologies inc) - 30% Fetal bovine serum, 1% BSA, 0.9% methylcellulose, 10^{-4}M 2-mercaptoethanol and 2mM glutamine. After thorough mixing, this 1ml assay was seeded into one well within a humidified 24 well plate.

4.7 T cell proliferation assays.

Thymocytes and splenic T cells - depleted of erythrocytes by lysis and of B cells by adherence to nylon wool - were cultured in 200μl of base medium in

microtiter wells at a density of between $4x10^3$ and $5x10^5$ per well. Cells were stimulated with anti-CD3 (145-2C11) monoclonal antibody (0.36μg/well) and 5ng/ml PMA (Sigma), or with 5μg/ml conA (conA), or with 5ng/ml PMA only as a control. 48 hours after stimulation, cells were labelled for 16 hours with 1μCi/well of ^{3}H-thymidine (Amersham) before harvest. Cellular DNA containing the incorporated radioactivity was precipitated on glass fibre filter paper and susequently counted by liquid scintillation.

4.8 Thymocyte adhesion assays.

$1x10^6$ thymocytes were cultured in 200μl base medium in flat bottomed 96 well plates overnight (18-24 hours), then gently pipetted with a wide bore pipette tip. Homotypic adhesion was guaged by counting cells in a haemocytometer.

For the antibody blockade experiment, the protocol of Abraham *et al.* (Abraham *et al.*, 1990) was followed. Briefly, $5x10^5$ thymocytes were seeded onto a monolayer of confluent NIH3T3 cells in 96 well plates after a 30 minute preincubation with each antibody. The thymocytes and 3T3 cells were incubated at 37°C for 2 hours in the presence of 50-200μg/ml of each respective antibody, then gently washed with warm medium, methanol fixed, stained with crystal violet and adherent lymphocytes were counted over 10 different fields.

5. Animal procedures.

5.1 Production of transgenic mice.

Fertilised (CBA/Ca x C57/B10)F2 mouse oocytes were microinjected with each construct after removal of all vector sequences. This was acheived for Ly-6E.1/*lacZ*, Ly-6E.1/*myc* and Ly-6E.1/*bcl-2* by digestion with Not1 and gel purification to remove pPolyIII sequences. For the generation of XN lines, containing a truncated Ly-6E.1/*lacZ*, pL6LZ was digested with Xba1 and Not1 and the injection fragment purified away from both pPolyIII and 3' Ly-6E.1 sequences. The CD2/Ly-6E.1 microinjection fragment was released from the plasmid vector by digestion with Sal1 and Not1. Positive founder animals were bred with (CBA/Ca x C57/B10)F1 mice and lines were generally maintained as heterozygotes. DNA slot blot analysis or Southern analysis was used to assess the integration patterns of the transgenes, and to identify the transgenic mice within a litter.

5.2 Bone marrow transplantation.

Donor cells for the generation of radiation chimaeras were manipulated *ex vivo* in L-15 medium/5%FCS and were finally suspended in sterile PBS (500μl per recipient). The cells were then injected intravenously into the tail vein of 4-6 month old female (CBA/Ca x C57/B10)F1 recipient mice. The recipients had previously

been exposed to a split dose of 1000 rads of a ^{60}Co source and were housed in positive pressure cabinets. The recipient mice were fed on sterile feed and received 1.6g/l neomycin in their drinking water. All animal procedures were in accordance with the Animals Scientific Procedures Act, 1986, UK.

6. DNA probe list (for Southern and northern blots).

lacZ: 1.1Kb BamH1-EcoRV

Thy-1: 1.2Kb Xba1-Nru1 (Spanopoulou *et al.*, 1988)

Ly6E.1: 760bp EcoR1 (LeClair *et al.*, 1986)

actin: 1.8Kb BamH1-Sal1 (Gunning *et al.*, 1983)

c-myc: 4.8Kb BamH1-Xba1 (Spanopoulou, *et al.*, 1989)

bcl-2: 865bp EcoR1-HindIII (Nunez *et al.*, 1990)

CD28: 374bp RT-PCR prod. sense; GAC GTG GAA GTC TGT GTC GGG
anti-; CAT GGT AGT CAC TTG AAG GAG (Gross *et al.*, 1990)

B29: 470bp RT-PCR prod. sense; GCA GTG ACC TGC CAC TGA ATT
anti-; AGC CTT GCC GTC ATC CTT GTC (Goodman *et al.*, 1993)

CD2: (gift D. Abraham)

ODC: (gift D. Abraham)

ADH: (gift D. Abraham)

γIFN: (gift D. Abraham)

S1 probes: *Ly-6E.1* - 813bp EcoR1-Nde1 fragment, spanning boundary of intron 3 and exon 4 (Khan *et al.*,1990).

β-actin - 240bp BamH1-Ava1 fragment (Gunning *et al.*,1983).

7. Reagent List.

APES:-2% 3-aminopropyltriethoxysilane (Sigma) in acetone.

Denhardt's (100x):- 2% Ficoll, 2% BSA, 2% polyvinyl pyrollidone.

DNA hyb. mix:- 3x SSC, 0.1% SDS, 10x Denhardts, 10% Dextran sulphate, 50μg/ml denatured salmon sperm DNA.

DNA pre-hyb. mix:- 3x SSC, 0.1% SDS, 10x Denhardts, 50μg/ml denatured salmon sperm DNA.

Embryo wash solution:- 0.02% NP40 (BDH, Poole,UK) in PBS

FACS fix:- 1% formaldehyde in PBSa.

FACS media:- 500ml αMEM, 5% fetal calf serum, 0.01% NaAzide.

Glucomix (10x):- 500mM glucose, 100mM EDTA, 250mM Tris pH 8.0.

Kinase buffer (5x):- 250mM Tris pH9.0, 250mM $MgCl_2$, 50mM DTT, 50μg/ml BSA.

LB broth:- 1% bactotryptone, 0.5% bacto-yeast extract, 1% NaCl, adjust final pH to 7.5 with NaOH.

Ligation buffer (10x):- 500mM Tris pH 7.4, 100mM $MgCl_2$, 10mM DTT, 10mM spermidine, 10mM ATP, 1mg/ml BSA.

MOPS (10x):- 0.2M MOPS, 80mM NaOAc, 10mM EDTA.

Northern loading dye:- bromophenol blue added to each sample.

OLB (2x):- 100mM Tris pH 7.6, 100mM NaCl, 20mM $MgCl_2$, 200μg/ml gelatin, 40μM each of dCTP, dGTP, dTTP, 20μg/ml random hexamers.

PBSa (PBS):- 171mM NaCl, 3.3mM KCl, 10.1mM Na_2HPO_4, 1.8mM KH_2PO_4.

PCR mix (2x):- 2x cambio PARR™ buffer, 0.4mM of each of dATP, dTTP, dCTP, & dGTP, add taq polymerase last 1U/reaction.

Phenol:- Phenol (equilibrated with 0.1M Tris pH 8.0), 0.1% 8-hydroxy-quinoline.

Phenol/Chloroform:- 1:1 (v/v) ratio.

PI buffer:- 50μg/ml propidium iodide, 0.1% sodium citrate, 0.1% triton-X in H_2O.

Proteinase K:- 10mg/ml proteinase K, 10mM Tris pH 8.0, 1mM EDTA, 0.5% SDS.

RNA hyb. mix:- 60% formamide, 5x SSC, 0.1% BSA, 0.1% Ficoll 400, 0.1% polyvinyl pyrolidone, 20mM sodium phosphate buffer pH 6.8, 1% SDS, 7% dextran sulphate, 100μg/ml denatured salmon sperm DNA, 100μg/ml tRNA(bakers yeast), 10μg/ml poly A.

RT buffer (10x):- 50mM Tris pH 7.6, 60mM KCl, 10mM $MgCl_2$, 1mM dNTPs, 1mM DTT, 1U/μl RNasein, 50μg/ml actinomycin D.

S1 digestion buffer:- 0.28M NaCl, 50mM NaOAc, 4.5mM $ZnSO_4$.

S1 hyb. buffer:- 40mM PIPES pH 6.4, 1mM EDTA, 0.4M NaCl, 80% formamide.

S1 loading buffer:- 7M urea, 0.05% xylene cyanol, 0.05% bromophenol blue.

SSC (20x):- 3M NaCl, 0.3M Na_3Citrate

TAE (50x):- 2M Tris pH 8.0, 50mM EDTA, 1M glacial acetic acid.

Tail mix buffer:- 50mM Tris pH 8.0, 100mM EDTA, 100mM NaCl.

TBE (10x):- 0.89M Tris pH 7.4, 0.89M boric acid, 10mM EDTA.

TE pH 7.4/8.0:- 10mM Tris pH 7.4/8.0, 1mM EDTA.

TEN:- 10mM Tris pH 7.4, 1mM EDTA, 0.1M NaOH.

X-gal fixation buffer:- 1% formaldehyde, 0.2% glutaraldehyde, 2mM $MgCl_2$, 5mM EGTA, 0.02% NP40 in PBS.

X-gal stain buffer:- 5mM $K_3Fe(CN)_6$, 5mM $K_4Fe(CN)_6.3H_2O$, 2mM $MgCl_2$, 0.01% Na deoxycholate, 0.02% NP40, 1mg/ml X-gal.

RESULTS

1. Ly-6E.1/*lacZ* transgenic mice.

1.1 Introduction.

Haematopoietic stem cell enrichment procedures rely upon the depletion of cells bearing specific antigens to mature, lineage commited cells prior to a round of positive selection. There are only a few gene products that are known to be expressed on haematopoietic stem cells and, consequently, few candidate genes are suitable for use in molecular targeting experiments. Haematopoietic stem cells express Thy-1, c-kit, CD34 and Sca-1 (Spangrude *et al.*, 1988; Müller-Seiburg *et al.*, 1986; Ikuta *et al.*, 1990; Ikuta & Weissman, 1992; Krause *et al.*, 1994) although none of these antigens exclusively mark stem cells. Of these candidates, only the gene encoding Sca-1 appears to be useful for transgenic mouse studies. The Thy-1 gene has previously been introduced into transgenic mice (Dzierezak *et al.*, 1993) but the expression of a HSV *tk* gene under its control had no effect on haematopoietic stem cells in these experiments, possibly due to an absence of stem cell specific expression at sufficiently high levels. The c-kit gene encompasses 70Kb of the genome, with putative regulatory elements spread even further, thus is not readily manipulated for transgenic experiments (Gokkel *et al.*, 1992); the CD34 gene has only recently been characterised (May & Enver, 1995) and the elements necessary to regulate expression *in vivo* are not yet known. Consequently, the Ly-6E.1 gene,

encoding Sca-1 (van der Rijn *et al.*, 1989), is the most suitable candidate, being the most extensively characterised at the molecular level (Sinclair & Dzierzak, 1993; Sinclair *et al.*, 1996). Furthermore, the Ly-6E.1 gene product, Sca-1, has been most widely used in, and is essential for, haematopoietic stem cell enrichment procedures (Spangrude *et al.*, 1988; Uchida & Weissman, 1992; Uchida *et al.*, 1994).

It is likely that for *in vivo* expression of the Ly-6E.1 gene, both 5' and 3' flanking genomic regions will be required for transgenic experiments. *In vivo* expression of Ly-6 genes is reduced if either 5' (Philbrick *et al.*,1990) or 3' (Weissman, pers. comm.) elements are absent. Recent *in vitro* deletion analysis of the cloned Ly-6E.1 gene has demonstrated the importance of the 3' flanking region containing several DNase1 hypersensitive sites in obtaining high levels of expression in stably transfected haematopoietic cell lines (Sinclair, Daly & Dzierzak, 1996). Taken together, these data suggest that a full length genomic construct containing both 5' and 3' sequences will be the most likely to give high levels of tissue specific expression in transgenic mice. Thus for the *in vivo* studies presented in this thesis, the original Ly-6E.1 genomic clone (Sinclair & Dzierzak, 1993) containing the entire coding and intronic sequences of the Ly-6E.1 gene with 3.5 Kb of 5' and 8 Kb of 3' flanking sequence (14 Kb BamH1 subclone) was reconstructed with a unique cloning site (Cla1) inserted into the first, untranslated exon (figure 1). Although the construct used for *in vitro* deletion analysis lacked intronic sequences and the most 5' region of the Ly-6E.1 gene, the 14 Kb subclone, when stably transfected into MEL cells demonstrated comparable levels of Ly-6E.1 protein expression when compared with endogenous Ly-6A.2 (Sinclair & Dzierzak, 1993) and, therefore, represents a likely candidate transgene to recapitulate endogenous Ly-6E.1 expression *in vivo*.

In general, transgenic mice are generated and maintained on an outbred genetic background. The F1 hybrid vigour associated with outbred animals enables efficient superovulation resulting in the production of large numbers of fertilised oocytes (Hogan *et al.*, 1986). In addition, the oocytes obtained from outbred animals are more robust and less likely to become damaged during the microinjection process than those isolated from inbred animals. One drawback arising from the use of outbred mice for transgenesis is a lack of strain specific markers in the resulting hybrid transgenic mice. To enable expression from the Ly-6E.1 transgene to be specifically detected above the background of the endogenous Ly-6E.1/A.2 genes, the constructs for production of transgenic mice were engineered to express a heterologous gene product instead of Ly-6E.1. A number of reporter genes have been used previously to discriminate transgenic from endogenous gene expression. Each reporter molecule possesses particular properties enabling detection and/or quantitation of expression. Some examples of these include; human growth hormone (hGH), engineered to produce a stable mRNA, whilst protein expression is detected by a specific radioimmunoassay (Swift *et al.*, 1989); enzymes such as chloramphenicol acetyltransferase (CAT)(Gorman *et al.*, 1982) and firefly luciferase (Crenshaw *et al.*, 1989) which can be detected with a high degree of sensitivity; alkaline phosphatase (Fields-Berry *et al.*, 1992) and β-galactosidase (Cui *et al.*, 1994), which enable *in situ* detection at the level of single cells; and cell surface proteins from other species, such as human CD2 (Elliot *et al.*, 1995) and Thy-1 (Kollias *et al.*, 1987), which can be detected at the cell surface by specific monoclonal antibodies and FACS analysis.

The expression of hormones, or cell surface proteins can be useful markers, but may exert some unwanted, unpredictable, physiological effects in the transgenic animals, a most dramatic example being the giant transgenic mice expressing hGH (Morello *et al.*, 1986). As Ly-6E.1 is expressed on the haematopoietic stem cell, an exquisitely sensitive cell type, and is likely to be involved in the development and modulation of the haematopoietic system, the reporter gene for monitoring Ly-6E.1 expression should preferably be as inert as possible. In addition, it is not always desirable to make cell lysates for enzyme/hormone assays. A marker gene which can be used to detect cells whilst keeping them intact, *in situ* and preferably alive could have many advantages. A suitable candidate is the bacterial *lacZ* gene, which encodes the glycolytic enzyme, β-galactosidase. As a non-mammalian gene, nucleic acid probes do not cross react with any endogenous DNA and RNA present in cells of the mouse, facilitating accurate quantitative and qualitative molecular analyses. There are no reports that the enzyme affects cellular physiology and, more importantly, there are several well characterised methods for detecting and quantitating expression of β-galactosidase.

Of particular interest, in light of these studies, is the use of the β-galactosidase transgene to visualise the spatial and temporal expression patterns of genes during embryonic development, making use of the blue colouration developed by the precipitation of the substrate, X-gal, after hydrolysis by β-galactosidase. Recent examples of such transgenic experiments include the studies of the tissue and developmental stage specific regulatory elements of the myogenin (Yee & Rigby, 1993) and nestin (Zimmerman *et al.*, 1994) genes. The expression of Ly-6E.1 during ontogeny of the mouse has not been investigated and an approach based upon Ly-

6E.1/*lacZ* transgenes would enable the spatial and temporal pattern of Ly-6E.1 expression to be determined without the potential problems associated with binding of probes to mRNAs of other Ly-6 family members when using *in situ* hybridisation techniques or cross reaction of antisera when performing immunohistochemistry. If the 14Kb BamH1 subclone is able to recapitulate the expression pattern of Ly-6E.1 in Ly-6E.1/*lacZ* transgenic mice, X-gal staining of transgenic embryos will be used to provide evidence of potential embryonic sites of Ly-6E.1 expression and perhaps of the embryonic origin of haematopoietic stem cells.

The *lacZ* gene has also been used to specifically mark the movement of donor cells in chimaeric mice (Wilson *et al.*, 1995). In these studies, the *tail-less* mutation was shown to be the result of a defect in cell migration. Similarly, *lacZ* could prove a useful marker in radiation chimaeras following bone marrow transplantation of transgenic cells. The Ly-6E.1/*lacZ* transgene could serve as a molecular marker for donor derived cells as well as providing a means to specifically locate individual cells within chimaeric animals. In addition, the use of the non-toxic fluorescent β-galactosidase substrate, fluorescein di-β-D-galactopyranoside (FDG) allows the staining of live cells. In combination with fluorescence activated cell sorting (FACS), this substrate allows the separation and recovery of viable *lacZ* expressing cells (Nolan *et al.*, 1988; Krasnow *et al.*, 1991). Recently, this technique has been applied to the selection of embryonic stem cells transiently transfected with a *lacZ* gene prior to the generation of germ line contributing chimaeric mice (Fiering *et al.*, 1995). Thus FACS/FDG sorting appears to have no adverse affects on a totipotent embryonic cell type. It will be interesting to determine whether the FDG substrate will facilitate the sorting of haematopoietic stem cells from Ly-6E.1/*lacZ* transgenic mice. Indeed

the transplantation of such sorted cells into lethally irradiated recipient mice is the only direct test for transgene expression in haematopoietic stem cells. This transgenic approach may serve as a useful tool for haematopoietic stem cell manipulation and may circumvent the need to select haematopoietic stem cells with an antibody (Sca-1) that may disrupt the normal physiology and deliver mitogenic signals to the cell.

1.2 Generation of Ly-6E.1/*lacZ* transgenic mice (BL mice).

In order to determine whether the cloned 14 Kb Ly-6E.1 gene was capable of functioning *in vivo*, the β-galactosidase reporter gene was inserted into the first, untranslated exon of Ly-6E.1 to enable detection of expression from the transgene. The cloning strategies employed are represented in figure 1. To facilitate the introduction of reporter genes into the complete 14 Kb cloned Ly-6E.1 genomic fragment, pL6Cla was generated by linking pCla1, consisting of the 5' 3.5 Kb fragment of the Ly-6E.1 gene with a unique Cla1 site engineered into the first exon (Sinclair, Daly & Dzierzak, 1996), to a 12.3 Kb EcoR1(partial) - Sph1 fragment from pAB14 containing the remaining 3' part of the Ly-6E.1 gene in pPolyIII. The Sma1 restriction site 3' of the *lacZ* gene in p610ZA (gift D. Meijer) was converted into a Nar1 site using synthetic oligonucleotides, enabling the isolation of *lacZ* coding and SV40 polyadenylation sequences as a 3.6 Kb Nar1 fragment which was then cloned into the unique Cla1 site in pL6Cla to generate pL6LZ. The orientation of the insert was checked by restriction digest using EcoRV, an enzyme whose site is asymetrically located within the *lacZ* gene.

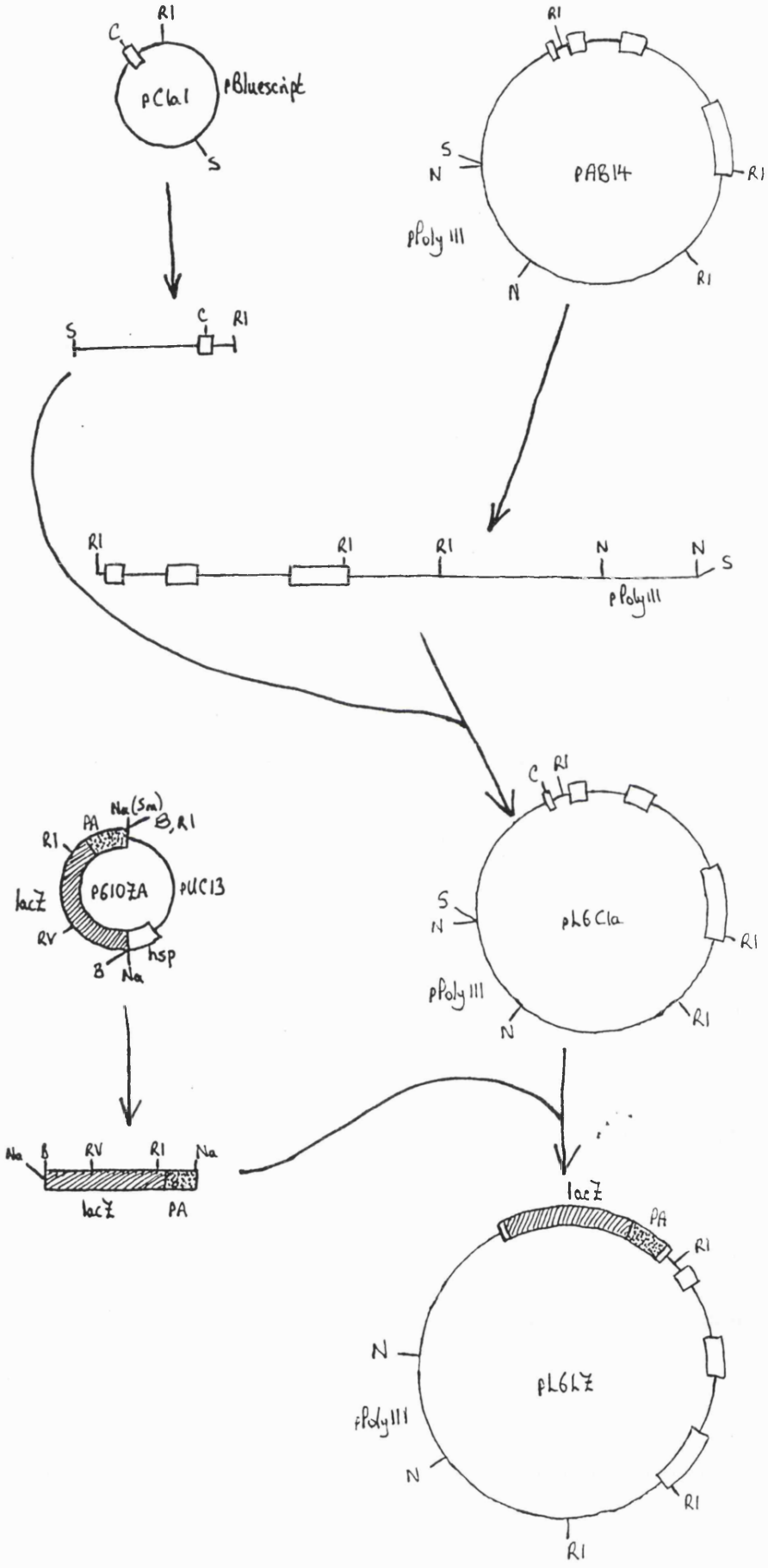
Figure 1. Cloning strategy used to generate the Ly-6E.1/*lacZ* construct.

Schematic representation of the cloning steps involved in linking pCla1 and pAB14 to generate pL6Cla and subsequent insertion of a Nar1 flanked *lacZ* gene into the unique Cla1 cloning site within the first exon of Ly-6E.1.

pCla1 = 3.5 Kb BamH1-EcoR1 Ly-6E.1 fragment with unique Cla1 restriction site within first exon (Sinclair *et al.*, 1995); pAB14 = original 14 Kb BamH1 subclone of Ly-6E.1 in pPolyIII (Sinclair & Dzierzak, 1993); p610ZA = *lacZ* gene with SV40 polyadenylation signal downstream of hsp67 promoter in pUC13 (gift. D. Meijer); pL6LZ = Ly-6E.1/*lacZ* construct in pPolyIII, from which a 17.6 Kb Not1 fragment was used for microinjection to generate transgenic mice.

Restriction enzyme sites: R1 = EcoR1; C = Cla1; S = Sph1; N = Not1; B = BamH1; Sm = Sma1; Na = Nar1; RV = EcoRV. PA = SV40 polyadenylation signal.

For generation of Ly-6E.1/*myc* and Ly-6E.1/*bcl-2* constructs, the *c-myc* and *bcl-2* genes respectively were flanked with Cla1 compatible restriction sites and cloned into pL6Cla as with *lacZ*.


C
R1
pCla1
pBluescript
S
S
C
R1
R1
S
N
pPoly III
N
PA814
R1
R1
R1
R1
N
N
S
pPoly III
Na(Sm)
B, R1
PA
R1
lacZ
RV
B
Na
P6107A
pUC13
hsp
Na
B
RV
R1
Na
lacZ
PA
C
R1
S
N
pPoly III
N
pL6Cla
R1
R1
lacZ
PA
R1
N
pPoly III
N
pL6lacZ
R1
R1

To verify that the resulting Ly-6E.1/*lacZ* transgene (pL6LZ) was capable of producing functional *lacZ* protein, it was transfected into an Ly-6E.1$^+$ cell line, NIH3T3. pPolyIII vector sequences were removed entirely by digestion with Not1 and the resulting 17.6 Kb fragment (figure 2A) was purified by preparative gel electrophoresis prior to being cotransfected into NIH3T3 cells with a neomycin resistance expression vector. G418 resistant clones transfected with control pAB14 (figure 2B) or with pL6LZ (figure 2C) were stained with X-gal. Only transfectants containing pL6LZ show expression of functional β-galactosidase.

For the production of transgenic mice, the 17.6 Kb Not1 Ly-6E.1/*lacZ* fragment (figure 2A) was microinjected into pronuclei of fertilised (CBA/Ca x C57/B10) F2 oocytes. 30 oocytes not visibly damaged by microinjection were transferred into one oviduct of each pseudopregnant female recipient. In general, one of three pseudopregnant recipients produced offspring, of which approximately one of four carried the transgene. Litter size of founder mice ranged between 1 and 5. Genomic DNA prepared from tail biopsies obtained from founder animals 10 days *post partum* was screened by southern blot for the presence of the *lacZ* transgene. Of a total of 34 founder mice born after microinjection of Ly-6E.1/*lacZ*, five were positive for the *lacZ* transgene. From these five transgenic founders, a total of four Ly-6E.1/*lacZ* lines of transgenic mice were generated. Figure 2D shows a southern blot, used to determine transgene copy number, of BamH1 restricted genomic DNA from transgenic animals of Ly-6E.1/*lacZ* lines probed with a *lacZ* probe and a *Thy-1* probe. BamH1 digestion of Ly-6E.1/*lacZ* yields a 3.6Kb *lacZ* fragment, the intensity of which is normalised for loading using the hybridisation signal of a 14Kb fragment of the endogenous *Thy-1* gene. After adjusting for loading differences, copy number

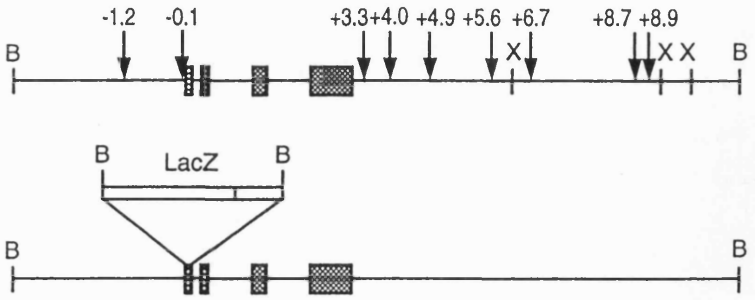
Figure 2. *LacZ* expression from the Ly-6E.1/*lacZ* construct and the generation of transgenic mice.

A. Diagram of the Ly-6E.1/*lacZ* construct compared with the Ly-6E.1 gene showing the location, in Kb, of DNase1 hypersensitive sites relative to transcriptional start site (after Sinclair & Dzierzak, 1993). B = BamH1 and X = Xba1 restriction sites.

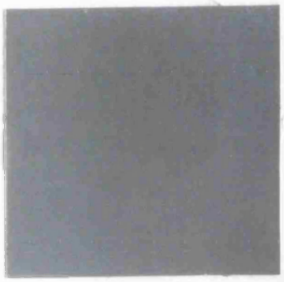
B&C. X-gal stained G418 resistant colonies of NIH3T3 cells after transfection with SV40neo alone (B.) or Ly-6E.1/*lacZ* plus SV40neo (C.).

D. Southern blot of 5μg BamH1 digested DNA from lines BL1a, BL1b, BLR1, BL7 and BL19 run on a 1% agarose/TAE gel. BamH1 digestion removes the *lacZ* gene and SV40 polyadenylation signal from Ly-6E.1/*lacZ* as a 3.3 Kb fragment and cleaves the endogenous *Thy-1* gene, used as a loading control in this case, to yield a 14 Kb fragment. After blotting, the filter was hybridised with ^{32}P-labelled *lacZ* (1.1 Kb EcoRV-BamH1) and *Thy-1* (1.2 Kb Xba1-Nru1) probes. Known amounts of pL6LZ in 5μg of non-transgenic DNA was used for copy number controls. The numbers to the right of the blot correspond to the distance migrated by BstEII digested λ DNA size marker.

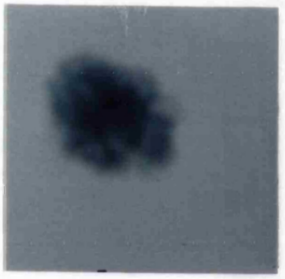
A



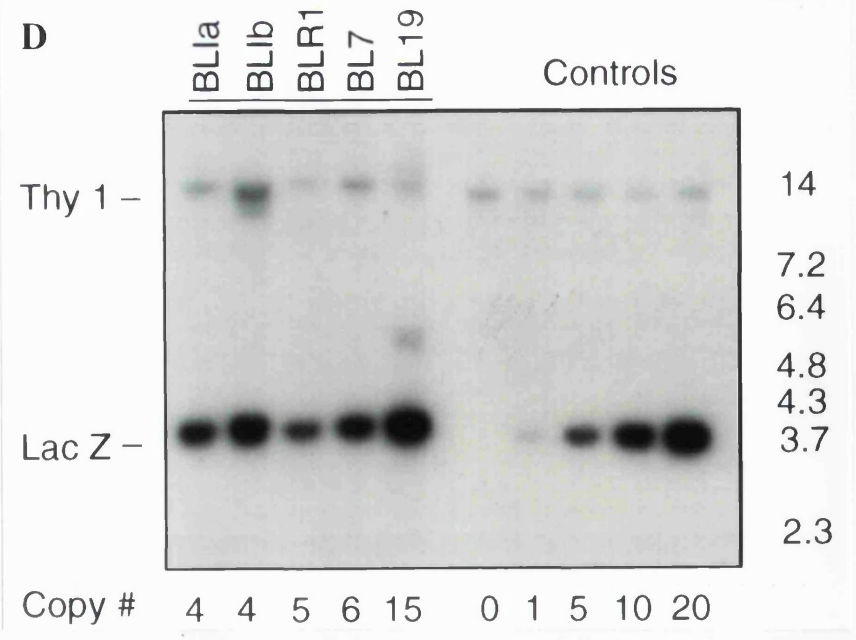
B SV40neo + pAB14



C SV40neo + pL6LZ



D



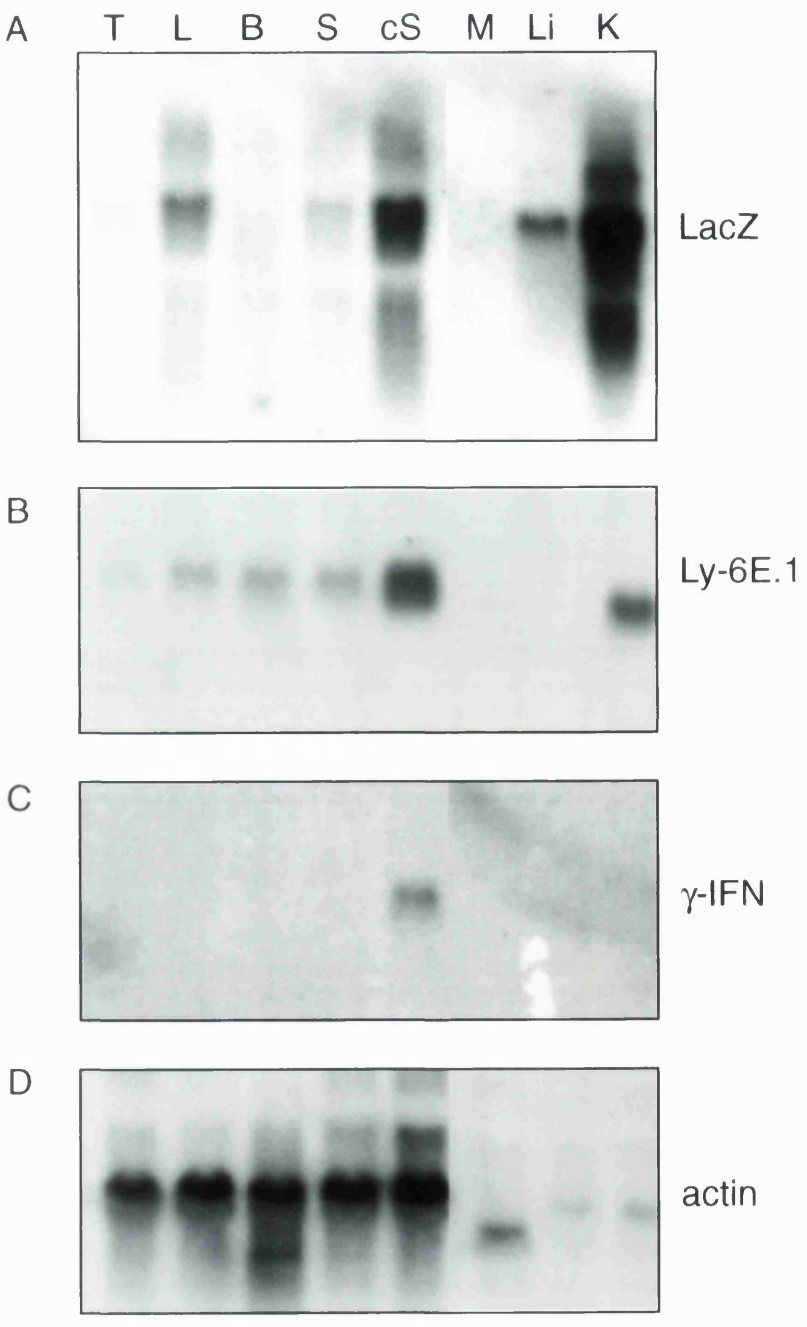
was determined based upon comparison with controls generated from dilutions of pL6LZ representative of specific numbers of transgene copies per genome (1pg plasmid gives an equivalent hybridisation signal to 1 transgene copy in 1μg genomic DNA). Quantitation of hybridising signals was carried out using a Phosphorimager (Molecular Dynamics). From such calculations, the number of copies per genome were estimated as follows: BL1a, 4; BL1b, 4; BL7, 6 and BL19, 15. In addition, a single transgenic founder, BLR1 containing 5 copies of the transgene was included in the analysis but this animal was unable to establish a transgenic line and was considered to be sterile. Copy number analysis of genomic DNA from all BLR1 tissues analysed was performed to determine the level of chimaerism in each. A similar level of transgene contribution (5 copies) was found in each tissue, confirming that BLR1 was not mosaic, ie, not chimaeric for transgenic/non-transgenic cells in any tissue (data not shown).

1.3 Ly-6E.1/*lacZ* mRNA expression analysis.

If the cloned Ly-6E.1 gene was being faithfully expressed *in vivo*, the pattern of *lacZ* expression seen should mirror that of the endogenous Ly-6E.1/A.2 genes across a range of tissues. Northern blot analysis was performed on RNA from all Ly-6E.1/*lacZ* lines and on founder BLR1. Figure 3A shows a representative northern blot of RNA isolated from a panel of adult tissues from a BL1a mouse demonstrating low level expression of *lacZ* in thymus, lymph node, bone marrow, spleen and liver with higher levels in splenocytes which had been activated for 48 hours with

Figure 3. Northern blot analysis of Ly-6E.1/*lacZ* transgenic line BL1a.

A. 15μg of total RNA prepared from thymus (T), lymph node (L), bone marrow (B), spleen (S), conA-treated splenocytes (cS), muscle (M), liver (Li) and kidney (K) was separated through a 1% formaldehyde gel, blotted and hybridised with a ^{32}P-labelled *lacZ* probe. The same filter was sequentially stripped and reprobed with the following probes: B. Ly-6E.1 cDNA; C. γ-interferon; and D. β-actin as a loading control.


A
T
L
B
S
cS
M
Li
K
LacZ
B
Ly-6E.1
C
γ-IFN
D
actin

concanavalin A (conA) and in kidney. When analysed for the expression of the endogenous Ly-6E.1 gene by reprobing the blot with a 760bp EcoR1 Ly-6E.1 cDNA, this pattern of low haematopoietic expression and high expression in the kidney and conA treated spleen is once again apparent (figure 3B). The Ly-6E.1 probe always displays a stronger hybridising signal than *lacZ*. This may be due to the absence of certain genetic elements from the transgene, or possibly reflects differences in mRNA stability or some bias towards detection or preparation of smaller mRNA species. In addition, subtle differences between the Ly-6E.1:*lacZ* mRNA ratio are apparent. Comparison of BL1a lymph node with spleen shows similar levels of Ly-6E.1 mRNA, whereas the level of *lacZ* mRNA is much higher in lymph node. A similar phenomenon is apparent for kidney and conA treated spleen from BL1a mice. These discrepancies in Ly-6E.1:*lacZ* mRNA may be an indication that the cloned Ly-6E.1 gene does not completely recapitulate endogenous gene expression or they may reflect some experimental variations such as differential stability of mRNA species in different tissues or the cross hybridisation of the Ly-6E.1 cDNA probe with Ly-6 family members exhibiting different tissue specificities of expression. A similar expression profile was seen for all lines (table 2). All animals containing the Ly-6E.1/*lacZ* transgene displayed a correspondence of *lacZ* expression with that of the endogenous Ly-6E.1/A.2 (Table 2). In general, high levels of expression of both Ly-6E.1/*lacZ* transgene and the endogenous Ly-6E.1 gene were found in adult kidney and activated lymphocytes, with low levels being detected in the haematopoietic organs, such as thymus, spleen, lymph nodes and bone marrow. As the Ly-6E.1 gene is upregulated upon T cell activation and it has been shown that the gene is γ-interferon inducible *in vitro*, the northern blot of BL1a RNA was also analysed for

Table 2. Summary of tissue specificity of Ly-6E.1/*lacZ* RNA expression in BL transgenic mice.

15μg of total RNA prepared from lymph node (LN), bone marrow (BM), thymus (Th), spleen (Sp), conA-treated spleen (cSp), muscle (M), liver (Li), kidney (K) was analysed by northern blot for Ly-6E.1/*lacZ* expression. Relative levels of *lacZ* hybridisation were determined using a phosphorimager and are shown compared with that of BL1a kidney. +++++ = strong hybridisation (BL1a kidney), +/- = weak hybridisation, - = no signal detected, n.d. = not done.

Line	Tissue (lacZ mRNA)							
	LN	BM	Th	Sp	cSp	M	Li	K
BL1a	+	+/-	+/-	+/-	++	-	+	+++++
BL1b	n.d.	+/-	+/-	+/-	n.d.	-	-	+++++
BLR1	+/-	-	n.d.	-	n.d.	-	-	+++
BL7	n.d.	-	+/-	+/-	+	-	-	+++
BL19	n.d.	++	++	++	n.d.	-	+	+++++

γ-interferon expression. Figure 3C shows the parallel upregulation of γ-interferon with both Ly-6E.1 and *lacZ* in concanavalin A treated spleen cultures. This suggests that both the endogenous Ly-6E.1 gene and the Ly-6E.1/*lacZ* transgene are γ-interferon inducible and upregulated upon T cell activation.

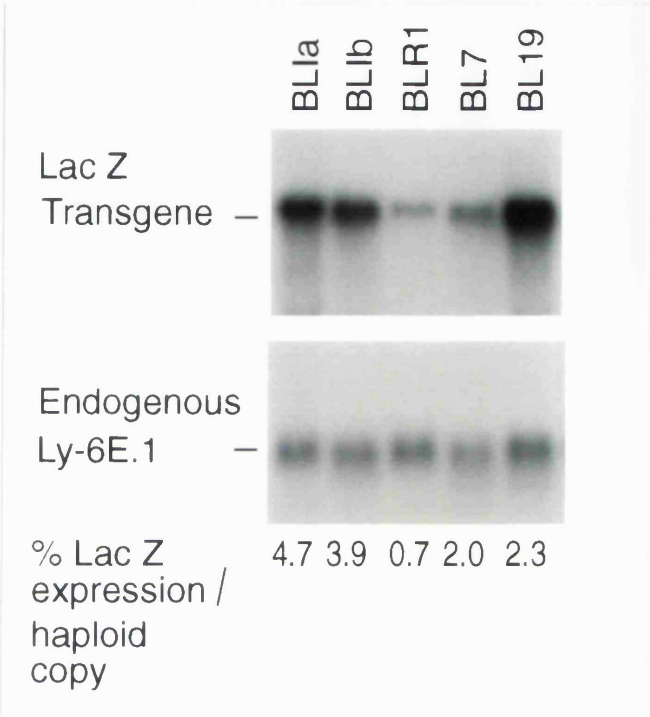
Northern blot analysis was undertaken to assess the relationship between Ly-6E.1/*lacZ* transgene copy number and level of expression. Figure 4A shows a northern blot of RNA isolated from adult kidney from the four lines and one founder of Ly-6E.1/*lacZ* transgenic mice. Kidney RNA was used because it was shown to exhibit the highest levels of endogenous Ly-6E.1/A.2 RNA expression and the highest levels of transgene RNA expression. This facilitated the most accurate quantitation of expression levels to be carried out. Quantitation of transgene expression was performed in the following way: The *lacZ* and Ly-6E.1 hybridising signals in each RNA sample were normalised using the level of expression of the housekeeping gene, GAPDH, and then expressed as a ratio of *lacZ* (transgene) : Ly-6E.1 (endogenous). This comparison of Ly-6E.1/*lacZ* with the endogenous Ly-6E.1/A.2 mRNA confirmed that expression levels increased with increasing copy number (figure 4B). This supports the idea that expression of the Ly-6E.1/*lacZ* transgene is copy number dependent, ie, that the 14 Kb fragment contains regulatory elements such that the surrounding chromatin does little to influence the expression of the transgene. This finding suggests that the transgene is not lacking any regulatory elements and that differences in the ratio of Ly-6E.1:*lacZ* mRNA between tissues could be due to tissue specific differential stability of one RNA species. Alternatively, expression in some tissues of an Ly-6 family member that crosshybridises to the Ly-6E.1 probe may distort the Ly-6E.1:*lacZ* mRNA ratio.

Figure 4. *LacZ* mRNA expression in Ly-6E.1/*lacZ* transgenic kidney.

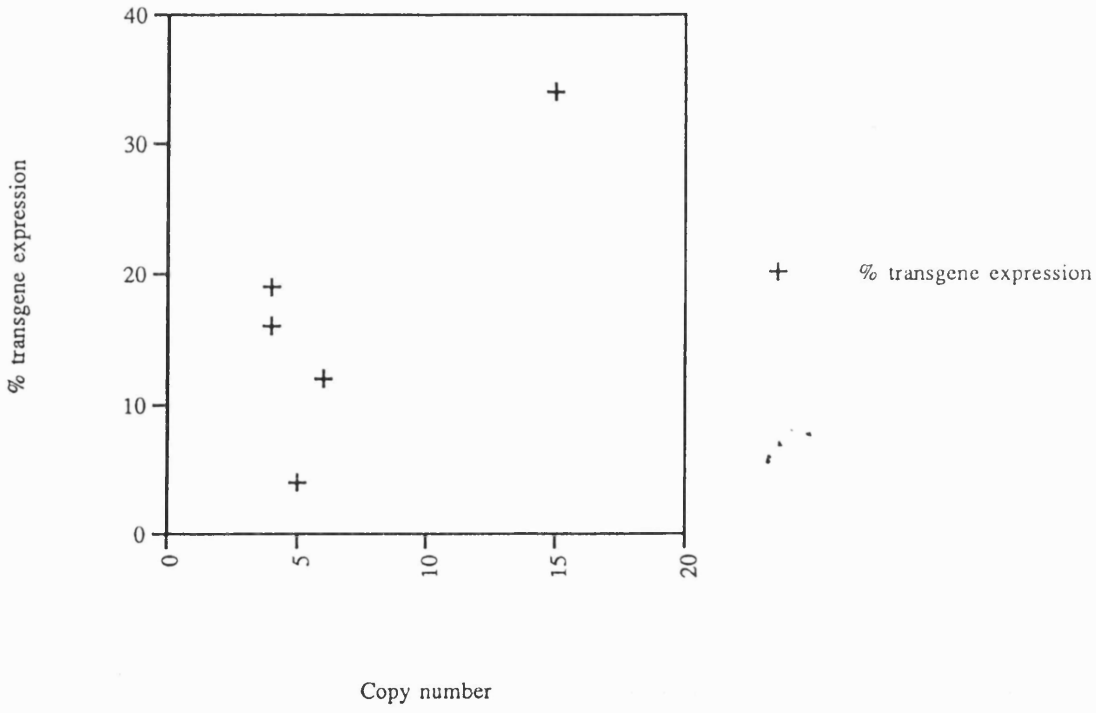
A. 5μg of total kidney RNA from transgenic mice of BL1a, BL1b, BLR1, BL7 and BL19 lines was subject to northern blot analysis and hybridised with ^{32}P-labelled *lacZ* and Ly-6E.1 probes. After normalisation of loading using a GAPDH control probe (not shown), the intensity of the *lacZ* hybridising signal was compared with that of Ly-6E.1 using a phosphorimager and expressed as a percentage of Ly-6E.1 expression.

B. Graph of % *lacZ* expression in kidney *versus* transgene copy number for the Ly-6E.1/*lacZ* mice analysed in A; BL1a (4 copy), BL1b (4 copy), BLR1 (5 copy), BL7 (6 copy) and BL19 (15 copy).

A. Northern blot of RNA from BL transgenic kidney.



B. Transgene expression versus copy number in BL kidney.



1.4 Visualisation of Ly-6E.1/*lacZ* expression using X-gal staining.

The expression pattern of the Ly-6E.1/*lacZ* transgene was examined further utilising X-gal staining. Since high levels of Ly-6E.1/*lacZ* mRNA expression were found in adult kidney, initial X-gal staining experiments were performed on the urinogenital system from a day 15p.c. Ly-6E.1/*lacZ* transgenic mouse of the BL1b line. Figure 5a shows transgene expression in epididymis and kidney but not testis. To examine if this transgenic expression pattern recapitulates the endogenous Ly-6E.1 expression pattern, S1 nuclease protection analysis was performed using a probe specific for the endogenous Ly-6E.1 mRNA. High levels of Ly-6E.1 expression were observed in the kidney and epididymis but not the testis (figure 5b). Control mRNAs from NIH3T3 and MEL cells cultured in the presence or absence of γ-interferon (gift A. Sinclair) are included and demonstrate that Ly-6E.1 is expressed in NIH3T3 but not in MEL cells and is upregulated in both cell types after treatment with γ-interferon. Thus, β-galactosidase expression from the Ly-6E.1/*lacZ* transgene in the urinogenital system precisely correlates with endogenous Ly-6E.1 expression.

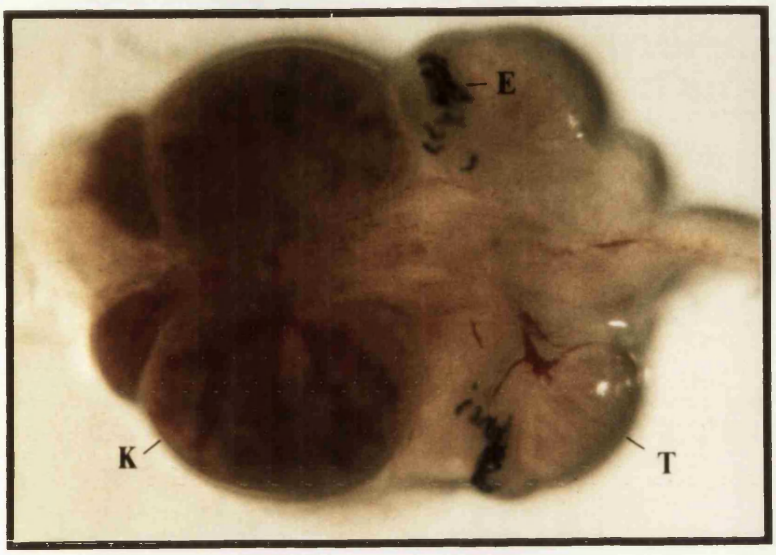
X-gal staining of haematopoietic cells was performed to verify that the expression of Ly-6E.1/*lacZ* is induced upon lymphocyte activation in all Ly-6E.1/*lacZ* transgenic lines. Figure 6 shows a representative example of X-gal stained thymocytes (counterstained with eosin) before and after 48 hour cultivation in the presence of concanavalin A. In this case the animals were of the BL7 line. Activated T cells can be readily distinguished from their resting counterparts because they exhibit an increase in size as they begin to divide rapidly. As shown in figure 6D, it is these larger, activated cells from the BL7 transgenic thymus which have

Figure 5. *LacZ* expression in Ly-6E.1/*lacZ* transgenic urinogenital system.

a. X-gal stained urinogenital system from a day 15.5 p.c. Ly-6E.1/*lacZ* transgenic embryo of the BL1b line. K = kidney, T = testis and E = epididymis.

b. S1 nuclease protection analysis of the expression pattern of Ly-6E.1 in urinogenital tissues from a non-transgenic mouse. RNA from testis, epididymis, kidney and control NIH3T3 and MEL cell lines (+/- γ-interferon) was hybridised with ^{32}P end-labelled Ly-6E.1 and β-actin probes prior to S1 nuclease digestion and analysis on an 8% denaturing acrylamide gel.

a.


E
K
T

b.

