EXPRESSION AND FUNCTION OF HOX HOMEOBOX TRANSCRIPTION FACTORS IN EARLY HEMATOPOIESIS

by

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ABSTRACT

The Hox genes, first identified in Drosophila, are now recognized as key determinants of mammalian development. Recent evidence of Hox gene expression in leukemic cell lines have suggested that these transcription factors also play important roles in regulation of hematopoiesis. Using an improved RT/PCR technique which enables the generation of extended-length and representative cDNA from fewer than 1,000 cells, the expression pattern of Hox genes in highly purified primitive hematopoietic subpopulations was examined. Two different approaches were used. The first exploited the presence of a highly conserved region in the DNA-binding domain of Hox genes that could be targeted with degenerate primers for amplification from cDNA obtained from each purified population and the amplified produced were subsequently subcloned and sequenced. Over 150 Hox sequence containing clones were characterized. *HOXA9, A6, A5, A4, A2, B3, B7, B9* and *C9* were detected in a subpopulation which was highly enriched for very primitive cells detected as long-term culture-initiating cells (LTC-IC) and depleted for clonogenic progenitors. *HOXA10, A9, A7, A5, A4* and *B7* were detected in a subpopulation highly enriched for clonogenic myeloid progenitors, and *HOXA10, A9, A7, A6, A5, B7* and *C8* were found in an erythroid progenitor enriched subpopulation. These data suggested that HOX A cluster genes are widely expressed amongst early hematopoietic subpopulations; in contrast, some genes of the B cluster appear to be restricted to the more primitive LTC-IC containing subpopulation. To better assess the potential differential expression of Hox genes, the initial amplified cDNA from five purified $CD34^+$ subpopulations of bone marrow cells was analyzed by Southern blot using probes for specific Hox genes. With this approach, it was possible to show that expression of *HOXB3* and *B4* was

markedly higher (up to 40 fold) in the most primitive subpopulation than in the more mature subpopulations whereas that of other Hox genes such as *HOXA10, A9* and *B9* was constant in all populations. Taken together, these data suggest that many Hox genes are active in early hematopoiesis and that some of them (i.e. *HOXB3* and *B4*) are possible candidates for regulating stem cell function. To gain further insight into the role Hox genes may play in early hematopoiesis, the effect of overexpression of *HOXB4* was studied in a murine model using a myeloproliferative sarcoma-based retroviral vector carrying the human *HOXB4* cDNA under the control of the 5' viral long terminal repeat. Overexpression of *HOXB4* had proliferative effects on clonogenic progenitors, day 12 CFU-S and cells with marrow repopulating ability (MRA) as assessed by colony replating or recovery from seven day liquid cultures (up to 200 fold over neo-transduced control). To study the possible effects of *HOXB4* overexpression on hematopoietic cells maintained for prolonged periods in vivo, *HOXB4* or neo-transduced marrow cells were transplanted into lethally irradiated syngeneic recipients and reconstitution of various hematopoietic populations analyzed. At 20 weeks post-transplantation, recipients of *HOXB4*-transduced marrow had ~5 fold more clonogenic progenitors per femur than neo controls. To determine if *HOXB4* overexpression affected the expansion of the earliest hematopoietic stem cells (HSC), their numbers were determined by using the competitive repopulation unit (CRU) assay. CRU numbers in recipients of *HOXB4*-transduced bone marrow cells had recovered to 140% of normal levels found in untransplanted mice or some 50 fold higher than in recipients of neo-transduced marrow; all recipients of *HOXB4*-transduced marrow however had normal peripheral blood counts. Southern blot analysis of unique proviral integration patterns in DNA isolated from bone marrow and thymus of secondary recipients confirmed that *HOXB4*-transduced

hematopoietic repopulating cells were totipotent and that they extensively self-renewed. This dramatic effect of *HOXB4* on HSC self-renewal was maintained in serial transplantation experiments. Together, these results indicate *HOXB4* to be an important regulator of very early but not late hematopoietic cell proliferation. The ability of *HOXB4* to reverse the severe decline in HSC numbers suggest an exciting new approach to the controlled amplification of genetically modified hematopoietic stem cell populations.