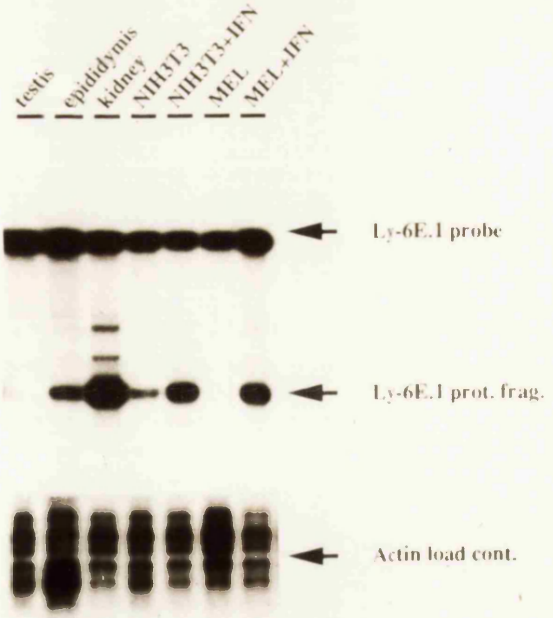

testis
epididymis
kidney
NIH3T3
NIH3T3+IFN
MEL
MEL+IFN
Ly-6E.1 probe
Ly-6E.1 prot. frag.
Actin load cont.

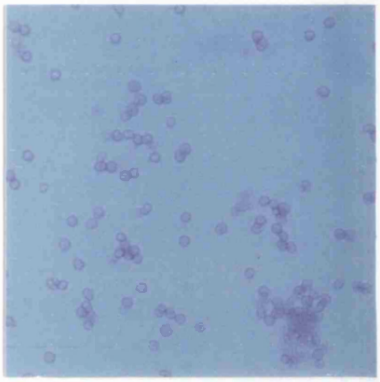
Figure 6. *LacZ* expression in resting and activated Ly-6E.1/*lacZ* transgenic thymocytes.

Thymocytes from BL7 transgenic and a non-transgenic littermates were cultured for 48 hours both with (C. & D.) and without (A. & B.) concanavalin A at 2μg/ml prior to fixation and staining with X-gal. Cells were visualised after cytospin preparation and eosin counterstaining.

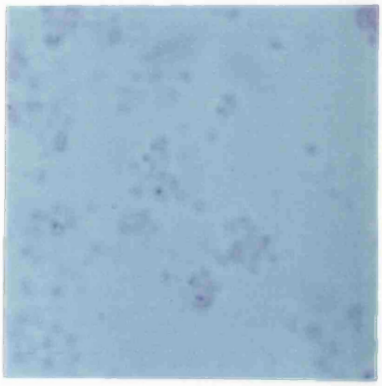
non transgenic thymocytes

BL7 transgenic thymocytes

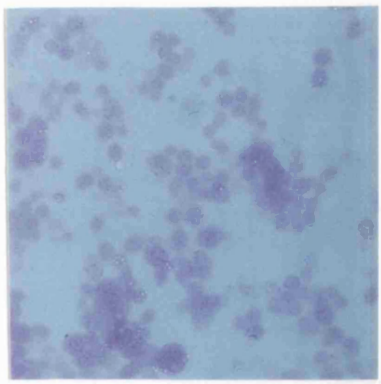
A. untreated



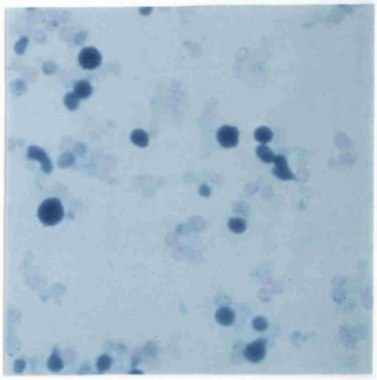
B. untreated



C. +conA



D. +conA



X-gal stained thymocytes +/- activation.

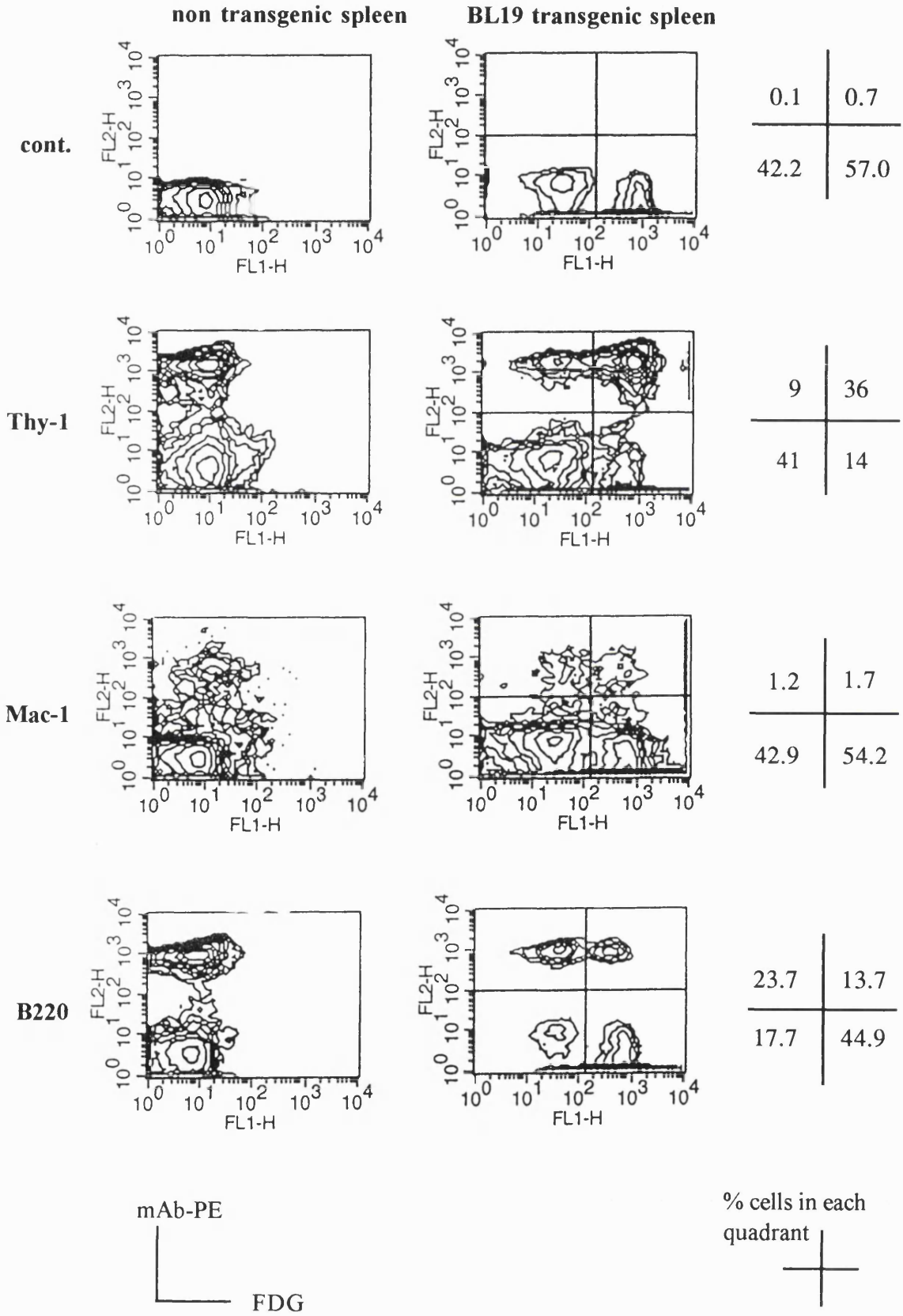
stained with X-gal . Activated thymocytes from the non-transgenic littermate (figure 6C) are completely negative for X-gal staining. This data provides strong evidence that the transgene is expressing faithfully in the haematopoietic system at the level of the individual cell and, as with endogenous Ly-6E.1, is upregulated upon lymphocyte activation.

1.5 FACS/FDG analysis of Ly-6E.1/*lacZ* expression within the haematopoietic system.

To analyse in detail the expression of Ly-6E.1/*lacZ* in cells of the haematopoietic system, the fluorescein-coupled β-galactosidase substrate FDG was used to stain cells and fluorescence activated cell sorting (FACS) was employed to analyse the stained population. The expression of Ly-6E.1/*lacZ* can be specifically localised to particular subsets of cells by double staining haematopoietic cells with both FDG and fluorescent antibodies to particular antigens. This not only verifies that the Ly-6E.1/*lacZ* transgene expresses in the same subsets of cells as the endogenous protein but also indicates which subsets of cells can be targeted with the Ly-6E.1 expression cassette. Figure 7 shows FDG staining (Ly-6E.1/*lacZ*) in subsets of splenocytes from BL19 offspring. Normal and Ly-6E.1/*lacZ* transgenic spleens were homogenised to a single cell suspension and stained with FDG and either PE-conjugated Thy-1 (T cell specific), B220 (B cell specific) or Mac-1 (macrophage specific) monoclonal antibodies. Cells which fall within the upper right quadrant exhibit both green and red fluorescence, ie, $FITC^+$ and PE^+, therefore these cells are

Figure 7. FACS plot analysis of *lacZ* expression in subsets of Ly-6E.1/*lacZ* transgenic splenocytes.

Splenocytes from BL19 Ly-6E.1/*lacZ* transgenic and non-transgenic littermates were first loaded with FDG and then kept ice cold whilst stained with PE conjugated monoclonal antibodies specific for T cells (Thy-1), Macrophages (Mac-1) or B cells (B220). Control samples consisted of splenocytes stained with FDG alone. 10^4 cells were analysed using a Becton Dickinson FACScan. Contour FACS plots are shown, displaying PE fluorescence from each antibody on the ordinate and green fluorescence from FDG on the abscissa. The percentage of splenocytes falling into each quadrant is shown for BL19 transgenic cells to the right of the FACS plots.

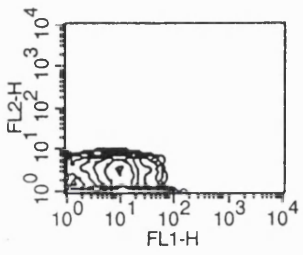
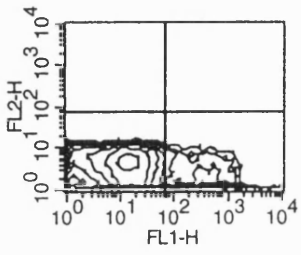
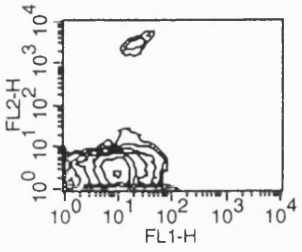
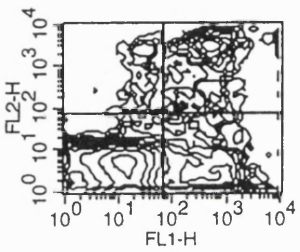
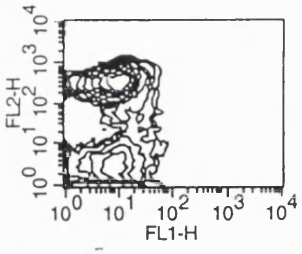
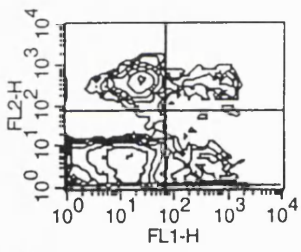
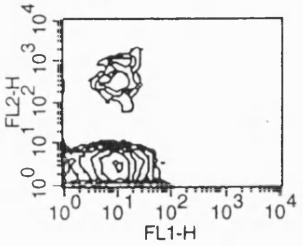
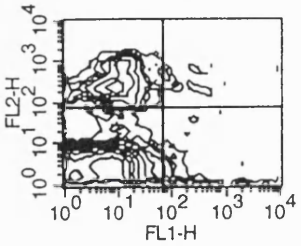
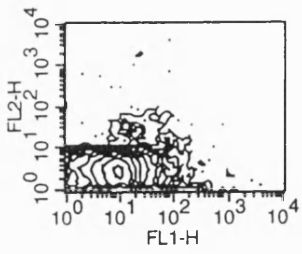
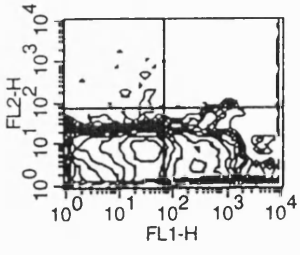

non transgenic spleen
BL19 transgenic spleen
cont.
Thy-1
Mac-1
B220
FL2-H
FL1-H
0.1
0.7
42.2
57.0
9
36
41
14
1.2
1.7
42.9
54.2
23.7
13.7
17.7
44.9
mAb-PE
FDG
% cells in each quadrant

positive for both the cell surface marker and transgenic β-galactosidase expression. Thus, *lacZ* expression can be detected in splenic T and B cells as well as macrophages. This data is consistent with previously published Ly-6E.1 expression profiles (see table 1 and references therein) in which not all T cells, B cells and macrophages express Ly-6E.1 and suggests that these cells are mature and activated.

Based on the fact that the Ly-6E.1 gene encodes Sca-1, a marker of the bone marrow haematopioetic stem cell, and that Ly-6E.1/*lacZ* expression can be detected in bone marrow by northern blot and X-gal staining (not shown), FDG staining was employed in combination with FACS analysis to identify precise subsets of lacZ positive cells in bone marrow. Figure 8 shows FACS plots of bone marrow cells from non-transgenic and Ly-6E.1/*lacZ* transgenic animals of the BL19 line stained with FDG and PE conjugated monoclonal antibodies specific for Thy-1, B220, Mac-1 and c-kit. As was shown with splenocytes, *lacZ* expression is detected on some T cells, B cells and macrophages within the bone marrow compartment. However, *lacZ* is expressed in far fewer macrophages and B cells in the bone marrow compared with splenic macrophages and B cells (compare upper left and upper right quadrants of figures 7 and 8). Approximately one third of splenic B cells and more than half of splenic macrophages are positive for *lacZ* expression whereas less than one tenth of bone marrow B cells and only one third of bone marrow macrophages express *lacZ*. These differences in expression are to be expected if one considers the different types of B cells and macrophages present in spleen and bone marrow. B cells and macrophages found in the spleen are more likely to be mature, activated cells than those in the bone marrow where they originate and consequently are more likely to be expressing Ly-6E.1 and *lacZ*. Intriguingly, a small population of c-kit expressing

Figure 8. FACS plot analysis of *lacZ* expression in subsets of Ly-6E.1/*lacZ* transgenic bone marrow cells.

Bone marrow cells from BL19 Ly-6E.1/*lacZ* transgenic and non-transgenic littermates were permeablised and incubated with FDG and subsequently stained with PE conjugated monoclonal antibodies specific for T cells (Thy-1), Macrophages (Mac-1), B cells (B220) or the c-kit receptor tyrosine kinase. Control samples consisted of bone marrow cells stained with FDG alone. 10^4 cells were analysed using a Becton Dickinson FACScan. Contour FACS plots are shown, on which, PE fluorescence from each antibody = ordinate and green fluorescence from FDG = abscissa. The percentage of bone marrow cells in each quadrant is shown for BL19 transgenic cells to the right of the FACS plots.

	non transgenic bone marrow	BL19 transgenic bone marrow	
cont.			0.0 | 0.1 79.7 | 20.2
Thy-1			0.8 | 4.5 80.6 | 14.1
Mac-1			16.3 | 6.0 63.4 | 14.3
B220			6.1 | 0.5 92.0 | 1.4
c-kit			0.1 | 0.2 74.6 | 25.1

mAb-PE

FDG

% cells in each quadrant

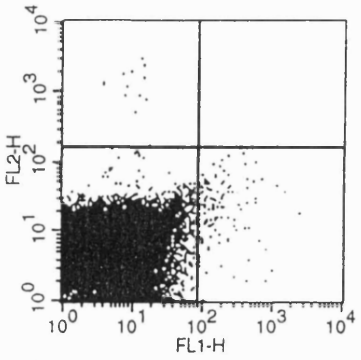
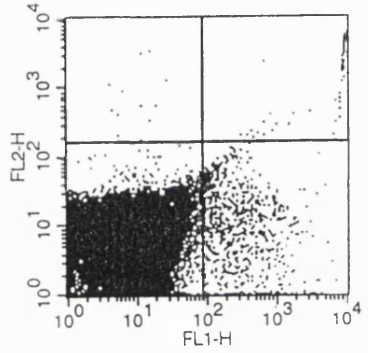
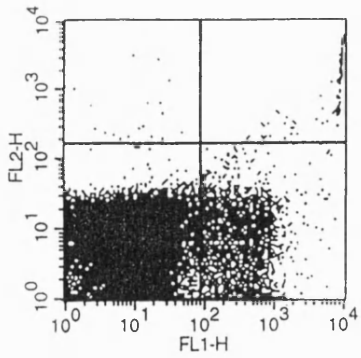
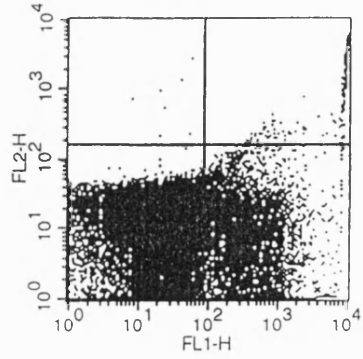
cells seem to be FDG$^+$. In the bone marrow, c-kit$^+$ cells are usually regarded as haematopoietic precursor cells. As cells mature along different lineages, c-kit is downregulated, though not as rapidly as is Sca-1, remaining on the surface of precursor cells that have already become Sca-1$^-$ (Okada *et al.*, 1992; Li & Johnson, 1995). The discovery of some cells that express *lacZ* and are c-kit$^+$ in Ly-6E.1/*lacZ* bone marrow suggests they may be immature haematopoietic progenitor cells. Thus, the cell surface phenotype of FDG$^+$ cells in the bone marrow of Ly-6E.1/*lacZ* transgenic mice corresponds to that reported for Ly-6E.1$^+$ non-transgenic bone marrow cells.

To determine whether transgene copy number correlated with levels of Ly-6E.1/*lacZ* expression in haematopoietic tissue, splenocytes from transgenic animals of several BL lines were examined utilising FDG staining and FACS analysis. In this experiment, mice of the BL1a line were interbred to generate homozygous animals with a transgene copy number of 8 and were compared with BL7 (6 copies) and BL19 (15 copies) mice. FACS-FDG analysis of splenocytes (figure 9) demonstrated that the percentage of FDG positive cells increased with increasing transgene copy number. As with *lacZ* mRNA expression levels in kidney (figure 4), this data on β-galactosidase activity supports the suggestion that the Ly-6E.1/*lacZ* transgene is copy number dependent and independent of the influences of the surrounding chromatin at the site of transgene integration.

To directly determine whether all Sca-1$^+$ bone marrow cells express Ly-6E.1/*lacZ* in transgenic mice, transgenic bone marrow was stained with a combination of FDG and a PE conjugated Sca-1 monoclonal antibody and examined by FACS analysis. Figure 10A shows that not all FDG$^+$ cells express Sca-1, suggesting that

Figure 9. FACS plot analysis of *lacZ* expression in whole Ly-6E.1/*lacZ* transgenic spleen.

Spleens from age and sex-matched BL7, BL1a homozygous, BL19 and non-transgenic Ly-6E.1/*lacZ* mice were homogenised on ice and loaded with FDG. *LacZ* expression was detected as green fluorescence (abscissa) using a Becton Dickinson FACScan and displayed on FACS dot plots. The percentage of FDG positive spleen cells determined for each animal is shown.

	FDG stained splenocytes	% FDG positive	transgene copy no.
non transgenic		0.28	0
BL7		1.69	6
BL1a (hom)		7.88	8
BL19		17.73	15

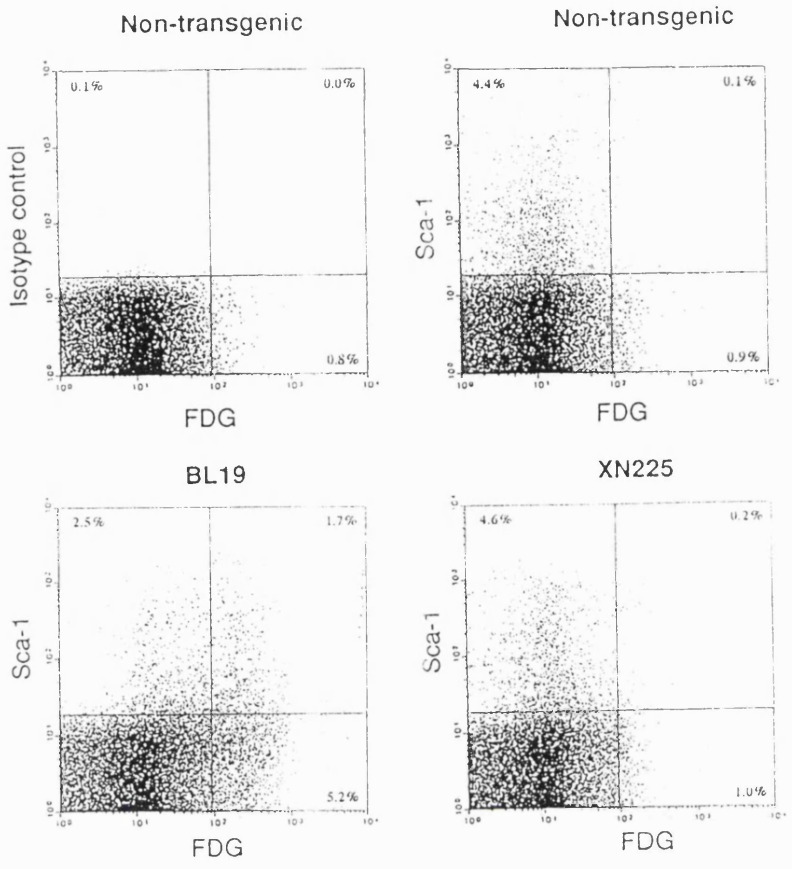
FDG

Figure 10. Direct comparison of *lacZ* expression with Sca-1 in Ly-6E.1/*lacZ* transgenic bone marrow.

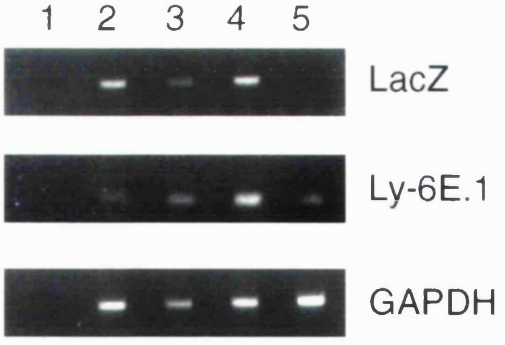
A. FACS plot analysis of Sca-1 (PE, ordinate) and *lacZ* (FDG, abscissa) expression on bone marrow cells of age and sex-matched transgenic and non-transgenic mice.

B. RT-PCR analysis of *lacZ* and Ly-6E.1 expression in FACS sorted populations of bone marrow cells. Lane 1, whole, non-transgenic bone marrow; Lane 2, whole, BL19 Ly-6E.1/*lacZ* transgenic bone marrow; Lane 3, BL19 Sca-1^+, FDG^+ cells (upper right quadrant); Lane 4, BL19 Sca-1^-, FDG^+ cells (lower right quadrant); Lane 5, no cells RT-PCR control. For each sample, RNA from between 4-8x10^5 cells was reverse transcribed and an equal volume of the RT product, in each case, was subject to PCR to detect expression of *lacZ*, Ly-6E.1 and, as a control for the RT step, GAPDH. Primers to both Ly-6E.1 and GAPDH failed to amplify genomic sequences, thereby excluding the possibility of contaminating DNA within the RNA preparation. Amplified products were visualised on 1.5% ethidium bromide stained agarose/TAE gels.

A


Non-transgenic
Non-transgenic
Isotype control
0.1%
0.0%
0.8%
4.4%
0.1%
0.9%
Sca-1
FDG
BL19
XN225
2.5%
1.7%
5.2%
4.6%
0.2%
1.0%
Sca-1
FDG

B


1 2 3 4 5
LacZ
Ly-6E.1
GAPDH

there may be ectopic expression of the Ly-6E.1/*lacZ* transgene within the bone marrow. An alternative explanation could be that post-transcriptional differences between endogenous Ly-6E.1 and transgenic Ly-6E.1/*lacZ* may result in the detection of $FDG^+Sca\text{-}1^-$ cells. In order to investigate this further, $FDG^+Sca\text{-}1^+$ and $FDG^+Sca\text{-}1^-$ cells were sorted from BL19 bone marrow and RNA prepared from the sorted populations as well as from unsorted Ly-6E.1/*lacZ* transgenic and non-transgenic bone marrow cells as controls. RT-PCR analysis (Figure 10B) revealed that both the Ly-6E.1/*lacZ* transgene and the endogenous Ly-6E.1 gene were expressed in $FDG^+Sca\text{-}1^-$ cells at the level of mRNA. This result strongly suggests that the transgene is, indeed recapitulating the endogenous gene expression pattern and that the $FDG^+Sca\text{-}1^-$ cells arise as a result of some post transcriptional mechanism, possibly due to delayed transport/processing of the GPI-linked Ly-6E.1 protein.

1.6 The Ly-6E/*lacZ* construct expresses in haematopoietic stem cells.

In addition to providing a sensitive technique for the analysis of *lacZ* expression at the level of the single cell in combination with specific monoclonal antibodies, FDG is non-toxic and its use requires no cellular fixation. Consequently cells remain viable and functional after staining. This property allows the question of whether Ly-6E.1/*lacZ* is expressed on haematopoietic stem cells to be directly addressed. Haematopoietic stem cells are defined by their ability to reconstitute lethally irradiated transplantation recipients. Therefore to prove *lacZ* expression in

haematopoietic stem cells, bone marrow cells from Ly-6E.1/*lacZ* transgenic mice were stained with FDG. Stained cells were FACSorted into positive and negative populations and transplanted into irradiated mice to test for haematopoietic reconstitution. If the Ly-6E.1/*lacZ* transgene were expressed in haematopoietic stem cells, the positive population of cells would be enriched for haematopoietic activity, whilst the negative would be depleted of this activity.

Figures 11A and 11B show typical FDG staining profiles of control non-transgenic and Ly-6E.1/*lacZ* bone marrow from a homozygous animal of the BL1a transgenic line. Between 10% and 20% of total bone marrow cells stain positive with FDG. This is also observed with animals of BL19 (figure 12B) and BL1b (not shown) transgenic lines. Positive fluorescent cells were separated from non-fluorescent cells using a FACSTAR cell sorter. A "window" of cells corresponding to those at the border between positive and negative cells was left uncollected in order to minimise cross contamination of sorted populations. Positive and negative sorted cells were injected intravenously into irradiated recipient mice at serial dilutions, in triplicate for each cell number. In addition, FDG stained but unsorted bone marrow was also injected at serial dilutions to control for any adverse affects of FDG staining on the haematopoietic potential of the bone marrow.

As all donor cells, regardless of β-galactosidase expression, derive from Ly-6E.1/*lacZ* transgenic bone marrow and only differ with respect to expression of the transgene, any donor cells present in the recipient animals will be marked with *lacZ*. The haematopoietic potential of sorted populations was determined by analysing peripheral blood genomic DNA from recipient mice for the presence of donor *lacZ* sequences at one and four months post-transplantation. The results of two such

Figure 11. Bone marrow transplantation to test for haematopoietic stem cell specific expression of *lacZ* in Ly-6E.1/*lacZ* transgenic mice I.

A. FACS histogram of control non-transgenic bone marrow stained with FDG. Ordinate = cell number, abscissa = green (FDG) fluorescence intensity.

B. FACS histogram of homozygous BL1a Ly-6E.1/*lacZ* transgenic bone marrow stained with FDG. Ordinate = cell number, abscissa = green (FDG) fluorescence intensity. Note increased number of cells exhibiting FDG fluorescence of greater than 10^2 units. Bars below the abscissa correspond to the parameters used for FACS sorting FDG^+ and FDG^- cells.

C. PCR detection of *lacZ* donor specific sequences in peripheral blood of non-transgenic irradiated recipient mice. The arrows link the sorted cells with DNA samples from irradiated mice that received the stated number of cells from each population. Peripheral blood genomic DNA was isolated 4 months post-transplantation and subject to PCR amplification using primers specific for *lacZ* and, as a loading control, the endogenous myogenin (myo) gene. Control lanes consist of appropriate dilutions of genomic DNA from a BL1a transgenic mouse mixed with non-transgenic genomic DNA.

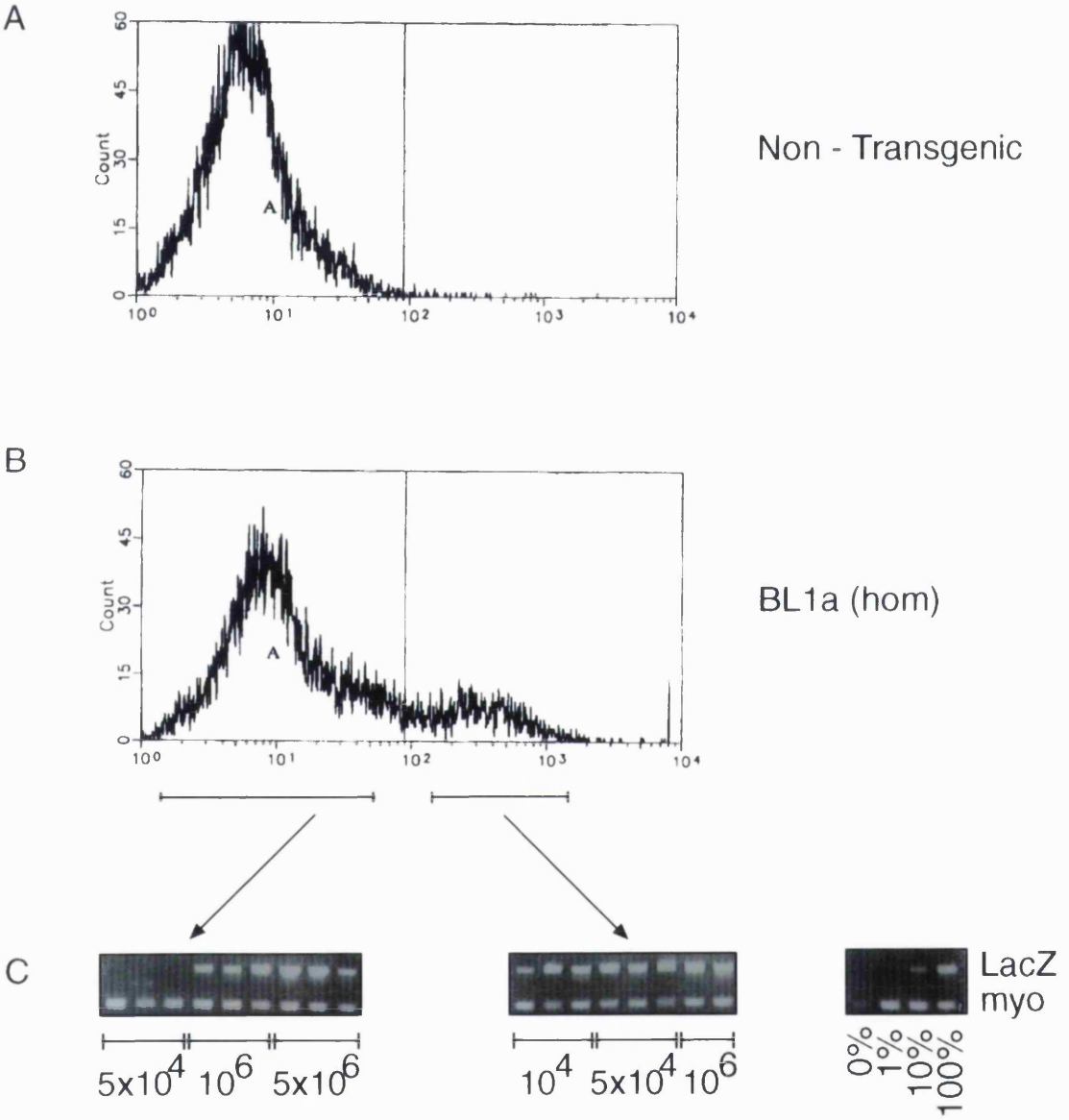
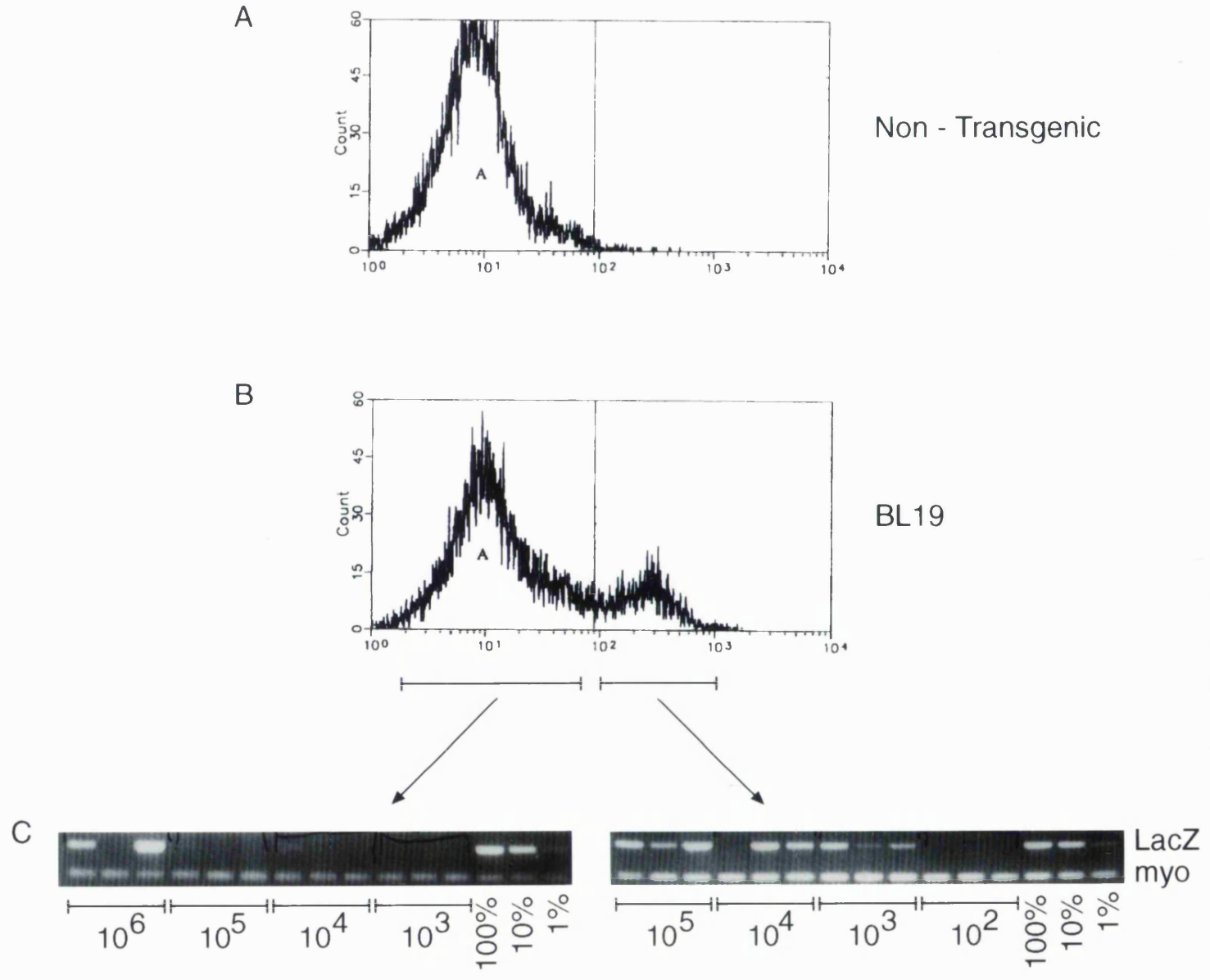

A
Count
60
45
30
15
0
A
10^0 10^1 10^2 10^3 10^4
Non - Transgenic
B
Count
60
45
30
15
0
A
10^0 10^1 10^2 10^3 10^4
BL1a (hom)
C
$5x10^4$ 10^6 $5x10^6$
10^4 $5x10^4$ 10^6
LacZ
myo
0%
1%
10%
100%

Figure 12. Bone marrow transplantation to test for haematopoietic stem cell specific expression of *lacZ* in Ly-6E.1/*lacZ* transgenic mice II.

A. FACS histogram of control non-transgenic bone marrow stained with FDG. Ordinate = cell number, abscissa = green (FDG) fluorescence intensity.

B. FACS histogram of heterozygous BL19 Ly-6E.1/*lacZ* transgenic bone marrow stained with FDG. Ordinate = cell number, abscissa = green (FDG) fluorescence intensity. Note increased number of cells exhibiting FDG fluorescence of greater than 10^2 units. Bars below the abscissa correspond to the parameters used for FACS sorting FDG^+ and FDG^- cells.

C. PCR detection of *lacZ* donor specific sequences in peripheral blood of non-transgenic irradiated recipient mice. The arrows link the sorted cells with DNA samples from irradiated mice that received the stated number of cells from each population. Peripheral blood genomic DNA was isolated 4 months post-transplantation and subject to PCR amplification using primers specific for *lacZ* and, as a loading control, the endogenous myogenin (myo) gene. Control lanes consist of appropriate dilutions of genomic DNA from a BL19 transgenic mouse mixed with non-transgenic genomic DNA.


A
Count
A
Non - Transgenic
B
Count
A
BL19
C
LacZ
myo
10^6
10^5
10^4
10^3
100%
10%
1%
10^5
10^4
10^3
10^2
100%
10%
1%

experiments carried out independently - using mice of the BL1a and BL19 lines are shown in figures 11C and 12C, respectively. Both figures show levels of donor derived repopulation at 4 months post-transplantation. In both cases peripheral blood genomic DNA from each individual (non-transgenic) recipient mouse was subjected to PCR analysis using primers specific for *lacZ* sequences and the endogenous myogenin gene as an internal control for the amount of DNA present. A clear and substantial enrichment of haematopoietic stem cell activity, as determined by long term repopulation of peripheral blood, was found in recipients injected with FDG^+ sorted cells. Haematopoietic engraftment could be detected in recipients of as few as 10^3 FDG^+ cells, whilst very little haematopoietic reconstituting activity is associated with FDG^- cells and only detectable with 10^6 transplanted FDG^- cells. Although the PCR analysis suggests that up to 100% of peripheral blood DNA is donor derived (figures 11C and 12C), the most stringent assay for haematopoietic stem cells requires the demonstration of multilineage reconstitution. To demonstrate this, a recipient that received $5x10^4$ FDG^+ bone marrow cells from the BL1a derived transplant was sacrificed at 10 months post-transplantation and subsets of spleen cells were analysed for *lacZ* expression. Figure 13 shows FACS profiles of spleen cells stained with FDG and with PE conjugated antibodies against Thy-1 (T cell specific), B220 (B cell specific) and Mac-1 (macrophage specific). *LacZ* expression was found in cells of the T and B lymphocyte and macrophage lineages, indicating that the haematopoietic reconstitution from $lacZ^+$ cells was multilineage. The percentage of FDG^+ cells in the spleen of the radiation chimaera generated from a BL1a homozygous mouse is similar to the percentage found in previously analysed BL1a homozygotes (see figure 9), suggesting that all splenocytes are transgenic in the

Figure 13. Analysis of multilineage reconstitution by *lacZ*$^+$ FACS sorted bone marrow in BL1a radiation chimaera.

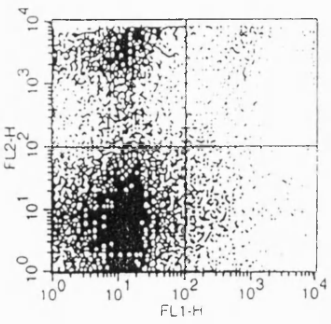
A. Splenocytes from a BL1a(hom) derived radiation chimaera were loaded with FDG and then kept ice cold whilst stained with PE conjugated monoclonal antibodies specific for T cells (Thy-1), Macrophages (Mac-1) or B cells (B220). 10^4 cells were analysed using a Becton Dickinson FACScan. FACS dot plots are shown, on which PE fluorescence from each antibody is displayed on the ordinate and green fluorescence from FDG on the abscissa. The percentage of splenocytes falling into each quadrant is shown to the right of the FACS plots.

B. Southern blot of 5μg DNA from tissues of BL1a(hom) derived radiation chimaera. Genomic DNA from thymus (T), spleen (S), bone marrow (BM), purified B cells and purified macrophages (mac) was digested with BamH1 and run on a 1% agarose/TAE gel. BamH1 digestion removes the *lacZ* gene and SV40 polyadenylation signal from Ly-6E.1/*lacZ* as a 3.3 Kb fragment and cleaves the endogenous *Thy-1* gene into a 14 Kb fragment. After blotting, the filter was hybridised with ^{32}P-labelled *lacZ* (1.1 Kb EcoRV-BamH1) and, as a loading control, *Thy-1* (1.2 Kb Xba1-Nru1) probes. Genomic DNA from a known BL1a transgenic mouse was used as a positive control (+ve). The size discrepancy between *lacZ* hybridising bands from purified cell DNA and from tissue is due to an artefact within the gel, as a similar retardation can be seen for the *Thy-1* specific signal.

A

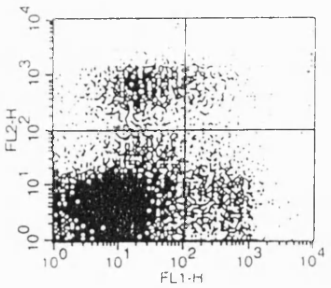
BL1a radiation chimaera - splenocytes

Thy-1



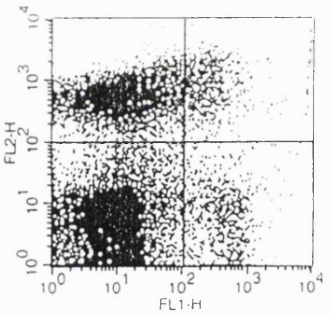
17.83	2.1
76.1	3.97

Mac-1



6.99	1.31
84.81	6.89

B220



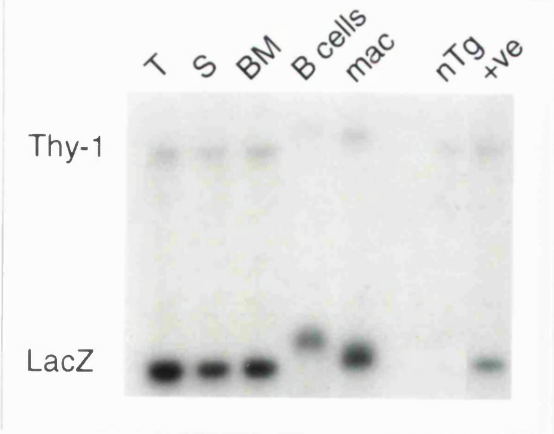
32.9	3.73
59.11	4.26

mAb-PE

FDG

% cells in each quadrant

B



chimaera and that reconstitution is 100%. In addition, genomic DNA was prepared from thymus, spleen, lymph nodes, purified macrophages and purified B cells of the reconstituted animal and subjected to Southern blot analysis. Figure 13B shows such a blot probed for the *lacZ* transgene and the endogenous *Thy-1* gene as a control, demonstrating the presence of the transgene in T cells (thymus), B cells and macrophages, further supporting notion that reconstitution was 100%, ie, the entire haematopoietic system of the recipient was reconstituted by transplanted *lacZ*$^+$ bone marrow cells. These sorting experiments provide direct evidence that Ly-6E.1/*lacZ* is expressed in haematopoietic stem cells and that the Ly-6E.1 transgene can be used to express heterologous genes with a view to manipulating the transplantable bone marrow haematopoietic stem cell.

The degree of enrichment of haematopoietic stem cells within the FDG positive sorted cells compared with negative suggests that the Ly-6E.1/*lacZ* transgene is expressed on nearly all stem cells in the outbred (CBA/Ca x C57/B10) F1 transgenic mice. The combination of Ly-6E.1/*lacZ* and FDG sorting provides a means to enrich haematopoietic stem cells from bone marrow without the use of antibodies. This is advantageous since the binding of antibodies may alter the very cells they are intended to purify, by delivering mitogenic signals.

1.7 X-gal staining of Ly-6E.1/*lacZ* expression during ontogeny.

The expression pattern of Ly-6E.1 during embryonic development has not been investigated. Conventional techniques such as *in situ* hybridisation to mRNA and immunohistochemistry are of limited use due to the size of the Ly-6 superfamily

and the degree of homology that exists between members. Conventional probes could cross react with several family members and yield potentially confusing results. Having demonstrated that the Ly-6E.1/*lacZ* transgene can recapitulate the endogenous Ly-6E.1 gene expression pattern, *lacZ* expression in embryos of Ly-6E.1/*lacZ* transgenic mice was used for the examination of the expression pattern of Ly-6E.1 in the developing embryo. Of particular interest is the expression pattern between day 10p.c. and day 11p.c. when definitive haematopoiesis is thought to originate within the body of the embryo. Recent data has suggested that mammals may be similar to other vertebrates in that they exhibit primitive and definitive haematopoiesis, the former associated with the yolk sac and the latter originating in dorsal, mesodermal tissue within the embryo corresponding to a region including the dorsal aorta, gonads and mesonephros (AGM) in mice (reviewed by Dzierzak & Medvinsky, 1995). Sca-1 is a marker of bone marrow haematopoietic stem cells (definitive) but not yolk sac (primitive) and there is some evidence from RT-PCR analysis of dissected embryos that Sca-1 is expressed in the AGM region (Dzierzak *et al.*, 1995). If Sca-1 is a marker of definitive haematopoiesis, analysis of X-gal stained Ly-6E.1/*lacZ* embryos could enable the visualisation of the first definitive haematopoietic stem cells within the embryo.

Representative whole mount X-gal stained embryos of the BL1b line from day 9.5p.c. to day 11.5p.c. are shown in figure 14a. Intense X-gal staining is clearly visible in the tail at all these stages and is found in embryos of all BL lines. Blue staining is visible within one hour of addition of the X-gal substrate, suggesting that the Ly-6E.1/*lacZ* transgene is expressed to high levels. The staining at day 9.5p.c. was specifically located in the hindgut and did not extend into the fore-gut. The

Figure 14. X-gal staining of wholemount BL1b Ly-6E.1/*lacZ* transgenic embryos.

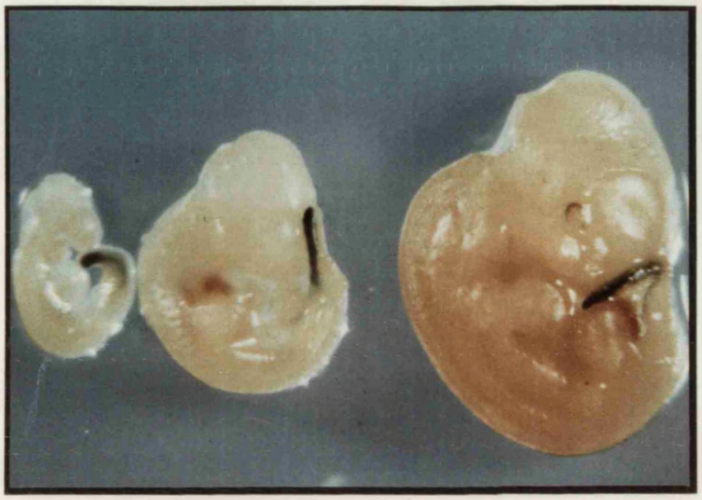
Whole mount X-gal stained embryos isolated at: a. days 9.5, 10.5 & 11.5 p.c.

b. Whole mount X-gal stained Ly-6E.1/*lacZ* embryo at day 11.5 p.c. A small piece of the body wall has been removed after fixation and staining to expose the underlying mesonephric region. M = mesonephros.

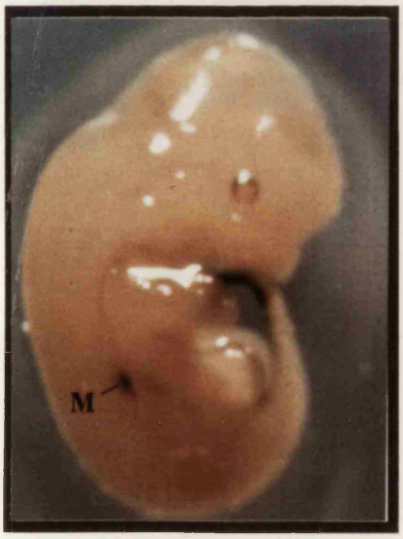
c. Partially dissected X-gal stained Ly-6E.1/*lacZ* transgenic embryo at day 11.5 p.c. The limb buds, somites, head and upper body have been removed to enable *lacZ* expression in the tail, hindgut, gut hernia and anterior mesonephric region to be clearly seen. G = gut hernia (contiguous with the hindgut endoderm), M = mesonephros.

The X-gal staining pattern observed for animals of the BL1b line, shown here, was reproduced by transgenic embryos of lines BL1a, BL7 and BL19.

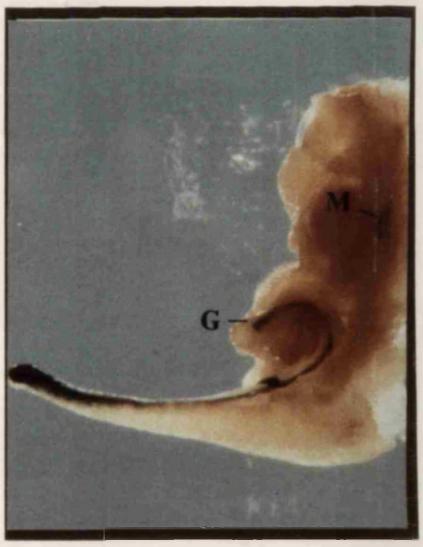
a.



b.



c.



staining remains intense within the endoderm of the hindgut between days 8.5p.c. and 12.5p.c. and can also be seen in mesodermal tissue and notochord. As embryos age past day 11.5p.c., the staining recedes posterially until approximately day 14p.c. when only weak, diffuse specks of staining are visible at the tail tip (not shown).

Closer examination of day 11.5p.c. embryos revealed another site of intense X-gal staining, in a region containing the mesonephros. Figures 14b and 14c show partly dissected day 11.5p.c. embryos of the BL1b line. Intense X-gal staining was observed in the mesonephros (M) and in the tail extending into the gut hernia (G). Temporal analysis of X-gal staining in the mesonephros demonstrated that it was first visible at day 10.5p.c. often only in one mesonephric tubule and became increasingly widespread in the anterior mesonephros between days 10.5 and 12.5p.c. when the staining receded anteriorly towards the tip of the mesonephros. Transverse sections through the AGM region clearly show the X-gal staining within the mesonephric tubules (M) and the fact that not all tubules of the mesonephros stain (Figure 15). This temporal pattern of X-gal staining within the mesonephros is intriguing as it coincides with the acquisition of haematopoietic stem cell activity by this region (Medvinsky *et al.*, 1993; Müller *et al.*, 1994).

Specific X-gal staining above background is not detectable in yolk sac at any stage in development from day 8p.c. to birth (not shown). Analysis of whole mount tissues cannot rule out the possibility that a small number of isolated or weakly expressing *lacZ*$^+$ cells are present in yolk sac. However, the absence of Ly-6E.1/*lacZ* expression in yolk sac during mid-gestation is consistent with the absence of Sca-1 (Huang & Auerbach, 1993) and Ly-6E.1 (Dzierzak *et al.*, 1995) expression at day 10p.c. and with the primitive nature of yolk sac haematopoiesis.

Figure 15. Transverse sections through X-gal stained Ly-6E.1/*lacZ* transgenic embryos.

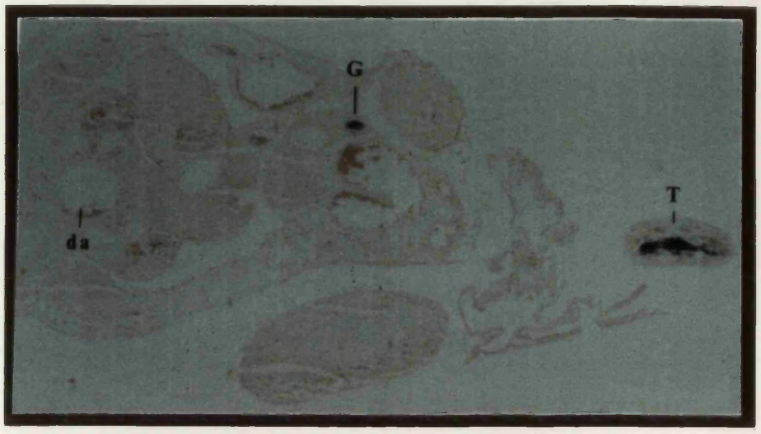
Fixed and stained embryos were dehydrated and mounted in paraffin wax prior to sectioning. 8μm dewaxed sections were rehydrated, counterstained with eosin, mounted in DPX mountant and photographed on a Zeiss Axiophot microscope.

a. Transverse section through X-gal stained BL1b embryo at day 11.5 p.c. da = dorsal aortal, G = gut endoderm, T = tail.

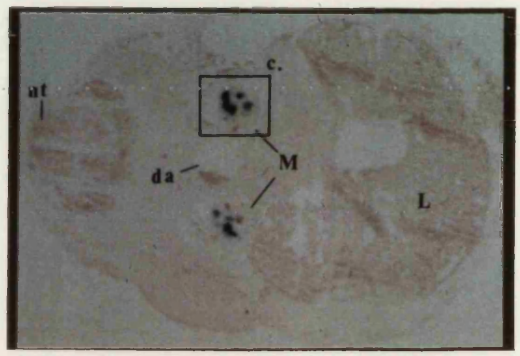
b. Transverse section through the same X-gal stained BL1b embryo as in a. This section is derived from a more anterior location in the embryo and clearly shows the *lacZ* expression in the mesonephric region. nt = neural tube, L = liver, da = dorsal aorta and M = mesonephros.

c. Higher magnification of the boxed area in plate b. showing details of X-gal staining within mesonephric tubules.

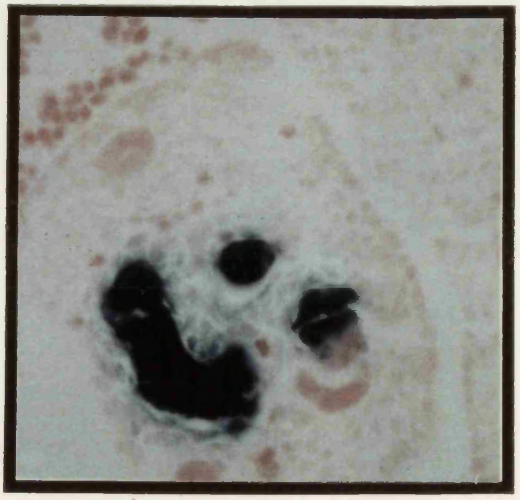
a.



b.



c.



2. Deletion analysis of the Ly-6E.1/*lacZ* transgene.

2.1 Introduction.

The results of the previous section have demonstrated that the cloned 14 Kb Ly-6E.1 gene recapitulates the expression pattern of the endogenous Ly-6E.1 gene *in vivo* and directs a specific, reproducible pattern of lacZ expression during embryonic development. The Ly-6E.1/*lacZ* construct described provides a suitable starting point for studying the genetic regulatory elements of the Ly-6E.1 gene that direct expression *in vivo*. In order to identify tissue and developmental stage specific regulatory elements, regions upstream or downstream of the Ly-6E.1 gene can now be deleted or mutated and these constructs used to generate transgenic mice. The resulting levels and pattern of expression in transgenic mice can be compared with those obtained from the full length 14 Kb clone to determine regions important for directing high levels of tissue-specific expression, in particular to haematopoietic stem cells.

In addition, it is important to determine whether the Ly-6E.1 expression vector could be reduced in size whilst maintaining faithful expression in Sca-1^+ cells. A 14 Kb expression cassette is difficult to manipulate and is too large to be accomodated by retroviral vectors. If the Ly-6E.1 transgene could be minimalised, whilst retaining specific expression properties, the haematopoietic system alone could be targeted by infecting bone marrow and generating chimaeric mice with transgenic bone marrow. Retroviral delivery of such a construct to bone marrow cells would circumvent the

potential problems associated with Ly-6E.1 directed expression of the heterologous gene in Sca-1$^+$ non-haematopoietic cells, such as the kidney, as in chimaeric mice, only the haematopoietic system would be transgenic.

To determine whether regions flanking the Ly-6E.1 gene were required for the *in vivo* expression described for BL Ly-6E.1/*lacZ* transgenic mice, deletion analysis was initiated. It was decided to initiate *in vivo* deletion analysis by removing the most distal 3' region, encompassing three DNaseI hypersensitive sites, +6.7, +8.7 and +8.9, a total of 4 Kb of sequence from the full length Ly-6E.1/*lacZ* construct. Figure 16A illustrates a map of the construct which is depicted in relation to the genomic Ly-6E.1 clone showing the DNaseI hypersensitive sites.

Previously, *in vitro* transfection studies (Sinclair *et al.*, 1996) identified several potential regulatory elements located 3' to the Ly-6E.1 gene which were important for high levels of expression in transfected MEL cells. Most significantly, the removal of the most distal 2Kb, including DNaseI hypersensitive sites +8.7 and +8.9, resulted in a 10 fold reduction in the level of expression per transfected copy, whilst removal of a further 2.5Kb, including DNaseI hypersensitive site +6.7, only resulted in a 2 fold decrease per transfected copy (Sinclair *et al.*, 1996). This data suggests that the most important elements within the 3' region are contained within the most distal 2Kb, most likely corresponding to DNaseI HSSs +8.7 and +8.9. Due to the design of reporter constructs used in the transfection studies, two regions were omitted from all constructs, upstream of -1.8Kb and the region encompassing the gene itself from +0.02Kb to +2.94Kb. Although no DNaseI hypersensitive sites have been located in these regions, the possibility remains that they include some regulatory elements. The location of regulatory elements 3' to the Ly-6E.1 gene

suggested by *in vitro* studies is consistent with many other genes expressed in the haematopoietic system that require 3' genetic elements for their expression, such as: β-globin (Antoniou *et al.*, 1988; Behringer *et al.*, 1987); lysozyme (Bonnifer *et al.*, 1990); CD2 (Greaves *et al.*, 1989); CD3 (Clevers *et al.*, 1989); the T cell receptor genes (Leiden, 1993) and another stem cell antigen, CD34 (May & Enver, 1995).

The results obtained for the 14 Kb Ly-6E.1 clone suggest that this clone may contain a dominant, locus controlling element (Dillon & Grosveld, 1993) although copy number dependent and position independent expression was not consistently observed for all BL lines. However, a consistent expression pattern was maintained in haematolymphoid cells, kidney, tail and mesonephros in all lines, suggesting some resistance to chromosomal position effect silencing. The 14Kb Ly-6E.1 subclone could contain some dominant regulatory element representing an example of a regulatory element of "lower order" than an LCR (May & Enver, 1995). Tapscott *et al.* (1993) postulated the existence of chromatin dependent elements with different degrees of penetrance which would be influenced to varying degrees by the surrounding chromatin and the strength of regulatory elements nearby.

The characterisation of the tissue and stage specific genetic regulatory elements of the Ly-6E.1 gene will also increase our understanding of the way in which the complex pattern of Ly-6E.1 expression is controlled across several diverse tissue types. For example, what are the tissue specific genetic regulatory elements of the Ly-6E.1 gene and do similar factors and pathways act on the Ly-6E.1 gene in different tissues and at different stages of development and ? If haematopoietic stem cell specific regulatory elements could be identified, retroviruses and transgenes targeting stem cell specific expression could be generated. In addition, a knowledge

of the transcription factors and signal transduction pathways that are active in haematopoietic stem cells could provide important information on the nature of the molecular events within these cells, such as events leading to multipotency and self-renewal.

2.2 Generation of Ly-6E.1/*lacZ* transgenic mice lacking the 3' region of the Ly-6E.1 transgene (XN mice).

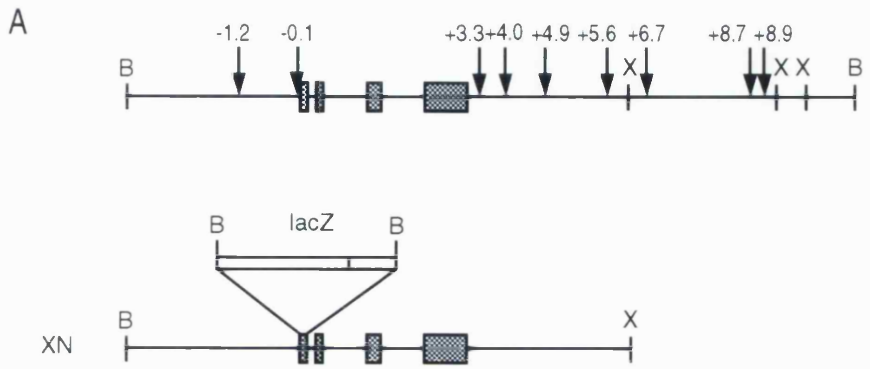
In order to investigate the role of potential regulatory elements located within the 3' region of the Ly-6E.1 gene in directing levels and patterns of expression *in vivo*, transgenic mice were generated with a truncated Ly-6E.1/*lacZ* construct (figure 16A). This transgene is lacking the distal 4 Kb of the Ly-6E.1 gene, a region encompassing DNase1 hypersensitive sites +6.7, +8.7 and +8.9. The microinjection fragment was isolated from pL6LZ by digestion with Xba1 and Not1 restriction enzymes and purified away from 3' Ly-6E.1 sequences and the pPolyIII vector by preparative gel electrophoresis. The 13.6 Kb Xba1-Not1 Ly-6E.1/*lacZ* fragment was microinjected into pronuclei of fertilised (CBA/Ca x C57/B10)F2 oocytes. A total of 72 founder mice were born after transfer of microinjected oocytes into pseudopregnant recipients. Ten were positive for the *lacZ* transgene (not shown). Of these 10 transgenic animals, referred to as XN mice because they contain an Xba1-Not1 construct, 7 were analysed in detail. One transgenic founder died unexplainedly and could not be analysed, another failed to produce transgenic

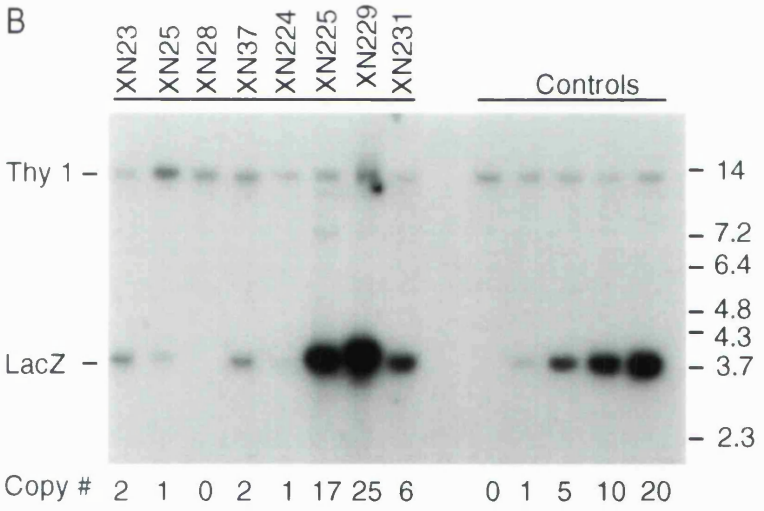
Figure 16. Molecular analysis of transgenic mice containing a truncated Ly-6E.1/*lacZ* construct.

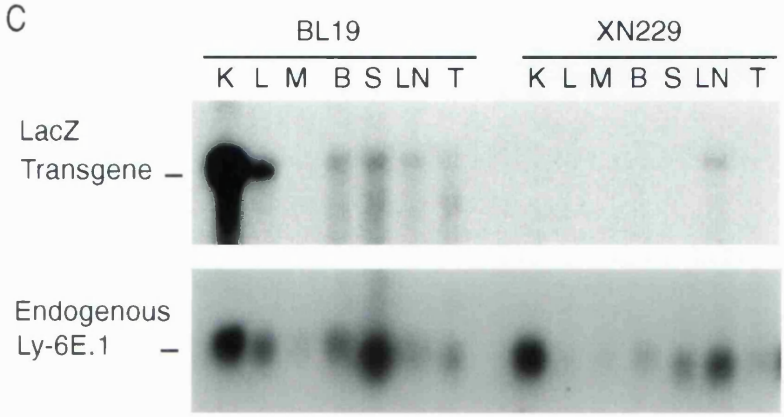
A. Diagram of the truncated XN Ly-6E.1/*lacZ* construct compared with the Ly-6E.1 gene showing the location, in Kb, of DNase1 hypersensitive sites relative to the transcriptional start site (after Sinclair & Dzierzak, 1993). B = BamH1 and X = Xba1 restriction sites.

B. Southern blot of 5μg BamH1 digested DNA from lines XN23, XN25, XN28, XN37, XN224, XN225, XN229, XN231 run on a 1% agarose/TAE gel. BamH1 digestion removes the *lacZ* gene and SV40 polyadenylation signal from Ly-6E.1/*lacZ* as a 3.3 Kb fragment and cleaves the endogenous *Thy-1* gene, used as a loading control in this case, to yield a 14 Kb fragment. After blotting, the filter was hybridised with ^{32}P-labelled *lacZ* (1.1 Kb EcoRV-BamH1) and *Thy-1* (1.2 Kb Xba1-Nru1) probes. Known amounts of pL6LZ in 5μg of non-transgenic DNA was used for copy number controls. The numbers to the right of the blot correspond to the distance migrated by BstEII digested λ DNA size marker.

C. Northern blot analysis of *lacZ* expression in transgenic lines BL19 (15 copy) and XN229 (25 copy). 15μg of total RNA prepared from thymus (T), lymph node (LN), spleen (S), bone marrow (B), muscle (M), liver (L) and kidney (K) was separated through a 1% formaldehyde gel, blotted and hybridised with a ^{32}P-labelled *lacZ* probe. The same filter was then stripped and reprobed with a ^{32}P-labelled Ly-6E.1 cDNA probe.


A
-1.2
-0.1
+3.3+4.0
+4.9
+5.6
+6.7
+8.7
+8.9
B
X
X X
B
B
lacZ
B
B
X
XN


B
XN23
XN25
XN28
XN37
XN224
XN225
XN229
XN231
Controls
Thy 1
LacZ
14
7.2
6.4
4.8
4.3
3.7
2.3
Copy #
2 1 0 2 1 17 25 6
0 1 5 10 20


C
BL19
XN229
K L M B S LN T
K L M B S LN T
LacZ
Transgene
Endogenous
Ly-6E.1

offspring, presumably as a result of mosaicism and was not analysed and a third contained a single copy of the transgene that showed evidence of rearrangement by Southern blot analysis (data not shown).

Figure 16B shows a Southern blot of genomic DNA isolated from the 7 transgenic mice analysed. As previously described (figure 2), the DNA was digested with BamH1 and the resulting southern blot filter was probed with a 1.1 Kb EcoRV-BamH1 *lacZ* probe and a 1.2 Kb Xba1-Nru1 *Thy-1* probe. Copy numbers were calculated, after correction of *lacZ* hybridising signals with the *Thy-1* loading control, by comparison with control samples containing specific amounts of pL6LZ. Six transgenic lines were established; XN23, XN37, XN224, XN225, XN229 and XN231 containing 2, 2, 1, 17, 25 and 6 copies of the transgene per genome respectively (Figure 16B). One transgenic mouse, XN25, containing 1 copy of the transgene, failed to establish a line but was included in the analyses as it showed no evidence of mosaicism in any tissue (data not shown). XN28 was negative for *lacZ* DNA in this blot and showed evidence of transgene rearrangement when DNA was digested with other enzymes.

2.3 Reduced *lacZ* mRNA expression in adult tissues of XN mice.

To investigate the effect of removing the region encompassing DNase1 hypersensitive sites +6.7, +8.7 and +8.9 from the Ly-6E.1/*lacZ* construct on transgene expression *in vivo*, total RNA from all XN transgenic lines and XN25 and XN28 founder animals was subjected to northern blot analysis. mRNA expression

from the truncated Ly-6E.1/*lacZ* transgene was compared with that from the endogenous Ly-6E.1 gene. Figure 16C shows a northern blot probed for *lacZ* and Ly-6E.1 mRNA comparing transgene expression in tissues from a 15 copy BL19 animal and a 25 copy XN229 animal, the highest copy number XN transgenic line. Despite possessing a greater number of transgene copies per genome and exhibiting comparable levels of endogenous Ly-6E.1 expression to BL19 in each tissue, *lacZ* mRNA expression is only detectable in XN229 lymph node. Further, detailed analysis of XN229 lymph nodes at the level of the single cell showed no β-galactosidase expression in lymphocytes (figure 18). The *lacZ* hybridisation is presumably the result of ectopic expression in the lymph node stroma. Figure 17 shows a northern blot of RNA isolated from various tissues from lines XN23, XN37, XN224, XN225 and XN231. On each individual blot, samples from a BL19 animal containing 15 copies of the transgene are included as a positive control for *lacZ* mRNA. Figure 17A shows a comparison between XN224, containing a single copy, XN231, containing 6 copies of the transgene and BL19, containing 15. No *lacZ* expression is found in either of the XN lines, even in those tissues expressing high levels of Ly-6E.1, such as kidney and conA treated spleen. Further evidence for a lack of expression from the truncated construct is presented in figure 17C in which XN225 and BL19 are compared. Despite similar copy numbers, 17 and 15 respectively, *lacZ* expression is detectable in BL19 spleen and is completely lacking in all XN225 tissues. Northern blot analysis confirmed an absence of Ly-6E.1-specific *lacZ* expression in all XN transgenics with the exception of XN23, in which *lacZ* expression was detected in the kidney only (figure 17B). The expression seen

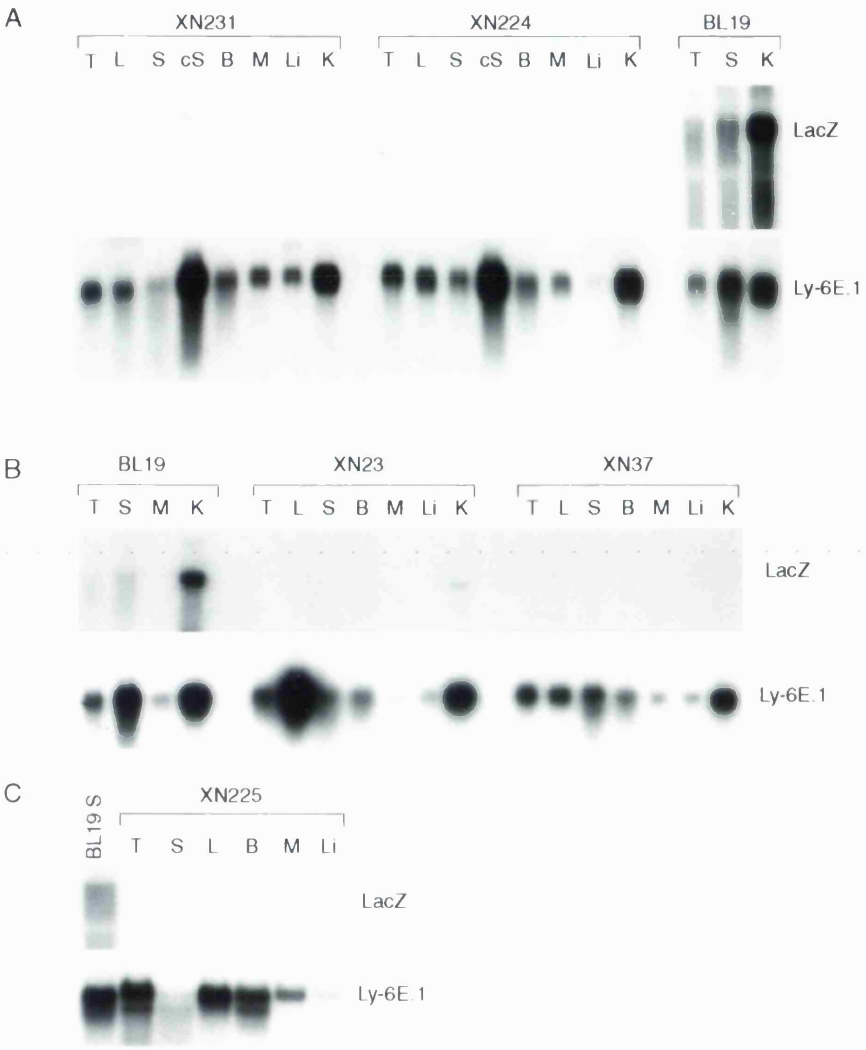
Figure 17. Northern blot analysis of *lacZ* expression compared with endogenous Ly-6E.1 expression in lines of XN Ly-6E.1/*lacZ* transgenic mice.

RNA from a panel of tissues from transgenic mice containing the truncated Ly-6E.1/*lacZ* construct was analysed by northern blot, hybridising with a ^{32}P-labelled *lacZ* probe. To control for endogenous Ly-6E.1 mRNA in each lane, each blot was stripped and reprobed with an Ly-6E.1 cDNA probe. RNA samples prepared from BL19 tissues were included on each blot as a positive control for *lacZ* hybridisation. T = thymus, L = lymph node, S = spleen, cS = conA-treated splenocytes, B = bone marrow, M = muscle, Li = liver and K = kidney.

A. XN231 and XN224 northern blot.

B. XN23 and XN37 northern blot.

C. XN225 northern blot.


A
XN231
T L S cS B M Li K
XN224
T L S cS B M Li K
BL19
T S K
LacZ
Ly-6E.1
B
BL19
T S M K
XN23
T L S B M Li K
XN37
T L S B M Li K
LacZ
Ly-6E.1
C
BL19 S
XN225
T S L B M Li
LacZ
Ly-6E.1

in XN23 kidney could be the result of a transgene independent, position effect, such as insertion of the transgene close to a kidney specific enhancer within the genome. Alternatively, it is possible that the basic elements necessary for kidney specific expression are retained in the truncated Ly-6E.1/*lacZ* construct and some other elements of the gene are missing such as a powerful enhancer or insulator sequence, which renders the transgene highly susceptible to silencing as is seen in the other XN transgenic lines of mice.

2.4 β-galactosidase assays for Ly-6E.1/*lacZ* transgene expression in XN mice.

Expression of *lacZ* in the haematopoietic system of XN transgenic animals was also investigated at the level of the individual cell using the β-galactosidase assays of X-gal or FDG staining. Figure 18 shows FACS profiles of cell number *vs* FDG fluorescence (*lacZ* expression) for the 25 copy XN229 thymus, spleen, lymph node and bone marrow. Cells from non-transgenic and 15 copy BL19 transgenic animals are shown for comparison. The percentage of background positive cells is almost identical between XN229 and non-transgenic animals in thymus, spleen, lymph node and bone marrow, confirming the findings of the northern analysis that in animals of the high copy line XN229, there is no haematopoietic expression of *lacZ*. Similarly, thymus, spleen, lymph node and bone marrow cells from the 17 copy XN225 transgenic line were analysed by FACS/FDG and compared with non transgenic and BL19 FDG stained cells (figure 19). As with XN229 haematopoietic cells, no FDG staining above non-transgenic background was observed, once again confirming the

Figure 18. FACS analysis of *lacZ* expression in haematolymphoid organs of XN229 transgenic mice.

10^6 cells from XN229 (25 copy) transgenic thymus, spleen, lymph nodes and bone marrow were stained with FDG to detect *lacZ* expression. 10^4 were analysed using a Becton Dickinson FACScan. Histogram plots of cell number (ordinate) *versus lacZ* expression (FDG fluorescence, abscissa, logarithmic scale) are shown. Cells isolated from thymus, spleen, lymph nodes and bone marrow of non-transgenic and BL19 transgenic mice were similarly stained with FDG to serve as negative and positive controls, respectively. The numbers above the bars on each plot correspond to the percentage of cells falling within that fluorescence range for each tissue.

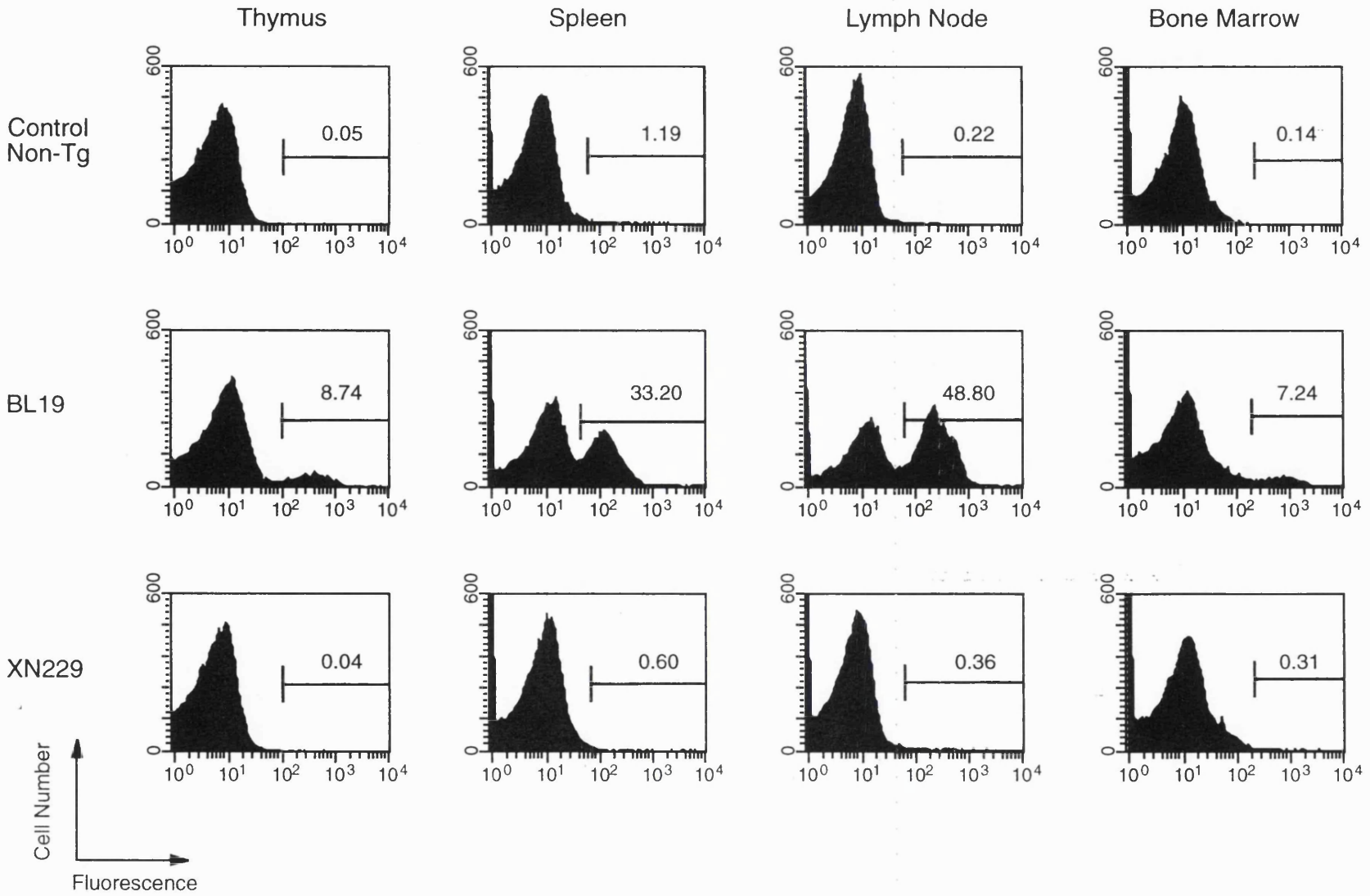
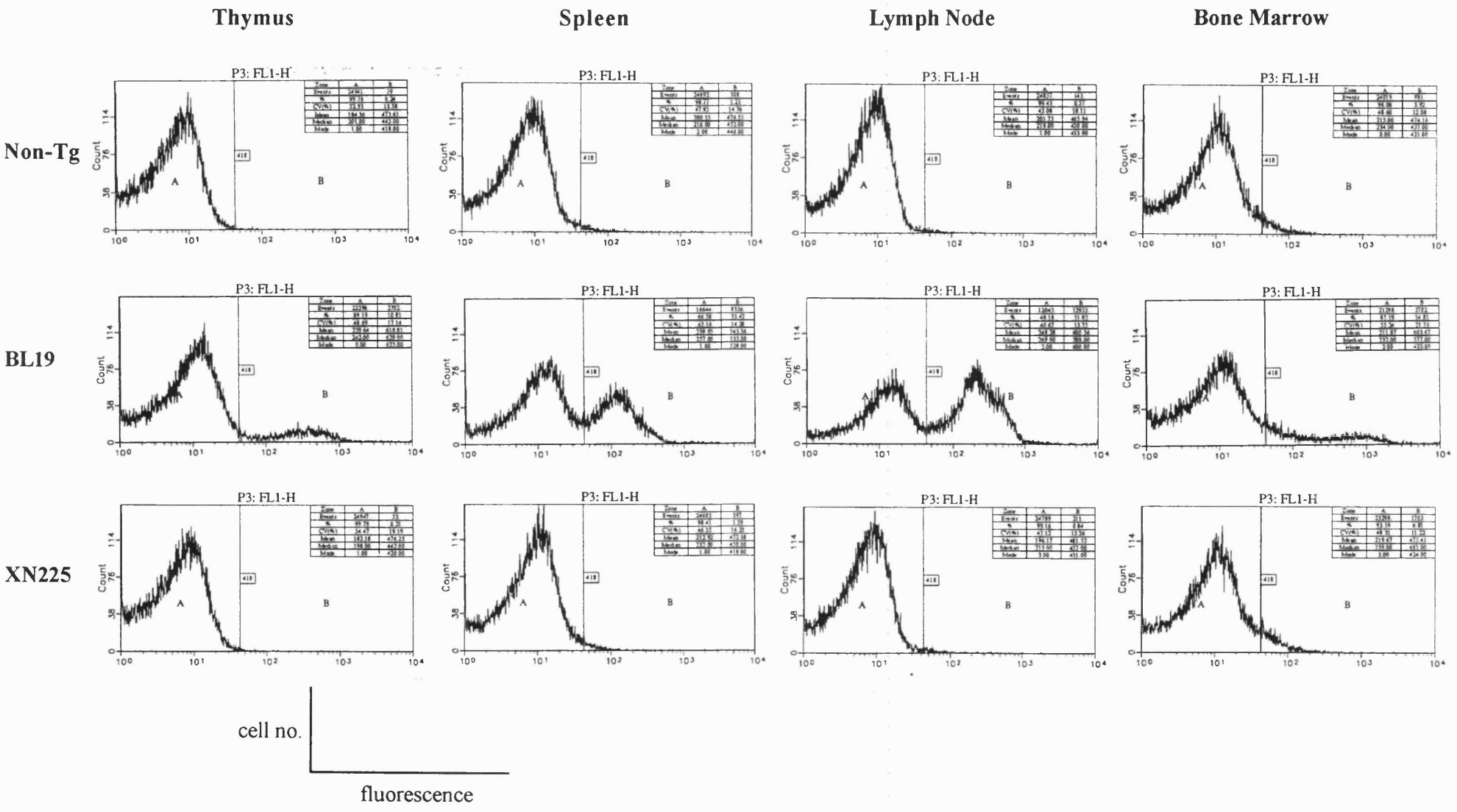

Thymus
Spleen
Lymph Node
Bone Marrow
Control Non-Tg
BL19
XN229
0.05
1.19
0.22
0.14
8.74
33.20
48.80
7.24
0.04
0.60
0.36
0.31
600
0
10^0 10^1 10^2 10^3 10^4
Cell Number
Fluorescence

Figure 19. FACS analysis of *lacZ* expression in haematolymphoid organs of XN225 transgenic mice.

10^6 cells from XN225 (17 copy) transgenic thymus, spleen, lymph nodes and bone marrow were stained with FDG to detect *lacZ* expression. 10^4 were analysed using a Becton Dickinson FACScan. Histogram plots of cell number (ordinate) *versus lacZ* expression (FDG fluorescence, abscissa, logarithmic scale) are shown. Cells isolated from thymus, spleen, lymph nodes and bone marrow of non-transgenic and BL19 transgenic mice were similarly stained with FDG to serve as negative and positive controls, respectively. The percentage of cells exhibiting fluorescence greater than 418 units is given in the insert to each histogram, region B, to enable comparison between samples.


Thymus
Spleen
Lymph Node
Bone Marrow
Non-Tg
BL19
XN225
P3: FL1-H
Count
cell no.
fluorescence

northern blot result that XN transgenic mice show no haematopoietic specific expression of the truncated Ly-6E.1/*lacZ* transgene.

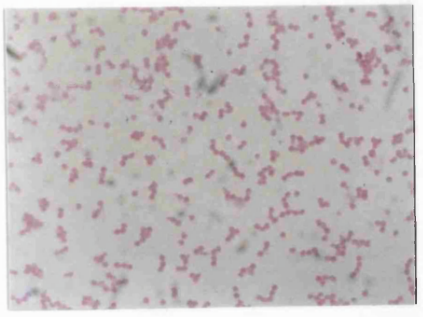
X-gal staining of lymphocytes before and after activation, however, showed *lacZ* expression in lymphocytes of one XN line. Cytological analysis of X-gal stained XN37 thymocytes and splenocytes showed rare blue cells. The number of stained cells increased upon activation, presumably as a result of Ly-6E.1-specific, γ-interferon induction. X-gal staining of XN23 and XN37 thymocytes before and after activation is shown in figure 20. In contrast, XN23 thymocytes show no X-gal staining even after activation. As with XN23 kidney specific expression, the haematopoietic specific expression displayed by XN37 can be explained either by a fortuitous position effect resulting in activation inducible lymphoid expression or that the elements required for haematopoietic specific expression are retained in the XN construct. These two observations suggest that the removal of DNase1 hypersensitive sites +6.7, +8.7 and +8.9 does not affect the basal elements required for kidney or haematopoietic specific expression from the Ly-6E.1 gene. In addition, it appears that the 3' region of the Ly-6E.1 gene does not contain a simple enhancer element, as expression in kidney and haematopoietic cells is readily detectable in the 2 copy lines XN23 and XN37 respectively but not in XN231, XN225 or XN229 mice containing 6, 17 and 25 transgene copies respectively.

Figure 20. *LacZ* expression in resting and activated XN transgenic thymocytes containing a truncated Ly-6E.1/*lacZ* construct.

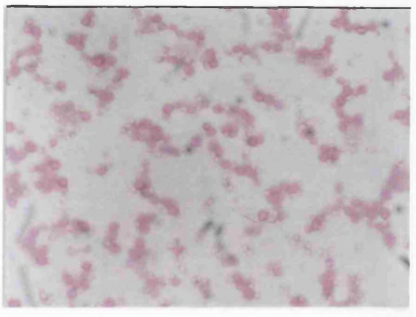
Thymocytes from XN23 and XN37 transgenic mice were cultured for 48 hours both with (C. & D.) and without (A. & B.) concanavalin A at 2 μg/ml prior to fixation and staining with X-gal. Cells were visualised after cytospin preparation and eosin counterstaining.

XN23 transgenic thymocytes

A. untreated

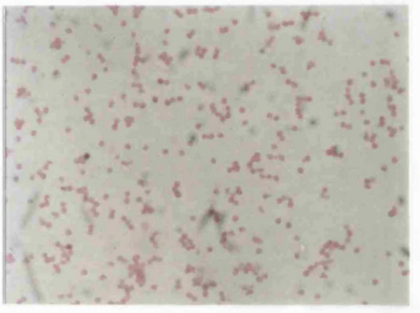


C. +conA

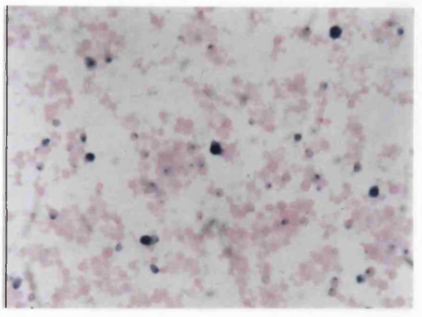


XN37 transgenic thymocytes

B. untreated



D. +conA



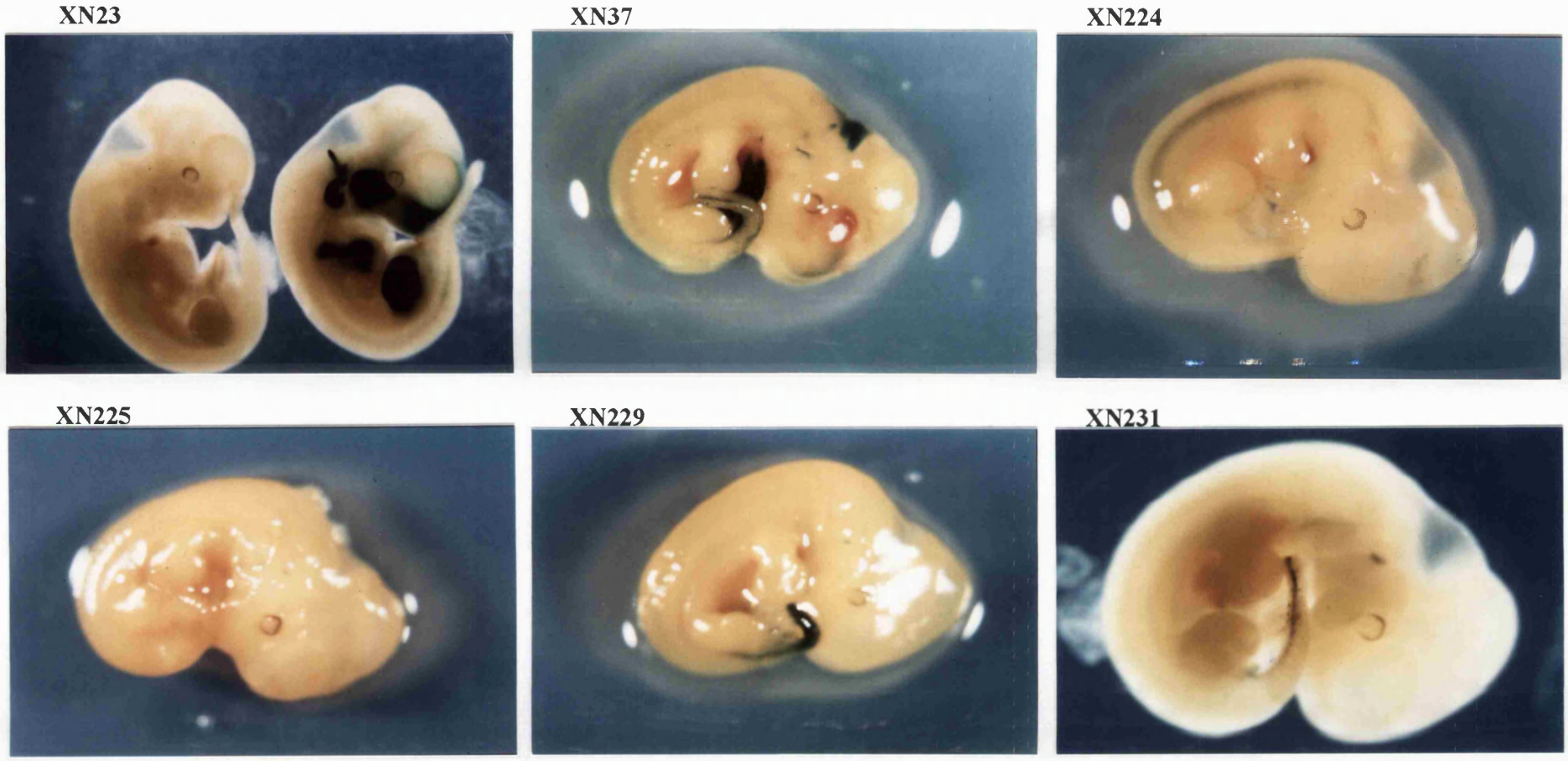
X-gal stained XN thymocytes +/- activation.

2.5 X-gal staining of XN Ly-6E.1/*lacZ* embryos.

The full length Ly-6E.1/*lacZ* construct exhibited reproducible, high level, tissue specific expression in mid-gestation embryos in all 4 BL transgenic lines with minimal background, ectopic expression. In order to determine whether the removal of sequences including DNase1 hypersensitive sites +6.7, +8.7 and +8.9 affects the spatial pattern of transgene expression, embryos containing the truncated Ly-6E.1/*lacZ* construct were stained with X-gal at day 11p.c. Stained whole mount embryos at day 11p.c. from all XN transgenic lines are shown in figure 21. For comparison, the first panel in figure 21 shows a non-transgenic X-gal stained day 11p.c. embryo demonstrating the absence of non-specific staining and a XN23 transgenic embryo displaying extensive ectopic X-gal staining. Mesonephros specific expression was observed only in embryos of one line, XN23. In addition, XN23 adults alone exhibit kidney specific expression of *lacZ*, suggesting that common genetic regulatory elements may be used, or that there is a direct relationship between $lacZ^+$ mesonephros cells and $lacZ^+$ kidney cells, although anterior mesonephros cells do not give rise to adult renal structures (A. Medvinsky, pers. comm. and Kaufman, 1992). Intense X-gal staining in the tail, similar to that observed for BL transgenic lines was observed only in XN229 transgenic embryos (figure 19), although weaker tail staining can be detected in XN37 and XN231 embryos. Upon closer inspection, whole mount X-gal stained XN229 embryos display tail staining in hindgut endoderm, mesoderm and notochord as is seen in BL embryos, whereas XN37 embryos display only notochord and mesodermal staining and XN231 embryos display only endodermal staining. No Ly-6E.1 specific adult expression, however,

Figure 21. X-gal staining of XN truncated Ly-6E.1/*lacZ* containing embryos.

Embryos were isolated from all breeding lines of XN transgenic mice (XN23, XN37, XN224, XN225, XN229, and XN231) at day 11 p.c., fixed and stained with X-gal. For reference, the embryo of line XN23 is depicted adjacent to a non-transgenic embryo that does not exhibit X-gal staining.



X-gal stained d11.5 p.c. XN transgenic embryos

is seen in either XN229 or XN231 lines. In addition, embryos containing XN constructs seem to be more susceptible to position effects, exhibiting stronger and more widespread ectopic expression than embryos of BL Ly-6E.1/*lacZ* transgenic lines.

Although not providing conclusive evidence, analysis of transgenic mice containing the truncated Ly-6E.1/*lacZ* construct suggests that the basic elements directing Ly-6E.1 specific expression may still be present in the XN construct. The probability that specific aspects of the BL expression pattern could arise in a cohort of fewer than ten XN animals by chance is miniscule. Each of the broad classes of expression detected in full-length Ly-6E.1/*lacZ*-containing transgenic mice is seen in XN mice, although not coincident in animals of the same line, ie, XN229 = tail only (hindgut, notochord and mesoderm); XN23 = mesonephros and kidney; XN37 = tail mesoderm, notochord and activated lymphocytes; XN231 = hindgut only. It is possible that the element deleted from the BL construct renders the transgene less susceptible to position effects from the surrounding genetic elements at the site of integration. Alternatively, it may be a powerful enhancer element that, in the BL mice masks position effects and its absence renders expression in XN mice too low to be detectable in most situations.

3. Ly-6E.1/*myc* transgenic mice.

3.1 Introduction.

Cell lines are a useful tool for the study of rare cell populations, providing relatively large amounts of material enabling biochemical studies to be undertaken on homogeneous cell populations. In an attempt to generate haematopoietic precursor cell lines for such biochemical studies and to investigate the role of the cell cycle in haematopoietic homeostasis *in vivo*, Ly-6E.1/*c-myc* transgenic mice were generated.

The *c-myc* gene is the cellular homologue of *v-myc*, a viral oncogene associated with avian retroviruses that has been shown to cause leukaemias and carcinomas (Cole, 1986). Genetic abnormalities found in human Burkitt's lymphoma and rodent plasmocytomas have been reported in which the *c-myc* gene is translocated into the immunoglobin locus, effectively placing *c-myc* under genetic control of the immunoglobin heavy chain enhancer (Cory, 1986). Under experimental conditions, transgenic mice expressing *c-myc* controlled by the immunoglobin heavy chain enhancer are predisposed to malignancy in pre-B and mature B lymphocytes, suggesting an important role for *c-myc* in the control of cell proliferation (Adams *et al.*, 1985; Langdon *et al.*, 1986). The *c-myc* gene encodes a transcription factor that forms heterodimers (Blackwood & Eisenman, 1991). The protein binds to DNA, possesses leucine zipper domains similar to those of the Fos, Jun and CREB transcription factors and basic-helix-loop-helix domains similar to those of MyoD, and

is an "immediate early growth response" factor, being rapidly induced upon mitogen stimulation of quiescent cells (Landschultz *et al.*, 1988; Murre *et al.*, 1989).

Studies on the function of *c-myc* demonstrated that it has a role in both cell proliferation and programmed cell death, apoptosis (Evan *et al.*, 1992). Whilst induction of *c-myc* expression is sufficient to drive quiescent cells into the cell cycle (Eilers *et al.*, 1991), specific inhibition of *c-myc* expression by the addition of antisense oligonucleotides demonstrated that it is essential for cell proliferation (Heikkila *et al.*, 1987; Loke *et al.*, 1988). As *c-myc* is both necessary and sufficient for cell proliferation, it can be thought of as a single step to cellular transformation. The induction of an apoptotic pathway by *c-myc* expression may provide an inbuilt safeguard against the potentially oncogenic effects of a single mutation deregulating its expression. Thus, it is thought that cells expressing *c-myc* are primed for programmed cell death, the avoidance of which relies upon a further, positive survival signal (Evan *et al.*, 1992). This is consistent with the theory that apoptosis is the default pathway for all cells to follow unless averted by survival factors (Raff, pers. comm.).

The oncogenic properties of *c-myc* have been utilised for tissue directed oncogenesis experiments using both retroviral and transgenic approaches (Langdon *et al.*, 1986; Stewart *et al.*, 1984; Leder *et al.*, 1986; Alexander *et al.*, 1987). In some of these studies, *c-myc* expressing cells have been maintained *in vitro* and yielded stable cell lines (Adams *et al.*, 1985; Spanopoulou *et al.*, 1989; Harris *et al.*, 1988). Targeted expression of *c-myc* using the regulatory elements of the Thy-1 gene resulted in the production of both T lymphoid and thymic epithelial cell lines (Spanopoulou *et al.*, 1989). The unexpected transformation by *c-myc* of thymic

epithelial cells is an example of targeted oncogenesis providing a convenient source of rare cell types for detailed biochemical analysis. Similarly, Ly-6E.1/*myc* transgenic mice may allow the characterisation and manipulation of rare Ly-6E.1$^+$ cells, such as haematopoietic progenitors, *in vitro*. The high degree of penetrance of *c-myc* in targeted oncogenesis experiments is demonstrated by the fact that 9 out of 10 independent Thy-1/*myc* transgenic mice displayed thymic tumours (Spanopoulou *et al.*, 1989).

As suggested by the results of the Thy-1/*myc* study, *c-myc* induced transformation is not restricted to cells of the lymphoid compartment. Transgenic mice expressing *c-myc* under control of the erythroid specific GATA-1 regulatory elements exhibit a severe, early onset erythroleukemia (Skoda *et al.*, 1995) whilst *c-myc* expression can induce mammary tumours when targeted to the mammary gland in transgenic mice (Schoenberger *et al.*, 1988). However, not all *c-myc* expressing transgenic mice develop tumours and it seems that certain tissues and organs are more susceptible to *c-myc* induced transformation than others (Adams & Cory, 1991; Roland & Morello, 1993). The precise cell type in which the transgene is expressed and the level of expression are crucial factors to the outcome of targeted oncogenesis experiments.