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ABBREVIATIONS

AMV	avian myeloproliferative virus
ATCC	American Tissue Culture Collection
bp	base pair
BD	Becton Dickinson
BFU-E	burst forming unit-erythroid
BMT	bone marrow transplantation
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CFU	colony forming unit
CFU-E	colony forming unit-erythroid
CFU-GEMM	CFU- granulocyte, erythrocyte, macrophage, megakaryocyte
CFU-GM	CFU-granulocyte, macrophage
CFU-S	colony forming unit-spleen
cGy	centiGray
CLL	chronic lymphocytic leukemia
CRU	competitive repopulation unit
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
dGTP	deoxyguanosine triphosphate
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DTT	1,4-dithioerythritol
dTTP	deoxythymidine triphosphate
Epo	erythropoietin
ES	embryonic stem (cells)

FAB	French American British
FCS	fetal calf serum
5-FU	5-fluorouracil
G-CSF	granulocyte-colony-stimulating factor
GM-CSF	granulocyte-macrophage-colony-stimulating factor
HGF	hematopoietic growth factor
HSC	hematopoietic stem cell
HXM	hypoxanthine-xanthine-mycophenolic acid
IAP	intracisternal A particle
IL	interleukin
KCl	potassium chloride
LTC	long-term culture
LTC-IC	long-term culture-initiating cell
LTR	long terminal repeat
MDR-1	multi-drug resistance-1 or P-glycoprotein
ß-ME	β-mercaptoethanol
$MgCl_2$	magnesium chloride
MIP-1α	macrophage inhibitory protein-1 alpha
MRA	marrow repopulating ability
mRNA	messenger ribonucleic acid
MSCV	murine stem cell virus
NCS	newborn calf serum
OSM	oncostatin-M
pgk	phosphoglycerate kinase
PHA	phytohemagglutinin
R-PE	R-phycoerythrin
RNA	ribonucleic acid

RU	repopulation unit
S.D.	standard deviation
SCCM	spleen cell conditioned medium
SDS	sodium dodecyl sulfate
Taq	Thermus Aquaticus
TBP	Tata-binding protein
TdT	terminaldeoxyribonucleotidyl transferase
TGF-β1	tumor growth factor-beta 1
U	units

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CHAPTER 1

Introduction

1.1 Overview

Every day billions of mature specialized cells found in the peripheral blood of a normal human adult need to be replaced. The burden of the life-long production of these mature elements is carried out by a small number of primitive bone marrow cells constituting less than 0.01% of the total marrow. These primitive cells have the key properties of being able to undergo self-renewal, that is divide and give rise to progeny cells with essentially the same biological properties or they can differentiate and give rise to "committed progenitors" that progressively acquire the specialized characteristics of one of the 10 lineages which constitute human hematopoiesis. This impressive machinery appears to be highly controlled at multiple levels by both positive and negative regulators and, under normal circumstances, assures the life long maintenance of various peripheral blood cell numbers within a relatively narrow range.

While as reviewed below, much progress has now been made in identifying a variety of cytokines that can regulate the cycling status of primitive hematopoietic cells, little is known yet about the genetic mechanisms responsible for the self-renewal and differentiation outcomes of these early cells. Accumulating evidence however points to transcription factors such as Ikaros, PU-1 and GATA-2 as key regulators of early hematopoietic cells function. A group of transcription regulators initially described in the fruit fly as master regulators of embryonic cell proliferation and differentiation was recently found to be expressed in hematopoietic cell lines. These genes contain a helix-turn-helix DNA-binding domain termed the "homeobox" and by analogy to their

function in Drosophila, it has been hypothesized that homeobox genes may also play central roles in the regulation of early hematopoiesis.

As a test of this hypothesis, the research described in this thesis was aimed at identifying whether and which homeobox genes are differentially expressed in normal primitive hematopoietic cells and, subsequently testing the possibles roles such genes may play in early hematopoiesis using retroviral gene transfer to engineer overexpression in murine bone marrow cells. Following a brief review of the current understanding of the organization and regulation of the hematopoietic system and its cellular constituents, the next sections of the introduction focus on two topics that are central to this work: Hox homeobox genes and hematopoietic stem cells.