To utilise the cell transformation potency of *c-myc* for the analysis of haematopoietic stem and progenitor cells, transgenic mice were generated in which *c-myc* expression was under control of the regulatory elements of the 14 Kb Ly-6E.1 gene. The tissue specific expression pattern of the Ly-6E.1 transgene should yield cell lines transformed by *c-myc* which will be representative of immature haematopoietic stem and progenitor cells. Detailed biochemical analyses and

comparisons between such cell lines may suggest precursor/progeny relationships within the heirarchy of differentiating haematopoietic stem cells.

In addition to the generation of cell lines, the analysis of Ly-6E.1/*myc* transgenic mice may provide an insight into haematopoietic stem cell maintenance *in vivo*. The mechanisms involved in the lifelong maintenance of the haematopoietic system remain unclear (Orlic & Bodine, 1994; Spangrude *et al.*, 1991; Ogawa, 1993; Lord & Dexter, 1995). One theory is that a cohort of haematopoietic stem cells exist as a pool of quiescent cells which seed the bone marrow during development and are sequentially, clonally activated throughout adult life (Kay, 1965; Hellman *et al.*, 1978; Brecher *et al.*, 1986). If there exists a quiescent pool of haematopoietic stem cells residing in the adult bone marrow that are awaiting recruitment, the expression of *c-myc* may drive these cells into cell division and to differentiate. However, if the haematopoietic stem cell pool were not sustained by progenitors capable of continually seeding this compartment, the reservoir of haematopoietic stem cells in Ly-6E.1/*myc* transgenic mice may be depleted. In addition, a greater number of commited progenitors should be detectable as the haematopoietic stem cell is forced into cell proliferation and differentiation.

3.2 Generation of Ly-6E.1/*myc* transgenic mice.

In order to express the *c-myc* proto-oncogene in Ly-6E.1 expressing cells, in particular transplantable bone marrow haematopoietic stem cells, pL6Cla was utilised as an expression vector. The expression pattern of this vector has already been

described for Ly-6E.1/*lacZ* transgenic mice. The strategy employed to generate the Ly-6E.1/*myc* construct was essentially similar to that used to introduce the *lacZ* gene into pL6Cla (see figures 1 and 22A). The *c-myc* insert was obtained from p1271myc, a plasmid containing a fragment of genomic DNA encompassing exons two and three of the *c-myc* gene, which has previously been used for targeted oncogenesis (Spanopoulou *et al.*, 1989). This plasmid was modified using blunt ended and BamH1 oligonucleotide linkers to create Nar1 restriction sites at the Stu1 and BamH1 sites respectively. The BamH1 site 3' of *c-myc* exon three was reformed to enable the orientation of the *c-myc* gene within pL6Cla to be determined during subsequent cloning steps. The 4.8 Kb Nar1 fragment consisting of *c-myc* exons two and three, including the ATG translational start site and the polyadenylation signal, was cloned into the Cla1 site within the first, untranslated, exon of Ly-6E.1 to generate pLM12. The assymetrically located BamHI restriction site enabled the orientation of the *c-myc* gene to be determined.

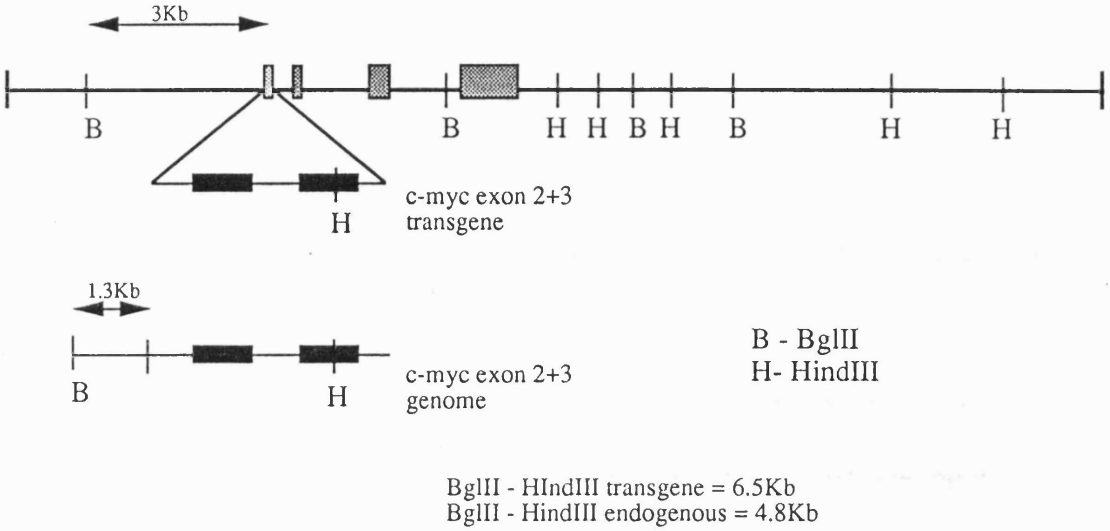
The Ly-6E.1/*myc* construct was purified away from pPolyIII vector sequences by Not1 digestion and preparative gel electrophoresis. The resulting 18.8 Kb fragment was microinjected into fertilised (CBA/Ca x C57/B10)F2 oocytes. Of 15 founder animals born, 4 were positive for the transgene, designated LM1, LM5, LM7 and LM8. Figure 22B shows a southern blot of genomic DNA prepared from tail biopsies from Ly-6E.1/*myc* founder mice probed with the entire 4.8 Kb *c-myc* fragment. Restriction digest with with BglII and HindIII enables discrimination between the transgenic murine *c-myc*, producing a 5.7 Kb hybridising band, and the endogenous gene's signal at 4 Kb. The endogenous 4 Kb hybridising band also serves as an internal loading control. By comparing the intensity of the endogenous

Figure 22. Ly-6E.1/*myc* construct and the generation of transgenic mice.

A. Diagram of the Ly-6E.1/*myc* construct highlighting the BglII polymorphism which allows discrimination between endogenous murine *c-myc* sequences and *c-myc* sequences present within the transgene.

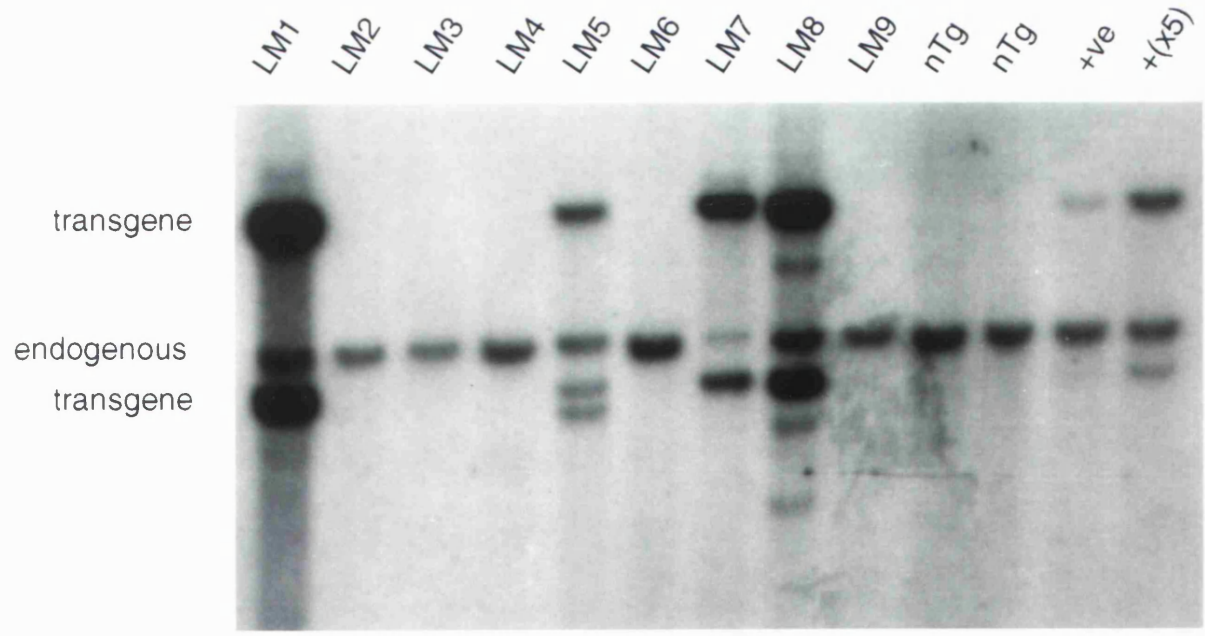
B. Southern blot of 10μg of tail genomic DNA isolated from Ly-6E.1/*myc* founder mice LM1-LM9, digested with HindIII and BglII. Doubly digested DNA was run on a 1% agarose/TAE gel, transferred to a nylon membrane and hybridised with a ^{32}P-labelled *c-myc* probe (4.8 Kb Xba1-BamH1). The 4.8 Kb hybridising endogenous *c-myc* band serves as a loading control and, by comparing the intensity of this signal with that of the transgene-specific band at 6.5 Kb, using a phosphorimager, the copy number of each positive founder was determined. Positive control samples consist of the Ly-6E.1/*myc* plasmid mixed with non-transgenic DNA. The transgenic-specific hybridising fragment slightly smaller than the endogenous *c-myc* band corresponds to the HindIII-BglII fragment encompassing part of *c-myc* exon 3 and exons 2 and 3 of the Ly-6E.1 transgene. The copy number of each positive founder animal was approximately; LM1 = 30 copies, LM5 = 4 copies, LM7 = 20 copies and LM8 = 10 copies.

A. Ly-6E.1/c-myc construct



BglII - HIndIII transgene = 6.5Kb
BglII - HindIII endogenous = 4.8Kb

B. Southern Blot of Ly-6E.1/myc Founder mice.



c-myc band (two copies) with that of the transgene using a Phosphorimager, the copy numbers of the founders LM1, 5, 7 and 8 was estimated to be 30, 4, 20 and 10 respectively. Of these founders, only LM5 bred to establish a line.

3.3 A severe phenotype exhibited by Ly-6E.1/*myc* founder transgenic mice.

The three positive founder animals containing the highest number of transgene copies all became chronically sick by five weeks of age (figure 23A). LM1 (30 copies) died suddenly at three weeks of age before any analysis could be performed and both LM7 (20 copies) and LM8 (10 copies) were moribund at 5 weeks of age and had to be sacrificed due to the severity of their health status. LM7 and LM8 both displayed the same gross phenotype as LM1, being severely runted and in poor condition. Neither LM7 nor LM8 reached sexual maturity, so could not be used to establish transgenic lines. Internal examination revealed an almost complete absence of lymphoid organs; thymus, spleen and lymph nodes. In particular, the thymuses from transgenic founders consisted almost entirely of connective tissue and contained very few lymphocytes in comparison with non-transgenic littermates. LM7 thymus consisted of 1.75×10^6 cells compared with 2×10^8 cells for LM4 thymus. In addition, LM7 and LM8 displayed solid kidney tumours. Figure 24 shows a photograph of a kidney isolated from non-transgenic LM6 and, at the same magnification and illumination, a tumourous kidney from LM8 transgenic founder.

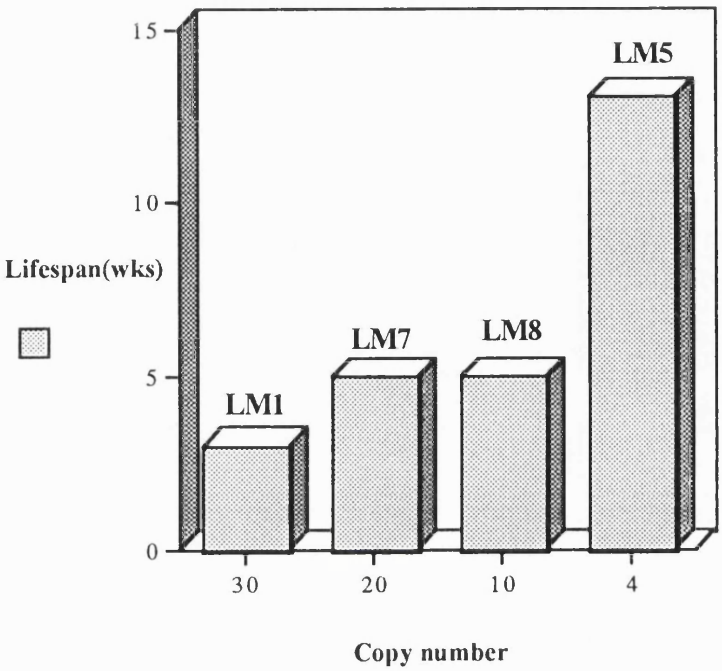
In order to investigate the molecular events which may have contributed to the gross phenotype of LM7 and LM8 founders, RNA was isolated for northern blot

Figure 23. Lifespans of Ly-6E.1/*myc* transgenic mice.

A. Graph depicting the lifespans, in weeks, of the four Ly-6E.1/*myc* transgenic founder mice.

B. Graph depicting the lifespans, in weeks, of 12 individual transgenic Ly-6E.1/*myc* mice of the LM5 line. Four litters of mice from heterozygous LM5 matings with non-transgenic females were monitored. All 12 transgenic mice died prematurely, whilst all of their non-transgenic littermates (16 animals) remained healthy throughout.

A. Lifespans of Ly-6E.1/*myc* transgenic founders.



B. Lifespans of LM5 transgenic offspring.

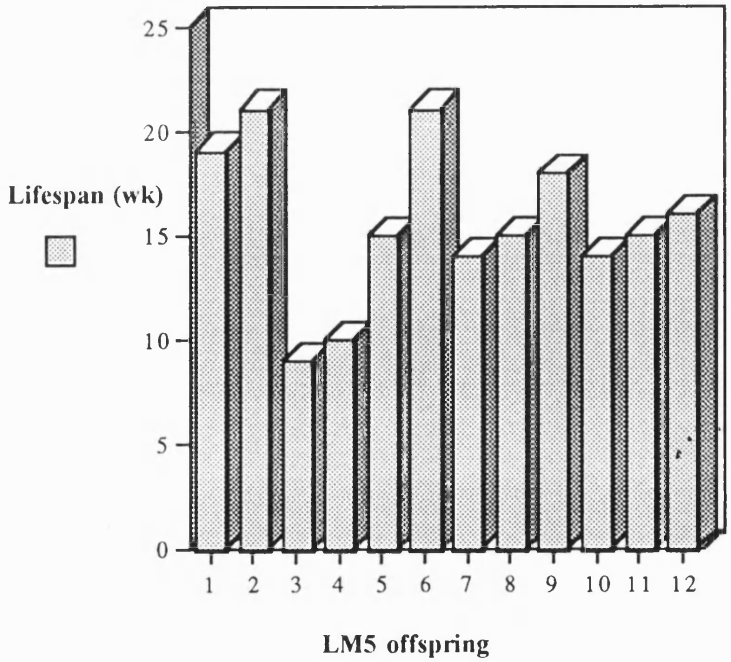
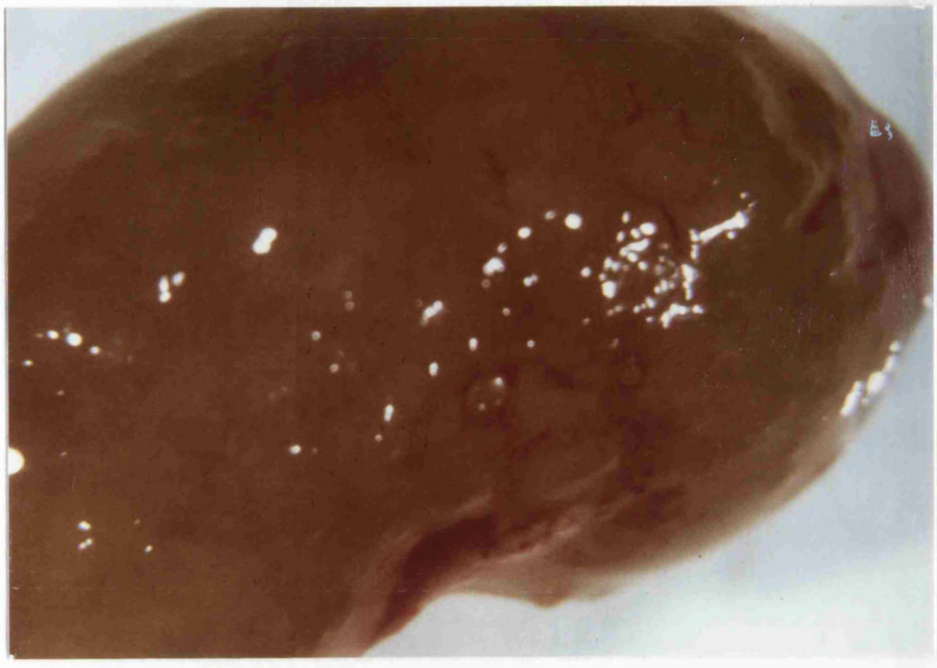


Figure 24. Solid kidney tumours develop in high copy Ly-6E.1/*myc* transgenic mice.

A. Tumourous kidney from LM7 Ly-6E.1/*myc* transgenic founder. The kidneys of LM8 displayed the same phenotype.

B. Normal kidney from LM4, non-transgenic founder littermate of LM7, photographed at the same magnification and with the same illumination as in A.

A



B

analysis from spleen, muscle, liver and kidney of these mice and from LM4 and LM6, sex matched non-transgenic littermates as controls. Figure 25 shows a northern blot comparing the levels of several specific mRNAs, chosen as markers of specific cell types, in LM7 and LM8 tissues with those in non-transgenic littermates LM4 and LM6. The level of *c-myc* mRNA is dramatically upregulated in kidney (Figure 25a) of LM7 and LM8 mice. Upon closer analysis, the size of the *myc* transcript derived from the transgene is smaller than the endogenous transcript in tissues from non-transgenic mice. The difference is due to the transgenic *c-myc* mRNA lacking the 5' untranslated first exon.

Rehybridising the same northern blot with an Ly-6E.1 cDNA probe showed there to be an inverse correlation between levels of *c-myc* mRNA and that of endogenous Ly-6E.1 (figure 25b). This may reflect a direct effect of *c-myc* repressing Ly-6E.1 gene expression in a similar manner to *c-myc* induced transcriptional repression of the *neu* gene (Suen & Hung, 1991). Alternatively, the reduction in Ly-6E.1 mRNA may be due to changes in cellularity within tissues, such as a reduction in Ly-6E.1$^+$ cells due to *c-myc* induced apoptosis. Further analysis of kidney and liver mRNA demonstrates reduced levels of Ly-6E.1 expression in transgenic founders, supporting the notion that *c-myc* induces changes in cellularity within these tissues. In figure 25c, the level of mRNA encoding the urea cycle enzyme ornithine decarboxylase (ODC) in the kidney demonstrated a dramatic reduction to between 4-8% that found in normal kidney in both transgenic founders compared with non-transgenic littermates. It is possible that this severe reduction in ODC level led to renal dysfunction resulting in the premature death of high copy number founder animals and may be a reflection of severely altered kidney

Figure 25. Northern blot analysis of Ly-6E.1/*myc* transgenic mice.

10μg of total RNA prepared from spleen, muscle, liver and kidney was separated through a 1% formaldehyde gel, blotted and hybridised with a ^{32}P-labelled *c-myc* probe (a.). The tissues were obtained from Ly-6E.1/*myc* founder animals; 4 = LM4, 6 = LM6, 7 = LM7 and 8 = LM8.

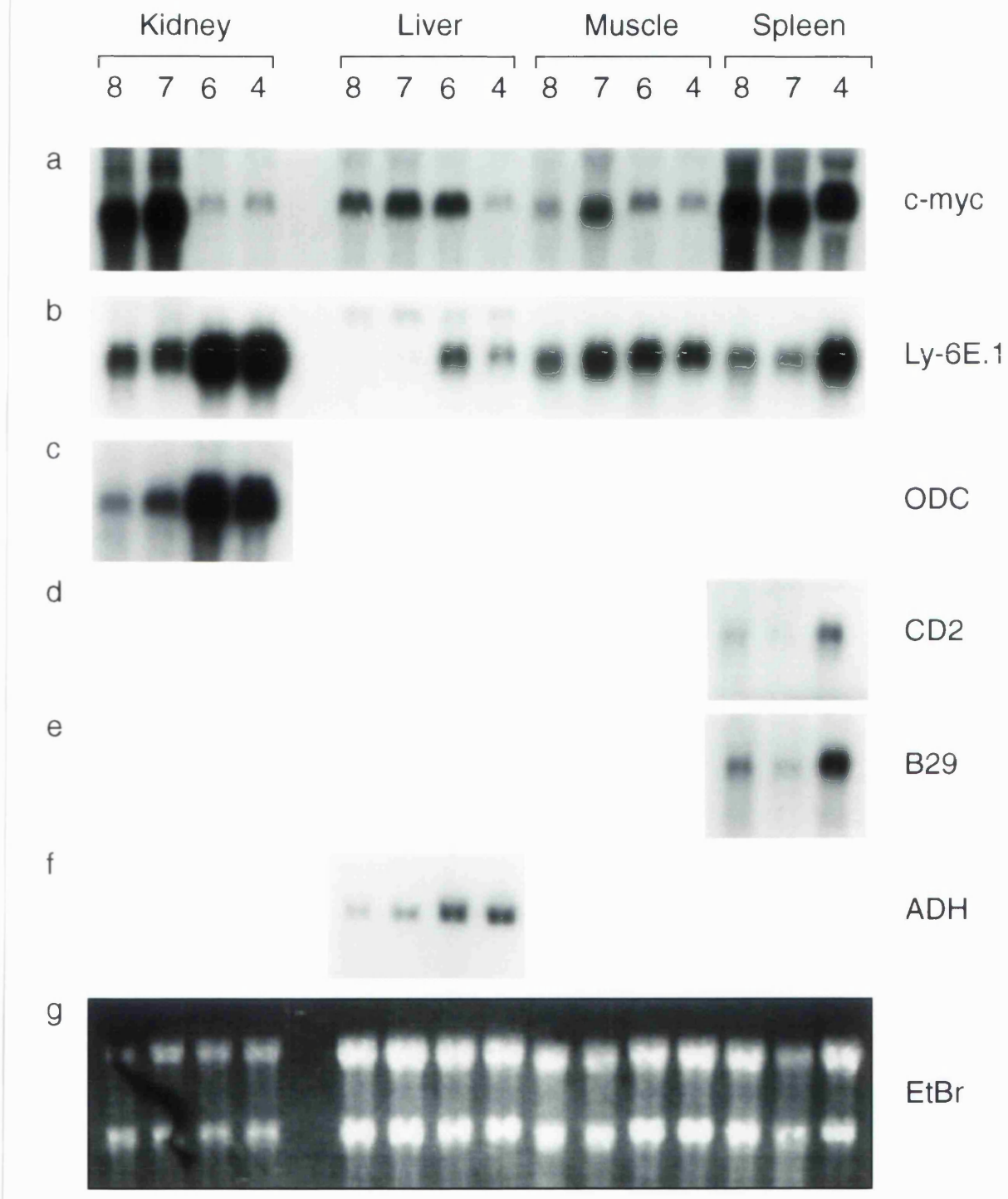
b. The same filter was stripped and reprobed with a ^{32}P-labelled Ly-6E.1 cDNA probe.

c. The panel of the filter consisting of kidney RNA was again stripped and reprobed with a ^{32}P-labelled probe specific for ornithine decarboxylase (ODC).

d. & e. The panel of the filter consisting of spleen RNA was sequentially stripped and reprobed for the T cell specific CD2 mRNA (d.) and for the B cell specific B29 mRNA (e.)

f. The panel of the filter consisting of liver RNA was stripped and reprobed with a ^{32}P-labelled probe specific for alcohol dehydrogenase (ADH).

g. Ethidium bromide stained northern blot gel, prior to transfer. The 18S and 28S ribosomal RNA bands demonstrate the integrity of the RNA samples and serve as a control for equal loading.


Kidney
Liver
Muscle
Spleen
8 7 6 4
8 7 6 4
8 7 6 4
8 7 4
a
c-myc
b
Ly-6E.1
c
ODC
d
CD2
e
B29
f
ADH
g
EtBr

cellularity. The changes in ODC mRNA level is an unexpected observation as ODC expression is usually upregulated in tumours and is a target for *c-myc* induced transcriptional activation (Packham & Cleveland, 1994). A further abnormality associated with the Ly-6E.1/*myc* transgenic mice is evident in the liver. Figure 25f shows the same northern blot reanalysed for levels of alcohol dehydrogenase (ADH) mRNA. There is a 3-10 fold reduction in ADH mRNA level in Ly-6E.1/*myc* transgenic liver in LM7 and LM8 mice as compared with non-transgenic littermates, despite the overtly normal gross appearance of the organ. In summary, there is clearly altered cellularity within transgenic kidney, however no obvious differences are apparent in liver. It is unclear whether the reduction in ODC and ADH levels is a result of changes in cellularity of the organ, or direct downregulation of the expression of these genes by myc. However, the reduction in expression of these two enzymes could contribute significantly to the reduced life expectancy of high copy number Ly-6E.1/*c-myc* transgenic mice. Each of the 5 different gene transcript probes used to analyse the Ly-6E.1/*c-myc* founder northern blot show a decrease in mRNA level in transgenic compared with non-transgenic tissues. It is unlikely that *c-myc* is repressing transcription of all of these genes as, in most cases, *c-myc* is thought to act as a transcriptional activator. Therefore, the altered mRNA levels in Ly-6E.1/*myc* transgenic mice suggest *c-myc* is likely to be affecting the normal development of certain tissues, including the haematopietic system.

The only transgenic founder animal from which a line could be derived was the LM5 founder which contained approximately 4 copies of the transgene. This line was routinely maintained in the heterozygous state through the male line to avoid the problems associated with fostering pups orphaned as a result of the shortened life

expectancy of LM5 mice. The gross phenotype of LM5 transgenic mice resembled that of the high copy number founders in that the haematopoietic organs were smaller than in non-transgenic littermate controls. Transgenic LM5 animals had small thymuses and spleens (figure 26) although the reduction in cellularity was less severe than that observed for LM7 and LM8, being approximately five fold less than their non-transgenic littermates (table 3). However, the kidneys showed no evidence of solid tumours and animals of this line reached ages of between 10 and 20 weeks (Fig. 23B). The cause of this much reduced life expectancy displayed by LM5 mice was not determined, *post mortem* analysis of LM5 transgenic animals revealed a small thymus and spleen but no gross morphological or anatomical abnormalities to either liver or kidney were apparent. LM5 Ly-6E.1/*myc* transgenic mice are currently being interbred to generate mice homozygous for this transgene, in order to determine whether there is an increase in the severity of the phenotype after effectively doubling the transgene copy number.

3.4 Small lymphoid organs in Ly-6E.1/*myc* transgenic mice.

The reduction in thymic cellularity in Ly-6E.1/*c-myc* transgenic mice was unexpected. Transgenic mice expressing *c-myc* in all thymocytes under control of Thy-1 regulatory elements displayed enlarged thymuses and often developed thymic tumours (Spanopoulou *et al.*, 1989). To investigate this penomenon further in LM5 mice at the level of specific lymphocyte subsets, FACS analysis was performed. Figure 27A shows FACS profiles of thymocytes from LM5 transgenic and non

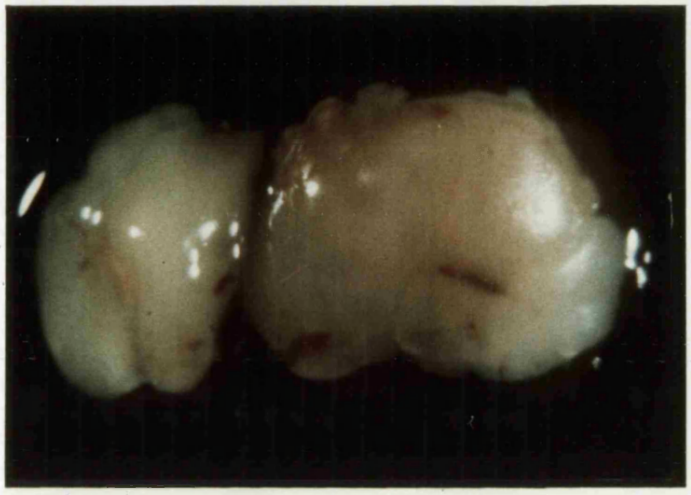
Figure 26. Reduction in size of Ly-6E.1/*myc* lymphoid organs.

a. Thymuses from 5 week old LM5 (left) and non-transgenic (right) littermates.

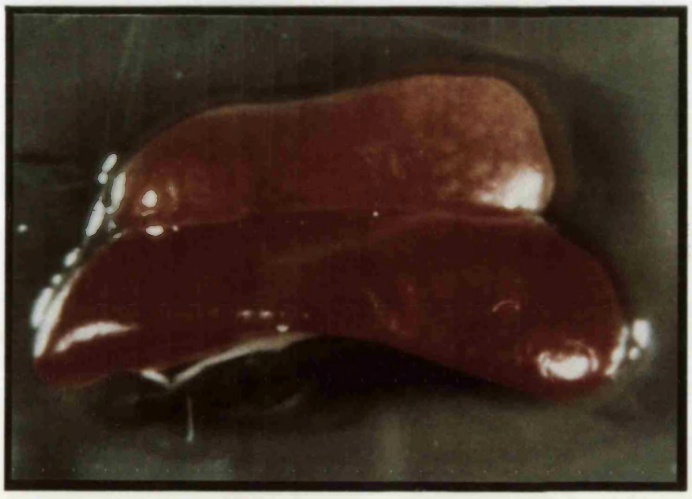
b. Spleens from the same 5 week old LM5 (upper) and non-transgenic (lower) littermates.

Lymphoid organs in Ly-6E.1/myc transgenic mice are small

a. Thymus - LM5(left) vs normal(right)



b. Spleen - LM5(above) vs normal(below)



* LM5 is a low copy line. Size differences become more pronounced with copy number - high copy mice are almost athymic/asplenic.

Table 3. Absolute cell numbers within lymphoid organs of LM5 Ly-6E.1/*myc* transgenic mice.

Thymus (a.) and spleen (b.) from LM5 and non-transgenic Ly-6E.1/*myc* littermates were homogenised to single cell suspension and accurately counted. These cell counts are from the individual samples analysed by FACS in figure 27 and are representative of all LM5 mice analysed.

-

a) Thymus	non transgenic	1.03×10^8		
	LM5 transgenic	0.21×10^8		

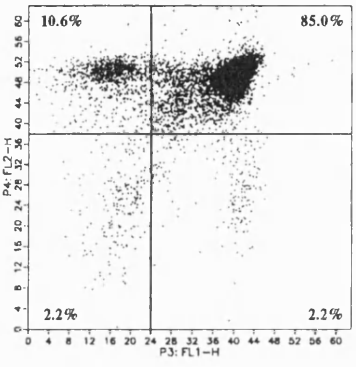
		$CD4^+$	$CD8^+$	$B220^+$
b) Spleen	non transgenic	3.5×10^6	1.4×10^6	2.7×10^9
	LM5 transgenic	1.6×10^6	1.1×10^6	7.4×10^6

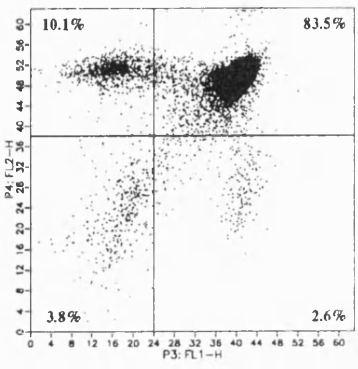
Figure 27. Distribution of CD4/CD8 T cell subsets in thymus and spleen of Ly-6E.1/*myc* transgenic mice.

Thymocytes (A) and splenocytes (B) from LM5 transgenic and non-transgenic littermates were stained for CD4 (PE-ordinate) and CD8 (FITC-abscissa). 10^4 cells were analysed using a Beckton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale, with percentages of each subset of cells indicated in the relevant quadrant.

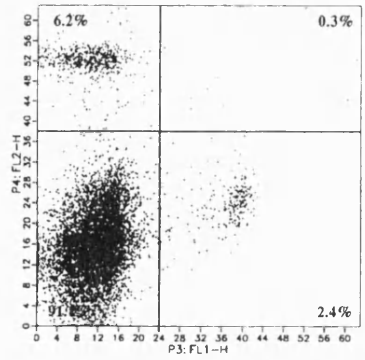
Non transgenic. **LM5 transgenic.**

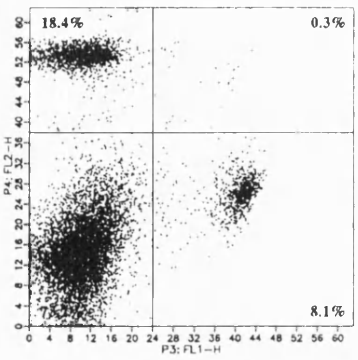
A. Thymus

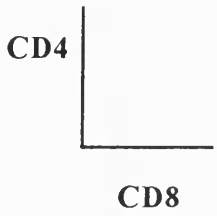




B. Spleen







-transgenic littermates stained with antibodies specific for CD4 (PE, ordinate) and CD8 (FITC, abscissa). The percentage of cells in each of the different CD4 and CD8 T cell subsets within the thymus was the same between transgenic and non transgenic littermates. This suggested that, although transgenic thymuses were reduced in size, there was no apparent subset perturbation during T cell development. As all T cell subsets are reduced by the same amount it seems likely that the deficiency in Ly-6E.1/*myc* transgenic thymopoiesis is at the level of thymic progenitors or precursors, possibly occurring at a pre-thymus stage of T cell development. This deficiency could be at the level of a T lymphoid commited progenitor, the haematopoietic stem cell or may be due to a deficiency in thymic colonisation or homing/migration of pre-T cells.

The spleens of Ly-6E.1/*myc* transgenic mice were also analysed in detail using FACS. Figure 27B shows FACS profiles of splenocytes from LM5 transgenic and non-transgenic littermates stained with CD4-PE and CD8-FITC monoclonal antibodies. Unlike the thymus, the CD4/CD8 profile of Ly-6E.1/*myc* spleen is perturbed compared with non transgenic littermate controls. There is an increase in the percentages of both CD4 positive and CD8 positive cells (helper and cytotoxic T cells respectively). However, when the reduced cellularity of the spleen is accounted for, it can be seen that there are decreases in the absolute number of both T cells and B cells within the spleen (table 3). The most dramatic reduction occurs in the B cell compartment. Transgenic LM5 animals possess fewer than 50% the number of $B220^+$ cells compared with non-transgenic littermates. It is this reduction in B cell number that produces FACS plots showing relatively higher percentages of CD4 and CD8 positive cells. The reduction in the number of both peripheral T and B cells in

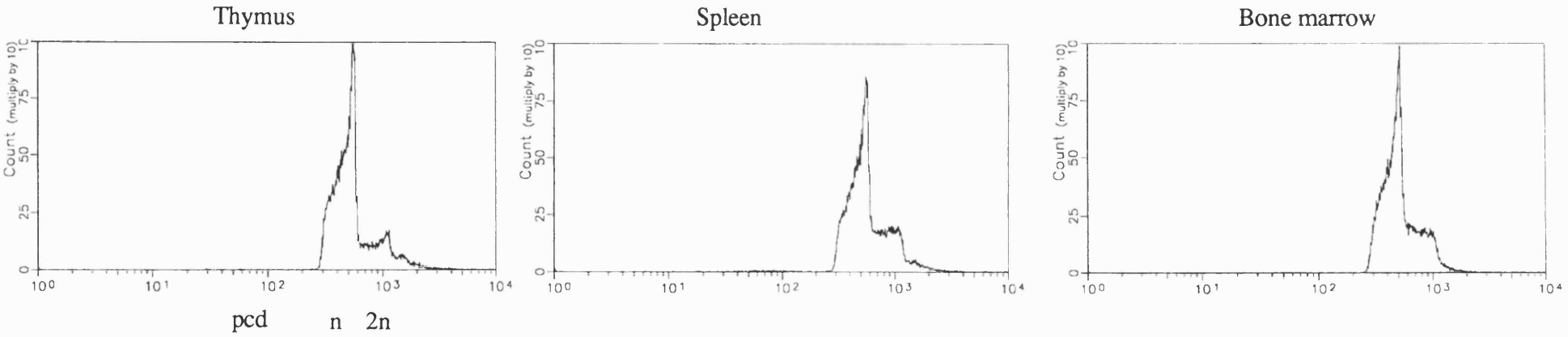
LM5 transgenic mice is a less severe example of the same phenotype exhibited by LM7 and LM8 transgenic founder mice. Correlating with reduced numbers of T and B cells, the levels of CD2 (T cell specific) and (B29 B cell specific) mRNA are also reduced in LM7 and LM8 transgenic spleens compared with non transgenic spleens, as shown in figures 25d and 25e.

C-myc has been implicated in the induction of apoptosis (Evan et al 1992). To investigate whether the reduction in thymic and splenic cellularity was a result of apoptosis, propidium iodide (PI) staining was performed on permeablised thymocytes, splenocytes and bone marrow cells from transgenic and normal control littermates. FACS analysis of permeablised, propidium iodide stained cells allowed the DNA content of the cells to be visualised (figure 28). Characteristic peaks corresponding to cells containing n and 2n amounts of DNA were clearly visible in both transgenic and non-transgenic samples. There was no evidence of the broad hypodiploid DNA peak characteristic of apoptosis (pcd = programmed cell death) in any of the cell populations from LM5 transgenic mice. Apoptotic cells usually exhibit fluorescence of between 10 and 100 units due to DNA fragmentation. Given the relatively low sensitivity of PI staining for the detection of apoptosis, it is possible that the apoptotic death of a small number of progenitor cells could go undetected and lead to the dramatic reduction in T and B cell numbers characteristic of Ly-6E.1/*c-myc* transgenic mice.

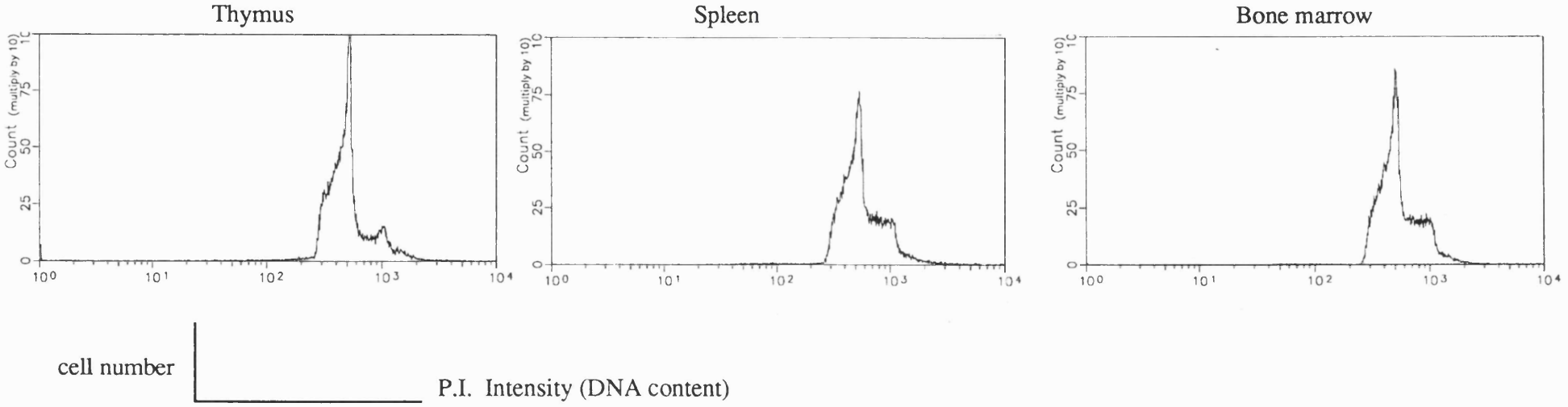
Figure 28. FACS analysis for apoptosis in Ly-6E.1/*myc* transgenic mice using propidium iodide.

Thymocytes, splenocytes and bone marrow cells from LM5 transgenic (B) and non-transgenic (A) littermates were stained overnight in a hypotonic solution containing propidium iodide (PI), then analysed using a Becton Dickinson FACScan. Histogram plots were obtained of cell number (ordinate) *versus* PI fluorescence (abscissa). The relative fluorescence intensities of cells with a DNA content indicative of resting cells (n), actively cycling cells (2n) and cells undergoing programmed cell death, apoptosis, (pcd) are shown on the non-transgenic thymus histogram.

A. Non transgenic



B. LM5 transgenic



3.5 Altered cellularity in Ly-6E.1/*myc* bone marrow.

As both lymphoid compartments were perturbed in Ly-6E.1/*myc* transgenic mice, bone marrow was examined to determine whether any other haematopoietic lineages were affected. Figure 29 shows bone marrow cells from the LM8 transgenic founder and a non-transgenic littermate, LM6, stained with eosin and thiazine. An examination report on these preparations by the Royal Veterinary College, UCL, London is presented in figure 29A. In summary, LM8 bone marrow displays normal numbers of erythroid, myeloid and megakaryocytic cells with a subtle decrease in progranulocytes. No evidence of neoplastic cells was observed in transgenic bone marrow when compared with a non-transgenic littermate (courtesy Dawn Loser, RVC, UCL, London). To provide more detailed analysis of haematopoietic cells in Ly-6E.1/*c-myc* transgenic bone marrow, FACS analysis was performed with subset specific, fluorescent labelled antibodies on LM5 bone marrow cells. Figure 30 shows FACS profiles of bone marrow cells from LM5 transgenic animals and non-transgenic littermates using antibodies specific for B cells (figure 30A) and macrophages (figure 30B). As the absolute number of cells in the bone marrow was not significantly different between transgenic and non-transgenic littermates, the percentages of each cell subset can be directly compared. There are 50% fewer B220 positive cells in the bone marrow of transgenic animals compared with normal littermates (figure 30A). Since splenic B cells are known to be derived from B cell progenitors in the bone marrow, this data suggests that the B cell deficiency observed in the spleen results from an effect of *c-myc* expression on B cell precursors or haematopoietic stem cells.

Figure 29. Cytological analysis of Ly-6E.1/*myc* bone marrow.

A. Cytology report from Royal Veterinary College on bone marrow smears from LM6 and LM8, normal and transgenic Ly-6E.1/*myc* founder animals.

Cytospin preparations of LM6 (B.) & LM8 (C.) bone marrow cells stained with Diff-Quick. 10^5 bone marrow cells were PBS washed, resuspended in 100μl PBS and centrifuged onto a Cytospin slide. Air-dried slides were methanol fixed, stained with "Diff-Quick" (eosin and thiazine) and mounted in DPX mountant.

DEPARTMENT OF PATHOLOGY AND INFECTIOUS DISEASES

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University of London

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Hatfield Herts AL9 7TA
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Fax 0707 661464

A.

Colin Miles
Gene Structure and Expression
National Inst for Medical Research
The Ridgeway
Mill Hill
NW7 1AA

DL/DRM

11 May 1995

LABORATORY EXAMINATION REPORT

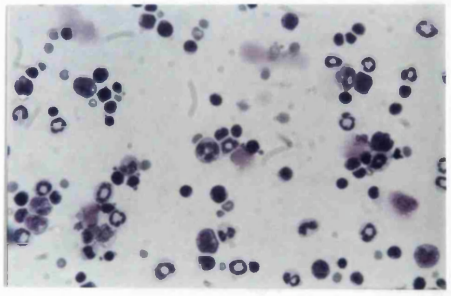
Yr ref: #6 (LM6) #8 (LM8) Transgenic mouse and non-transgenic littermate
Our ref: 226/0595
Sample: Bone marrow smears

Cytology report:
In both number 6 & 8 there are adequate numbers of cells of various stages of the erythroid, and myeloid series.
The M:E ration is ± 1:1.
There appears to be a fractional difference in that the #6 shows slightly more progranulocytes than #8. This difference is extremely subtle.
There also appear to be adequate numbers of megakaryocytes.
No neoplastic cells can be seen.
It is our opinion that these marrows are normal.

Yours sincerely

Dawn Löser BVSc MRCVS

B.



C.

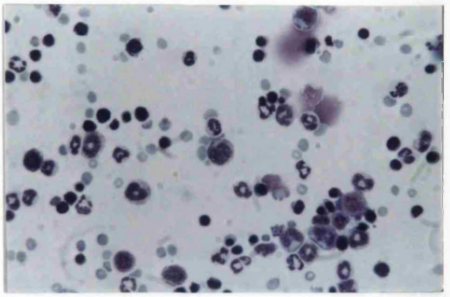
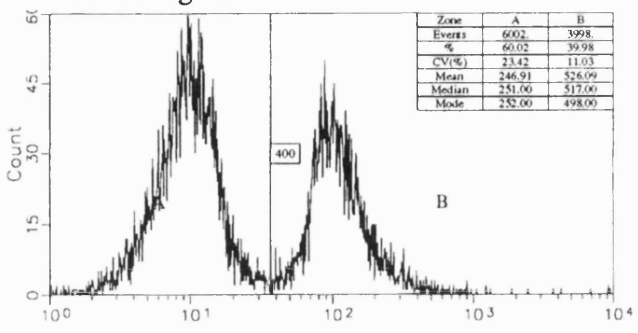


Figure 30. FACS analysis of B cells and macrophages in Ly-6E.1/*myc* bone marrow.

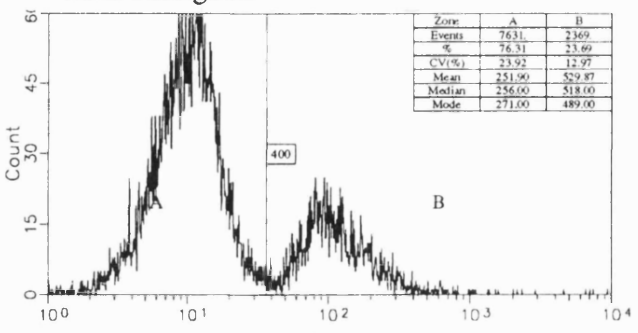
Bone marrow cells from LM5 transgenic and non-transgenic littermates were stained with B220-FITC to detect B cells (A.) and with Mac-1-PE to detect macrophages (B.). 10^4 cells were analysed using a Becton Dickinson FACScan and histogram plots of cell number (ordinate) *versus* relative fluorescence intensity (abscissa, logarithmic scale) were generated. Cells considered to be expressing each particular antigen exhibit fluorescence greater than 400 units and are located in region B. The number and percentage of cells within region B for each sample is indicated within the histogram.

A. B cells in LM5 transgenic and non transgenic bone marrow.

Non transgenic



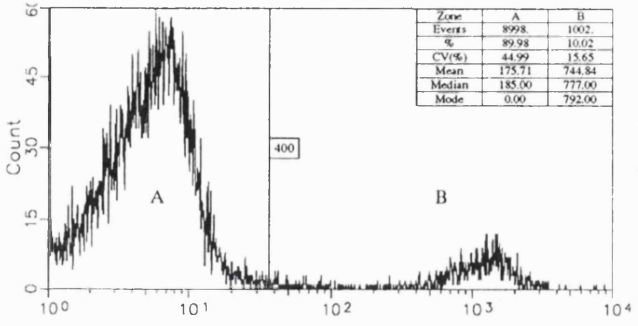
LM5 transgenic



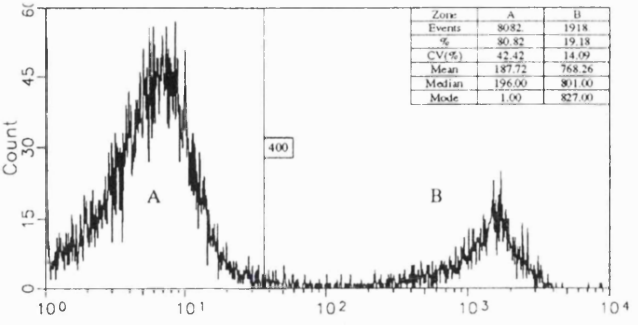
B220-FITC

B. Macrophages in LM5 transgenic and non transgenic bone marrow.

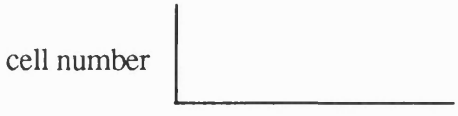
Non transgenic



LM5 transgenic



Mac-1-PE



In general, the lymphoid deficiencies found in both Ly-6E.1/*myc* transgenic T cells and B cells appear to be the result of abnormal development from a progenitor cell.

The other significant difference between transgenic and normal bone marrow is that the Ly-6E.1/*myc* bone marrow contains twice the number of Mac-1 positive cells (figure 30B). Mac-1 is generally thought of as a marker for mature macrophages but there are recent reports that haematopoietic stem cells in foetal liver and AGM are Mac-1$^+$ (Morrison *et al.*, 1995; Sanchez, pers. comm.). Therefore, the increased number of Mac-1$^+$ cells in LM5 bone marrow may represent a population of progenitor cells. However, there is no corresponding increase in the numbers of Sca-1$^+$ cells in bone marrow (not shown), which would be expected if Ly-6E.1/*myc* were inducing the expansion of a progenitor population. FACSorting of these Mac-1$^+$ cells and functional assays of their potential is required to determine whether they represent an expanded progenitor cell population or are simply a result of compensation for the lack of B220$^+$ cells.

3.6 Ly-6E.1/*myc* bone marrow fails to differentiate normally.

In order to gauge the functional significance of the phenotypic differences observed in Ly-6E.1/*myc* bone marrow, methylcellulose cultures designed to detect myelo-erythroid progenitors were set up using either transgenic or non-transgenic bone marrow. Serial dilutions of bone marrow cells were cultured in methyl cellulose supplemented with erythropoietin and pokeweed mitogen spleen cell conditioned medium (PWM SCCM). Colony forming frequency was determined by counting

macroscopic colonies after 10 to 14 days. The cell types differentiating in the cultures were determined morphologically, after cytological staining of cytospin preparations. Bone marrow from non-transgenic LM5 littermates gave rise to between 10 and 30 colonies per initial 10^5 cells seeded whilst the frequency of transgenic colonies was between 40 and 50 per 10^5 cells seeded (figure 31). Non-transgenic bone marrow produced the normal variety of myeloid colonies, erythroid colonies and complex mixed colonies (figure 32A). In contrast, transgenic bone marrow yielded generally uniform colonies with a distinct morphology that were smaller, more homogeneous and more compact than non-transgenic colonies (figure 32B). No morphologically distinguishable myeloid, erythroid, mixed or blast colonies were visible from cultured transgenic bone marrow. In this assay, myeloid and erythroid colonies are thought to form from commited progenitor cells, whereas mixed colonies are thought to be derived from a more immature progenitor cell type which is capable of multilineage differentiation. Detailed cytological analysis revealed altered subset cellularity within the transgenic colonies (figures 32C and 32D) which corresponds to differences in colony morphology. Ly-6E.1/*myc* bone marrow appears to differentiate exclusively into macrophage-like cells *in vitro*, in the near absence of other differentiated cell types. Since these *in vitro* growth conditions favour myelopoiesis, this observation may be an extreme case of the situation *in vivo*, in which twice the number of macrophages can be detected in fresh transgenic bone marrow.

To determine whether the apparent block in multilineage haematopoiesis seen in the methylcellulose assay affected the differentiation of bone marrow *in vivo*, the capacity for Ly-6E.1/*myc* bone marrow to reconstitute the haematopoietic system of

Figure 31. Methyl cellulose colony forming potential of Ly-6E.1/*myc* bone marrow.

Bone marrow cells from 2 LM5 transgenic and 2 non-transgenic littermates were seeded, each in triplicate, at $5x10^3$, 10^4, $5x10^4$ and 10^5 cells per ml in methyl cellulose culture supplemented with pokeweed mitogen spleen cell conditioned medium and erythropoietin. Colonies of cells were scored after ten days, data for each individual mouse pooled and expressed in the form of number of colonies per 10^5 bone marrow cells.

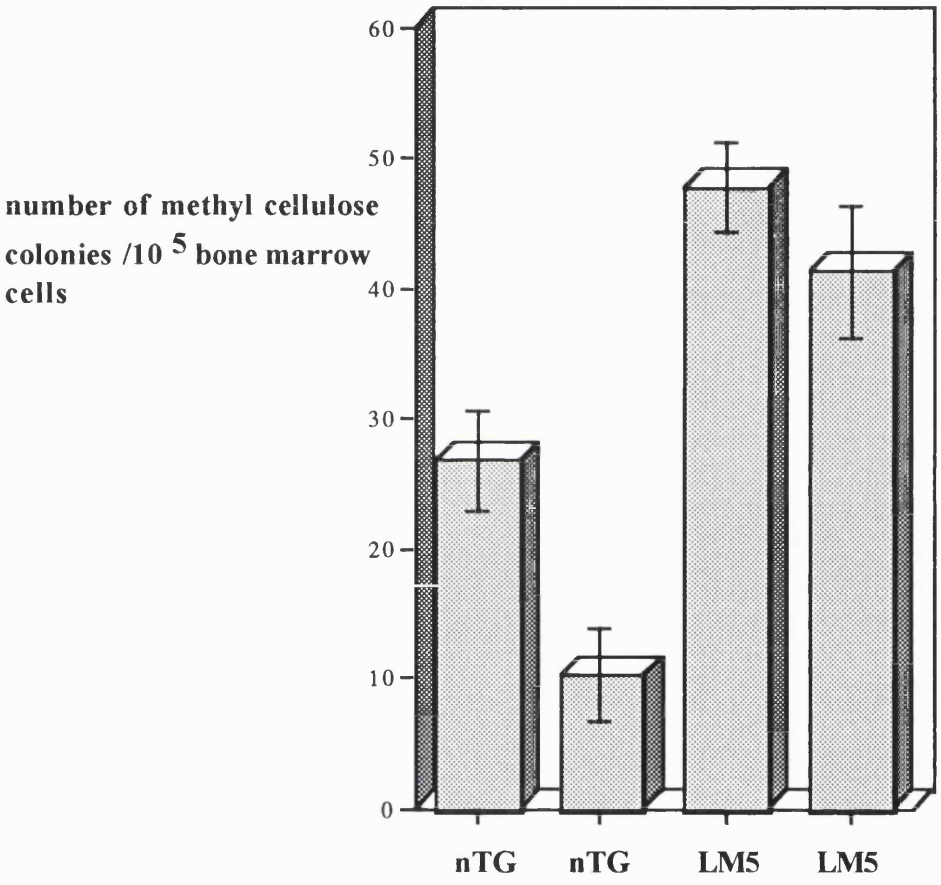
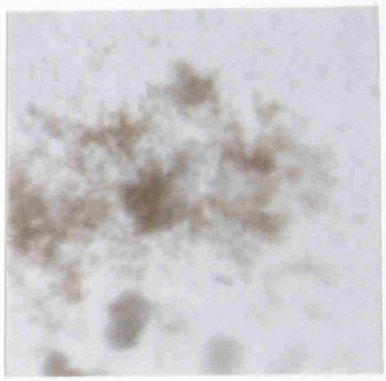

number of methyl cellulose colonies /10 5 bone marrow cells
60
50
40
30
20
10
0
nTG
nTG
LM5
LM5

Figure 32. Cytological analysis of Ly-6E.1/*myc* methyl cellulose colonies.

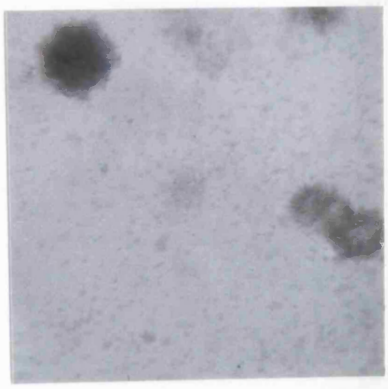
A. & B. Methyl cellulose colonies from non-transgenic and LM5 transgenic bone marrow, respectively.

C. & D. Cytological analysis of cells within non-transgenic and LM5 transgenic derived colonies, respectively. Colonies were pooled from 1ml of culture medium, PBS washed and 10% of each was centrifuged onto a Cytospin slide. Air-dried slides were methanol fixed, stained with "Diff-Quick" (eosin and thiazine) and mounted in DPX mountant.

A. Non transgenic

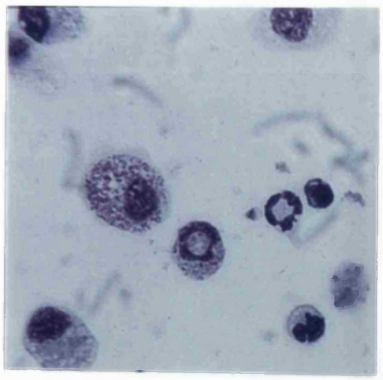


B. LM5 transgenic

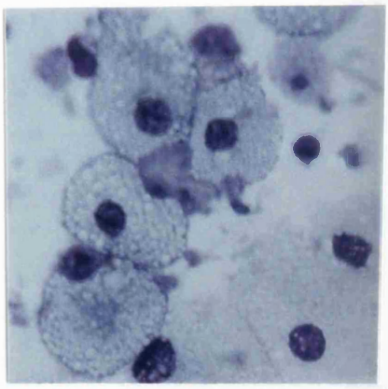


Macroscopic morphology of methyl cellulose colonies.

C. Non transgenic



D. LM5 transgenic



Cytological analysis of cells within colonies.

an irradiated mouse was investigated. Bone marrow from male LM5 transgenic and non-transgenic littermates was transplanted into irradiated female recipient mice at serial dilutions from $2x10^6$ to $2x10^3$. The level of haematopoietic reconstitution was determined at one and four months post-transplantation by PCR detection of male specific sequences in peripheral blood genomic DNA of the female recipients Figure 33 shows the level of male-derived cells in the peripheral blood of bone marrow transplant recipients at 4 months post-transplantation. The reconstitution profiles are similar for both transgenic and non-transgenic donor bone marrow cells, with reconstitution readily detectable from $2x10^4$ and greater cell numbers in both cases. However, at one month post transplant, male signal was detectable from transgenic bone marrow at $2x10^3$ donor cells but not from non-transgenic. This signal is not present at 4 months post transplant, suggesting either that Ly-6E.1/*myc* bonemarrow displays greater short term haematopoietic reconstitution potential in comparison with non-transgenic bone marrow, or that differentiated Ly-6E.1/*myc* transgenic cells selectively survive in recipients and remain detectable at 1 month post-transplantation. However, there is no difference in long term reconstitution potential. To determine whether Ly-6E.1/*myc* haematopoietic stem cells were fully functional in terms of giving rise to multilineage reconstitution, FACS analysis was performed on haematopoietic cells from transplant recipients. The peripheral lymphoid organs of Ly-6E.1/*myc* chimaeras displayed no gross abnormalities compared with chimaeras generated from normal bone marrow (not shown) and the cellularity of the thymus was similarly normal (discussed in results section 4.5). Figure 34 shows a FACS comparison between recipients of $2x10^6$ non-transgenic bone marrow cells and $2x10^6$ LM5 bone marrow cells. Both animals were fully reconstituted (100%), as

Figure 33. Long term haematopoietic reconstitution by Ly-6E.1/*myc* bone marrow cells.

Lethally irradiated female mice were injected with male bone marrow cells from LM5 Ly-6E.1/*myc* transgenic and non-transgenic littermates at dilutions of cells from $2x10^6$ to $2x10^3$. Haematopoietic repopulation at 4 months post-transplantation was determined by male specific PCR of peripheral blood genomic DNA from the female radiation chimaeras. Comparison of the intensity of Y-chromosome specific signal with that obtained from standards containing known ratios of male:female DNA enabled the level of repopulation to be estimated.

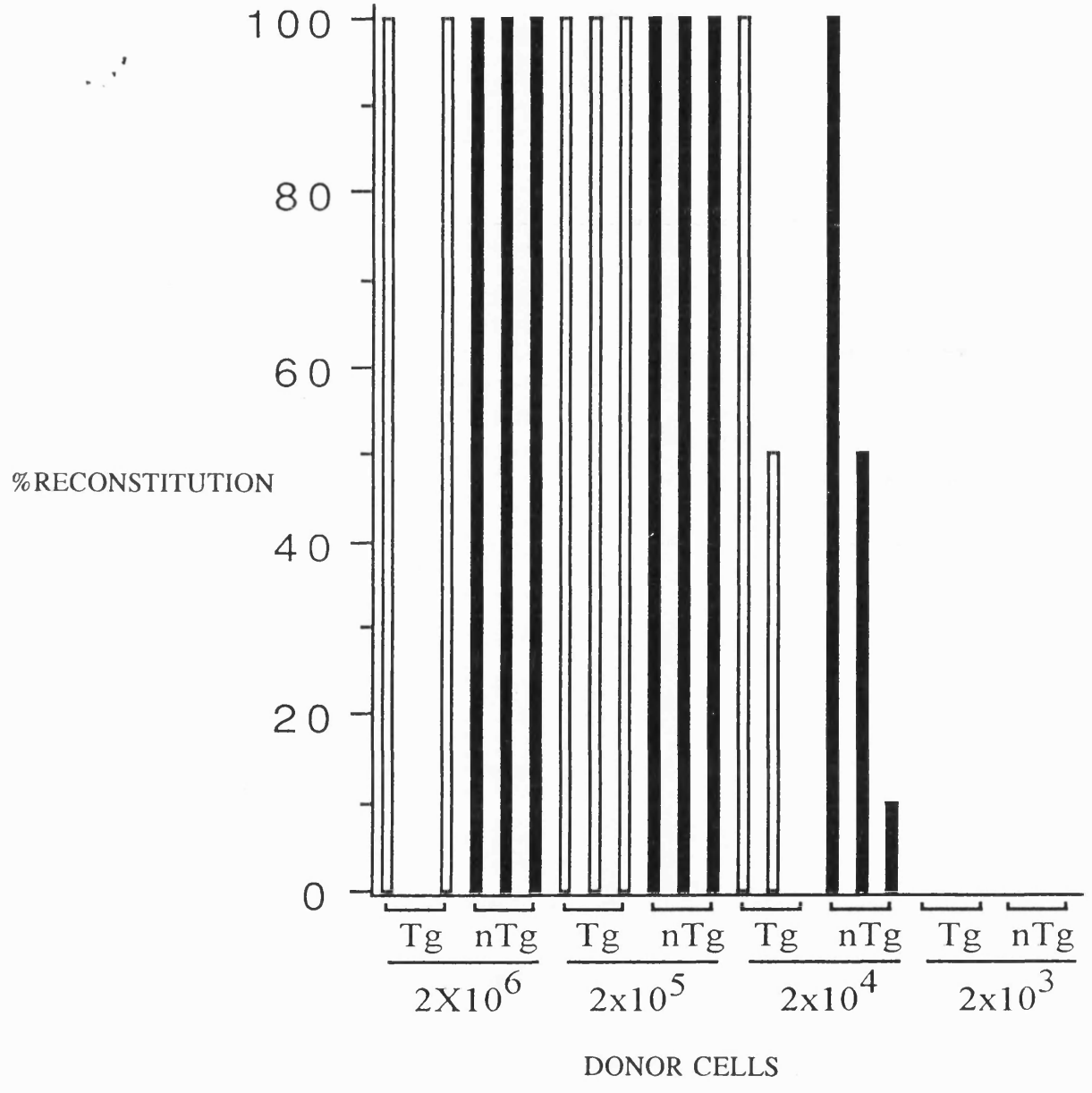
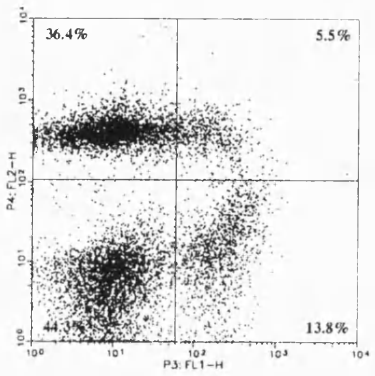

100
80
60
40
20
0
%RECONSTITUTION
Tg
nTg
Tg
nTg
Tg
nTg
Tg
nTg
2X10^6
2x10^5
2x10^4
2x10^3
DONOR CELLS

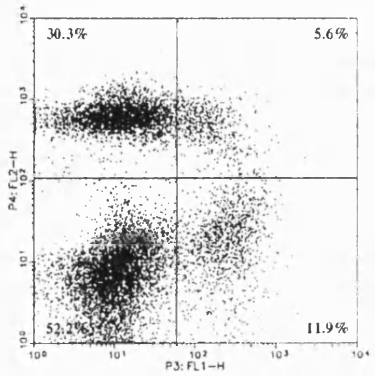
Figure 34. Multilineage FACS analysis of bone marrow cells from Ly-6E.1/*myc* radiation chimaera.

Bone marrow cells from radiation chimaeras, both Ly-6E.1/*myc* and non-transgenic derived, exhibiting 100% repopulation of peripheral blood as determined by PCR were analysed by FACS. 10^6 bone marrow cells were stained with monoclonal antibodies specific for A. erythroid cells (Ter119-PE, ordinate) and T cells (CD3-FITC, abscissa); B. Ly-6A/E (Sca-1-PE, ordinate) and B cells (B220-FITC, abscissa); and C. macrophages (Mac-1-PE, ordinate) and the tyrosine kinase c-kit (FITC, abscissa). 10^4 cells were analysed using a Becton Dickinson FACScan. FACS dot plots with logarithmic axes for fluorescence are shown. The percentage of bone marrow cells expressing each antigen is shown within each quadrant of the FACS plots. Note: In these FACS analyses, the normal radiation chimaeras used as controls are not littermates and have been extensively manipulated. For these reasons, there is a high degree of variation between individuals which prevents conclusions being drawn regarding subtle differences in cellularity.

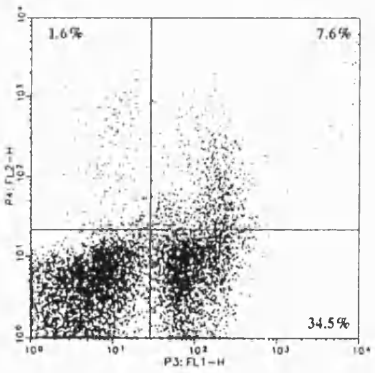
Normal radiation chimaera **Transgenic radiation chimaera**

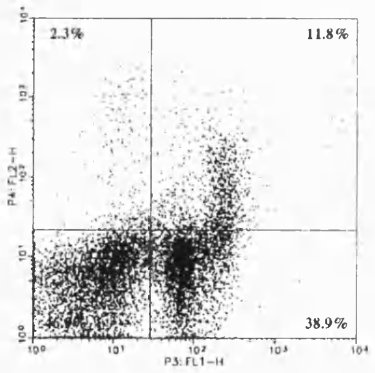
A. Ter119-PE + CD3-FITC



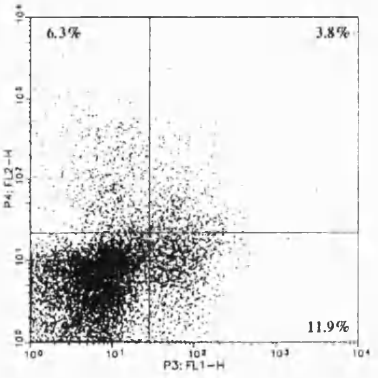


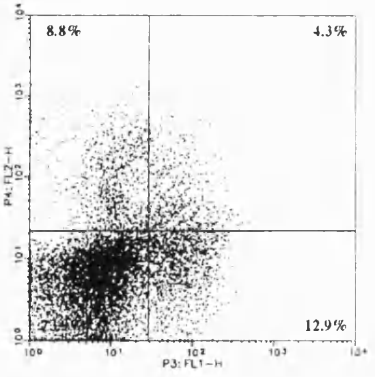
B. Sca1-PE + B220-FITC





C. Mac1-PE + c-kit-FITC





PE fluorescence

FITC fluorescence

determined by peripheral blood genomic DNA PCR analysis and, as shown in figure 34, both show normal numbers of T cells ($CD3^+$), B cells ($B220^+$), macrophages ($Mac\text{-}1^+$) and erythroid cells ($Ter119^+$). In addition, Sca-1 and c-kit expression, two markers associated with primitive haematopoietic cells, is also normal. These functional and phenotypic studies suggest a *c-myc* induced expansion of haematopoietic progenitor cells without an effect on long term reconstitution potential. In order to test the self-renewing potential of Ly-6E.1/*myc* transgenic bone marrow, it would be interesting to perform serial transplantation experiments.

4. Ly-6E.1/*bcl-2* transgenic mice.

4.1 Introduction.

The *bcl-2* proto-oncogene was initially identified by virtue of its involvement in the most common chromosomal translocation associated with human lymphoid malignancies, the t(14;18). (Fukuhara *et al.*, 1979; Levine *et al.*, 1985; Yunis *et al.*, 1987) This translocation is found in 20% of diffuse and approximately 85% of follicular B cell lymphomas and, in a similar fashion to *c-myc* translocation in Burkitt's lymphoma, occurs early in B cell development at the pre-B cell stage during immunoglobin heavy chain gene rearrangement (Bakhski *et al.*, 1987; Cleary & Sklar, 1985; Tsujimoto *et al.*, 1985). The translocation effectively brings the *bcl-2* gene from chromosome 18 into the immunoglobin heavy chain locus in chromosome 14, resulting in a *bcl-2*/immunoglobin gene fusion on chromosome 14. The resulting derivative chromosome gives rise to a fusion mRNA between the entire *bcl-2* coding region and part of the immunoglobin heavy chain gene under the transcriptional control of the immunoglobin heavy chain enhancer (Cleary *et al.*, 1986; Seto *et al.*, 1988). High levels of the fusion mRNA are found in lymphoma cells along with inappropriately high levels of bcl-2 protein (Graninger *et al.*, 1987).

Biochemical and cell biological analysis showed the *bcl-2* gene product is a 26Kd protein associated with intracellular membranes, in particular mitochondrial membranes (Chen-Levy *et al.*, 1989; Hockenbery *et al.*, 1990). In contrast to most proto-oncogenes, bcl-2 does not exhibit the characteristics of a transcription factor or

a tyrosine kinase. However, *bcl-2* does appear to play a vital role in the normal physiological homeostasis of non-transformed mammalian cells. Mice lacking a functional *bcl-2* gene have been generated by gene targeting and despite exhibiting normal lymphoid development, show a degeneration of the lymphoid system as well as abnormalities to their kidneys and hair (Nakayama *et al.*, 1993; Veis *et al.*, 1993). In general, *bcl-2* and the other members of its family of proteins are involved in cell survival via the regulation of apoptosis (reviewed by Cory, 1995). As can be seen in mice made deficient in *bcl-2* or *bcl-x*, programmed cell death (pcd) plays a vital role in many cellular processes and the dysregulation of normal apoptosis often causes dramatic developmental defects (Nakayama *et al.*, 1993; Veis *et al.*, 1993; Motoyama *et al.*, 1995). It is possible that apoptosis is involved in the early events of haematopoiesis, possibly even playing a role in haematopoietic stem cell survival. *Bcl-2* is highly expressed in progenitor cells of both lymphoid (Cory, 1995) and erythroid lineages (Bonati *et al.*, 1996) and downregulated in lymphocytes undergoing selection and erythrocytes undergoing maturation. More direct evidence for an important role for apoptosis in primitive haematopoietic cells comes from the study of mice made deficient in the bcl-2 family member, bcl-x. In the absence of a functional *bcl-x* gene, mice display a haematopoietic deficiency brought on by death of immature haematopoietic cells within the foetal liver (Motoyama *et al.*, 1995).

The over expression of *bcl-2* in cell lines extends cellular survival by the inhibition of programmed cell death without inducing proliferation. This may provide some indication as to how *bcl-2* may function as a proto-oncogene. If a dysfunctional cell that would under normal circumstances be eliminated by apoptosis, were to express high levels of *bcl-2*, it could be rescued, for example, in cases of neoplasia.

In particular, *bcl-2* prolongs the short term survival of many factor dependent haematopoietic cell lines (Vaux *et al.*, 1988). This phenomenon can be observed in IL-3 dependent mast cell, prolymphocyte and promyelocyte lines after growth factor deprivation (Nunez *et al.*, 1990). In addition, the overexpression of *bcl-2* can enhance the survival of GM-CSF or IL-4 dependent cells but not IL-2 or IL-6 dependent cells (Nunez *et al.*, 1990). The ability of *bcl-2* to abrogate certain factor dependencies is clearly of potential use in the generation of haematopoietic cell lines. However, not all growth factor dependencies can be overcome by bcl-2 suggesting that bcl-2 independent survival mechanisms exist and that certain cell types are refractory to the protective effects of bcl-2.

When considering the self-renewal of pluripotent haematopoietic stem cells, it remains unclear whether the signal for the pluripotent stem cell to undergo commitment and produce mature haematopoietic cells is instructive or stochastic (Ogawa, 1993). Considering that many other biological processes are based upon stochastic events, for example, the generation of antibody diversity, it is possible that the commitment of haematopoietic stem cells could also occur via stochastic mechanisms (see Ogawa, 1993 for review). One hypothesis could be that haematopoietic stem cells are continually undergoing assymetric cell division, generating a daughter cell which is commited to differentiating whilst self-renewing to produce another stem cell (see Lord & Dexter, 1995 for review). If there was no demand for further haematopoietic cells, the commited daughter cell would not develop and may apoptose. However, upon demand, the daughter cell may receive survival signals which could take the form of haematopoietic growth factors or some specific cell-cell interaction. Apoptosis is a common feature of selection processes,

for example, during T cell selection in the thymus, *bcl-2* expression is upregulated within positively selected cells, whilst negatively selected cells are eliminated by programmed cell death (von Boehmer & Kisielow, 1990; Swat *et al.*, 1991; Huesman *et al.*, 1991). If an analogous process were occuring during early haematopoietic differentiation in the bone marrow, it may be possible to manipulate this through *bcl-2* over expression. To examine this possibility, Ly-6E.1/*bcl-2* transgenic mice were generated in which *bcl-2* expression was directed to haematopoietic stem cells for the perturbation of normal stochastic processes and to facilitate the derivation of haematopoietic stem and progenitor cell lines.

In addition, the Ly-6E.1 gene will direct *bcl-2* expression to certain mature T and B cells and all activated lymphocytes, enabling the examination of T and B lymphopoiesis. Previous studies of *bcl-2* transgenic mice, attempting to mimic the naturally occuring t(14;18) in B cells, showed that there was an accumulation of small non-cycling B cells in these animals which showed an increased capacity for survival *in vitro*. The lifespan *in vivo* of all classes of B lymphoid cells was prolonged by high levels of *bcl-2* expression in transgenic mice (McDonnell *et al.*, 1989; McDonnell *et al.*, 1990; Strasser *et al.*, 1990).

Of perhaps greater interest is the study of T lymphopoiesis in Ly-6E.1/*bcl-2* transgenic mice. Approximately 50% of immature $CD4^-CD8^-$ double negative thymocytes and 50% of mature single positive thymocytes are Ly-6E.1 positive. At the double positive stage of T cell development, downregulation of Ly-6E.1 occurs and only 2-3% of $CD4^+/CD8^+$ double positive cells express Ly-6E.1 (Codias *et al.*, 1989; Bamezai *et al.*, 1995; Spangrude *et al.*, 1988). It has been postulated that the downregulation of Ly-6E.1 is crucial to the process of T cell development (Bamezai

et al., 1995) and that it is the few double positive cells that express Ly-6E.1 which are selected to become functional T cells. It will, therefore, be interesting to determine whether Ly-6E.1/*bcl-2* expression perturbs T cell development. In addition, the expression of *bcl-2* under control of the Ly-6E.1 gene in transgenic mice may help to resolve some of the controversy surrounding the role of *bcl-2* in T cell selection. Both Strasser *et al.* and Siegel *et al.* demonstrated some inhibition of negative selection in transgenic mice expressing *bcl-2* in the thymus (Strasser *et al.*, 1991; Siegel *et al.*, 1992) though in these cases, expression was controlled by the immunoglobin heavy chain enhancer. In contrast, transgenic mice expressing *bcl-2* under control of the T cell specific p56*lck* promoter showed no evidence of impaired negative selection (Sentman *et al.*, 1991). T cell development in *bcl-2* null mutant mice also appears normal (Nakayama *et al.*, 1993; Veis *et al.*, 1993). Therefore although *bcl-2* does not appear to be necessary for normal T lymphopoiesis, apoptosis is a vital part of the correct selection process.

4.2 Generation of Ly-6E.1/*bcl-2* transgenic mice.

In order to examine the effects of directed expression of *bcl-2* to Ly-6E.1 positive cells, in particular to transplantable bone marrow haematopoietic stem cells, a *bcl-2* cDNA was cloned into the ClaI site of pL6Cla (figure 1), in an analogous fashion to *lacZ* and *c-myc* (in results sections 1 and 3). Briefly, pmB2, containing the *bcl-2* cDNA cloned into bluescript (Nunez *et al.*, 1990), was digested with SmaI and blunt ended synthetic oligonucleotide linkers were inserted to create a NarI

restriction site. In the resulting plasmid, designated pB4, the *bcl-2* cDNA is flanked by Nar1 and Cla1 restriction sites. Partial Nar1 digestion followed by Cla1 digestion and purification of the 865bp fragment yielded the *bcl-2* cDNA flanked by Cla1 compatible sites which was cloned into pL6Cla to generate pL6B2. The orientation of the insert was verified by restriction digest with HindIII, a restriction enzyme whose site is located assymetrically within the *bcl-2* cDNA (figure 35A).

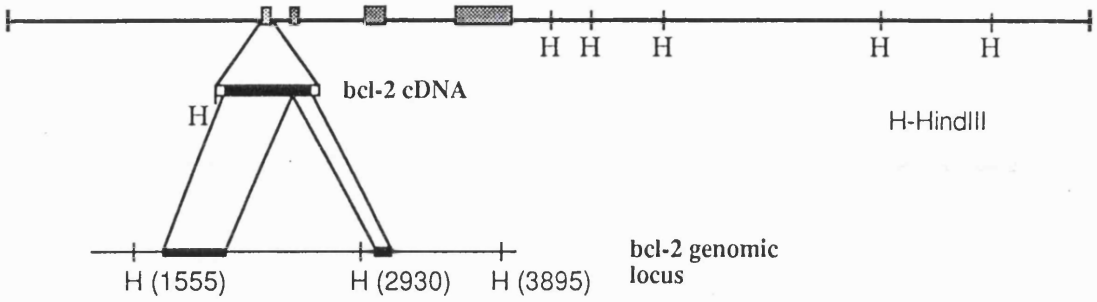
A 14.9Kb Not1 fragment from pL6B2 consisting of Ly-6E.1/*bcl-2* was microinjected into the pronuclei of fertilised (CBA/Ca x C57/B10)F2 mouse oocytes and out of a total of 11 founder mice born, 3 carried the transgene as determined by southern blot analysis (figure 35B). For detection of Ly-6E.1/*bcl-2* transgenic mice by southern blot, genomic DNA prepared from tail biopsies was digested with HindIII. Figure 35A shows how HindIII digestion enabled discrimination between the transgene and the endogenous murine *bcl-2* gene. Briefly, the genomic *bcl-2* locus has an intronic HindIII site, at position 2930, which is not present in the cDNA. This results in a smaller fragment being detected for the endogenous gene (1.4 Kb hybridising band) as compared with the transgene (4.7 Kb hybridising band) when using the entire 865 bp *bcl-2* cDNA as a probe. Using a Phosphorimager to compare the hybridising signals of the endogenous gene (2 copies per genome) with the transgene specific band, the copy number of the three transgenic founders 469, 471 and 479 was determined to be approximately 5, 30 and 20 respectively. All three transgenic founder mice were healthy and displayed normal life expectancies. However, only founders 471 and 479 bred to establish lines. Founder 469, a female, produced four litters, in which no transgenic pups were detected was presumed to be highly mosaic for the transgene and was not analysed further. The phenotypes of

Figure 35. Ly-6E.1/*bcl-2* construct and the generation of transgenic mice.

A. Diagram of the Ly-6E.1/*bcl-2* construct highlighting the intronic HindIII polymorphism between genomic DNA and cDNA of murine *bcl-2*, which allows discrimination between endogenous *bcl-2* and the Ly-6E.1/*bcl-2* transgene on southern blots.

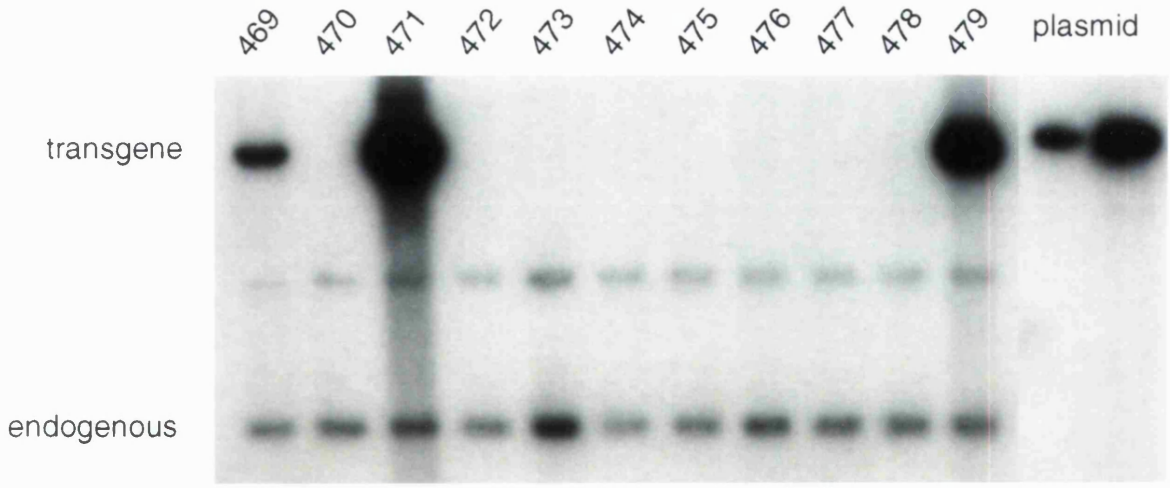
B. Southern blot of 10μg of tail genomic DNA isolated from Ly-6E.1/*bcl-2* founder mice 469-479, digested with HindIII, separated on a 1% agarose/TAE gel, transferred to a nylon membrane and hybridised with a ^{32}P-labelled *bcl-2* cDNA probe. The 1.4 Kb hybridising endogenous *bcl-2* band serves as a loading control and, by comparing the intensity of this signal with that of the transgene-specific band at 4.8 Kb, using a phosphorimager, the copy number of each positive founder was determined to be approximately: 469 = 5 copy; 471 = 30 copy; and 479 = 20 copy. Positive control samples consist of the Ly-6E.1/*bcl-2* plasmid digested with HindIII. The weakly hybridising band present in all tail samples is likely to be a *bcl-2* related gene with some sequence homology to the cDNA probe.

A. Ly-6E.1/bcl-2 construct



HindIII transgene = 4.7Kb
HindIII genomic = 1.4Kb

B. Southern Blot of Ly-6E.1/bcl-2 Founder mice.



lines 471 and 479 were*p883Xidentical*the following results are taken from both of these lines.

To verify that the Ly-6E.1/*bcl-2* transgene was expressing *in vivo*, northern blot analysis was performed. Total RNA from thymus, spleen, liver and kidney of transgenic (+) and non-transgenic (-) littermates from founder 479 offspring was analysed (figure 36). Specific signal hybridising to the *bcl-2* cDNA probe was observed only for RNA prepared from transgenic tissues. The same filter was reprobed for GAPDH mRNA as a control for equal loading of transgenic and non-transgenic RNA for each tissue. No endogenous *bcl-2* mRNA was detectable in this analysis, presumably because of expression at much lower levels than the multiple copies of the Ly-6E.1/*bcl-2* transgene.

4.3 Enlarged lymphoid organs in Ly-6E.1/*bcl-2* transgenic mice.

Transgenic animals of both lines 471 and 479 showed no reduction in lifespan, remaining healthy and indistinguishable from their non-transgenic littermates beyond six months of age. Previous transgenic studies, in which *bcl-2* was expressed under control of either the T cell specific p56*lck* or immunoglobin Eμ regulatory elements resulted in mice with enlarged lymphoid organs. These studies are suggestive of *bcl-2* mediated protection from programmed cell death. As the Ly-6E.1 transgene directs heterologous gene expression to mature lymphoid cells, in addition to immature precursors, the lymphoid organs of Ly-6E.1/*bcl-2* transgenic mice were examined for phenotypic evidence of *bcl-2* expression. Figure 37 shows the thymus and a lymph

Figure 36. Northern blot analysis of Ly-6E.1/*bcl-2* transgenic mice.

10μg of total RNA prepared from thymus, spleen, liver and kidney was separated through a 1% formaldehyde gel, blotted and hybridised with a ^{32}P-labelled *bcl-2* cDNA probe (a.). The tissues were obtained from transgenic (+) and non-transgenic (-) Ly-6E.1/*bcl-2* mice of the 479 line. To demonstrate equal loading of RNA between transgenic and non-transgenic lanes, the filter was stripped and reprobed with a ^{32}P-labelled GAPDH probe (b.).

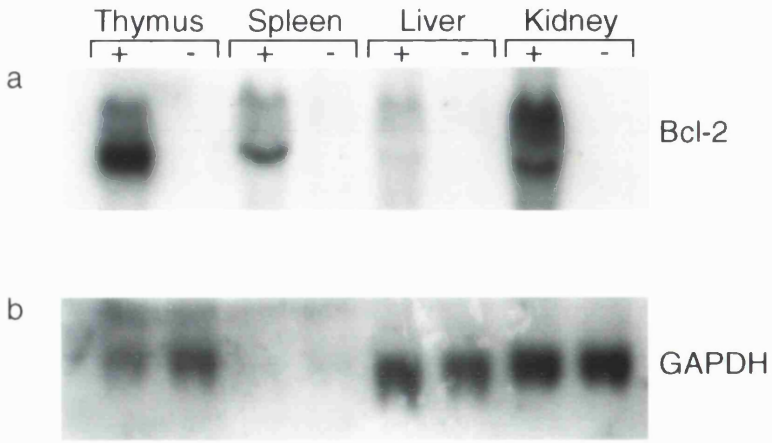

Thymus
Spleen
Liver
Kidney
+ - + - + - + -
a
Bcl-2
b
GAPDH

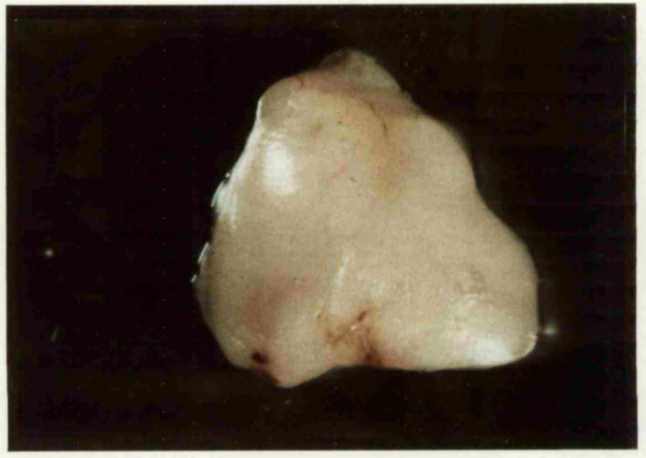
Figure 37. Enlarged lymphoid organs in Ly-6E.1/*bcl-2* transgenic mice.

a. & b. Thymuses from 6 week old non-transgenic (left) and Ly-6E.1/*bcl-2* transgenic (right) littermates of the 471 line pictured at the same magnification and illumination.

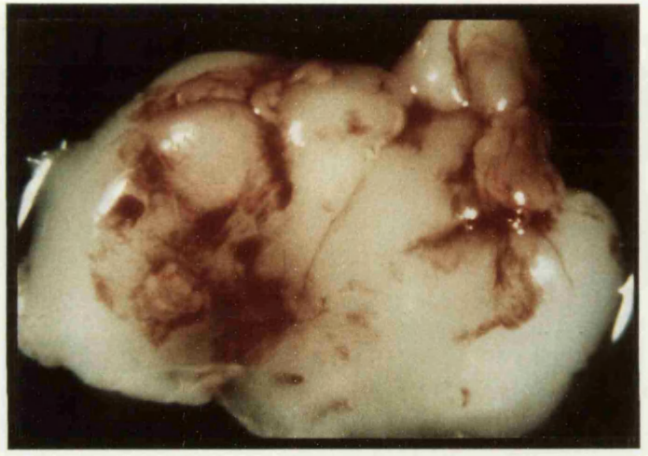
c. Lymph nodes from the same 6 week old non-transgenic (left) and transgenic (right) littermates.

Enlarged lymphoid organs in Ly-6E.1/bcl-2 transgenic mice

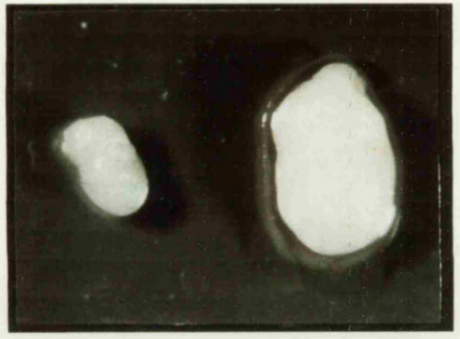
normal thymus



transgenic thymus



lymph node - normal(left) vs transgenic(right)



node isolated from a six week old transgenic animal of the 471 line and a non-transgenic littermate. The degree of enlargement of each organ was determined by homogenisation to single cell suspension and accurately counting absolute cell number from two transgenic and two non-transgenic littermates (table 4). In agreement with the results of published studies with *bcl-2* expressing transgenic mice, the thymus in Ly-6E.1/*bcl-2* transgenic mice displayed the most modest enlargement, being approximately 50% larger than wild type littermate controls. By comparison, spleen and lymph nodes exhibited a greater enlargement and were found to be 2.5 times and 7 times larger respectively in transgenic animals. Although previous studies suggest *bcl-2* preferentially acts to prolong the survival of peripheral lymphocytes, any *bcl-2* mediated effects observed in the peripheral lymphoid system in Ly-6E.1/*bcl-2* transgenic mice would be further enhanced by the fact that the transgene is upregulated upon lymphocyte activation and by γ-interferon.

In order to determine precisely which populations of lymphoid cells were affected by expression of the transgene, FACS analysis was performed on single cell suspensions obtained from thymus, lymph node and spleen of Ly-6E.1/*bcl-2* transgenic mice and non-transgenic littermates. Figure 38A shows the FACS profiles of thymocytes from transgenic and normal Ly-6E.1/*bcl-2* littermates stained with monoclonal antibodies specifically recognising the CD4 and CD8 antigens. During T cell development in the thymus, cells exhibit characteristic expression patterns of the CD4 and CD8 glycoproteins as they pass through development. The most immature thymocytes are $CD4^-$ and $CD8^-$. T cells then express both CD4 and CD8 simultaneously as they undergo positive and negative selection during which useless and harmful T cell clones are eliminated. The cells which survive selection, T cell

Table 4. Absolute cell numbers within haematolymphoid organs of Ly-6E.1/*bcl-2* transgenic mice.

Thymus, spleen, lymph nodes and bone marrow were recovered from two transgenic Ly-6E.1/*bcl-2* mice of the 471 line and two non-transgenic littermates. Accurate cell counts were performed on single cell suspensions from each tissue using trypan blue exclusion. There was no difference between the proportion of cells staining blue from either transgenic or non-transgenic tissues, indicating a similar viability for lymphocytes from either Ly-6E.1/*bcl-2* or non-transgenic mice.

Table 4: 471 transgenic and non-transgenic hematopoietic organs. Absolute cell numbers

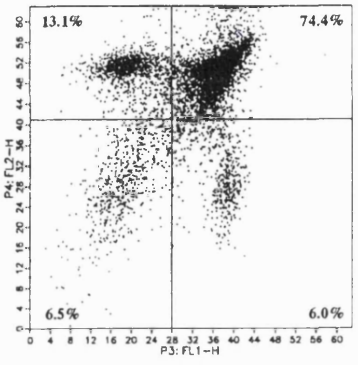
	Thymus	Lymphnodes	Spleen	Bone marrow
Non transgenic 1	$1.60x10^8$	$1.08x10^7$	$1.10x10^8$	$4.7x10^7$
Non transgenic 2	$2.12x10^8$	$1.45x10^7$	$1.30x10^8$	$4.9x10^7$
Transgenic 1	$2.55x10^8$	$1.35x10^8$	$2.60x10^8$	$5.0x10^7$
Transgenic 2	$2.57x10^8$	$5.82x10^7$	$3.41x10^8$	$5.3x10^7$

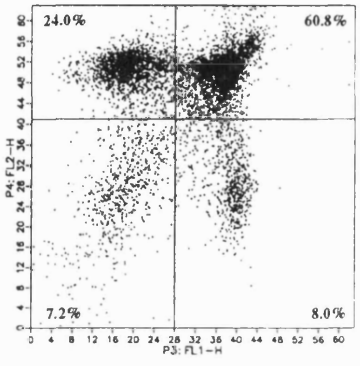
Figure 38. Distribution of CD4/CD8 T cell subsets in thymus and lymph nodes of Ly-6E.1/*bcl-2* transgenic mice.

Thymocytes (A) and lymph node lymphocytes (B) from 471 transgenic and non-transgenic littermates were stained for CD4 (PE-ordinate) and CD8 (FITC-abscissa). 10^4 cells were analysed using a Beckton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale, with percentages of each subset of cells indicated in the relevant quadrant.

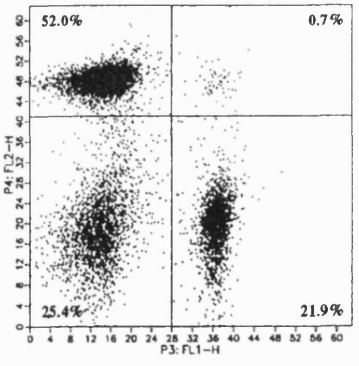
Non transgenic. **Ly-6E.1/*bcl-2* transgenic**

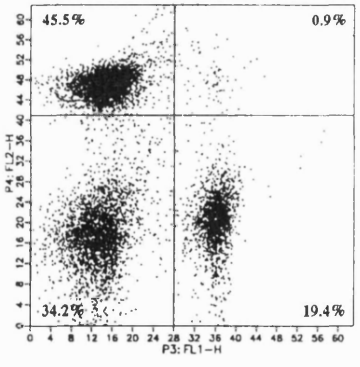
A. Thymus

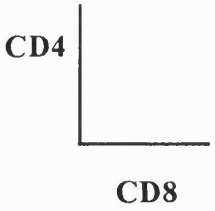




B. Lymph node







clones capable of recognising foreign antigens, then downregulate either CD4 or CD8 to become single positive cells which leave the thymus to become peripheral helper ($CD4^+$) or cytotoxic ($CD8^+$) T cells. It is notable that there is an approximate 10% decrease in the percentage of CD4/CD8 double positive cells in Ly-6E.1/*bcl-2* transgenic thymus compared with a normal littermate and a 50% increase in CD4 and CD8 single positive cells (figure 38A). As the absolute numbers of both $CD4^+CD8^+$ double positive and single positive cells is greater in transgenic mice than in non-transgenic littermates, this alteration in CD4/CD8 profile suggests that thymic selection has been perturbed in Ly-6E.1/*bcl-2* transgenic mice and supports the notion that *bcl-2* is an important factor involved in T cell selection. The abnormal thymus in Ly-6E.1/*bcl-2* transgenic mice also provides indirect evidence of the importance of Ly-$6E.1^+$ cells during T cell development. In normal animals, Ly-6E.1 is expressed on approximately 7% of total thymocytes and only on 2-3% of CD4/CD8 double positive cells, ie, Ly-6E.1 is downregulated as T cells undergo positive and negative selection. The phenotype of Ly-6E.1/*bcl-2* transgenic mice suggests that Ly-$6E.1^+$ cells represent a functionally important category of T cells within the thymus, alterations to the fate of which, for example by prolonging their lifespan with transgenic *bcl-2* expression, can disrupt thymic T cell development.

Analysis of peripheral $CD4^+$ and $CD8^+$ T cells (figure 38B) in the lymph nodes shows there to be a decrease in the percentage of $CD4^+$ T cells. There is a corresponding increase in the percentage of $CD4^-CD8^-$ cells, the majority of which are B cells. However, when the difference in the size of transgenic lymph nodes was compared with normal lymph nodes, the absolute number of both $CD4^+$ and $CD8^+$ cells was found to be between 3 and 10 fold greater in the periphery of Ly-6E.1/*bcl-2*

mice than in non-transgenic littermates and the absolute number of $CD4^-CD8^-$ cells is increased by up to 20 fold (table 5). As Ly-6E.1 is known to be expressed on both mature T cells and B cells, this observation may suggest that more peripheral B cells are Ly-6E.1^+ than T cells or, alternatively, may reflect an inherent sensitivity of B cells to the effects of *bcl-2*. Assuming that FDG expression in Ly-6E.1/*lacZ* transgenic mice provides an indication of the tissue specificity of the Ly-6E.1 cassette, elevated *bcl-2* expression should be found in 80% of Thy-1^+ mature T cells and only 40% of $B220^+$ mature B cells (see figure 7). In view of this, the preferential effect on B cells is probably due to an inherent propensity for *bcl-2* to mediate B cell survival, as is the case with follicular B cell lymphoma and the t(14;18).

To more directly examine the lymphoid populations in the enlarged spleen and lymph nodes of Ly-6E.1/*bcl-2* transgenic mice, FACS analysis was performed using monoclonal antibodies specific for T cell and B cell associated antigens. CD3 (T cell) and B220 (B cell) FACS profiles (figure 39) demonstrate that the percentage of B cells ($B220^+$) is increased in the peripheral lymphoid organs whilst the percentage of T cells ($CD3^+$) is decreased when compared with non-transgenic littermates. As the lymphoid organs significantly differ in size (table 4 and figure 37) compared with normal littermates, the precise cellular differences between transgenic and normal lymphoid organs become clear when considering the absolute numbers of each particular cell subset within the organs (table 5). In Ly-6E.1/*bcl-2* transgenic mice, the absolute number of splenic T cells is only slightly increased in transgenic animals ($<0.5x$), whereas the number of B cells is increased by 3-6 times ($1.5\text{-}2x10^8$) when compared with normal littermates ($3\text{-}6x10^7$). In addition, there are comparable

Table 5. Absolute numbers of lymphocyte subsets in Ly-6E.1/*bcl-2* peripheral lymphoid organs.

Combining the cell count data from table 4 with FACS analysis, the absolute number of a.) $CD4^+$, $CD8^+$ and $CD4^-CD8^-$ lymph node cells and b.) $CD3^+$ T cells and $B220^+$ B cells in spleen were calculated.