1.2 Hematopoiesis, its cellular constituents and regulation

1.2.1 Ontogeny of the hematopoietic system

In mammals, hematopoiesis appears to derive from the mesodermal layer of the forming embryo. Blood-forming cells originate in the yolk sac (extra-embryonic) and, at specific times, migrate to the fetal liver and ultimately to the bone cavities (bone marrow) (Moore and Metcalf, 1970; Toles et al., 1989). The extra-embryonic origin of the definitive hematopoietic system has recently been challenged however by studies showing a potential early intraembryonic site of hematopoiesis (Godin et al., 1993; Medvinsky et al., 1993).

1.2.2 Cellular constituents of the mammalian hematopoietic system

A multiplicity of both in vivo and in vitro assays are now available for the detection of hematopoietic cells found at various levels within the hematopoietic hierarchy (reviewed in Eaves, 1995). One of the first hematopoietic assays to be

developed was the spleen-colony-forming-unit (CFU-S) assay (Till and McCulloch, 1961). This assay detects cells present at a low frequency in murine bone marrow that, when intravenously injected into myelo-ablated recipients, can generate macroscopic hematopoietic nodules on the spleen 9-14 days later. These colonies were shown to be clonal (Wu et al., 1968), to contain multiple myeloid lineages (Till and McCulloch, 1961) and, importantly, in many cases were able to generate similar colonies upon transplantation into secondary recipients (Siminovitch et al., 1963).

CFU-S were thus initially considered as HSC because they shared important characteristics which were attributed to these cells including high proliferative potential, multipotentiality and self-renewal ability (Siminovitch et al., 1963). It is now clear that most CFU-S cells are committed myeloid progenitors (Jones et al., 1989) that can be physically separated from cells with greater long-term repopulating potential (Mulder and Visser, 1987; Spangrude et al., 1991; van der Loo et al., 1994; Visser and de Vries, 1988). Nevertheless studies focusing on CFU-S have played key roles in developing concepts of the organization and regulation of mammalian hematopoiesis (Till et al., 1964).

A wide range of myeloid cells with various potential for proliferation and differentiation can be quantitated based on their ability to form various types of colonies in semi-solid culture supplemented with different cocktails of crude or purified hematopoietic growth factors (reviewed in Eaves, 1995). Such cells have been operationally defined as "clonogenic progenitors" and have been instrumental in the characterization of cytokines acting on hematopoietic cells. Using unmanipulated bone marrow cells, most progenitors that can be recognized in these cultures are oligo- or uni-potential and have limited proliferative potential with no or minimal self-renewal potential. More recently

additional in vitro assays have opened up the identification of bone marrow cells with B but not T lymphoid potential (Suda et al., 1989).

Another group of in vitro assays aimed at detecting earlier myeloid cells rely on in vitro simulation of the bone marrow microenvironment by "feeder cells". This group of assays pioneered by Dexter (Dexter et al., 1977) is based on the concept that cells with limited proliferative potential (i.e. the clonogenic progenitors described above) will be "extinguished" after a prolonged period of culture (weeks) whereas clonogenic progenitors present at the end of these "long-term cultures" (LTC) must be derived from a more primitive population which can be accurately quantitated by limiting dilution analysis procedures (Ploemacher et al., 1989; Sutherland et al., 1990). More recently the possibility of detecting early lympho-myeloid hematopoietic cells was demonstrated by the identification of cells with myeloid and B lymphoid potential which can be maintained in a two-step long-term "switch" culture assay (Lemieux et al., 1995).

Other in vitro and in vivo assays that also appear to recognize primitive hematopoietic cells (Breems et al., 1994; Gordon et al., 1987; Harrison et al., 1993; Hodgson, 1979; Leary, 1987; McNiece et al., 1989; Ploemacher et al., 1991; Szilvassy et al., 1990) are listed in Figure 1.1 but are not explored in this thesis and therefore are not further described.