Table 5:

a) Lymph node:

	$CD4^+$	$CD8^+$	$CD4^-/CD8^-$
Non-transgenic 1	$5.6x10^6$	$3.3x10^6$	$2.0x10^6$
Non-transgenic 2	$9.4x10^6$	$3.9x10^6$	$4.6x10^6$
Transgenic 1	$5.4x10^7$	$3.9x10^7$	$4.0x10^7$
Transgenic 1	$2.7x10^7$	$1.1x10^7$	$2.0x10^7$

b) Spleen:

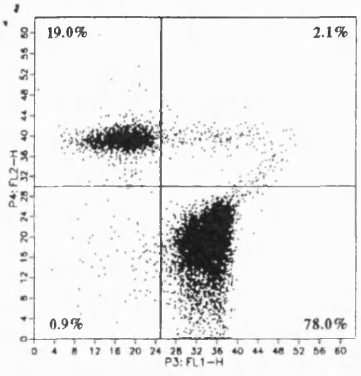
	$CD3^+$	$B220^+$
Non-transgenic 1	$6.1x10^8$	$2.6x10^7$
Non-transgenic 2	$4.3x10^8$	$6.1x10^7$
Transgenic 1	$5.7x10^8$	$1.6x10^8$
Transgenic 1	$8.1x10^8$	$2.0x10^8$

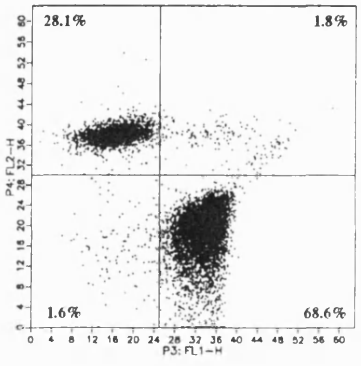
Figure 39. Distribution of T cells and B cells in the periphery of Ly-6E.1/*bcl-2* transgenic mice.

Lymph node lymphocytes (A.) and splenocytes (B.) from 471 transgenic and non-transgenic littermates were stained with monoclonal antibodies specific for B cells (B220-PE, ordinate) and T cells (CD3-FITC, abscissa). Relative fluorescence intensities are shown on a logarithmic scale, with percentages of each subset of cells indicated in the relevant quadrant.

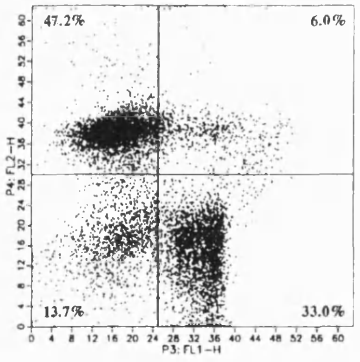
Non transgenic. **Ly-6E.1/*bcl-2* transgenic**

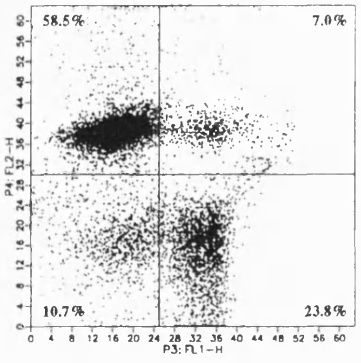
A. Lymph node

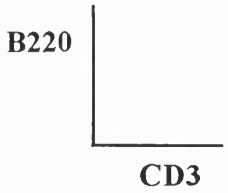




B. Spleen







increases in B cells (2-8 fold) in Ly-6E.1/*bcl-2* transgenic lymph node. However, in contrast to the spleen, there is a greater than 10 fold increase in the number of T cells in transgenic lymph node. The increase in T cell number in the lymph nodes above that seen in the spleen may be due to the fact that more T cells in the lymph node are in an activated state and expressing the Ly-6E.1/*bcl-2* transgene than in the spleen. It is interesting to note that *lacZ* expression is consistently higher in lymph nodes of Ly-6E.1/*lacZ* transgenic mice, where 50% of cells are positive, than in spleen where 30% of cells are positive (see figures 18 and 19), although more detailed analyses are required to precisely determine which cells are affected.

4.4 Altered cellularity in Ly-6E.1/*bcl-2* bone marrow.

To determine if *bcl-2* over expression had any effect on bone marrow haematopoietic stem cells, Ly-6E.1/*bcl-2* transgenic mice were analysed for perturbations to bone marrow haematopoiesis. Figure 40 shows bone marrow cells from non-transgenic (B), 471 transgenic (C) and 479 transgenic (D) Ly-6E.1/*bcl-2* mice stained with eosin/thiazine. An examination report on these preparations by the Royal Veterinary College, UCL, London is presented in figure 40A. In summary, Ly-6E.1/*bcl-2* transgenic bone marrow is normal with no evidence of neoplastic cells

Figure 40. Cytological analysis of Ly-6E.1/*bcl-2* bone marrow.

A. Cytology report from Royal Veterinary College on bone marrow preparations from 471, 479 and non-transgenic Ly-6E.1/*bcl-2* mice.

Cytospin preparations of non-transgenic (B.), 471 (C.) and 479 (D.) bone marrow cells stained with Diff-Quick. 10^5 bone marrow cells were PBS washed, resuspended in 100μl PBS and centrifuged onto a Cytospin slide. Air-dried slides were processed at the Royal Veterinary College.

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The Ridgeway
Mill Hill
NW7 1AA

APB/DRM

23 February 1996

LABORATORY EXAMINATION REPORT

Yr ref: ED 28, 32, 682 Mice
Our ref: 457/0296
Sample: Bone marrow smears (6 slides)

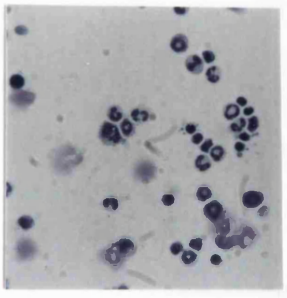
Cytology report:
The slides were stained with Diffquik.
The control cytospin has fewer cells than the two transgenic mice. However, an M:E ratio of about 2.5:1 is seen on all three slides. There are also 1% eosinophil cells.
The cell lines are differentiating normally and there are no signs of neoplastic cells.

These are normal findings for both the control and the transgenic mice.

pp A P Bjornson BVSc MRCVS

Charge: £30 + VAT - Invoice to follow

B.



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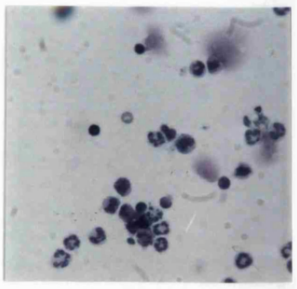

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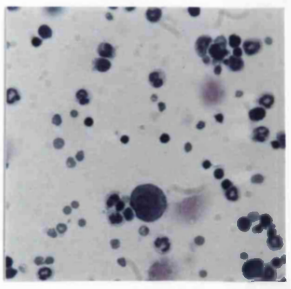


(Courtesy A P Bjornson, RVC, UCL, London). Further examination of bone marrow cells consisted of FACS analysis using the following antibodies: CD3, to specifically detect T cells; B220 for detection of B cells; and Mac-1 specific to macrophages. Figure 41 shows FACS profiles of Ly-6E.1/*bcl-2* transgenic and non-transgenic bone marrow. No significant differences in expression of CD3, B220 or Mac-1 were found between transgenic and normal littermates, hence Ly-6E.1/*bcl-2* bone marrow has normal profiles of T cells, B cells and macrophages. As the number of cells in Ly-6E.1/*bcl-2* transgenic bone marrow is equivalent to that in non transgenic controls, the percentages shown on the FACS plots are proportional to absolute cell number.

In contrast to Ly-6E.1/*myc* bone marrow, in Ly-6E.1/*bcl-2* transgenic bone marrow there are normal numbers of B cells and macrophages, as well as T cells, (compare figure 41 with figure 30). This suggests that the bone marrow cells which are sensitive to *c-myc* are relatively unaffected by *bcl-2* expression. In addition, it is worthy of note that although both Ly-6E.1/*myc* and Ly-6E.1/*bcl-2* transgenic mice exhibit perturbations to the B cell compartment these are only detectable in the bone marrow in the case of Ly-6E.1/*myc* transgenic mice, suggesting that each oncogene, in isolation, has a tropism for a different type of B cell, with *c-myc* affecting more primitive B cells than *bcl-2*.

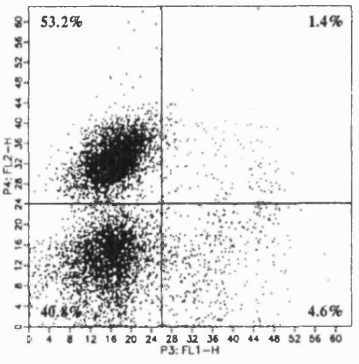
Although there are normal numbers of T cells, B cells and macrophages in Ly-6E.1/*bcl-2* bone marrow, FACS analysis using either Sca-1 or D7 monoclonal antibodies specific for the Ly-6E.1/Ly-6A.1 gene products shows a dramatic increase in the number of Sca-1 positive cells in Ly-6E.1/*bcl-2* bone marrow (figure 42). Either Sca-1 is upregulated resulting in an increase in expression at the cell surface, or there are changes to the cellularity of Ly-6E.1/*bcl-2* bone marrow resulting in

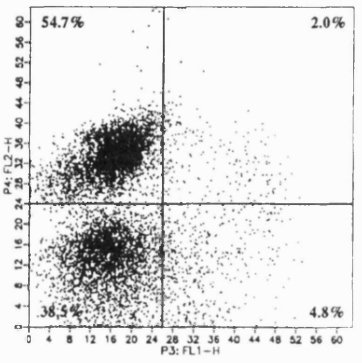
Figure 41. FACS analysis of T cells, B cells and macrophages in Ly-6E.1/*bcl-2* bone marrow.

Bone marrow cells from Ly-6E.1/*bcl-2* transgenic and non-transgenic littermates of line 471 were analysed by FACS. 10^6 bone marrow cells were stained with monoclonal antibodies specific for A. B cells (B220-PE, ordinate) and T cells (CD3-FITC, abscissa); and B. macrophages (Mac-1-PE, ordinate). 10^4 cells were analysed using a Becton Dickinson FACScan. FACS dot plots with logarithmic axes for fluorescence are shown. The percentage of bone marrow cells expressing each antigen is shown within each quadrant of the FACS plots.

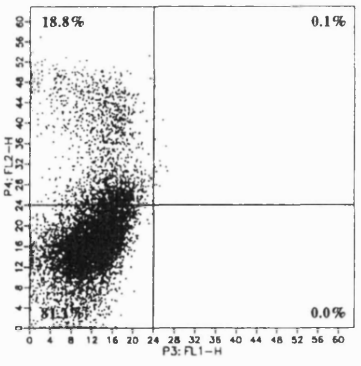
Non transgenic. **Ly-6E.1/*bcl-2* transgenic**

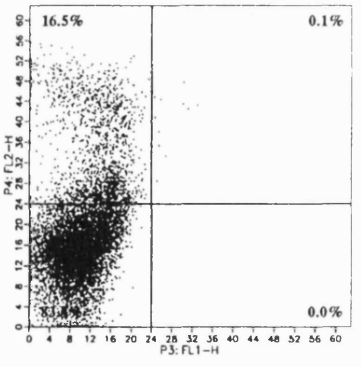
A. CD3-FITC + B220-PE





B. Mac-1-PE only





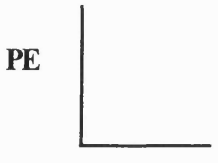
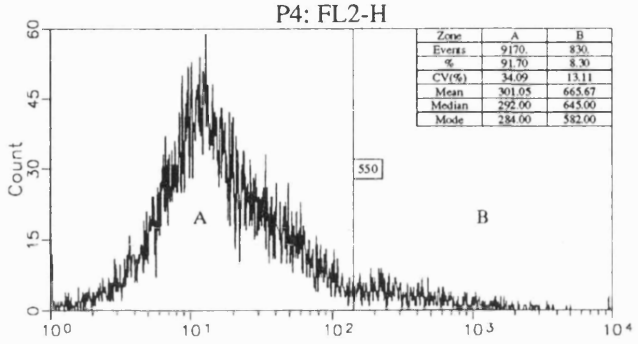


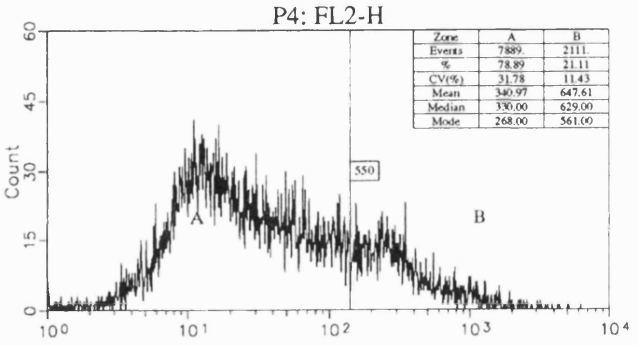
Figure 42. FACS analysis of Sca-1 expression in Ly-6E.1/*bcl-2* bone marrow.

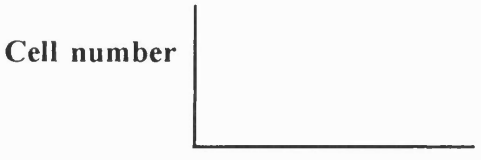
Bone marrow cells from transgenic and non-transgenic Ly-6E.1/*bcl-2* littermates were stained with Sca-1-PE to detect Ly-6E/A positive cells. 10^4 cells were analysed using a Becton Dickinson FACScan and histogram plots of cell number (ordinate) *versus* relative fluorescence intensity (PE, abscissa, logarithmic scale) were generated. A bar placed at 550 fluorescence units provides a reference point for comparison between normal and transgenic profiles. The number and percentage of cells within region B for each sample is indicated within the histogram.

Non transgenic bone marrow



Ly-6E.1/*bcl-2* transgenic bone marrow





more Sca-1 positive cells being present. There are no reports of *bcl-2* directly affecting the expression of other genes or proteins so these differences are likely to be due to changes in cellularity of the bone marrow which do not affect the overall numbers of $CD3^+$, $B220^+$ or $Mac\text{-}1^+$ cells. Simultaneous analysis of both Sca-1 and cell size by forward light scatter demonstrates that the Sca-1 positive cells in transgenic animals are both large and small. The large cells are probably monocytes, granulocytes and large blast cells, whilst the small cells are mainly lymphocytic cells and small blast cells. If *bcl-2* were affecting multiple Ly-6E.1^+ cell types, as is suggested by forward light scatter analysis, to the same extent, the percentages of various cell subsets would appear to be unchanged. However, determination of the precise effects of Ly-6E.1/*bcl-2* expression on the haematopoietic stem cell and bone marrow haematopoietic progenitors requires the use of functional assays.

4.5 Analysis of functional capability of Ly-6E.1/*bcl-2* bone marrow.

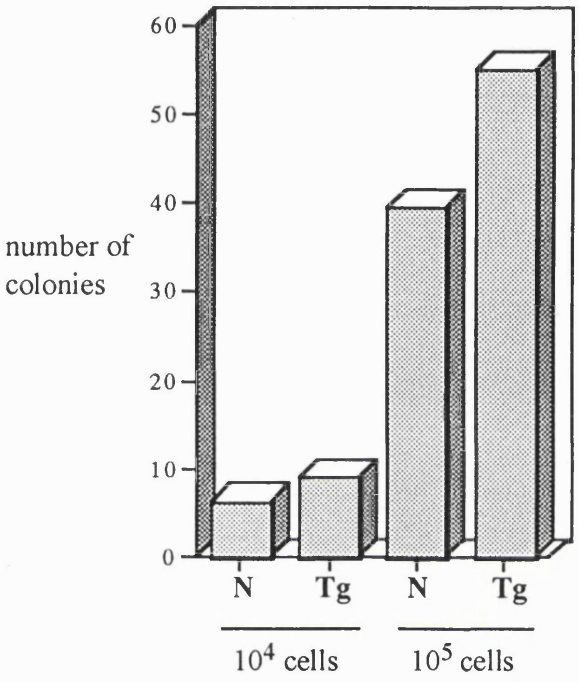
To gauge the myelo-erythroid differentiation potential of Ly-6E.1/*bcl-2* bone marrow, methyl cellulose colony forming assays were performed. Figure 43A shows that Ly-6E.1/*bcl-2* transgenic bone marrow has a slightly higher colony forming potential, yielding 55 colonies per 10^5 cells, as compared to 40 colonies per 10^5 derived from a non-transgenic littermate. The cellularity of transgenic colonies is similar to those derived from normal bone marrow (figure 43B) suggesting that the differentiation potential of haematopoietic stem cells in Ly-6E.1/*bcl-2* transgenic mice is normal but that the number of progenitors may be higher.

Figure 43. Methyl cellulose colony forming potential of Ly-6E.1/*bcl-2* bone marrow.

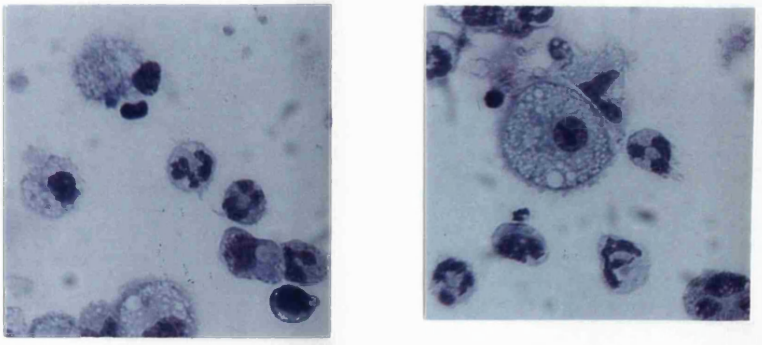
A. Bone marrow cells from transgenic and non-transgenic littermates were seeded, each in triplicate, at 10^4 and 10^5 cells per ml in methyl cellulose culture supplemented with pokeweed mitogen spleen cell conditioned medium and erythropoietin. Colonies of cells were scored after ten days and the average number of colonies from each is shown.

B. Cytological analysis of Ly-6E.1/*bcl-2* methyl cellulose colonies. Colonies were pooled from 1ml of culture medium, PBS washed and 10% of each was centrifuged onto a Cytospin slide. Air-dried slides were methanol fixed, stained with "Diff-Quick" (eosin and thiazine) and mounted in DPX mountant.

A.


60
50
40
30
20
10
0
number of colonies
N
Tg
N
Tg
10^4 cells
10^5 cells

B.

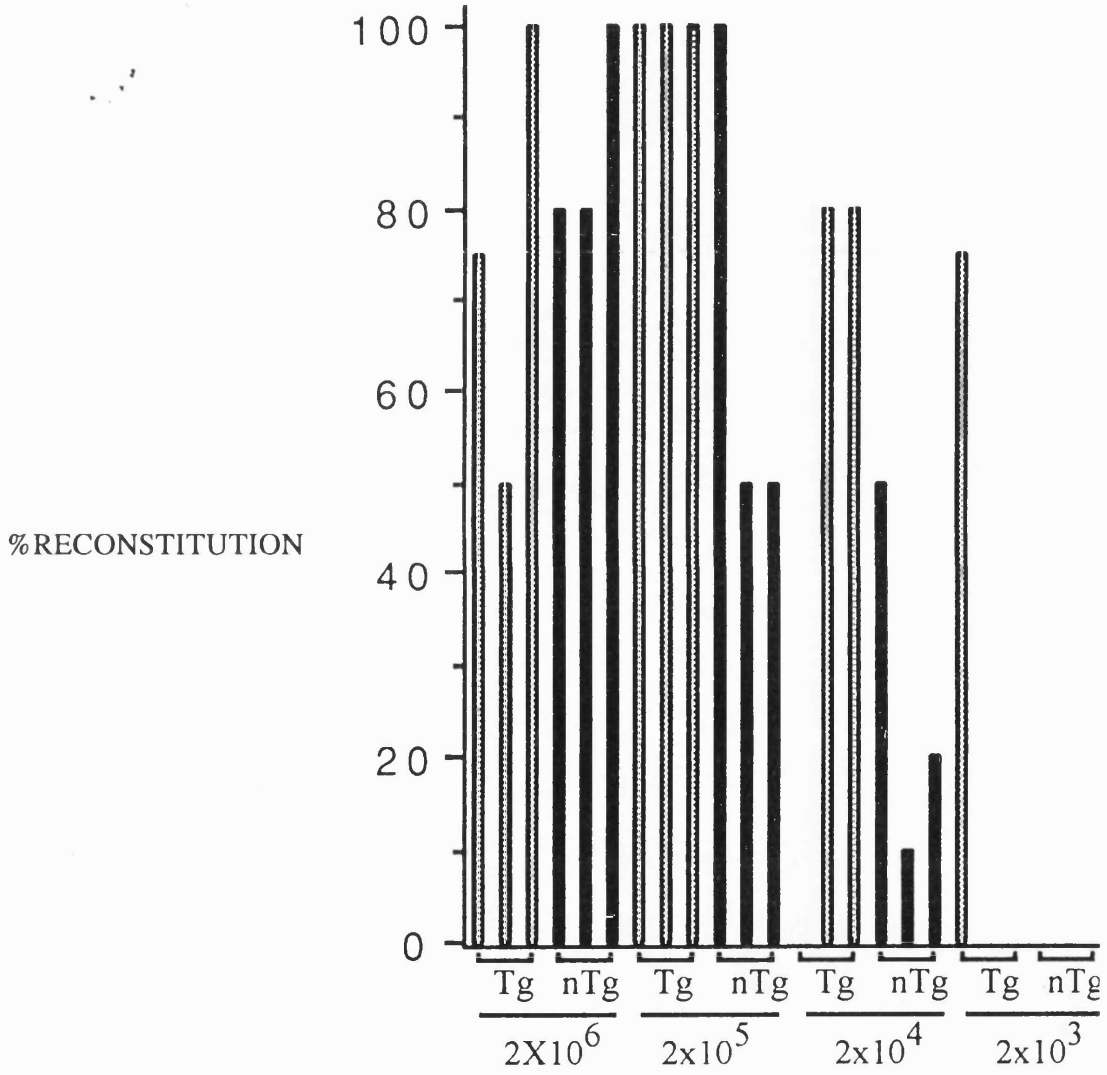

non Tg
Tg

To extend the methyl cellulose studies in order to consider all haematopoietic lineages and more immature haematopoietic progenitors in an *in vivo* situation, bone marrow transplantation and the generation of radiation chimaeras was performed. The experimental scheme employed was the same as for the analysis of Ly-6E.1/*myc* bone marrow. Male bone marrow from Ly-6E.1/*bcl-2* transgenic and normal littermates was used as a source of donor cells, the Y chromosome serving as a molecular marker. Unfractionated bone marrow cells from either transgenic or non-transgenic mice were injected into irradiated female recipient mice at serial dilutions from $2x10^6$ to $2x10^3$. Levels of engraftment were determined at one month and four months post transplantation by PCR anlaysis of peripheral blood DNA of recipients. Figure 44 shows that Ly-6E.1/*bcl-2* transgenic bone marrow has a slightly higher engraftment potential than non-transgenic bone marrow in this long term reconstitution assay. Although somewhat variable across triplicate samples, transgenic bone marrow generally exhibits higher levels of repopulation in recipients of $2x10^5$ and $2x10^4$ donor cells. The limiting dilution of transgenic donor cells is $2x10^3$, which gives one recipient repopulated to 75%, whereas no recipients of $2x10^3$ non-transgenic cells were positive. This data may support the hypothesis that the haematopoietic stem cell is dividing assymmetrically and that *bcl-2* transgene expression results in increased survival of daughter cells, resulting in increased long term reconstitution potential. Alternatively, the greater number of transgenic cells in the recipients may also be due to the inherent longevity of the *bcl-2* transgenic cells themselves.

In order to determine whether Ly-6E.1/*bcl-2* bone marrow possessed the potential to differentiate normally into all lineages of mature haematopoietic cell in

Figure 44. Long term haematopoietic reconstitution by Ly-6E.1/*bcl-2* bone marrow cells.

Lethally irradiated female mice were injected with male bone marrow cells from Ly-6E.1/*bcl-2* transgenic and non-transgenic littermates at dilutions of cells from $2x10^6$ to $2x10^3$. Haematopoietic repopulation at 4 months post-transplantation was determined by male specific PCR of peripheral blood genomic DNA from the female radiation chimaeras. Comparison of the intensity of Y-chromosome specific signal with that obtained from standards containing known ratios of male:female DNA enabled the level of repopulation to be estimated.


100
80
60
40
20
0
%RECONSTITUTION
Tg
nTg
Tg
nTg
Tg
nTg
Tg
nTg
2X10^6
2x10^5
2x10^4
2x10^3
DONOR CELLS

radiation chimaeras, FACS analysis was performed on cells isolated from the haematopoietic tissues of fully reconstituted mice (figure 45). Bone marrow cells were obtained from irradiated mice displaying 100% male signal in peripheral blood that had received either $2x10^6$ Ly-6E.1/*bcl-2* or non-transgenic donor cells and stained with several lineage specific monoclonal antibodies. T cells (CD3), B cells (B220), macrophages (Mac-1) and erythroid cells (Ter119) could be detected in chimaeric mice derived from Ly-6E.1/*bcl-2* as well as non-transgenic bone marrow, demonstrating that Ly-6E.1/*bcl-2* repopulation was multilineage. Detailed analysis of repopulated recipient mice showed a slight increase in the percentages of both B cells, from 40% to 55%, and macrophages, from 10% to 20%, in bone marrow. However, such differences in cellularity may reflect the normal variance between radiation chimaeras and should be examined in more recipient mice. Interestingly, the percentage of Sca-1 positive cells in the bone marrow was similarly elevated from 10% to 60% as was observed for primary Ly-6E.1/*bcl-2* transgenic mice.

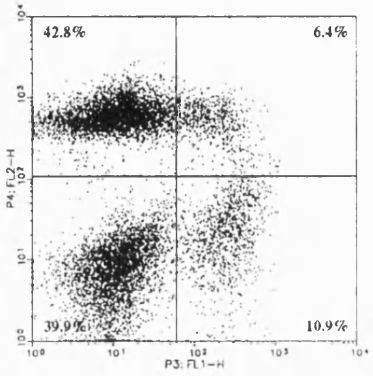
Figure 46 shows that thymic T cell development in non-transgenic and Ly-6E.1/*myc* derived radiation chimaeras is normal whereas in mice containing transgenic Ly-6E.1/*bcl-2* donor cells, T cell development is disrupted in a similar manner to that seen for primary Ly-6E.1/*bcl-2* transgenic thymocytes. Further analysis of these radiation chimaeras has also to the identification of a novel type of haematopoietic abnormality associated with Ly-6E.1 directed *bcl-2* expression. Figure 47 shows histogram plots of bone marrow cells from an Ly-6E.1/*bcl-2* radiation chimaera and, as controls, non-transgenic and Ly-6E.1/*myc* radiation chimaeras illustrating Ter119 expression. In both non-transgenic and Ly-6E.1/*myc* derived bone marrow, the percentage of erythroid cells is between 40 and 50%, as

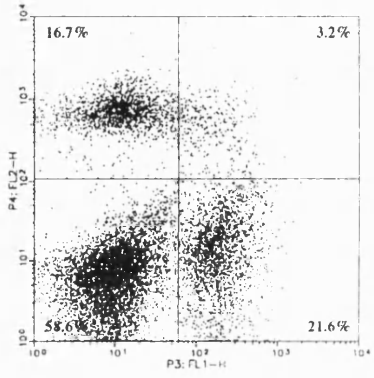
Figure 45. Multilineage FACS analysis of bone marrow cells from Ly-6E.1/*bcl-2* radiation chimaera.

Bone marrow cells from radiation chimaeras, both Ly-6E.1/*bcl-2* and non-transgenic derived, exhibiting 100% repopulation of peripheral blood as determined by PCR were analysed by FACS. 10^6 bone marrow cells were stained with monoclonal antibodies specific for A. erythroid cells (Ter119-PE, ordinate) and T cells (CD3-FITC, abscissa); B. Ly-6A/E (Sca-1-PE, ordinate) and B cells (B220-FITC, abscissa); and C. macrophages (Mac-1-PE, ordinate) and the tyrosine kinase c-kit (FITC, abscissa). 10^4 cells were analysed using a Becton Dickinson FACScan. FACS dot plots with logarithmic axes for fluorescence are shown. The percentage of bone marrow cells expressing each antigen is shown within each quadrant of the FACS plots.

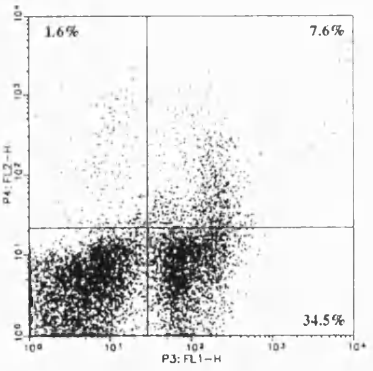
Normal radiation chimaera **Transgenic radiation chimaera**

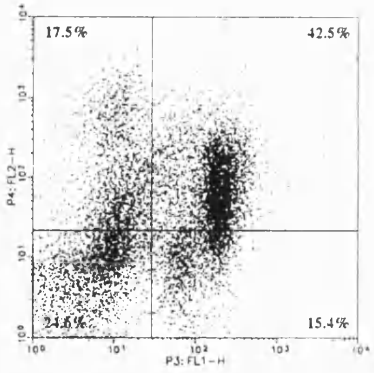
A. Ter119-PE + CD3-FITC



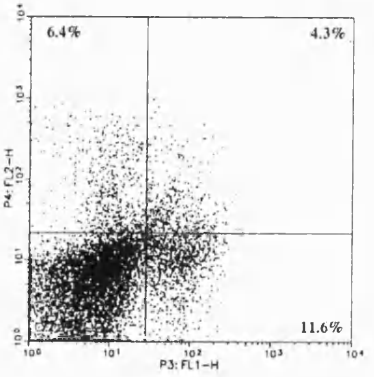


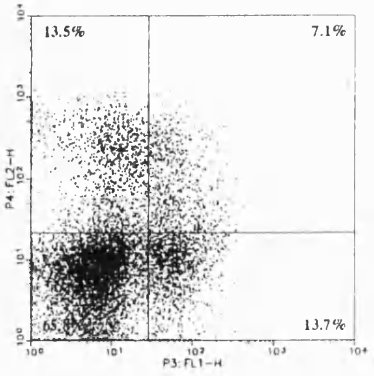
B. Sca1-PE + B220-FITC





C. Mac1-PE + c-kit-FITC





PE fluorescence

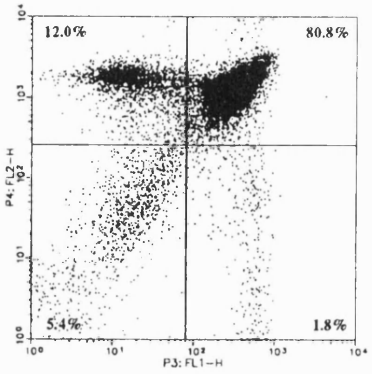
FITC fluorescence

determined by Ter119 expression whereas in Ly-6E.1/*bcl-2* derived bone marrow it is less than 20%. Reanalysis of primary Ly-6E.1/*bcl-2* transgenic mice showed that they also exhibit an erythroid deficiency in bone marrow, with similar percentages of Ter119$^+$ cells as the chimaeras (data not shown). The significance of the reduction in bone marrow Ter119$^+$ cells is unclear, as there is little evidence of any other erythroid deficiency (RVC report, figure 40). Despite this, however, Ter119$^+$ cells in Ly-6E.1/*bcl-2* bone marrow represent an unusual case in which expression of *bcl-2* does not appear to promote cell survival and further analysis may identify a potentially novel role for *bcl-2* in erythropoiesis. It may be that the downregulation in *bcl-2* expression observed as erythroid cells mature is important for their normal development and that these processes are blocked in Ly-6E.1/*bcl-2* transgenic mice.

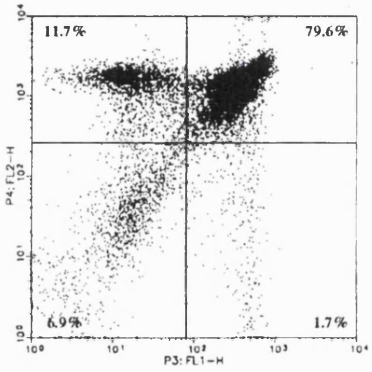
Figure 46. FACS analysis of CD4/CD8 T cells in thymus of radiation chimaeric mice.

Thymocytes from non-transgenic (A.), Ly-6E.1/*myc* (B.) and Ly-6E.1/*bcl-2* (C.) derived radiation chimaeric mice were stained for CD4 (PE-ordinate) and CD8 (FITC-abscissa). 10^4 cells were analysed using a Beckton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale, with percentages of each subset of cells indicated in the relevant quadrant.

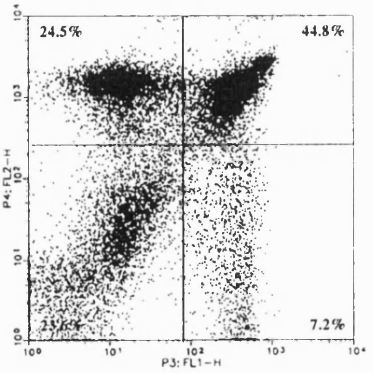
A. Normal radiation chimaera

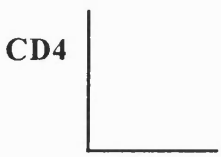


B. Ly-6E.1/c-myc radiation chimaera



C. Ly-6E.1/bcl-2 radiation chimaera



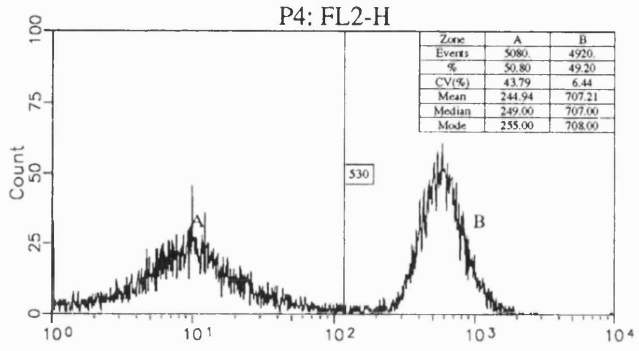


Thymus profiles

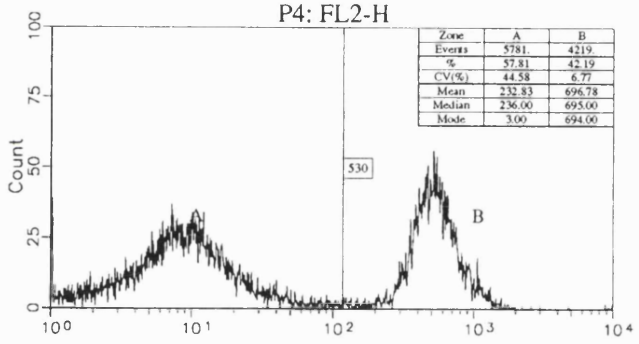
Figure 47. FACS analysis of Ter119 expression in bone marrow of radiation chimaeric mice.

Bone marrow cells from non-transgenic (A.), Ly-6E.1/*myc* (B.) and Ly-6E.1/*bcl-2* (C.) derived radiation chimaeric mice were stained with Ter119-PE to detect erythroid cells. 10^4 cells were analysed using a Becton Dickinson FACScan and histogram plots of cell number (ordinate) *versus* relative fluorescence intensity (PE, abscissa, logarithmic scale) were generated. Cells were considered positive for Ter119 expression if they exhibited a relative fluorescence intensity greater than 530 units. The number and percentage of positive cells, within region B., is shown on each histogram plot.

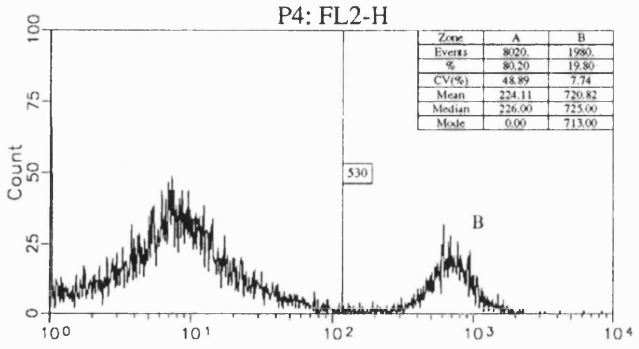
A. Normal radiation chimaera

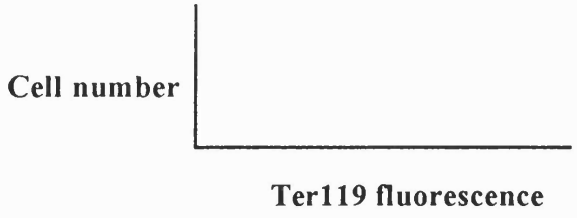


B. Ly-6E.1/c-myc radiation chimaera



C. Ly-6E.1/bcl-2 radiation chimaera





5. "Ly-6E.1/*myc* + Ly-6E.1/*bcl-2* " double transgenic mice.

5.1 Introduction.

Deregulated expression of the *c-myc* proto-oncogene is frequently associated with neoplasia (Cole, 1986) and although *c-myc* expression is both necessary and sufficient for entry into the cell cycle *in vitro*, a second, synergising event is thought to be required before cells exhibit uncontrolled malignant growth (see Hunter, 1991). A good example of this requirement for a secondary cooperating event is provided by the studies of Cole and co-workers. When a retrovirally delivered *c-myc* gene was expressed in monocytes, it did not directly induce neoplastic growth. The resulting *c-myc* expressing cells relied upon colony stimulating factor 1 (CSF-1) for growth and appeared to require a second mutation before exhibiting growth factor independence and transformation (Baumbach *et al.*, 1986). Subsequent studies of transformed cells identified a mutation causing a fusion between the CSF-1 gene and the platelet-derived growth factor β receptor gene (Eccles *et al.*, 1992). This mutation was strongly selected for and effectively overcame the CSF-1 dependency of *c-myc* expressing cells.

Most reports of successful targeted oncogenesis utilising *c-myc* have resulted in the derivation of haematolymphoid cell lines and the transformation of haematolymphoid cells (Adams & Cory, 1991; Roland & Morello, 1993; Spanopoulou *et al.*, 1989). Even when the MHC class 1 promoter was used, which directs expression to many adult tissue types, the major effects of *c-myc* over

expression were restricted to cells of the lymphoid compartment (Roland & Morello, 1993). Immature haematolymphoid progenitor cells have many characteristics that render them inherently suitable for *c-myc* induced transformation. They tend to be actively dividing and so have a higher probability of incurring DNA damage than resting cells and, perhaps more significantly, they are often performing somatic gene recombination, involving the expression of DNA recombinase enzymes. If a cooperating event is a prerequisite for full *c-myc* induced transformation, this may be facilitated by somatic recombination and explain why haematolymphoid cells are preferentially affected by *c-myc*. It is surely no coincidence that the majority of leukaemias involve somatic translocation and that many occur whilst immature haematolymphoid cells are undergoing selection (Rabbitts, 1994). In addition, immature haematopoietic cells express high levels of endogenous *bcl-2* (Bonati *et al.*, 1996; Cory, 1995) which may help abrogate growth factor requirements or directly block apoptosis.

Over expression of *bcl-2* alone is not sufficient to induce lymphoid malignancy, although inappropriately high level *bcl-2* expression confers an increased risk of malignant transformation, either by prolonging cell survival or overcoming growth factor dependency (Cory, 1995). For these reasons, high level *bcl-2* expression could be a useful attribute when attempting to derive haematopoietic cell lines from primary murine tissues.

While *bcl-2* expression inhibits apoptosis, the *c-myc* gene product can be a potent inducer of apoptosis. Fanidi *et al.* and Bissonnette *et al.* demonstrated that *bcl-2* directly cooperates with *c-myc* by specifically blocking *c-myc* induced apoptosis (Bissonette *et al.*, 1992; Fanidi *et al.*, 1992). The interaction between *c-myc* and *bcl-*

2 is novel amongst proto-oncogenes in that it does not morphologically transform cells nor does it induce focus formation in transfected fibroblasts (Fanidi *et al.*, 1992). The phenomenon of *c-myc* and *bcl-2* cooperation has been investigated *in vivo* using double transgenic, immunoglobulin heavy chain enhancer (Eμ)/*c-myc*/*bcl-2* mice (Strasser *et al.*, 1990). As with individual single transgenic animals, double transgenic mice displayed elevated numbers of lymphocytes. These lymphocytes were large, cycling cells similar to those found in Eμ/*myc* transgenic mice, in contrast with the smaller cells found in Eμ/*bcl-2* mice. In double transgenic Eμ/*c-myc*/*bcl-2* mice, the *c-myc* effects seem to be dominant, although these transgenic mice develop tumours at a higher frequency and have shorter lifespans than Eμ/*c-myc* mice. *Post mortem* analysis of Eμ/*c-myc*/*bcl-2* mice revealed disseminated malignant lymphoma in all cases, though the expanded B cell compartment was not itself malignant upon transplantation. The tumours isolated from Eμ/*c-myc*/*bcl-2* mice all had the surface phenotype Thy-1^{lo}, Sca-1^{+}, CD4^{+}, B220^{+}, probably representative of a immature lymphoid progenitor cell. The expression of mature lymphoid lineage markers and the fact that a B cell specific promoter was employed suggests this cell is not a transformed haematopoietic stem cell but is likely to be analogous to a commited lymphoid progenitor. In a separate study, H-2/*c-myc* transgenic mice produced primitive haematolymphoid tumours (Roland & Morello, 1993), providing another example of *c-myc* targeting immature haematopoietic cells. By using the Ly-6E.1 expression vector, double oncogene expression will be directed to transplantable haematopoietic stem cells. It is hoped that cell lines representative of the most immature haematopoietic stem cells will be isolatable from Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow.

5.2 Generation of double transgenic mice.

The proto-oncogenes *c-myc* and *bcl-2* have been shown to cooperate *in vivo* to transform immature haematolymphoid cells (Strasser *et al.*, 1990; Roland and Morello, 1993) and data presented in this thesis demonstrates that the Ly-6E.1 gene can direct heterologous gene expression to transplantable haematopoietic stem cells. In an attempt to isolate haematopoietic stem and progenitor cell lines by combining these two observations, Ly-6E.1/*bcl-2* and Ly-6E.1/*myc* transgenic mice were mated to generate transgenic animals expressing both proto-oncogenes within the same compartment. To achieve this, it is possible to microinject a mixture of DNA constructs to generate multiply transgenic animals. However, in this case, the initial generation of single transgenic animals which were then crossed with each other was undertaken for several reasons: i, to enable the single transgenic phenotype to be determined for both Ly-6E.1/*myc* and Ly-6E.1/*bcl-2*, thereby yielding valuable information about normal haematopoiesis and the potential roles of *c-myc* and *bcl-2* in haematopoietic stem cells; ii, to enable verification and characterisation of proper expression from both transgenes, prior to generating double transgenic animals; iii, in the event of embryonic lethality or other serious deformity caused by oncogenic cooperation, it would be possible to generate many embryos for analysis by breeding single transgenic animals; and iv, to eliminate the possibility of ectopic co-expression as each transgenic animal will possess a different transgene integration site and cooperation will only occur where the individual expression patterns overlap, ie, only in cells which are Ly-6E.1^{+}. In the studies presented here, male transgenic mice of the LM5 Ly-6E.1/*myc* line were mated with female transgenic mice of either the 471

or 479 Ly-6E.1/*bcl-2* lines to obtain doubly transgenic mice. It was necessary to use male LM5 transgenic mice in these breedings as LM5 female animals do not always survive until their offspring reach weaning age.

Figure 48A, B and C outlines the single step PCR based strategy for screening Ly-6E.1/*myc* x Ly-6E.1/*bcl-2* offspring for the presence of both transgenes. This strategy ulitised PE4 and PE5 oligonucleotides, located within exons 1 and 2 of the Ly-6E.1 gene, and cmex2.1, located within exon 2 of *c-myc* and allowed for the identification of all possible genotypes within the offspring. Figure 48D shows an example of such a PCR analysis of a litter from an Ly-6E.1/*bcl-2* x Ly-6E.1/*myc* mating. Non-transgenic animals exhibit a 315 bp endogenous Ly-6E.1 specific band only (N), which serves as an internal control for amplifiable genomic DNA; Ly-6E.1/*myc* transgenic mice exhibit the 315 bp endogenous control band plus a 150 bp *c-myc* fragment from the transgene (M); Ly-6E.1/*bcl-2* transgenic mice are characterised by the 315 bp control band plus a 1200 bp *bcl-2* fragment amplified from the Ly-6E.1/*bcl-2* transgene by PE4 and PE5 (B); and double transgenic offspring, containing both Ly-6E.1/*myc* and Ly-6E.1/*bcl-2* display all three bands (D).

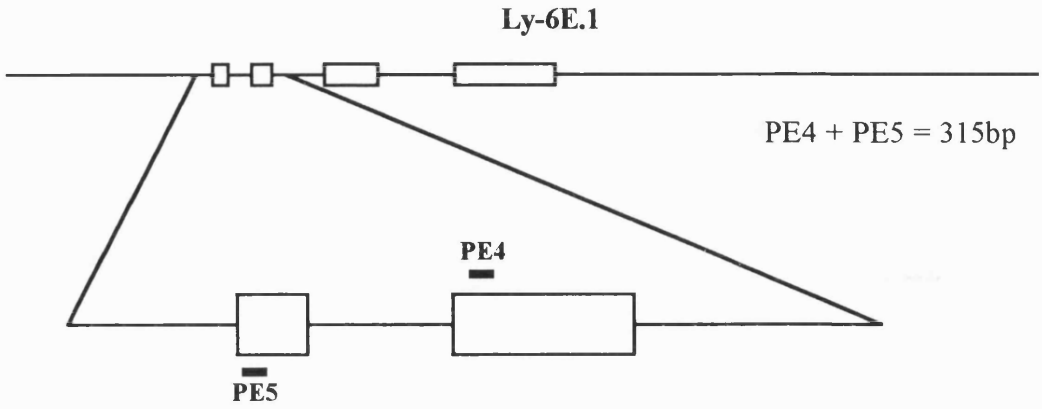
Normal Mendelian ratios of offspring and normal litter sizes were obtained from Ly-6E.1/*myc* x Ly-6E.1/*bcl-2* matings, suggesting that cooperation between *c-myc* and *bcl-2* in Ly-6E.1$^+$ cells did not cause embryonic lethality when highly co-expressed during development. Resulting double transgenic mice were overtly healthy and subsequent analysis was performed on mice between three and six weeks of age. Ly-6E.1/*myc*/*bcl-2* double transgenic mice showed no increased mortality compared with Ly-6E.1/*myc* mice of the LM5 line and their lifespan was between 15

Figure 48. Generation of Ly-6E.1/*myc*/*bcl-2* double transgenic mice.

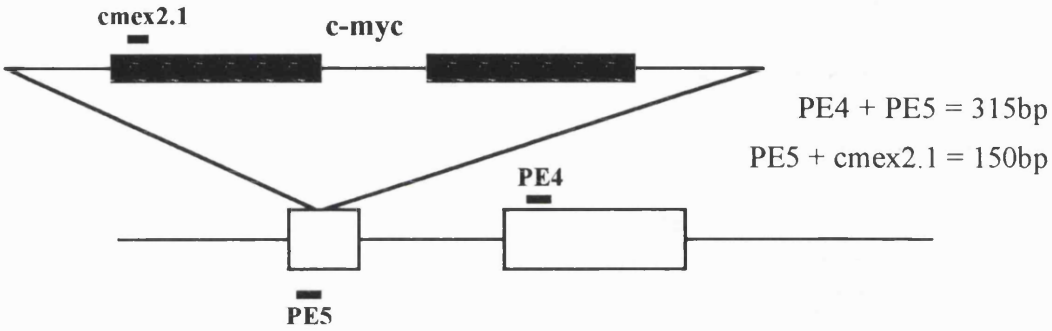
Diagramatic representation of A. Ly-6E.1 genomic region, B. Ly-6E.1/*myc* transgene and C. Ly-6E.1/*bcl-2* trangene showing oligonucleotides used in diagnostic PCR to genotype Ly-6E.1/*myc* X Ly-6E.1/*bcl-2* offspring and the sizes of PCR product generated by each primer pair.

D. Ethidium bromide stained agarose gel showing the result of PCR analysis of tail DNA from a typical litter of Ly-6E.1/*myc* X Ly-6E.1/*bcl-2* offspring. m = size marker, blank = no template, N = non-transgenic, B = Ly-6E.1/*bcl-2* transgenic, M = Ly-6E.1/*myc* transgenic and D = double transgenic.

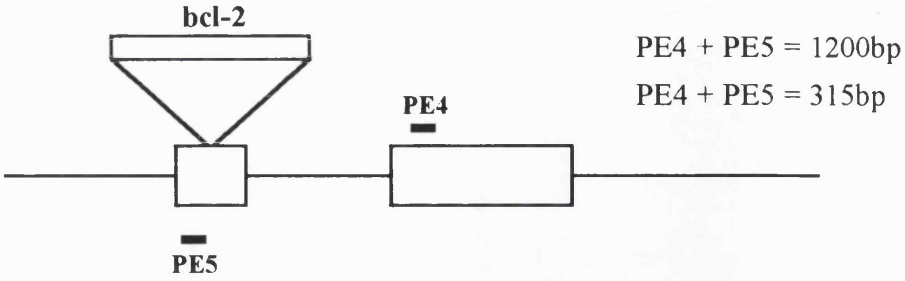
A. Endogenous Ly-6E.1 gene.



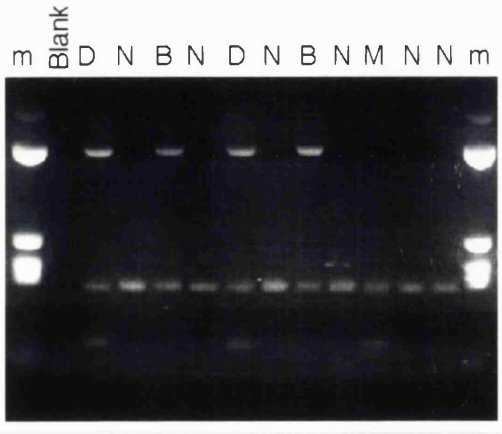
B. Ly-6E.1/*myc* transgene.



C. Ly-6E.1/*bcl-2* transgene



D. PCR analysis of double transgenic litter



and 20 weeks. Presumably, the mechanism involved in the premature death of LM5 mice is unaffected by *c-myc* and *bcl-2* cooperation, though further investigation into the cause of death in LM5 and double transgenic mice is required.