Although it is clearly possible to assay hematopoietic cells with different potential for proliferation and differentiation, it is important to stress that a specific cell can probably be detected in more than one of the available assays (Figure 1.1). As an example of this, a subset of clonogenic progenitors identified in a direct colony assay can also give rise to spleen colonies (Humphries et al., 1979).

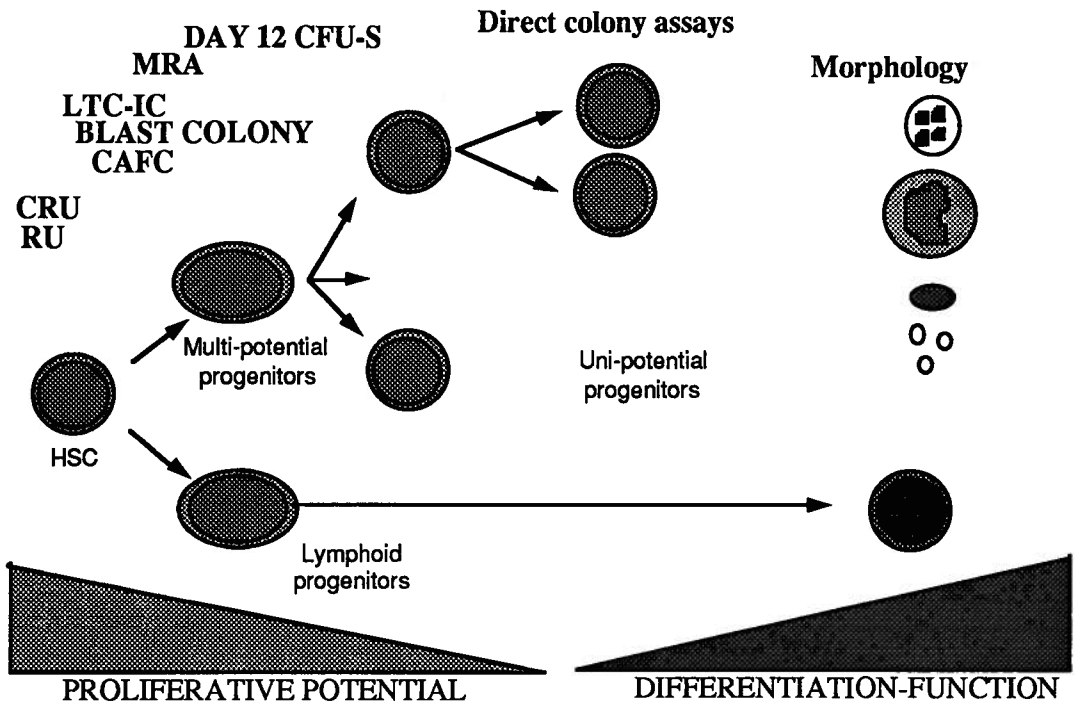

Figure 1.1 Schematic representation of the hematopoietic hierarchy.
Some of the assays used to evaluate various progenitor pools are depicted in bold. HSC, hematopoietic stem cells; MRA, cells with short-term marrow repopulating ability; BCA, blast colony assay; LTC-IC, in vitro assay for cells with ability to initiate in vitro long-term cultures; CAFC, in vitro assay for cobblestone forming area; CRU, competitive repopulation unit assay; RU, repopulation unit assay; day 12 CFU-S, colony-forming-unit spleen obtained 12 days after inoculation.

1.2.2.1 The hematopoietic stem cell (HSC)

1.2.2.1.1 Definition

The HSC can be operationally defined as a long-term repopulating cell which, in most cases, has lymphoid (T and B) and myeloid potential (Orlic and Bodine, 1994). Such a definition has relevance in the context of bone marrow transplantation (BMT) and gene therapy and will be retained through the thesis.

Early demonstration of the existence of such cells includes detection of common unique chromosomal markers in lymphoid and myeloid transplanted bone marrow cells (Abramson et al., 1977; Wu et al., 1968). Exploiting the ability

of retroviruses to randomly integrate in the genome, many investigators have now used replication-defective retroviral vectors to infect HSC and document by Southern blot analysis their lymphoid (T and B) and myeloid cell repopulating potential (Jordan and Lemischka, 1990; Keller and Snodgrass, 1990).

1.2.2.1.2 Quantitation of HSC

At the present time, it is not possible to directly quantitate HSC by the presence of unique or combinations of surface markers and physical properties although purification strategies based on such properties enable significant enrichment (discussed below) (Uchida et al., 1993). Detection and quantification of such cells therefore must still rely on functional assays (Orlic and Bodine, 1994).

Strict adherence to the rigorous operational definition of a long-term repopulating cell however requires that recipients are observed for prolonged periods of time (many months). One approach to accelerate evaluation of HSC has been the measurement of the graft radioprotective ability (Jones et al., 1989; Spangrude and Johnson, 1990). Such assays however primarily reflect the short-term radioprotective ability of the graft and further, it has now been documented that such cells can be physically separated from those with long-term repopulating ability (Jones et al., 1990). Life-sparing assays furthermore do not necessarily take into account host contributions to hematopoietic recovery which in some situations can be substantial.

One powerful approach to quantitate HSC based on principles of competitive repopulation was developed by Harrison ((Harrison et al., 1988); see Harrison et al., 1993 for review).This assay consists of comparing the long-term repopulating abilities of two populations of transplanted hematopoietic cells that can be distinguished by phenotypic, genetic or biochemical markers.

The first of these populations is called the "competitor" and normally consists of a predetermined number of normal bone marrow cells that, by themselves will readily repopulate the recipient. Various numbers of bone marrow cells from a second population termed the "donor" are simultaneously injected with high numbers (generally ~1 x 10^6) of competitors. The frequency of HSC or "repopulation units (RU)" in the "donor" is unknown and thus evaluated by the relative contribution to hematopoiesis of both cell populations by using the ratio of donor to competitor-derived hematopoiesis[1] (Harrison et al., 1993). In contrast to the CRU assay which uses limit dilution principles (see below), this procedure uses high cell numbers and thus reduces the demand for short-term repopulation imposed on the "tested" population. Therefore, this assay is particularly suited for the comparison of the relative numbers of RU in two different purified subpopulations of hematopoietic cells (Harrison et al., 1993).

In an effort to obtain a quick and accurate quantitative evaluation of HSC, Szilvassy et al., (Szilvassy et al., 1990) developed an assay that combines limiting dilution analysis procedures and competitive repopulation to quantitate HSC frequency in unknown "test" population. In its original description, various numbers of test cells were co-injected with "helper compromised" syngeneic cells into multiple irradiated recipients. The helper cells assure short term hematopoietic reconstitution and are said to be "compromised" because they have lost most of their long-term repopulation ability as a result of serial transplantation (see later in text for further explanation of this phenomenon). Because lympho-myeloid elements that originate from the "test" cells can be identified either by genetic markers (Y probe for male graft into female recipients (Szilvassy et al., 1990)) or by cell surface antigens (Ly5.1 or Ly5.2:

---

[1]The following formula is used to calculate RU frequency: RU= (% of hematopoiesis which is donor-derived / % competitor) x (# of competitor cells injected / 10^5)

(Rebel et al., 1994; Spangrude and Scollay, 1990; Szilvassy and Cory, 1993), it is possible to identify recipients that are significantly (i.e. >5%) repopulated by a lympho-myeloid "test" cell which contributes to long-term repopulation. The HSC operationally defined by this assay is termed a CRU (competitive repopulation unit) and its frequency is established from the proportion of mice that meet the repopulation criteria described above when limiting dilution is reached based on Poison statistics. More precisely, the exact frequency of CRU in a test population is: CRU frequency = 1 / (number of bone marrow "test" cells that repopulated exactly 63% of the irradiated recipients). Interestingly, CRU frequencies determined five weeks after transplantation were shown to be virtually identical to those obtained at later time points (Szilvassy and Cory, 1993) suggesting that this assay can be used to evaluate true long-term repopulating HSC after a little more than a month. For simplicity, the assay was recently modified to allow use of normal bone marrow cells (i.e. from unmanipulated normal donors) as helper cells and, after adjustment of the repopulation criteria, this has proved to be just as reliable as the method initially described ((Rebel et al., 1994), see also chapter 4).