5.3 Analysis of the lymphoid system in double transgenic mice.

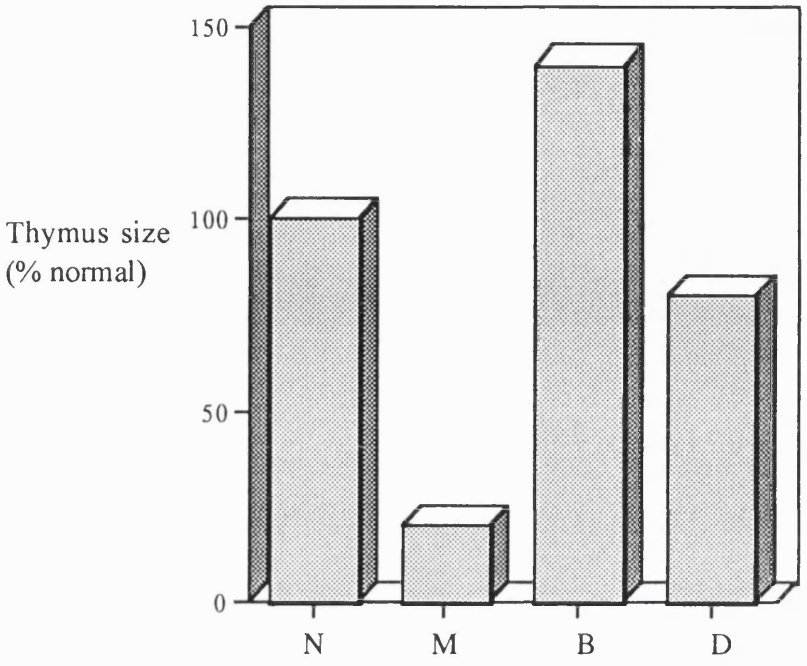
In addition to the isolation of cell lines, it was of interest to analyse the lymphoid compartment in double transgenic mice to determine the effects of oncogenic cooperation between *c-myc* and *bcl-2*. Upon examination of 6 week old double transgenic animals and non-transgenic littermates, no gross morphological or anatomical abnormalities were apparent in non-lymphoid organs. Accurate cell counts revealed no significant size difference between transgenic and normal thymus (figure 49A). However the spleen in double transgenic animals was enlarged approximately three fold (figure 49B). This is in contrast with Ly-6E.1/*myc* transgenic mice, which possess small spleens but is consistent with the increased spleen size in Ly-6E.1/*bcl-2* transgenic animals.

FACS analysis of CD4 and CD8 T cell profiles was performed on thymocytes and splenocytes from Ly-6E.1/*myc*/*bcl-2* double transgenic and non-transgenic littermates. As shown in figure 50A, there was a decrease in percentage of $CD4^+CD8^+$ double positive cells in the thymus when compared with a non-transgenic littermate. However, despite being of normal size, the double transgenic thymus was found to display a similar CD4/CD8 profile to that of a Ly-6E.1/*bcl-2* transgenic animal (compare figure 50A with figure 38A). This suggests that the thymic effects

Figure 49. Size of lymphoid organs in Ly-6E.1/*oncogene* transgenic mice.

Thymus (A.) and spleen (B.) from individual non-transgenic (N), Ly-6E.1/*myc* (M), Ly-6E.1/*bcl-2* (B) and double (D) transgenic littermates were homogenised to single cell suspension and cell accurately counted, with trypan blue exclusion. The size of each organ is displayed as a percentage, the non-transgenic organ being set to 100%

A.


Thymus size
(% normal)
150
100
50
0
N
M
B
D

B.

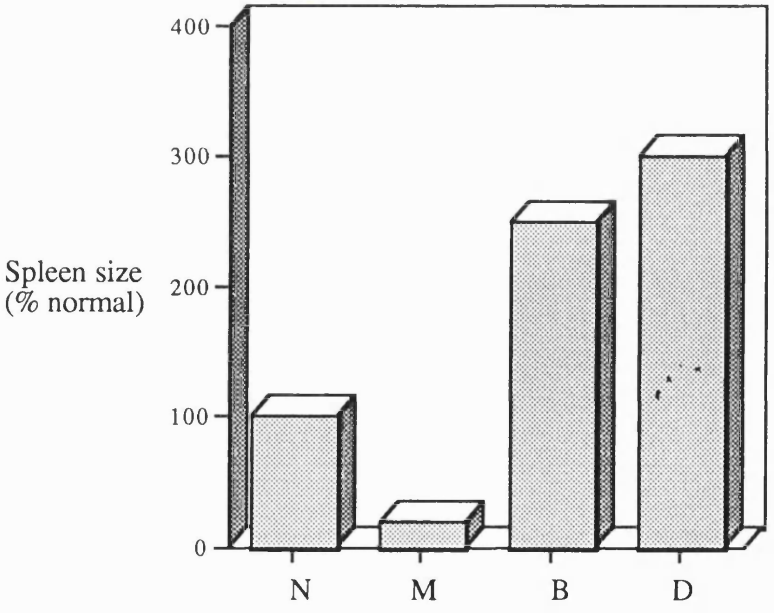
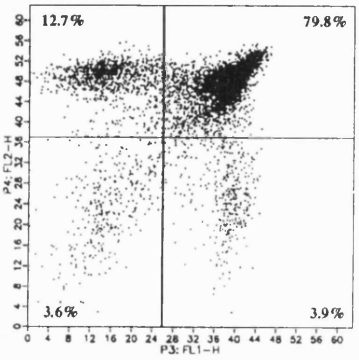

Spleen size
(% normal)
400
300
200
100
0
N
M
B
D

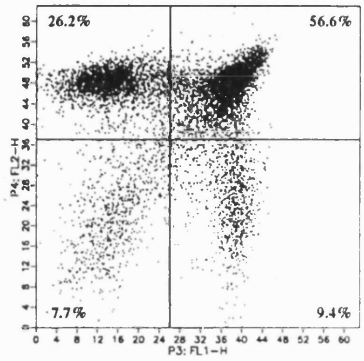
Figure 50. Distribution of CD4/CD8 T cell subsets in thymus and spleen of Ly-6E.1/*myc*/*bcl-2* double transgenic mice.

Thymocytes (A) and splenocytes (B) from double transgenic and non-transgenic littermates were stained for CD4 (PE-ordinate) and CD8 (FITC-abscissa). 10^4 cells were analysed using a Beckton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale, with percentages of each subset of cells indicated in the relevant quadrant.

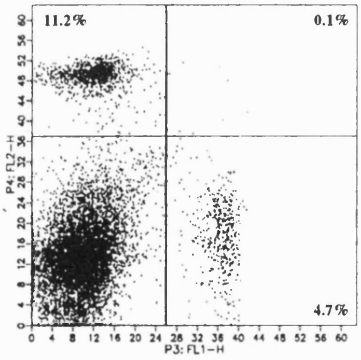
Non transgenic. **Ly-6E.1/*c-myc*/*bcl-2* double transgenic**

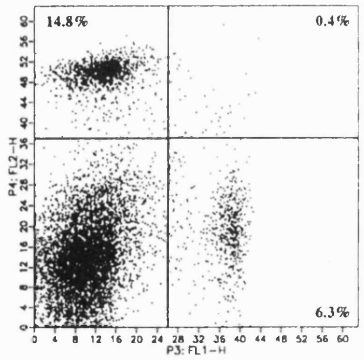
A. Thymus

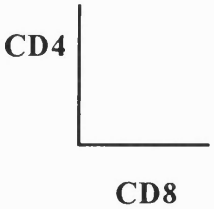




B. Spleen







of the Ly-6E.1/*myc* and Ly-6E.1/*bcl-2* transgenes are mutually exclusive in Ly-6E.1/*myc/bcl-2* double transgenic mice. The effects of *bcl-2* on T cell selection resulting in overproduction of $CD4^+$ and $CD8^+$ single positive cells is evident but in the absence of an enlarged organ. If *c-myc* were inducing apoptosis in a lymphoid progenitor cell, the rate of progenitor seeding of the thymus would be lowered and the effects of *bcl-2* induced enlargement negated. This also supports the hypothesis that the reduced thymic cellularity in Ly-6E.1/*myc* transgenic mice is not due to *c-myc* induced apoptosis during selection within the thymus. Again, if this were the case, *bcl-2* expression would abrogate the effect resulting in an enlarged thymus. In conclusion, the phenotype described for the double transgenic Ly-6E.1/*myc/bcl-2* thymus appears to be the product of each individual phenotype in the absence of oncogenic cooperation.

FACS analysis of the enlarged spleens of double transgenic animals compared with normal spleens revealed similar profiles for both CD4 and CD8 (figure 50B). Analysis of absolute cell number within each subset showed that the numbers of both T cells and B cells were increased by 3-4 fold in double transgenic mice compared with normal littermates. This phenotype is similar to that seen with Ly-6E.1/*bcl-2* transgenic mice. However, T cells seem to be more affected in double transgenic spleens, increasing in number by 3-4 fold, than in Ly-6E.1/*bcl-2* single transgenics where the increase in splenic T cell number is only 0.5 fold. Hence, splenic T cells in Ly-6E.1/*myc/bcl-2* mice may represent a population of cells in which there is cooperation between *c-myc* and *bcl-2*. In contrast to double transgenic mice containing *bcl-2* and *c-myc* transgenes expressed from the immunoglobin heavy chain

enhancer, Ly-6E.1/*c-myc* x Ly-6E.1/*bcl-2* double transgenic mice show no evidence of aggressive early onset lymphoid tumours.

5.4 FACS analysis of double transgenic bone marrow.

FACS analysis of Ly-6E.1/*myc/bcl-2* double transgenic bone marrow was performed using antibodies directed towards T cell, B cell and macrophage cell surface markers (figure 51). There was no dramatic increase in the number of B220 positive cells in bone marrow despite the increased numbers of T and B cells observed in the spleen. However the FACS profile of double transgenic bone marrow showed the appearance of a discrete population of cells expressing high levels of B220. The level of B220 expressed, in terms of relative fluorescence intensity, on this population of bone marrow cells is the same as that found on splenic B cells, suggesting that they may be a more mature population than the $B220^{lo}$ cells normally found in bone marrow. As double transgenic mice seem to overcome the *c-myc* induced reduction in bone marrow B cells, this $B220^{hi}$ population may represent an expanded B cell population in which *bcl-2* and *c-myc* are cooperating. It would be interesting to sort this distinct population of cells on the basis of high level B220 expression and carry out detailed analysis, such as *in vitro* culture or transplantation assays to determine whether they are the result of *bcl-2/c-myc* cooperation.

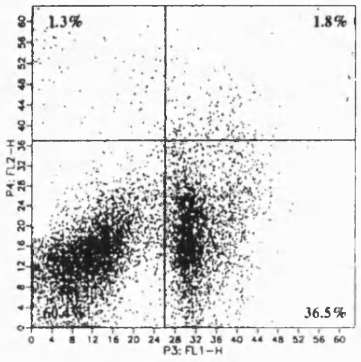
The other antibodies used to assess double transgenic bone marrow, Mac-1 and Sca-1, also show different staining patterns compared with normal (figures 51B and 52). There is a six-fold increase in the percentage of $Sca\text{-}1^{+}$ cells from

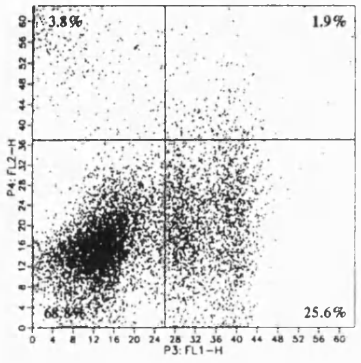
Figure 51. FACS analysis of T cells, B cells and macrophages in Ly-6E./1*myc*/*bcl-2* bone marrow.

Bone marrow cells from Ly-6E.1/*myc*/*bcl-2* double transgenic and non-transgenic littermates were analysed by FACS. 10^6 bone marrow cells were stained with monoclonal antibodies specific for A. T cells (Thy-1-PE, ordinate) and B cells (B220-FITC, abscissa); and B. macrophages (Mac-1-PE, ordinate). 10^4 cells were analysed using a Becton Dickinson FACScan. FACS dot plots with logarithmic axes for fluorescence are shown. The percentage of bone marrow cells expressing each antigen is shown within each quadrant of the FACS plots.

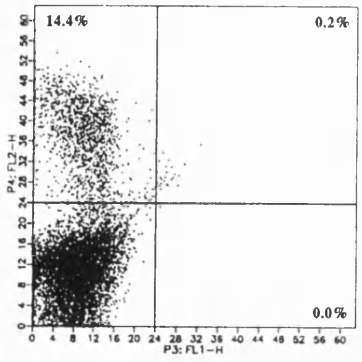
Non transgenic. **Ly-6E.1/*c-myc/bcl-2* double transgenic**

A. Thy1-PE + B220-FITC





B. Mac-1-PE only



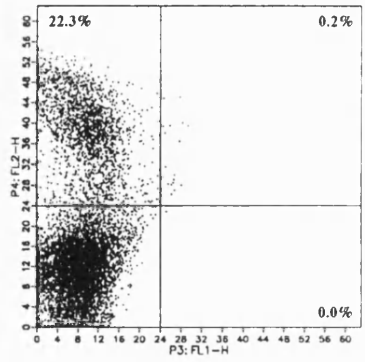
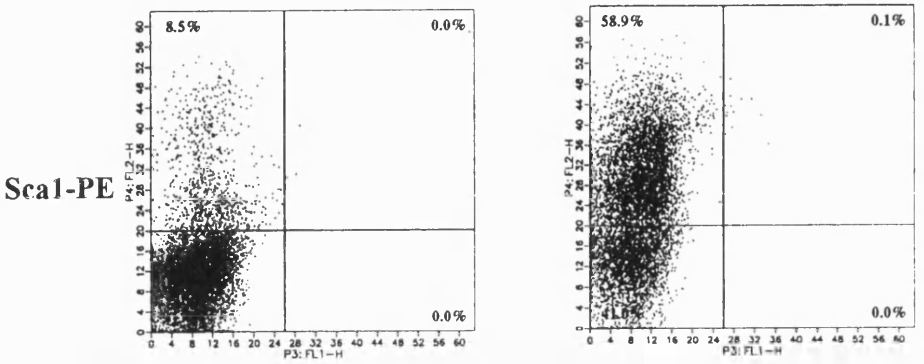


Figure 52. FACS analysis of Sca-1 expression in Ly-6E.1/*myc*/*bcl-2* bone marrow.

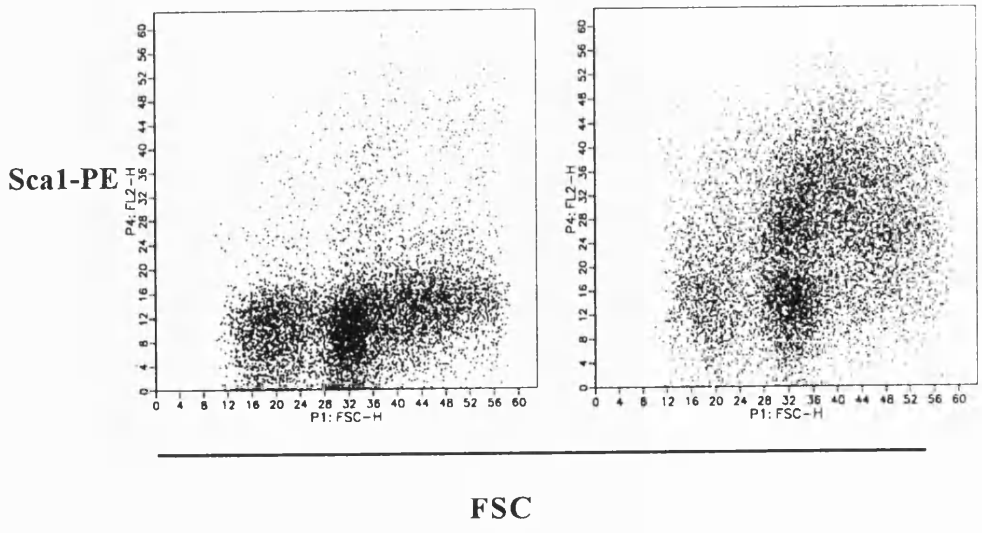
Bone marrow cells from transgenic and non-transgenic littermates were stained with Sca-1-PE to detect Ly-6E/A positive cells. 10^4 cells were analysed using a Becton Dickinson FACScan and FACS dot plots generated. A. Sca-1 (PE fluorescence, ordinate). The percentage of positive cells is displayed within upper left quadrant. B. FACS dot plots showing Sca-1 (PE, ordinate) in relation to cell size (forward angle light scatter, FSC, abscissa).

Non transgenic. **Ly-6E.1/*c-myc/bcl-2* double transgenic**

A.



B.



approximately 10% to 60% in the double transgenic bone marrow, resembling the situation in the bone marrow of Ly-6E.1/*bcl-2* single transgenic mice. This is in contrast to Ly-6E.1/*myc* bone marrow in which the percentage of Sca-1^+ cells is normal. As with Ly-6E.1/*bcl-2* bone marrow, the increase in Sca-1^+ cells is not restricted to cells exhibiting a particular forward angle light scatter (FSC) and thus is unlikely to represent an increase in a discrete population of cells. There is also a two-fold increase in the percentage of Mac-1^+ cells in double transgenic bone marrow. This resembles the FACS profile of Ly-6E.1/*myc* transgenic mice yet contrasts with the normal numbers of Mac-1^+ cells in the bone marrow of Ly-6E.1/*bcl-2* mice. These observations are again suggestive of mutually exclusive effects of *myc* and *bcl-2* oncogenes, with few indications of cooperation *in vivo*. The overall phenotype of double transgenic bone marrow is clearly complex, displaying some novel characteristics as well as some found in single transgenic animals. These observations suggest that the *c-myc* and *bcl-2* cooperation is not as potent *in vivo* as may have been expected from *in vitro* studies (Bisonette *et al.*, 1992; Fanidi *et al.*, 1992). Presumably the precise cell type is a crucial factor in determining the outcome of proto-oncogene expression. This is consistent with the lack of tumour development in Ly-6E.1/*myc/bcl-2* mice and previous transgenic studies in which all tumours isolated from Eμ/*myc/bcl-2* transgenic mice were of the same cell type (Strasser *et al.*, 1990).

5.5 Primary culture and the derivation of a novel cell line from double transgenic bone marrow.

Bone marrow cells from Ly-6E.1/*myc*/*bcl-2* double transgenic animals were cultured *in vitro* to determine whether the expression of both *c-myc* and *bcl-2* within the same cell, in particular the Sca-1 positive haematopoietic stem cell could facilitate the derivation of novel cell lines.

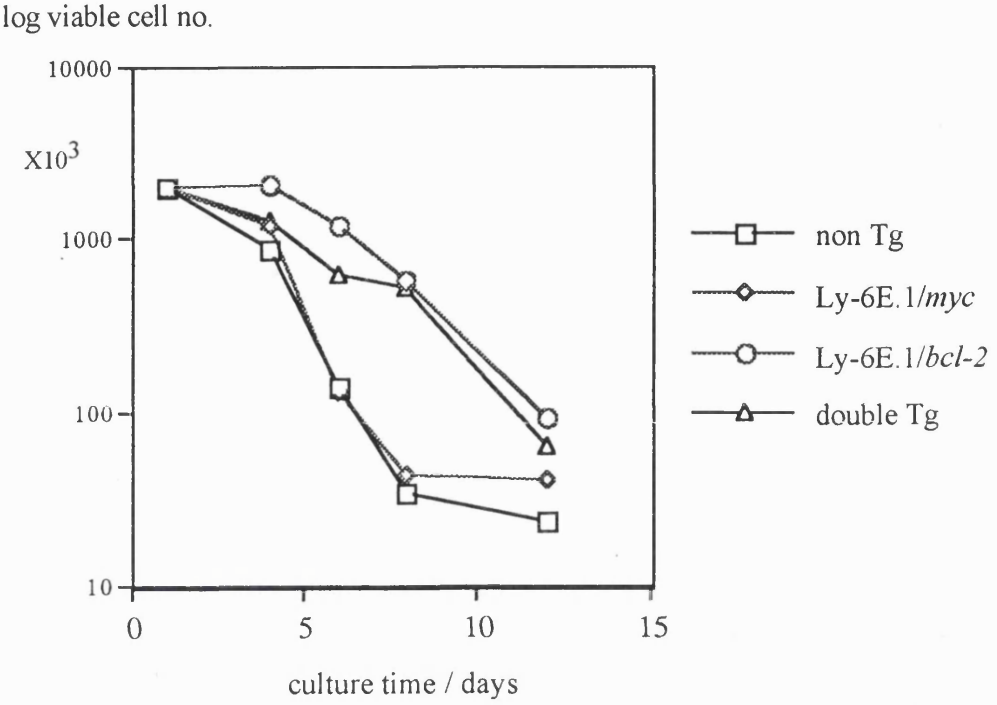
Assessment of *in vitro* growth potential was performed by culturing single cell suspensions of bone marrow from double transgenic, single transgenic and normal littermates in basic cell culture medium for two weeks without added haematopoietic growth factor supplements. Figure 53a shows cell viability profiles for cultures of non-transgenic, Ly-6E.1/*myc*, Ly-6E.1/*bcl-2* and Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow. Double transgenic and Ly-6E.1/*bcl-2* bone marrow both displayed enhanced survival characteristics compared with Ly-6E.1/*myc* and non-transgenic bone marrow, presumably due to the protective effects of high levels of *bcl-2* expression. Cultured cells were also tested for their ability to differentiate *in vitro* along the myelo-erythroid pathway. After two weeks of suspension culture, bone marrow cells were examined for colony formation in methyl cellulose supplemented with growth factors to determine whether haematopoietic potential was maintained in parallel with cell survival. Figure 53b shows cells from such methyl cellulose cultures. Several differences were evident when comparing cells derived from each transgenic bone marrow culture with the control bone marrow, which consisted of fresh non-transgenic bone marrow after a ten day methyl cellulose culture. Ly-6E.1/*myc* cultured bone marrow gave rise to cells morphologically

Figure 53. Analysis of Ly-6E.1/*oncogene* bone marrow *in vitro*.

a. Viability of bone marrow cells from non-transgenic, Ly-6E.1/*myc* transgenic, Ly-6E.1/*bcl-2* transgenic and double transgenic mice in basic medium with 10% FCS, β-mercaptoethanol and no growth factor supplements. Viable cells within each culture were counted over a 12 day period with trypan blue exclusion.

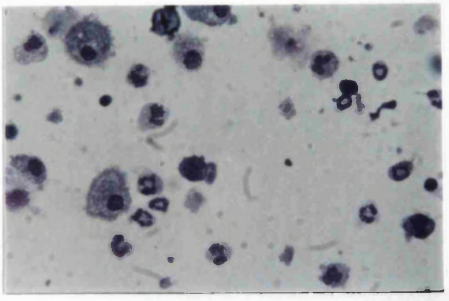
b. Assessment of haematopoietic potential remaining in cultured bone marrow cells using methyl cellulose colony forming assay. Cells from a. were seeded in triplicate at dilutions of between $5x10^3$ and 10^5 cells per ml in methyl cellulose culture supplemented with pokeweed mitogen spleen cell conditioned medium and erythropoietin. After ten days, methyl cellulose cultures from each individual were pooled and washed through several changes of PBS and centrifuged onto a Cytospin slide. Air-dried slides were methanol fixed, stained with "Diff-Quick" (eosin and thiazine) and mounted in DPX mountant. No cells were recovered from cultures inoculated with *in vitro* cultured non-transgenic bone marrow. Control = methyl cellulose culture inoculated with fresh, non-transgenic bone marrow at the same time as the *in vitro* cultured Ly-6E.1/*oncogene* bone marrow was seeded; e = eosinophilic cells; p = polymorphic cells; m = macrophage-like cells; b = blast-like cells.

a. viability of bone marrow cells *in vitro*

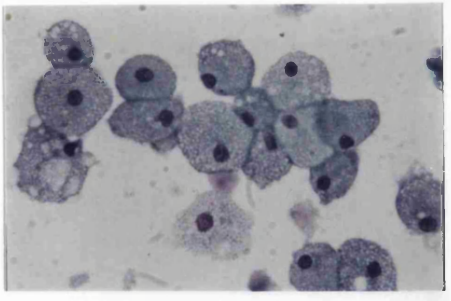


b. methylcellulose assay of cultured bone marrow from a.

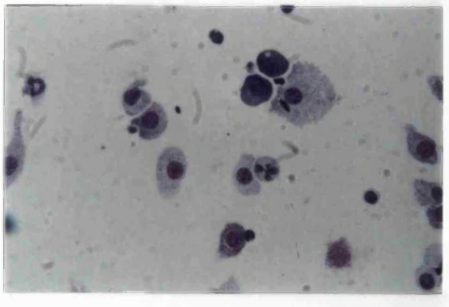
control



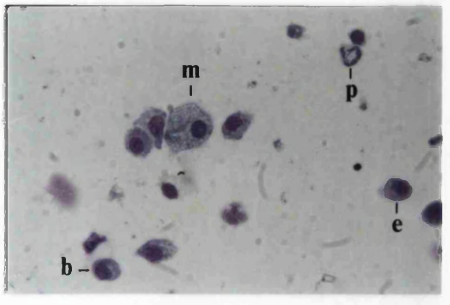
Ly-6E.1/*myc*



Ly-6E.1/*bcl-2*



double Tg



indistinguishable from those derived from fresh Ly-6E.1/*myc* bone marrow, that is, an overgrowth of the culture with large, macrophage-like cells. Ly-6E.1/*bcl-2* cultured bone marrow also gave rise to the same range of cells as seen in fresh non-transgenic and Ly-6E.1/*bcl-2* bone marrow in methyl cellulose culture, although there was an increase in the number of eosinophilic cells produced. Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow displayed many of the characteristics of normal non-transgenic bone marrow and Ly-6E.1/*bcl-2* cultured bone marrow. Eosinophilic cells, polymorphic cells, macrophage-like cells and blast-like cells were all produced by double transgenic bone marrow in methyl cellulose *post* culture without growth factors (figure 53b). Cells derived from both Ly-6E.1/*bcl-2* and Ly-6E.1/*myc*/*bcl-2* bear no resemblance to those from Ly-6E.1/*myc* after *in vitro* culture in the methyl cellulose assay.

To extend this study, the long term survival of double transgenic bone marrow was examined over a period of four weeks. Between two and three weeks, non-transgenic bone marrow cultures died, whilst the cell number was maintained in double transgenic cultures (figure 54a). After three weeks, the cell number in the double transgenic cultures began to increase and aliquots of cells were frozen or used for the following studies.

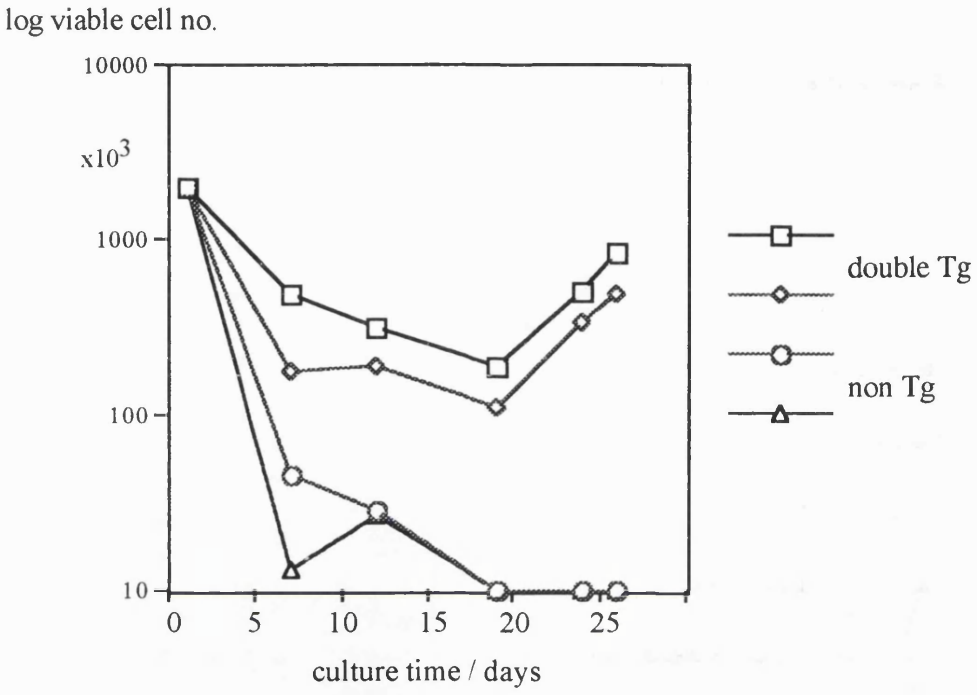
In addition to standard cell culture conditions, double transgenic bone marrow cells were given either leukaemia inhibitory factor conditioned medium (LIF), pokeweed mitogen spleen cell conditioned medium (SCM) or both sources of growth factors (not shown, see figure 55). These sources of growth factors were chosen either for their ability to maintain cells in an undifferentiated state, in the case of LIF, or as a rich source of haematopoietic growth factors, particularly IL-3 and GM-CSF,

Figure 54. Analysis of Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow *in vitro*.

a. Viability of bone marrow cells from non-transgenic and Ly-6E.1/*myc*/*bcl-2* double transgenic, mice. Cells from 2 individual double transgenic and 2 individual non-transgenic littermates were seeded in basic cell culture medium with 10% FCS, β-mercaptoethanol and no growth factor supplements. Viable cells within each culture were counted over a 4 week period with trypan blue exclusion.

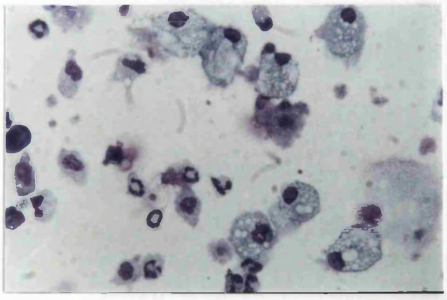
b. Assessment of haematopoietic potential remaining in cultured Ly-6E.1/*myc*/*bcl-2* transgenic bone marrow cells using methyl cellulose colony forming assay. Double transgenic cells from a. were seeded in triplicate at dilutions of between $5x10^3$ and 10^5 cells per ml in methyl cellulose culture supplemented with pokeweed mitogen spleen cell conditioned medium and erythropoietin. After ten days, methyl cellulose cultures from each individual were pooled and washed through several changes of PBS and centrifuged onto a "Cytospin" slide. Air-dried slides were methanol fixed, stained with "Diff-Quick" (eosin and thiazine) and mounted in DPX mountant. No cells were recovered from *in vitro* cultured non-transgenic bone marrow cells after 4 weeks, therefore, methyl cellulose cultures were not inoculated. control = fresh, non-transgenic bone marrow. Also shown are Cytospin preparations of methyl cellulose cultures inoculated with Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow cultured *in vitro* for 4 weeks with either LIF of SCM supplements.

a. long term viability of double Tg bone marrow *in vitro*

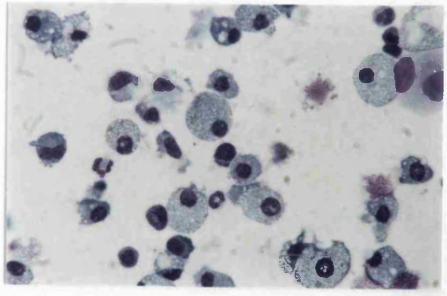


b. methylcellulose assay of 4 week double transgenic bone marrow cultures.

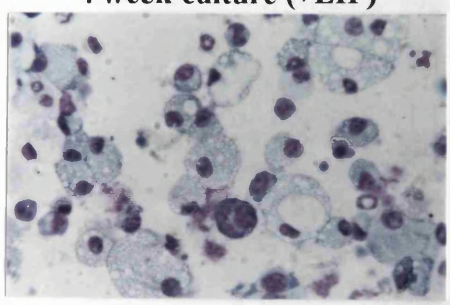
control



4 week culture



4 week culture (+LIF)



4 week culture (+SCM)

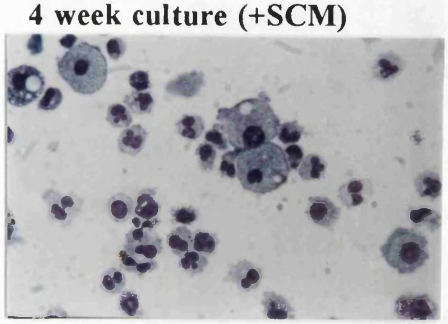
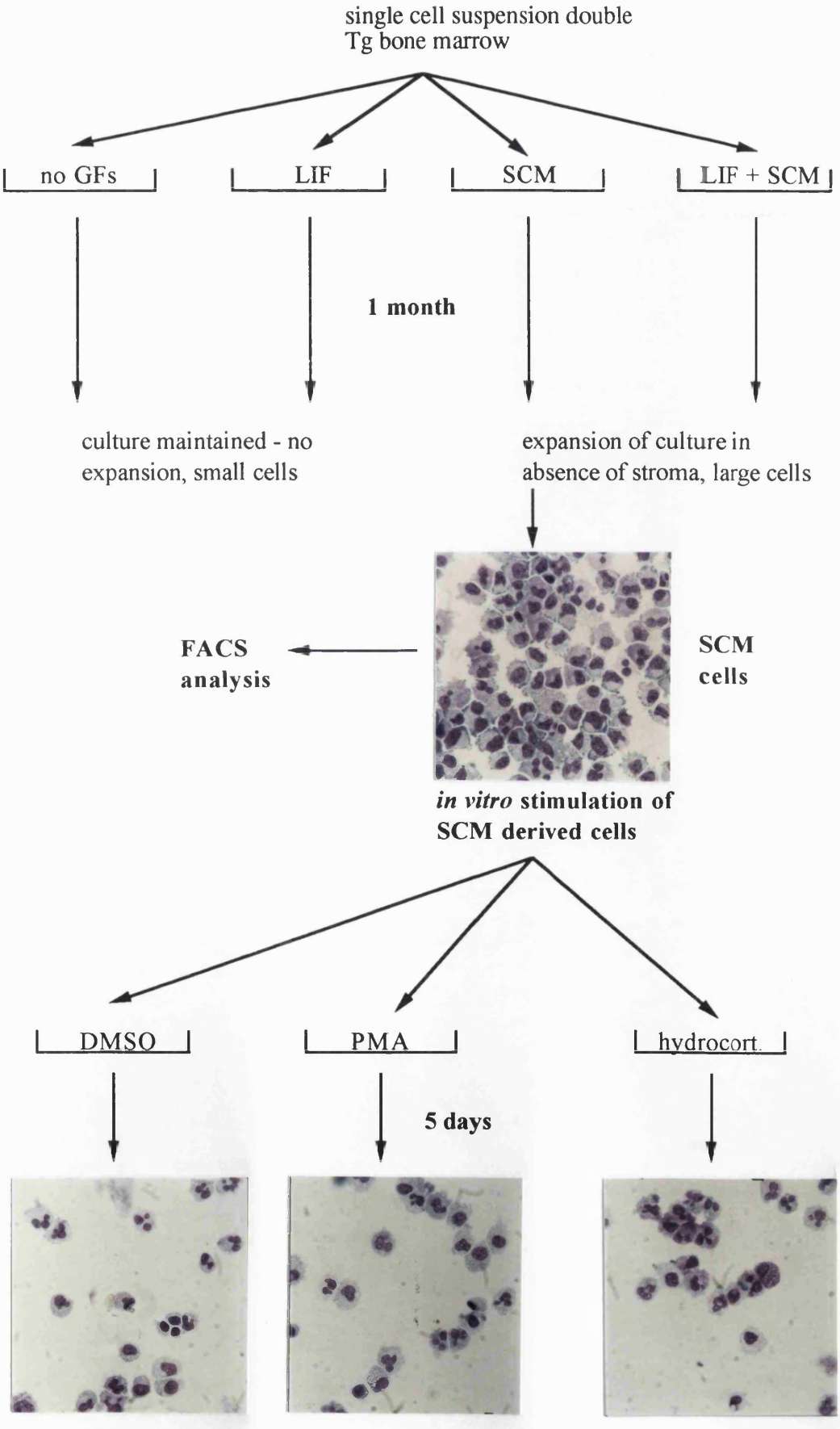


Figure 55. Derivation of a "cell line" from Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow.

Diagram summarising Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow cultures and the origin of SKP cells.


single cell suspension double Tg bone marrow
no GFs
LIF
SCM
LIF + SCM
1 month
culture maintained - no expansion, small cells
expansion of culture in absence of stroma, large cells
FACS analysis
SCM cells
in vitro stimulation of SCM derived cells
DMSO
PMA
hydrocort.
5 days

in the case of SCM. SCM supplemented growth conditions promoted the expansion of double transgenic bone marrow cells, whereas LIF supplemented cultures were merely maintained at approximately constant cell numbers, resembling cultures in basic cell culture medium only. SCM appears to have a dominant effect over LIF, as cells cultured in the presence of both LIF and SCM expand. The *in vitro* differentiation potential of these cells was investigated using the methyl cellulose colony formation assay (figure 54). Despite inducing an expansion of bone marrow cells in single cell suspension culture, SCM treatment appears to reduce the differentiation potential of the culture with the appearance of only very few macrophage-like or polymorphic cells. The majority of cells recovered from the methyl cellulose assays morphologically resembled the input cells derived from 4 week SCM supplemented *in vitro* cultures. As SCM is the main source of growth factors in the methyl cellulose assay, it may be that cells grown in SCM for four weeks are no longer responsive to it, or have already been induced to differentiate up to a certain point. However, Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow cultured without the addition of exogenous haematopoietic growth factors retained some ability to differentiate in methyl cellulose assays to generate macrophage-like cells, eosinophilic cells, polymorphic cells and blast cells. In general the morphology of cells recovered from cultures seeded with Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow after four week culture *in vitro* were similar to those cultures seeded with control, non-transgenic fresh bone marrow. This suggests that, in contrast to non-transgenic bone marrow, which does not survive beyond 2 weeks in culture without growth factor supplements, Ly-6E.1/*myc*/*bcl-2* bone marrow retains not only

viability but also the potential to differentiate normally *in vitro* into several haematopoietic cell types.

After extended *in vitro* culture, SCM derived cells do not differentiate in the methyl cellulose colony forming assay. The low level of differentiation observed in figure 54 for SCM derived cells at four weeks post-culture ceases to be detectable in methyl cellulose assays after six week *in vitro* culture with SCM.

In an attempt to induce suspension cultures of SCM derived cells to differentiate, they were treated with either the reducing agent DMSO, the phorbol esther PMA or the steroid hydrocortisone. The effect of these treatments on SCM derived cells is shown in figure 55. Only hydrocortisone appeared to have any effect, as determined by cellular morphology. Cultures supplemented with hydrocortisone demonstrated a low percentage of eosinophilic cells after a five day treatment. Removal of SCM did not induce differentiation but cell death.

The fact that SCM supplemented bone marrow cultures expanded over the course of the experiment enabled further analysis to be performed on these cells (see figure 55). SCM derived cells were subject to FACS analysis with a panel of monoclonal antibodies specific for a range of both mature and immature haematopoietic cell suface markers. Figure 56 shows the resulting FACS profiles, demonstrating that the SCM derived cultures were almost homogenous as only a single population is seen on each FACS profile. Although not formally cloned, this cell population is regarded as a cell line and will now be designated SKP (Sca-1, c-Kit and Pgp-1 positive cells). SKP cells lacked antigens associated with T and B lymphocytes (Thy-1 and B220), macrophages (Mac-1) and erythroid cells (Ter119)

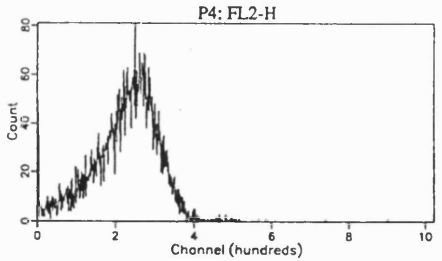
and are thus "lineage negative". Furthermore they express both Sca-1 and c-kit, markers of immature haematopoietic cells and haematopoietic stem cells.

In summary, double transgenic bone marrow cells displayed enhanced survival characteristics in the absence of growth factors whilst retaining some differentiation capability. Growth factor supplements were necessary for the expansion of double transgenic bone marrow cells in culture but seemed to reduce differentiation capacity. Thus *c-myc* and *bcl-2* expression in combination does not place an insurmountable block on differentiation despite allowing enhanced survival *in vitro*. It would be interesting to transplant both fresh and cultured Ly-6E.1/*myc*/*bcl-2* bone marrow into irradiated recipients to test for haematopoietic differentiation *in vivo*. In these studies, Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow has produced a cell line, SKP cells, with the cell surface phenotype of a haematopoietic stem cell and suggests that it is possible to expand haematopoietic stem or progenitor cells *in vitro* for further analysis.

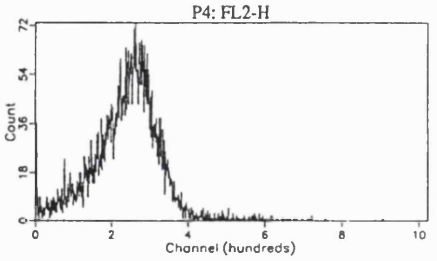
Figure 56. FACS analysis of SKP cells.

Analysis of SKP cell line with a panel of monoclonal antibodies recognising haematopoietic antigens. 10^4 SKP cells stained with; Thy-1-PE, Mac-1-PE, Ter119-PE, Sca-1-PE, HSA-PE, CD44-PE, B220-FITC and c-kit-FITC were analysed using a Becton Dickinson FACScan. Histogram plots of cell number (ordinate) *versus* fluorescence intensity (abscissa) were generated. SKP cells with the same growth, morphological and antigenic properties were simultaneously derived from 2 separate Ly-6E.1/*myc*/*bcl-2* double transgenic animals.

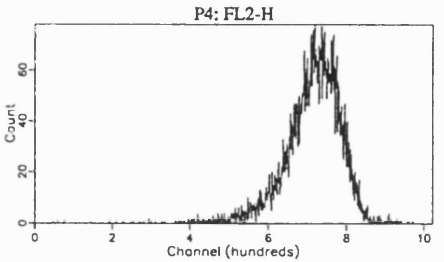
Unstained



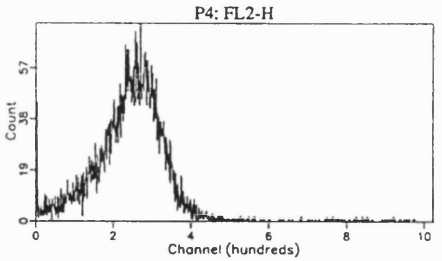
Ter119



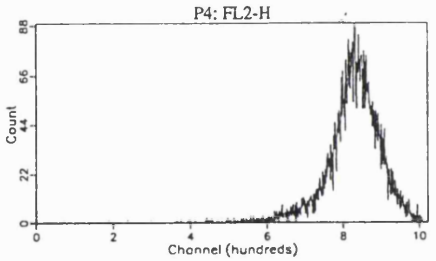
CD44 (PGP1)



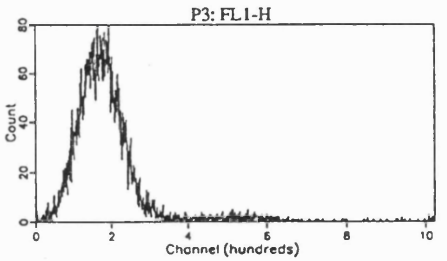
Thy1



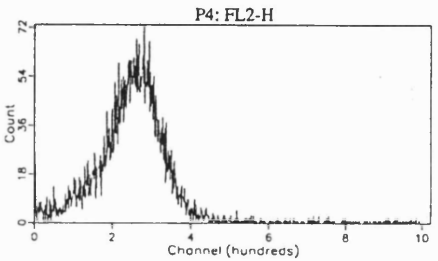
Sca1



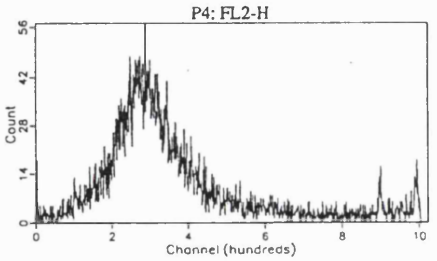
B220



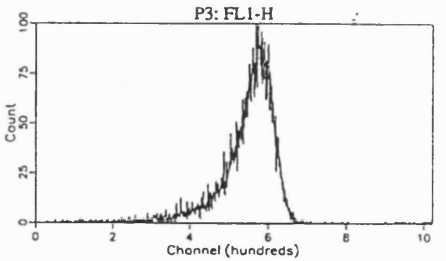
Mac1

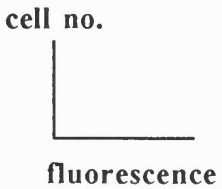


HSA



c-kit





DISCUSSION.

The work described in this thesis was undertaken to study the biology of haematopoietic stem cells, making use of the recently cloned Ly-6E.1 gene. The Ly-6E.1 gene encodes a cell surface protein found on haematopoietic stem cells that has been used in haematopoietic stem cell enrichment protocols. The reason for the interest in Ly-6E.1 gene is two fold, first, the study of the genetic regulatory mechanisms of this gene can potentially provide a means to study genetic mechanisms active in haematopoietic stem cells. Such studies could enable the processes of self-renewal and pluripotency of haematopoietic stem cells to be better understood. Second, this gene may be of use as a molecular tool for the manipulation of haematopoietic stem cells in both therapeutic or research settings. The experiments detailed in this thesis have attempted to address both of these aspects *in vivo* using transgenic mouse technology.

In sections 1 and 2 of the Results, I have described a study of the regulatory elements of the Ly-6E.1 gene important for *in vivo* expression. This work has identified an important transcriptional regulatory region of the gene which directs high level tissue specific expression and has also directly demonstrated the feasibility of using the Ly-6E.1 gene as a molecular tool to target heterologous gene expression to haematopoietic stem cells. In sections 3, 4 and 5 of the Results, I have described the use of the Ly-6E.1 gene to manipulate the haematopoietic system and facilitate the derivation of a cell line with a cell surface phenotype resembling haematopoietic stem cells.

1. The analysis of Ly-6E.1 gene expression *in vivo* using Ly-6E.1/*lacZ* transgenic mice.

The data presented in section 1 of the Results show that the 14 Kb BamH1 subclone of the Ly-6E.1 gene can direct expression of a linked *lacZ* reporter gene in transgenic mice. Qualitatively, *lacZ* expression can be detected in tissue and cell types that express the endogenous Ly-6E.1 gene, suggesting that the transgene contains the necessary elements with which to recapitulate the expression of the endogenous gene *in vivo*. High level Ly-6E.1/*lacZ* expression can be detected in kidney and activated lymphocytes whilst lower levels are found in liver and in haematopoietic and lymphoid tissues such as bone marrow, lymph nodes, spleen and thymus. The general pattern of transgene expression is consistent with the distribution of the endogenous Ly-6E.1 RNA (figure 3) and gene product (compare with table 1). In addition, the analysis of transgene expression in both kidney and spleen of several BL transgenic mice showed that *lacZ* expression increased with increasing transgene copy number. This observation implies that each copy of the transgene is expressing faithfully in each transgenic line, unaffected by its chromosomal location.

The fact that copy number dependency of Ly-6E.1 transgene expression is observed in both spleen and kidney suggests that the 14 Kb BL construct contains a genetic element similar to an LCR. The reduced expression exhibited by XN transgenic mice suggests that this element is located in the distal 3' region of the Ly-6E.1 gene. Previously, LCR elements have been found in the flanking regions of genes expressed in the haematopoietic system. LCRs, for example those of the

human β-globin and CD2 genes (Grosveld *et al.*, 1987; Greaves *et al.*, 1989), are similar to the 3' Ly-6E.1 element in that they are associated with DNase1 HSSs and direct tissue specific, copy number dependent expression in transgenic mice.

In addition to copy number dependency, further evidence that the 14Kb Ly-6E.1 subclone contains a genetic element capable of overcoming transcriptional repression due to position effects comes from the analysis of XN transgenic mice containing a truncated form of the Ly-6E.1 transgene lacking the most distal 3' end of the clone comprising DNase1 HSSs +6.7, +8.7 and +8.9. Seven independent transgenic mouse lines containing such a construct were analysed, with transgene copy number ranging from 1 to 25. No specific Ly-6E.1/*lacZ* expression was detectable by northern blot, even in high copy number transgenic mice, although some ectopic expression was observed. β-galactosidase assays on both adult and embryonic tissues, however, facilitated the detection of some *lacZ* expression. Despite the use of high copy number transgenic mice and sensitive FACS/FDG analysis, only one line demonstrated any haematopoietic specific expression, XN37 which contained 2 copies. Only one line exhibited *lacZ* expression in kidney, XN23 also with 2 copies. Curiously, XN23 was the only XN line in which embryos exhibited X-gal staining in the mesonephros. Although this may suggest some link between these $lacZ^+$ mesonephric cells and $lacZ^+$ kidney cells, the region in which they are found in the anterior mesonephros is not associated with development of the adult kidney (Kaufman, 1992; Medvinsky, pers. comm.).