Together, the availability of assays to identify and quantitate HSC has opened the way to characterize their properties, phenotype and regulation which will be discussed in the following sections.

1.2.2.1.2 Properties of HSC

1.2.2.1.2.1 Cycling status of HSC

One characteristic that distinguishes HSC from other more mature populations such as day 12 CFU-S is their cycling state. The majority of HSC under homeostatic condition are quiescent as elegantly demonstrated by studies

using an S phase-specific agent (5-FU) in which HSC detected by competitive repopulation assay but not CFU-S, are spared by a single injection of this agent (Hodgson and Bradley, 1979; Lerner and Harrison, 1990). Interestingly, 5-FU appears to recruit HSC into cycle since they become very sensitive to a second dose of 5-FU given 3 to 5 days later (Harrison and Lerner, 1991).

1.2.2.1.2.2 HSC self-renewal potential.

Several studies utilizing retroviral marking have now unequivocally demonstrated by the presence of identical proviral integration patterns in bone marrow and thymus tissue of primary and secondary recipients that HSC can self-renew (Jordan and Lemischka, 1990; Keller and Snodgrass, 1990; Lemischka et al., 1986).

Some HSC that have been "tracked" in these studies were proven to have extensive proliferative potential. Keller and Snodgrass, (1990) for example demonstrated that a single HSC could completely reconstitute all lineages of a recipient for more than 10 months and further that transplantation of this marrow into a secondary recipient resulted in almost complete reconstitution (>80%) by this same clone for another 17 months. Although studies such as these have clearly documented self-renewal division in the HSC compartment, they failed to quantitate the magnitude of self-renewal events because the absolute number of stem cell regenerated in recipients was not evaluated.

1.2.2.1.2.3 Repopulating potential of HSC.

Although HSC can self-renew, there is now considerable evidence to suggest that this property is not unlimited.

Transplantation of small (10^5) or moderate (10^7) numbers of murine bone marrow cells into lethally irradiated recipients rapidly results in normal bone marrow cellularity (~2-2.5 x 10^7 cells/femur) which persists for the life (>9 months) of the animal (Mauch and Hellman, 1989). Day-8 CFU-S also recover

to near normal levels after transplantation of 10^6-10^7 cells (Harrison et al., 1978; Mauch and Hellman, 1989) but are reduced to ~65% of normal values in recipients of 10^5 bone marrow cells (Mauch and Hellman, 1989). Although bone marrow cellularity and day-8 CFU-S are regenerated to normal numbers following BMT with ~10^6 cells or more, studies using competitive repopulation as described before (see section 1.2.2.1.2) have shown a dramatic (i.e. 3-20 fold) reduction in proliferative ability of HSC recovered even after a single transplantation of 10^5-10^7 cells (Harrison, 1982; Harrison et al., 1978). Interestingly, a lesser but still significant reduction was observed when a higher number of cells was transplanted (10^8 cells which represent about 1/2 of the hematopoietic system of an adult mouse). It has been argued that this loss of long-term repopulating ability may result from damage to the recipient's microenvironment inflicted by the conditioning regimen (i.e. irradiation, etc.). In order to exclude this possibility, experiments have also been conducted using a recipient mouse strain known to have a normal microenvironment but poorly competitive early hematopoietic cells (McCulloch et al., 1964) that are now known to have a defective c-Kit ligand receptor (Chabot et al., 1988). Transplantation into this anemic strain (W/W^V) can therefore be done without conditioning regimens and this was used by various investigators to prove that the loss of long-term repopulating ability of transplanted marrow is due not to a microenvironmental defect induced by the conditioning regimen (Gardner et al., 1988; Harrison, 1982) but rather to the transplanted cells themselves.

The mechanisms for this apparent reduction in repopulating ability following bone marrow transplantation is not clear. Whether this reduction is due to a depletion in the number of HSC or to a general decrease in their proliferative ability for example, remains unresolved.