XN transgenic embryos of all lines exhibited some degree of X-gal staining. All aspects of Ly-6E.1/*lacZ* expression seen in BL embryos that contain the full length construct can be detected in XN embryos, though never simultaneously in an

embryo of any one line. If the tail-specific expression of the BL Ly-6E.1/*lacZ* transgene is analysed in detail, it is seen to comprise three separate tissue specificities; notochord, endoderm of the hindgut and mesoderm. Each of these tissues alone can be found stained by X-gal in XN transgenic embryos. For example, *lacZ* expression is found in XN23 kidney and in day 11p.c. mesonephros, whereas in XN37 expression is found in activated lymphocytes and embryonic (day 11p.c.) notochord and mesoderm. Expression by the XN229 line is detectable in day 11p.c. notochord, endoderm and mesoderm, whilst in XN231 mice expression is only found in day 11p.c. endoderm. This data suggests that some of the necessary tissue specific elements of the Ly-6E.1 gene are retained in the truncated construct and that the full expression pattern is never seen because a regulatory element is absent which overcomes chromosomal position effects. The location of regulatory elements 3' to the Ly-6E.1 gene is consistent with many other genes expressed in the haematopoietic system that require 3' genetic elements for their expression, such as: β-globin (Antoniou *et al.*, 1988; Behringer *et al.*, 1987); lysozyme (Bonnifer *et al.*, 1990); CD2 (Greaves *et al.*, 1989); CD3 (Clevers *et al.*, 1989); the T cell receptor genes (Leiden, 1993) and another stem cell antigen, CD34 (May & Enver, 1995). In addition, it is unusual that no tissue specificity of expression is completely abolished upon removal of the 3' Ly-6E.1 element. This observation suggests that, rather than being an enhancer element, this region is likely to function as an insulator element, in a similar way to the special chromatin structure elements described in *Drosophila* (Kellum and Schedl, 1991).

The level of *lacZ* mRNA found in BL transgenic spleen and con A treated splenocytes was less than that found in lymph node and kidney, respectively, although

endogenous Ly-6E.1 mRNA levels were higher in these tissues. This finding could be interpreted as an example of inhibition of expression in the spleen by some genetic element near the site of transgene integration. However, this is inconsistent with data obtained from analyses of kidney and spleen from several lines of Ly-6E.1/*lacZ* mice. In both kidney and spleen, the expression of the transgene seems to display copy number dependency which suggests that the transgene is not affected by the integration site. A possible explanation for these observations is that *lacZ* and Ly-6E.1 mRNA species have different stabilities within different tissues, for example if Ly-6E.1 mRNA were much less stable in kidney than in con A treated spleen, relative to *lacZ*, the steady state levels would be proportionally lower on a northern blot, as is observed for Ly-6E.1/*lacZ* transgenic mice.

Further experiments are planned to investigate the regulatory elements 3' to the Ly-6E.1 gene in more detail. Initially, a 700bp fragment encompassing DNaseI HSSs +8.7 and +8.9 will be tested. Previously, HSS +6.7 was shown to have little effect on expression in stable transfection studies (Sinclair *et al.*, 1996). Thus it is assumed that HSS +6.7 is not involved in regulating high level tissue specific Ly-6E.1 expression. Two constructs will be employed to study DNaseI HSSs +8.7 and +8.9 in transgenic mice. The first will consist of a minimal hsp68 promoter linked to a *lacZ* reporter gene (gift, D. Meijer), 3' to which HSSs +8.7 and +8.9 will be added. X-gal staining of transgenic embryos will be used to determine whether this element can direct any tissue specificity to the construct and whether it can enhance expression. However, it is likely that this element is involved in isolating transgenes from the influence of chromatin at the integration site. To test this hypothesis, a minimal promoter with some tissue specificity will be employed. For example, the

133bp myogenin promoter can direct *lacZ* expression specifically to the myotome of the somites in 50% of transgenic embryos (Yee & Rigby, 1993). Such a 133bp myogenin/*lacZ* construct will be flanked with HSSs +8.7 and +8.9 of Ly-6E.1 at both 5' and 3' ends of the construct to determine whether this construct will now express in all transgenic embryos, independent of transgene integration site.

2. Haematopoietic stem cell specific reporter gene expression in Ly-6E.1/*lacZ* transgenic mice.

The tissue specific expression pattern of the Ly-6E.1 transgene suggests that Ly-6E.1/*lacZ* should be expressed on haematopoietic stem cells. As shown in section 1.6 of the Results, FACSorting followed by bone marrow transplantation of either FDG^+ or FDG^- cells directly demonstrated that the 14Kb subclone of Ly-6E.1 could direct *lacZ* expression to transplantable bone marrow haematopoietic stem cells. This was demonstrated with FDG sorted cells from homozygous mice of the BL1a and BL1b lines and heterozygous BL19 mice. As few as 10^3 FDG^+ cells were capable of long-term reconstitution, whilst at least 10^6 FDG^- sorted cells were required for this activity. This data corresponds to an enrichment in haematopoietic stem cell activity of 100 fold over unfractionated bone marrow, which usually requires 10^5 transplanted cells to achieve reconstitution (data not shown; Boggs *et al.*, 1982; Boggs *et al.*, 1984; Russel, 1979). However, it was not possible to FACSort for haematopoietic stem cells with FDG using bone marrow from 4 copy number animals as less than 1% of these bone marrow cells were FDG^+ (not shown), suggesting that

there is a threshold level of expression required to detect haematopoietic stem cells by this method.

A discrepancy between the expression of the transgene and the endogenous gene at the protein level is observed in bone marrow. Not all bone marrow cells co-express Sca-1 and *lacZ* (as determined by FDG staining). Sca-1$^+$, FDG$^-$ cells are readily detectable by FACS analysis and may represent cells that are expressing Ly-6A.2 and not Ly-6E.1 as the Sca-1 antibody does not discriminate between the two allelic gene products. The transgenic mice used in these studies were routinely maintained on an outbred genetic background which is heterozygous for the Ly-6E.1 and Ly-6A.2 alleles. An unexpected observation was that certain cells were FDG$^+$, Sca-1$^-$, suggesting that some genetic element close to the site of integration was causing the Ly-6E.1/*lacZ* transgene to be expressed in Ly-6E.1$^-$ cells. RT-PCR analysis of steady state mRNA levels within these cells, however, demostrated that they were expressing both Ly-6E.1 and *lacZ* mRNA. The differences in protein expression observed for these cells resulted from post-transcriptional differences between *lacZ* and endogenous Ly-6E.1. It may be that these FDG$^+$, Sca-1$^-$ cells have just begun to downregulate Ly-6E.1, or, conversely, are just about to become Sca-1$^+$ and may represent a very interesting transitional subpopulation of bone marrow cells.

Sorting bone marrow on the basis of Ly-6E.1/*lacZ* expression did not allow the recovery of all haematopoietic stem cells in the FDG$^+$ population, possibly as a result of the difference in protein expression discussed above. In addition, allelic variation in Sca-1 expression on haematopoietic stem cells (Jurecic *et al.*, 1993; Okada *et al.*, 1992; Spangrude & Brooks, 1993) may result in some haematopoietic stem cells being Sca-1$^+$ by virtue of Ly-6A.2 expression and therefore not expressing

Ly-6E.1/*lacZ*, whilst some may express low levels of *lacZ* which result in their contaminating the FDG$^-$ population. To address these questions, it would be necessary to fractionate bone marrow further either using double staining with Sca-1 antibody and FDG or a sort based on high, medium and low levels of *lacZ* expression.

The demonstration of Ly-6E.1/*lacZ* expression on haematopoietic stem cells presented in this thesis represents the first direct example of a transgenic construct with this property. As mentioned previously, such a construct has the potential to be used in haematopoietic stem cell enrichment procedures. It will be of interest to determine the level of enrichment obtained by FACS/FDG sorting following the exclusion of cells expressing markers of mature haematopoietic lineages. The resulting cells will not have been engaged by any antibodies during the enrichment procedure, unlike current protocols requiring positive selection with Sca-1. Such a population, enriched for haematopoietic stem cells without having potentially received some stimulus, could be useful for the study of the early molecular signalling events involved in haematopoietic stem cell differentiation.

In addition to enabling the FACSorting of specific cells, the *lacZ* transgene has been used, in combination with X-gal staining, to specifically mark cells and to study cell migration (Wilson *et al.*, 1995). Recent advances in developmental haematopoiesis have lead to the notion that mammalian haematopoiesis occurs in two waves, primitive and definitive in a similar manner to birds and amphibians. Definitive adult haematopoiesis appears to originate autonomously within the body of the embryo in the AGM region (Dzierzak & Medvinsky, 1995). The use of X-gal staining to analyse Ly-6E.1/*lacZ* expression during development has shown that

expression within the mesonephros coincides spatially and temporally with the onset of definitive haematopoiesis in the embryo. Long term repopulating haematopoietic stem cells are first detectable in the AGM region at late day 10p.c. (Müller *et al.*, 1994), whilst X-gal staining first appears in the anterior part of the mesonephros at day 10.5p.c. Further subdivision of the AGM region using haematopoietic stem cell assays has shown that long-term reconstituting activity is restricted to the anterior portion of the AGM region (A. Medvinsky, pers. comm.), coincident with X-gal staining in Ly-6E.1/*lacZ* embryos. The X-gal staining within the mesonephros recedes towards the anterior tip of the mesonephros between day 11.5 p.c. and day 12.5 p.c., which coincides with the time of foetal liver colonisation by haematopoietic stem cells. It is tempting to speculate that these Ly-6E.1/*lacZ*$^+$ cells play a role in one of the earliest stages of haematopoietic development. The lack of Ly-6E.1/*lacZ* expression in yolk sac further coincides with the association between Ly-6E.1 and definitive haematopoiesis, as yolk sac haematopoietic progenitor cells do not express Sca-1 (Huang & Auerbach, 1993).

If cells of the mesonephros from Ly-6E.1/*lacZ* transgenic embryos can be isolated in single cell suspension and stained with FDG, FACS/FDG sorting and transplantation should enable the direct assessment of their haematopoietic potential. In preliminary experiments (not shown), it was not possible to sort for haematopoietic stem cell activity from the AGM region of Ly-6E.1/*lacZ* transgenic embryos using FACS/FDG. The percentage of viable AGM cells staining with FDG was 0.1% (not shown) suggesting that the preparation of the cells was detrimental to staining, or that the cells of the AGM region are refractory to FDG staining. It is likely that a combination of the collagenase treatment required to prepare single cell suspension

from embryonic tissue and the hypotonic shock involved in FDG staining results in death of *lacZ* expressing cells. Experiments are planned using the Ly-6E.1 cassette to direct expression of the green fluorescent protein in transgenic mice (A. Holmes and E. Dzierzak. pers. comm.). It is hoped that AGM region cells from such transgenic mice will circumvent the problems associated with FDG staining and facilitate the FACS sorting and analysis of Ly-6E.1$^+$ cells from the AGM region.

The property of the cloned Ly-6E.1 gene to target heterologous gene expression to haematopoietic stem cells is not only of use in tracing stem cells and designing enrichment protocols but can also be used to manipulate haematopoietic stem cells in transgenic mice. Results of such experiments, designed to manipulate haematopoietic stem cells, are presented sections 3, 4 and 5 of the Results and will be discussed in the following sections.

3. Multiple defects in Ly-6E.1/*myc* transgenic mice.

The data presented in section 3 of the Results show that Ly-6E.1 directed expression of the *c-myc* proto-oncogene gave rise to numerous effects in transgenic mice. High copy number Ly-6E.1/*myc* transgenic mice had a considerably reduced life expectancy. The three highest copy number founder animals all became sick and died within 5 weeks of age. The two high copy number founder animals which were analysed *post mortem* were found to possess solid kidney tumours, severely reduced levels of both ODC and ADH mRNA and smaller haematolymphoid organs compared with normal age-matched non-transgenic animals.

The kidney is known to be the organ expressing the highest level of Ly-6E.1 mRNA in the mouse and therefore is likely to be the site of highest level *c-myc* expression. Northern blot analysis of RNA from a panel of Ly-6E.1/*myc* tissues verified this. Previous studies in which *c-myc* was expressed in kidneys of transgenic mice have produced differing results. Several lines of MHC class 1/*c-myc* transgenic mice showed no evidence of kidney tumours despite transgene expression in that organ (Roland & Morello, 1993), whereas an SV40/β-globin/*c-myc* construct routinely produced mice with tumourous kidneys and premature mortality. These mice became known as a mouse model of polycystic kidney disease and are referred to as SBM strains (Trudel *et al.*, 1991). These examples of transgenic mice expressing *c-myc* in kidney demonstrate that transformation is dependent upon and influenced by a number of factors such as the precise cell type expressing *c-myc* and the level of transgene expression. In the case of Ly-6E.1/*myc* transgenic mice, the absence of kidney tumours in LM5 mice is likely to be due to a sub-threshold expression level resulting from the low transgene copy number of this line. It is possible that the severe kidney phenotype exhibited by Ly-6E.1/*myc* mice is analogous to the SBM polycystic kidney. However it will be interesting to compare material from the high copy Ly-6E.1/*myc* transgenic founder mice with that from SBM mice to verify this as Ly-6E.1/*myc* mice may serve as a model for other forms of kidney neoplasia. As mentioned previously, the tumourous kidneys in Ly-6E.1/*myc* transgenic founder mice are unusual in that they show severely reduced levels of ODC mRNA, a phenotype not normally associated with high levels of *c-myc* expression or tumours in general (Auvinen *et al.*, 1992; Bello-Fernandez *et al.*, 1993).

The liver in Ly-6E.1/*myc* transgenic mice is macroscopically normal, consistent with the fact that *c-myc* rarely induces tumours in liver (Roland & Morello, 1993). However, the levels of ADH mRNA are less than 50% of those found in non-transgenic littermates which suggests that Ly-6E.1/*myc* has some liver specific effects. It is not known whether Ly-6E.1 is expressed at high levels on few hepatocytes, or at low levels on many as it is detectable at only modest levels by northern blot analysis of total RNA from whole liver. Further analysis, including histology and immunohistochemistry, will be required to determine the precise effects of Ly-6E.1/*myc* on the liver.

The haematolymphoid system in Ly-6E.1/*myc* mice displayed a surprising phenotype. In contrast to previous studies that have generated transgenic mice expressing *c-myc*, Ly-6E.1/*myc* transgenic mice exhibited small thymus, spleen and lymph nodes. Expression of *c-myc* directed to B cells under control of immunoglobulin enhancers resulted in transgenic mice with enlarged spleens and lymph nodes. These transgenic animals developed fatal B cell lymphoma affecting both pre-B cells and B cells, as well as more general B lymphoproliferation (Adams *et al.*, 1985). In another study where the expression of *c-myc* was restricted to thymocytes and T cells under control of the Thy-1 transcriptional unit, tumours affecting both transgenic thymocytes and thymic stroma were observed. These lymphoid tumours contained mostly cells showing the $CD4^+CD8^+$ double positive phenotype, with a productively rearranged TCRβ chain. Furthermore, 50% of tumours were able to grow as cell lines *in vitro* without exogenous growth factors (Spanopoulou *et al.*, 1989). In contrast, cells from the tiny thymus and spleen recovered from LM7 transgenic founder were unable to form cell lines in culture and

transgenic mice showed no evidence of the breathing difficulties associated with enlargement of their thymuses characteristic of Thy-1/*myc* mice.

Another unusual aspect of the Ly-6E.1/*myc* lymphoid phenotype is the normal CD4/CD8 profile of the thymus. Several lines of transgenic H-2/*myc* and Eμ/*myc* mice in which *c-myc* transgene expression was detected in the thymus showed significantly altered CD4/CD8 profiles (Roland & Morello, 1993). These observations suggest that, in Ly-6E.1/*myc* mice, the expression of *c-myc* in lymphocytes is not the cause of the reduction in lymphoid cell number as *c-myc* expression has been shown to cause lymphoproliferation and changes to CD4/CD8 profiles. As both B cells and all classes of T cells are altered in LM5 transgenic mice, Ly-6E.1/*myc* expression is most likely having an effect on a lymphoid progenitor cell, reducing the number of pre-T and pre-B cells which, once past a certain stage go on to develop normally.

Ly-6E.1/*myc* mice were generated primarily for the study of haematopoiesis, in particular to investigate the role of haematopoietic stem and progenitor cell quiescence in the maintenance of the haematopoietic system *in vivo*. Initial analysis of the bone marrow compartment in LM5 transgenic mice using FACS showed an increased percentage of Mac-1 positive cells. The reduction in T and B cells seen in LM5 transgenic mice could be sufficient to cause an apparent increase in the percentage of macrophages, however, the dramatic defect in myelo-erythropoiesis *in vitro* suggests there is a direct effect of Ly-6E.1/*myc* on macrophages. Despite a severe impairment of myelo-erythropoiesis *in vitro*, there was no significant perturbation in long-term reconstituting haematopoietic potential *in vivo*. This may reflect a particular growth factor requirement, induced by *c-myc* over expression,

which is only apparent *in vitro*. It may be that Ly-6E.1/*myc* expression is causing unwanted cell divisions in a quiescent bone marrow progenitor cell which may be masked in the LM5 transgenic mice *in vivo* by the presence of growth factors which prevent death, resulting in only a slight defect in lymphopoiesis. *In vitro*, however, most normal growth factors are lacking whereas myeloid specific factors, such as IL-3 and GM-CSF, are present in abundance due to the addition of SCM. The *in vitro* differentiation of LM5 bone marrow could have been forced along the macrophage lineage by a combination of *c-myc* induced cell division and growth factor mediated protection from apoptosis. The high level expression of *c-myc* in the high copy founder mice may have delivered a signal too potent for normal growth factor abrogation resulting in the severe lymphoid deficiency exhibited by LM7 and LM8.

The complex Ly-6E.1/*myc* phenotype could be due to *c-myc* expression in commited progenitors of multiple lineages but this is unlikely considering that Sca-1 is rapidly downregulated as haematopoietic stem cells differentiate (Okada *et al.*, 1992; Li & Johnson, 1995). A more likely and more simple explanation is that the Ly-6E.1/*myc* transgene is having an effect on the haematopoietic stem cell resulting in changes to multiple haematopoietic lineages.

LM5 transgenic mice have been bred for one year with no evidence of tumours, though their lifespan remains between 12 and 20 weeks. In order to try to recreate the severe kidney and haematopoietic phenotype of the high copy number Ly-6E.1/*myc* transgenic founder mice, animals of the LM5 line will be interbred to generate homozygous mice, thereby effectively increasing the transgene copy number to 8. If there is a threshold level of *c-myc* expression required for the severe phenotype, the homozygous animals may express *c-myc* at sufficiently high levels to

enable a detailed analysis to be made of the severe Ly-6E.1/*myc* phenotype. If they have severely reduced numbers of T and B cells, it may be possible to identify at which stage in lymphoid development the *c-myc* mediated defect occurs. In addition, such breeding would generate sufficient highly expressing animals to perform detailed studies of the kinetics and multipotency of haematopoietic reconstitution in radiation chimaeras generated from severely affected Ly-6E.1/*myc* transgenic bone marrow.

4. Ly-6E.1/*bcl-2* transgenic mice possess enlarged lymphoid organs and display an erythroid deficiency.

The thymus, spleen and lymph nodes of Ly-6E.1/*bcl-2* transgenic mice were found to be enlarged compared with normal littermates in the absence of other gross morphological/anatomical abnormalities. Normal numbers of offspring were produced from heterozygous transgenic matings and these mice remained overtly healthy beyond one year of age. Thus it would seem that Ly-6E.1 directed *bcl-2* expression causes no serious developmental effects outside the haematopoietic system. Presumably, although Ly-6E.1$^+$ cells in embryonic tail, mesonephros and adult kidney are expressing *bcl-2*, as indicated by X-gal staining of Ly-6E.1/*lacZ* mice, they are either insensitive to *bcl-2* or possess intrinsic mechanisms to counter any harmful effects of *bcl-2* expression.

Within the lymphoid system, Ly-6E.1/*bcl-2* expression induces profound changes and most are similar to those previously observed in other strains of *bcl-2* transgenic mice (reviewed by Cory, 1995). Briefly, the enlarged lymphoid organs

result from increased numbers of both mature, peripheral T and B cells. Ly-6E.1/*bcl-2* expression in the thymus most likely drives the positive selection of $CD4^+CD8^+$ double positive thymocytes producing an excess of single positive thymocytes. These observations are consistent with transgenic *bcl-2* expression in T and B cells and demonstrates that Ly-6E.1/*bcl-2* is being expressed and produces functional protein, resulting in a lymphoid phenotype characteristic of other *bcl-2* transgenic mice (Siegel *et al.*, 1992; Sentman *et al.*, 1991; Strasser *et al.*, 1991).

One unusual aspect of the phenotype of Ly-6E.1/*bcl-2* transgenic mice was a reduction in the percentage of bone marrow cells expressing the Ter119 erythroid specific antigen. In addition to a deficiency in Ter119 cells, bone marrow from Ly-6E.1/*bcl-2* transgenic mice was visibly paler than normal, providing further evidence of an erythroid deficiency. Since the kidney is the major site of erythropoietin production, one possible explanation is that the erythroid deficiency is an indirect effect of Ly-6E.1/*bcl-2* expression in the kidney. If Ly-6E.1/*bcl-2* were perturbing normal kidney homeostasis, resulting in a reduction in erythropoietin production, erythroid cells in the bone marrow would undergo apoptotic cell death (Cowling & Dexter, 1994). However, the fact that the phenotype is transplantable into mice with normal, non-transgenic kidneys rules out this last hypothesis and strongly suggests the erythroid deficiency in Ly-6E.1/*bcl-2* transgenic mice results from a cell autonomous effect of *bcl-2* expression.

Bcl-2 is usually thought of as a survival factor, hence the discovery of a reduction in erythroid cells upon enforced *bcl-2* expression is unusual. During normal erythropoiesis, *bcl-2* expression is downregulated as erythroid cells mature (Bonati *et al.*, 1996) and it may be that over expression of *bcl-2* in Ly-6E.1/*bcl-2*

transgenic mice prevents the maturation of an erythroid precursor by an unknown mechanism. Alternatively, Ly-6E.1/*bcl-2* expression at the level of the haematopoietic stem cell may affect immature haematopoietic cells by preventing the apoptotic death of a particular cell type which, in turn, hampers an assymetric cell division ultimately resulting in a deficiency in erythropoiesis. The availability of specific niches within bone marrow is thought to be vital for normal haematopoietic differentiation (reviewed in Lord & Dexter, 1995). If apoptosis of haematopoietic precursors were blocked by *bcl-2* expression in Ly-6E.1/*bcl-2* bone marrow, this could fill all niches with unwanted cells and impede normal haematopoietic differentiation, thus explaining how transgenic expression of a survival gene could result in a lineage deficiency.

5. Double transgenic Ly-6E.1/*myc*/*bcl-2* mice show little evidence of oncogenic cooperation.

Ly-6E.1/*myc*/*bcl-2* double transgenic animals had a similarly reduced lifespan to Ly-6E.1/*myc* single transgenic mice with no evidence of the severe, early onset lymphoid tumours found in Eμ/*myc*/*bcl-2* double transgenic mice (Strasser *et al.*, 1990). FACS analysis of haematopoietic cells in double transgenic animals revealed that most subsets of cells resembled either those in Ly-6E.1/*myc* or Ly-6E.1/*bcl-2* single transgenic mice. The exceptions to this were splenic T cells, which were present in greater numbers in double transgenic animals, and B cells within the bone

marrow, which contained an expanded population cells expressing high levels of B220. A similar population of $B220^{hi}$ cells was observed in bone marrow of Ly-6E.1/*bcl-2* radiation chimaeras, suggesting these cells accumulate as a result of Ly-6E.1/*bcl-2* expression. Ly-6E.1/*bcl-2* expression may be driving B cell selection, resulting in greater production of mature B cells in an analogous manner to the effects of *bcl-2* transgene expression in thymus (Linette *et al.*, 1994). As such a population of $B220^{hi}$ cells is not seen in Ly-6E.1/*bcl-2* bone marrow which shows no overall increase in B cells, *bcl-2* distorted B cell selection is unlikely. An alternative possibility to explain the origin of this population of cells is that they arise as a result of *bcl-2* expression in actively dividing B cells. In the case of Ly-6E.1/*myc*/*bcl-2* double transgenic cells, this proliferation could be stimulated by Ly-6E.1/*myc*, whilst in radiation chimaeras, it may result from the expansion of donor bone marrow. As transplanted Ly-6E.1/*bcl-2* transgenic bone marrow cells divide, their endogenous levels of *c-myc* will be upregulated possibly enabling its cooperation with transgenic *bcl-2* to give rise to $B220^{hi}$ cells also found in double transgenic bone marrow.

The apparent absence of haematolymphoid malignancy in Ly-6E.1/*myc*/*bcl-2* double transgenic mice is of interest. This data contrasts with the findings of Strasser *et al.*, who found a high incidence of lymphoid malignancy in Eμ/*myc*/*bcl-2* double transgenic mice (Strasser *et al.*, 1990) and may be due to the low copy number Ly-6E.1/*myc* transgenic line used to generate double transgenic mice. Although LM5 mice express sufficient *c-myc* to significantly perturb their haematopoietic system, a higher level may be required for full oncogenic cooperation with *bcl-2*. To investigate whether such a threshold exists, it would be of interest to generate double

transgenic animals both homozygous for the LM5 Ly-6E.1/*myc* and heterozygous for Ly-6E.1/*bcl-2*.

6. The generation of a cell line with characteristics of a haematopoietic stem cell.

The results presented in section 5.5 of the Results show that cells from Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow demonstrated prolonged survival when cultured *in vitro* compared with normal bone marrow whilst retaining some of the differentiation potential and characteristics of fresh bone marrow in the methyl cellulose culture assay. This suggests that double transgenic bone marrow may be useful for the study *in vitro* of haematopoietic differentiation and the effects of different growth factors on this process. Of more significance is the possibility of isolating a variety of haematopoietic cell lines from Ly-6E.1/*myc*/*bcl-2* bone marrow. Double transgenic bone marrow survives well *in vitro*, retains differentiation capability and responds to various culture conditions, suggesting that it may be possible to generate many different cell lines from Ly-6E.1/*myc*/*bcl-2* bone marrow.

For example, in one culture experiment, bone marrow cells from two individual double transgenic mice were cultured separately with PWMSCCM which stimulated a dramatic expansion in cell number compared with cells cultured in the absence of added growth factors. After six weeks, FACS analysis of the expanded cells in both cultures suggested that the bone marrow populations had become homogenous. Although not formally cloned, both populations consisted of cells with the surface phenotype Sca-1$^+$, c-kit$^+$, Pgp-1$^+$ and lin$^-$. The lineage-specific antibodies

used were Thy-1, B220, Mac-1 and Ter119. However, although able to be maintained *in vitro* for at least 4 weeks, Ly-6E.1/*myc*/*bcl-2* double transgenic bone marrow requires the addition of exogenous growth factors for extensive expansion. Combining cell cloning techniques with the controlled use of specific, purified growth factor combinations may facilitate the derivation and propogation of cell lines which may be representative of different stages of haematopoietic stem cell differentiation.

Such cell lines will enable biochemical studies to be performed which could facilitate the detailed analysis of cell specific nuclear factors within haematopoietic stem cells and haematopoietic progenitors and a further understanding of the transcriptional programmes involved in haematopoietic differentiation. Similarly, cell lines will assist the study of signal transduction pathways and their components, growth factors, receptors and cell surface markers on haematopoietic cells at different stages of differentiation.

7. Conclusion.

In summary, the work described in this thesis has clearly demonstrated that the Ly-6E.1/*lacZ* transgene expresses faithfully *in vivo* and requires a 3' genetic element for full activity. These findings will enable more detailed analyses to be conducted into the mechanisms regulating the expression of Ly-6E.1 *in vivo*, thereby allowing genetic regulatory mechanisms active in haematopoietic stem cells to be studied. In addition, to these broader implications, Ly-6E.1 transgenes will enable

investigations into aspects of the Ly-6 family such as allelic differences between Ly-6.1 and Ly-6.2 strains of mice.

The direct demonstration of haematopioetic stem cell specific expression by an Ly-6E.1 transgene suggests many potential uses for this cassette in the study haematopoiesis *in vivo*. The continued use of marker genes will enable haematopoietic stem cells to be visualised and may enable the developmental fate of the haematopoietic stem cell to be determined. Additionally, the haematopoietic stem cell could be manipulated by Ly-6E.1 directed expression of modifying genes. As described in this thesis, transgenic mice were generated in which the expression of proto-oncogenes was directed to haematopoietic stem cells. The resulting transgenic mice exhibited multiple haematopoietic deficiencies and facilitated the derivation of a cell line with a surface phenotype similar to that of the haematopoietic stem cell. These targeted oncogenesis experiments show that the Ly-6E.1 gene has great potential as a tool for the study of the haematopoietic stem cell differentiation and development.

APPENDIX - CD2/Ly-6E.1 TRANSGENIC MICE.

1. Introduction.

The cell surface expression of Ly-6E.1 and Ly-6A.2 is largely extinguished during T cell ontogeny within the thymus at the $CD4^+CD8^+$ stage of T cell development (Yeh *et al.*, 1986; Spangrude *et al.*, 1988). The downregulation of Ly-6E/A expression occurs at a specific stage of $CD4^-CD8^-$ T cell development, coincident with the loss of CD44 expression during the transition from $CD44^+CD25^+$ cells to $CD44^-CD25^+$ cells (Bamezai *et al.*, 1995). Bamezai *et al.* perturbed this normal series of events by constitutively expressing Ly-6A.2 at all stages of T cell development under the genetic control of the human CD2 LCR in transgenic mice. Ly-6A.2/CD2 transgenic mice exhibit a severe impairment in T cell production due to a block in T cell development at the time when Ly-6A.2 expression would normally be shut off (Bamezai *et al.*, 1995), suggesting that downregulation of Ly-6E/A is important for normal T cell development.

An additional difference between Ly-6A.2/CD2 transgenic and non-transgenic mice was the observation that transgenic thymocytes were prone to homotypic aggregation *in vitro* and could also form aggregates with normal T cells and B cells (Bamezai & Rock, 1995). This aggregation could be specifically blocked by anti-Ly-6A.2 antibodies, suggesting that ligands for Ly-6A.2 exhibit widespread expression throughout the lymphoid system.

Transgenic mice constitutively expressing Ly-6E.1 upon all T cells were generated and similarly examined but showed no evidence of impaired T cell development and no differences in the adhesive properties of their T cells compared with non-transgenic littermate controls. However, both thymocytes and peripheral T cells from CD2/Ly-6E.1 transgenic mice display impaired activation responses to both concanavalin A treatment and CD3ϵ crosslinkage.

2. Results.

The 760 bp Ly-6E.1 cDNA (LeClair *et al.*, 1986) was cloned as an EcoRI fragment into the human CD2 expression cassette p2629 (gift from D. Kioussis) and the orientation of the insert was determined by restriction analysis using NcoI and SstI digestion, both of which are assymetrically located within the Ly-6E.1 cDNA. The 3' LCR of the hCD2 gene from p2694 (gift from D. Kioussis) was added as a 4.5 Kb BamHI-NotI fragment to the hCD2 plasmid containing the Ly-6E.1 cDNA. The hCD2 regulatory elements direct expression of linked genes to all thymocytes and peripheral T cells in a position independent, copy number dependent manner (Greaves *et al.*, 1989).

Plasmid sequences were completely removed by restriction digest with SalI and NotI and the resulting 12 Kb CD2/Ly-6E.1 fragment (figure 57a) was gel purified prior to microinjection. A total of four transgenic founder mice were generated carrying the CD2/Ly-6E.1 construct; a female with approximately 10 transgene copies died at seven weeks of age without breeding; another female with approximately five

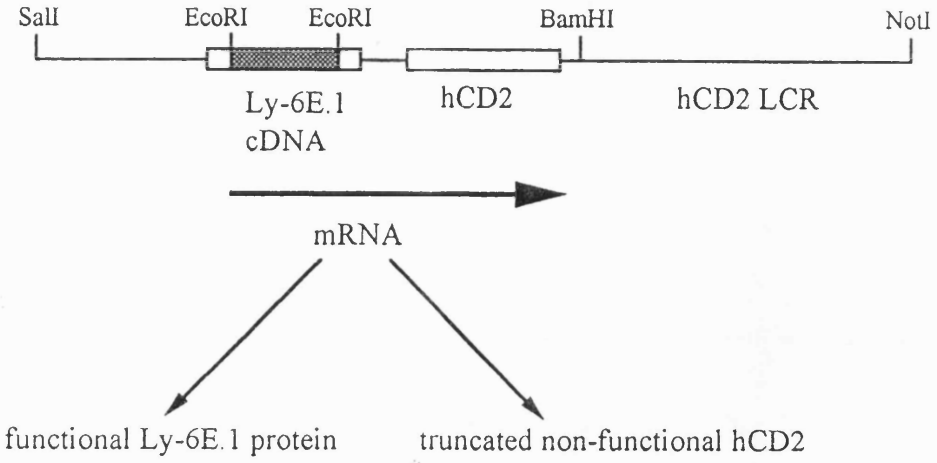
Figure 57. Generation of transgenic mice expressing Ly-6E.1 on all thymocytes.

a. CD2/Ly-6E.1 construct.

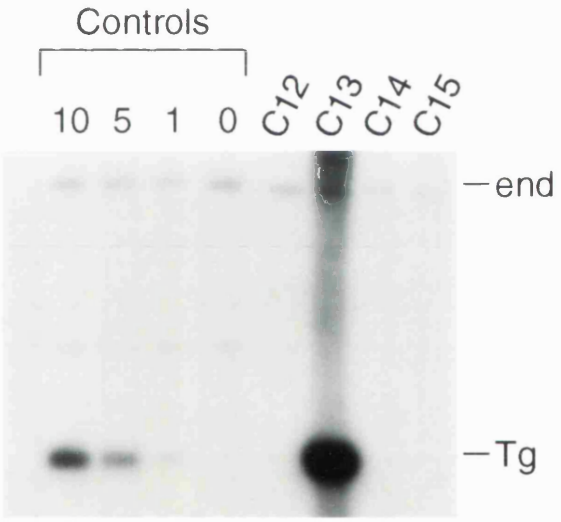
b. Southern blot of EcoR1 digested tail DNA from C12 - C15 founder mice probed with ^{32}P-labelled Ly-6E.1 cDNA. Control samples = dilutions of CD2/Ly-6E.1 plasmid in non-transgenic genomic DNA corresponding to approximately 10, 5, 1 and 0 transgene copies; end = endogenous Ly-6E.1 gene; Tg = Ly-6E.1 cDNA transgene.

c. & d. FACS dot plot of 10^4 thymocytes from non-transgenic (c.) and C13 transgenic (d.) littermates stained with FITC conjugated monoclonal antibody D7, specific for Ly-6E/A (abscissa).

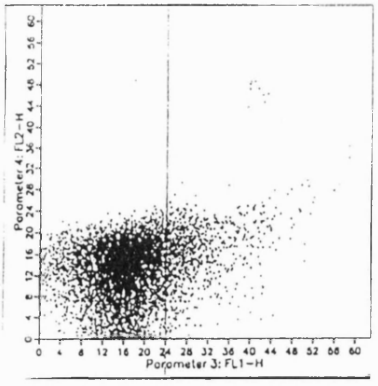
a. CD2/Ly-6E.1 construct



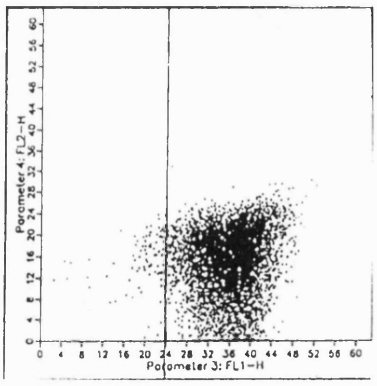
b. Southern blot of C13 founder



c. non-transgenic thymus



d. C13 thymus



D7-FITC

transgene copies bred but produced no transgenic offspring, presumably as a result of mosaicism; a male containing a single transgene copy bred to establish a line in which transgenic Ly-6E.1 cell surface protein expression could not be detected by FACS analysis in the thymus. As this line did not exhibit constitutive thymic Ly-6E.1 expression it was not analysed further; however, one male, containing at least 50 transgene copies (figure 57b), established a line which expressed high levels of Ly-6E.1 on all thymocytes (figure 57d), in contrast to non-transgenic littermates, in which the thymus comprises approximately 10% Ly-6E/A$^+$ cells (figure 57c).

To determine whether T cell development was impaired in CD2/Ly-6E.1 transgenic mice, transgenic and non-transgenic littermate control thymuses were analysed. There were no gross abnormalities evident in transgenic thymuses; the total cell number was normal (figure 59a) as were the CD4/CD8 profiles (figure 58b), suggesting that, in contrast to Ly-6A.2/CD2 mice, T cell development in CD2/Ly-6E.1 mice is normal. In view of the findings of Bamezai *et al.*, CD4$^-$CD8$^-$ double negative thymocytes were analysed in detail by three colour FACS analysis, to determine their CD44/CD25 profile, and found to be normal (figure 58d). There was no evidence of the dramatic block at the CD44$^+$ stage observed in Ly-6A.2/CD2 thymocyte development, suggesting that Ly-6E.1 and Ly-6A.2 may not be functionally equivalent in the thymus.

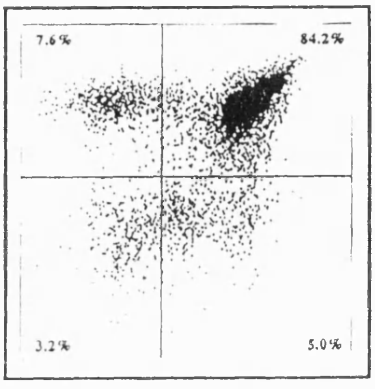
As all CD2/Ly-6E.1 thymocytes express high levels of Ly-6E.1 (figure 57d), their properties of adhesion were investigated, both in a simple homotypic adhesion assay and in a detailed study of adherence aimed at identifying specific cell-cell interractions. By counting at least 3000 thymocytes taken from six individual transgenic animals and five non-transgenic littermates, no evidence of homotypic

Figure 58. FACS plot analysis of thymocyte subsets in CD2/Ly-6E.1 transgenic mice.

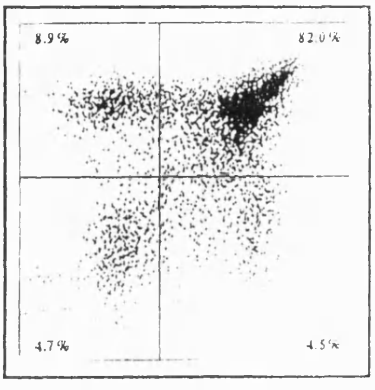
a. & b. Distribution of CD4/CD8 T cell subsets in thymus of C13 (b.) and non-transgenic (a.) littermates. Thymocytes were stained for CD4 (PE-ordinate) and CD8 (FITC-abscissa). 10^4 cells were analysed using a Beckton Dickinson FACScan. Relative fluorescence intensities are shown on a logarithmic scale, with percentages of each subset of cells indicated in the relevant quadrant.

c. & d. Analysis of CD4/CD8 double negative CD44/CD25 thymocyte subsets in CD2/Ly-6E.1 transgenic mice. Thymocytes were stained with anti-CD4, PE; anti-CD8, PE; anti-CD25, FITC; and anti-CD44, Cy-Chrome and analysed by FACScan analysis. PE staining cells were removed from the analysis, with the remaining CD4/CD8 negative cells being analysed for their expression of CD25 (FITC, abscissa) and CD44 (Cy-Chrome, ordinate).

a. non transgenic thymus



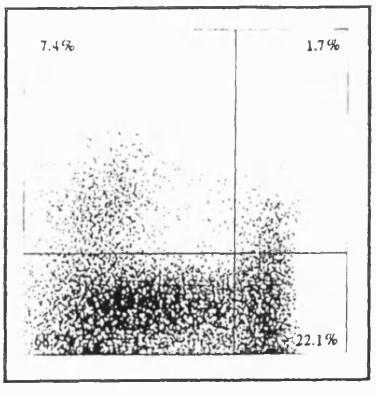
b. C13 thymus



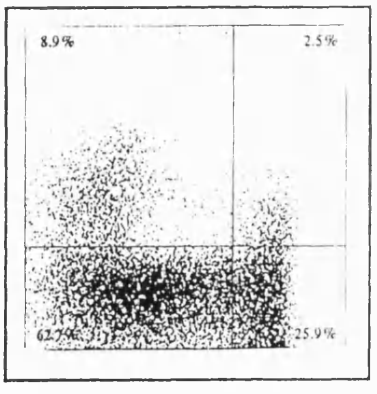
CD4

CD8

c. non transgenic CD4⁻CD8⁻ thymocytes



d. C13 CD4⁻CD8⁻ thymocytes



CD44

CD25

adhesion was found (figure 59b). Similarly, transgenic C13 thymocytes demonstrated similar adherence to NIH3T3 fibroblasts as non-transgenic thymocytes. The use of a panel of blocking antibodies also showed no difference between C13 and normal thymocyte adhesion (not shown). This data suggests that Ly-6E.1 does not bind a ligand expressed on thymocytes, in contrast to Ly-6A.2, nor does it bind a ligand on NIH3T3 cells.

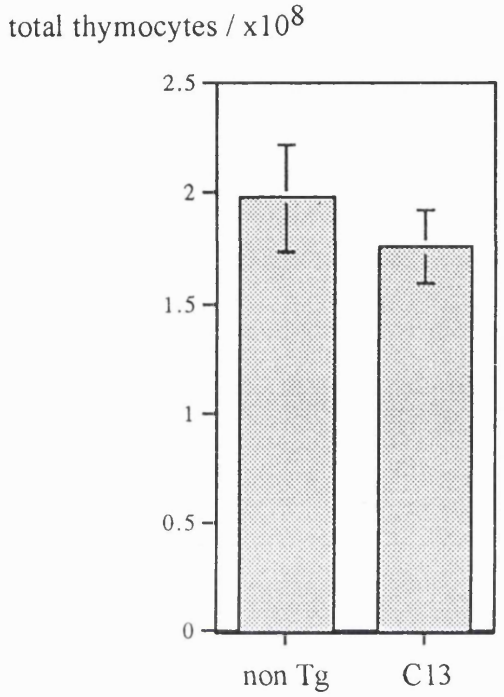
Ly-6E.1 and Ly-6A.2 have been implicated in lymphocyte activation by numerous *in vitro* assays both as activating agents (Malek *et al.*, 1986) and, more recently, in a repressive role (Codias *et al.*, 1992). In order to gain some insight into the possible role in lymphocyte activation of Ly-6E.1 *in vivo*, thymocytes and peripheral T cells from CD2/Ly-6E.1 transgenic mice were subject to a series of activation assays. Spleen and lymph node show normal CD4/CD8 profiles (not shown) consistent with the finding of normal T cell development in CD2/Ly-6E.1 transgenic thymus. However, thymocyte activation by both concanavalin A and immobilised anti-CD3ϵ antibody is impaired in transgenic, compared with non-transgenic cells (figure 60a). To examine peripheral T cell activation, splenic T cells, depleted of B cells and macrophages by adherence to nylon wool (>90% pure), were similarly treated with concanavalin A or immobilised anti-CD3ϵ antibody. CD2/Ly-6E.1 transgenic splenic T cells exhibited a greater reduction in proliferative response to concanavalin A and anti-CD3 than thymocytes (figure 60b). In particular, C13 transgenic splenic T cells were almost totally unresponsive to anti-CD3ϵ treatment, which is absolutely T cell specific, in contrast to splenic T cells from non-transgenic littermates which show vigorous proliferation in response to stimulation through CD3.

Figure 59. CD2/Ly-6E.1 thymuses are normal size and show no evidence of homotypic adhesion.

a. Thymuses from 5 transgenic and 6 non-transgenic littermates of the C13 line were homogenised to single cell suspension and accurate cell numbers obtained for each, using trypan blue exclusion.

b. $1x10^6$ thymocytes from (a.) were cultured over night in 200 μl of RPMI medium with 10% FCS in 96 well tissue culture plates and homotypic adhesion was determined by staining cells with crystal violet and counting aggregates in a haemocytometer.

a. thymus size



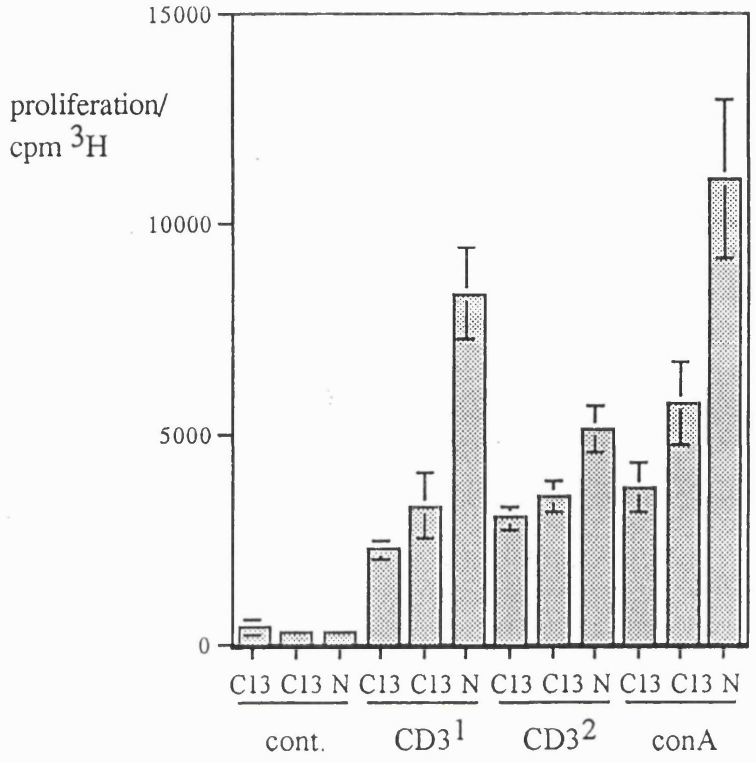
b. homotypic adhesion / per 600 thymocytes

C13	non Tg
2	0
1	1
0	2
1	4
4	3
3	

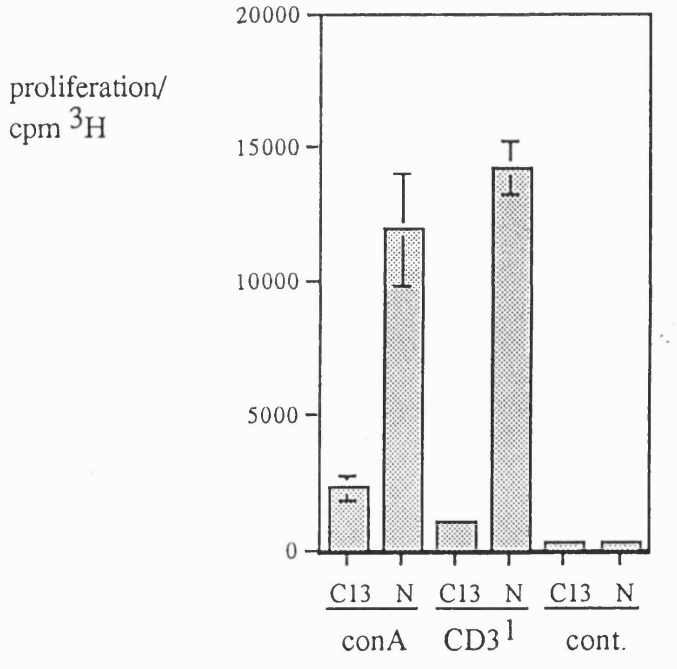
Figure 60. Activation assays using anti-CD3ϵ or conA on T lymphocytes from CD2/Ly-6E.1 transgenic mice.

Thymocytes (a.) and splenocytes (b.) from C13 transgenic (C13) and non-transgenic (N) littermates were cultured, in triplicate at least, at 10^6 cells/ml in 96 well tissue culture plates in RPMI supplemented with 10% FCS and 20mM β-mercaptoethanol. Activation was induced with either conA addition at 2μg/ml or anti-CD3ϵ coating of wells. After 48h, cells were pulsed for 16h with ^{3}H-thymidine and cell proliferation assessed in terms of ^{3}H-thymidine uptake. $CD3^1$ and $CD3^2$ correspond to two different preparations of anti-CD3 monoclonal antibody.

a. thymocyte activation assays



b. splenic T cell activation assays.



This data shows that, after thymic selection, peripheral T cells which overexpress Ly-6E.1 do not respond to either conA or anti-CD3ϵ activation.

3. Discussion.

The results of studies on CD2/Ly-6E.1 transgenic mice have demonstrated that ectopic expression of a transgenic Ly-6E.1 molecule on thymocytes does not affect normal T cell development. In particular, the downregulation of Ly-6E/A, previously thought to be necessary to reduce Ca^{2+} influxes and prevent thymocyte death at the double positive stage (Lee *et al.*, 1994) is not important for T cell development in the case of Ly-6E.1. In contrast, analogous Ly-6A.2/CD2 transgenic mice exhibit a block in T cell development resulting in a profound reduction in thymocyte number. These conflicting results suggest that Ly-6E.1 and Ly-6A.2 are not equivalent within the thymus, a notion supported by examination of their respective binding properties. Ly-6A.2/CD2 and not CD2/Ly-6E.1 thymocytes exhibit homotypic adhesion, suggesting that Ly-6A.2 binds a ligand expressed on all thymocytes, whilst Ly-6E.1 does not.

Despite overtly normal T cell development, CD2/Ly-6E.1 transgenic mice exhibit non-responsiveness in the peripheral T cell population. Splenic T cells and, to a lesser extent, thymocytes, show reduced proliferative responses to both concanavalin A and anti-CD3ϵ. This impairment is unlikely to be due to a direct block in CD3-mediated signalling as T cells develop normally in the presence of overexpressed Ly-6E.1 which wouldn't be expected if CD3 signalling was impaired

(Liu *et al.*, 1993). The Ly-6E.1 induced anergy in transgenic mice may be a reflection of the normal physiological role of Ly-6E.1 in the immune response. Ly-6E.1 is normally upregulated upon T cell activation (Codias *et al.*, 1989) and may provide a mechanism by which an ongoing immune response could be negatively regulated, thereby creating a regulatory feedback loop. Experimental evidence supporting this comes from studies in which a pool of activated T cells was sorted into Ly-6E/A$^+$ and Ly-6E/A$^-$ fractions. The Ly-6E/A$^+$ fraction did not secrete IL-2 when further stimulated, whereas the Ly-6E/A$^-$ fraction from the same pool did (Codias and Malek, 1993). To assess whether peripheral CD2/Ly-6E.1 T cells are permanently disabled, experiments are under way in which exogenous IL-2 is added to the activation assays to attempt to bypass the Ly-6E.1 mediated block.

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