One possibility to explain this loss in repopulating ability of transplanted HSC is that the proliferative stress imposed upon newly generated HSC shifts the balance from self-renewal division to differentiation and that the HSC pool is not fully recovered. One recent intriguing hypothesis to explain the decreased proliferative ability of transplanted HSC arises from accumulating evidence indicating that telomere length reduction occurs with every hematopoietic cell division and hence could contribute to the decreased proliferative potential of transplanted HSC (Vaziri et al., 1994). However, telomere shortening has not been demonstrated in the inbred mice typically used in experimental hematology (Greider, 1995; Kipling and Cooke, 1990).

Together these results have relevance to clinical bone marrow transplantation studies and in fact predict the possibility of a persistent decline in HSC in recipients. Since we are still in the early days of clinical BMT (~20-25 years), it will be important to continue to monitor recipients of a bone marrow graft in order to rule out that HSC depletion will not eventually lead to bone marrow failure.

1.2.2.1.2.4 Stem cell "aging".

The demonstrated decline in HSC repopulating ability after bone marrow transplantation raised the question whether such a phenomenon would also be observed in unmanipulated HSC derived from "old" untransplanted mice.

By using a technique of competitive repopulation, it was found that bone marrow from old mice (2 to 2.5 years) compete equally well against "competitor cells" as that of young (3-6 months) donors (Harrison, 1982). This equal competitiveness was demonstrated at various time points (including 18 months after transplantation). Furthermore, it was also shown that bone marrow cells derived from "old" mice performed equally in serial transplantation experiments

as those derived from younger animals. For example, repopulation levels below 5% are frequent after two serial transplantations with young or old cells.

These results however are at the population level and using cell numbers that are in large excess of the minimum requirements leave open the possibility that individual HSC may have finite self-renewal capacity but that in the normal lifespan their limit is not approached.

1.2.2.1.2.5 Heterogeneity of the HSC.

By several criteria, HSC appear to be heterogeneous. Fetal HSC, for example, manifest increased ability compared to adults to competitively repopulate myeloablated recipients (Rebel, 1995). HSC are also heterogeneous in their expression of surface molecules. Studies of Spangrude et al. (1988) and Uchida and Weissman (1992) for example showed that long-term repopulating cells are found within a minute (<0.05%) population of bone marrow cells that express Sca-1^{+}Thy1.1^{lo}Lin^{-} (see next section). Further purification of these cells into functionally distinct populations was possible based on their expression of Mac-1, CD4 and c-kit (Morrison and Weissman, 1994). Although the Sca-1^{+}Thy1.1^{lo}Lin^{-}c-kit^{+}Mac-1^{-}CD4^{-} population was shown to be highly enriched for long-term repopulating cells and fairly depleted of cells with short-term repopulating potential it was still possible to detect, albeit at lesser frequency, cells with long-term repopulating potential in the two other populations (Mac-1^{lo} and Mac-1^{lo}CD4^{lo}) (Morrison and Weissman, 1994) suggesting that HSC are heterogeneous in their expression of certain surface molecules (also reviewed in Uchida et al. (1993)).

Heterogeneity in the contribution of some HSC to specific lineages has also been documented. Jordan and Lemischka carefully followed, by retroviral tagging, the behavior over time of 142 HSC in 63 different recipients and found by blood sampling (from 4 to 16 months post-transplantation) that a significant

proportion of HSC were not contributing equally to lymphoid and myeloid repopulation and further that some HSC showed unstable behavior (i.e. an apparently myeloid-restricted clone can also show lymphoid differentiation at a later time point, etc.). Importantly however, the most frequent HSC behavior (35% of the clones) observed in their studies was that of a cell that persistently and equally repopulated the lymphoid and myeloid "arm" of the hematopoietic system.

HSC may be also heterogeneous in their potential for long-term reconstitution (proliferation?). Experiments utilizing retroviral tagging have revealed that some HSC only contribute to repopulation for the first 3-9 months that follow transplantation whereas others contribute for the duration of the recipient's life (Jordan and Lemischka, 1990; Keller and Snodgrass, 1994).

Whether the heterogeneity observed, however, represents true or intrinsic differences in potential versus differences in the expression of this potential (e.g. premature differentiation) due to stochastic processes remains unclear. Further clarification of the nature of the observed heterogeneity will undoubtedly benefit from studies utilizing improved purified preparations.

1.2.2.1.3 Purification of murine HSC

The isolation of pure HSC has been long sought by several groups as a way of ultimately defining / quantitating HSC by phenotype and facilitating more detailed characterization of their properties. Importantly, the availability of such purified cells would open the way to studies aimed at identifying the genetic determinants that characterize a stem cell. Numerous strategies to isolate these cells have been successfully explored. These strategies include: 1- purification by physical differences between HSC and more mature cells (Jones et al., 1989), 2- the presence of high levels of P-glycoprotein products on the surface

of these cells which is involved in exclusion of the vital dye rhodamine-123 (Ploemacher and Brons, 1988b; Spangrude and Johnson, 1990) and, 3- the use of differentially expressed surface molecules that can interact with various lectins (Ploemacher and Brons, 1988a) or that can be recognized by monoclonal antibodies (Morrison and Weissman, 1994; Spangrude et al., 1988; Szilvassy and Cory, 1993). The combined use of some of these approaches now allows the enrichment of HSC by hundreds of fold to levels of purity that can in some instances reach 10% (Morrison and Weissman, 1994) .

Although variability in the expression of some of the cell surface antigens can occur between different mouse strains (Spangrude and Brooks, 1993), the following surface markers are usually found on murine cells with long-term repopulating potential: Ly6A/E$^+$ (Sca-1), Thy-1^{lo}, lineage$^-$ (Mac-1$^-$, Gr-1$^-$, Ly-1$^-$, B220$^-$): (Spangrude et al., 1988) , wheat germ agglutinin$^+$, (Ploemacher and Brons, 1988a; Visser et al., 1984), c-kit$^+$ (Morrison and Weissman, 1994; Okada et al., 1992) and very likely CD34$^+$ (Krause et al., 1994).

1.2.2.2 Towards defining human HSC and their properties

The in vivo assays for murine HSC described above are not currently available for evaluation of human HSC. The recent development of immunocompromised mice which support the growth of normal human (Kamel-Reid and Dick, 1988; Kyoizumi et al., 1992; Lapidot et al., 1994) and leukemic (Lapidot et al., 1994) cells however, may now open the way for eventual in vivo quantitation of human HSC using a "humanized" CRU assay.

Methods for evaluation of primitive human hematopoietic cells have been developed based on the detection of cells capable of initiating and sustaining clonogenic progenitor output in appropriate long-term culture conditions

analogous to that described for mice in section 1.2.2. Evidence that cells which initiate these cultures (called LTC-IC for LTC-initiating cells) overlap with HSC include their relative insensitivity to cell-cycle agents and the possibility to selectively enrich these by purification strategies (see below). Further support comes from murine studies which have shown LTC-IC frequency to be similar to that of CRU (Lemieux et al., 1995) and also that CRU can self-renew under the same LTC conditions that enables detection of LTC-IC (Fraser et al., 1992).

Considerable progress has been made in the methods and reagents available for purification of primitive human bone marrow cells (reviewed in Lansdorp and Thomas, 1991). The discovery of a sialomucin surface antigen, CD34, (Civin et al., 1984) expressed on ~1-4% of bone marrow cells was a major step toward purification of primitive human bone marrow cells. Cells lacking CD34 fail to give rise to colonies in direct assay, cannot initiate long-term cultures and have no long-term repopulating ability as assessed in non-human primates (Berenson et al., 1988).

Following the discovery of the CD34 surface antigen, much effort has been invested in the further refinement of the separation of this population into functionally distinct subpopulations (Lansdorp et al., 1990). For example, it was shown that $CD34^+$ cells that co-expressed CD45RA and low levels of CD71 were highly and selectively enriched in granulocyte-macrophage clonogenic progenitors and that cells which did not express any of these two cell surface molecules were highly enriched for LTC-IC. Further discrimination of this $CD34^+CD45RA^-CD71^-$ population was recently made possible by the use of an antibody directed against the glycoprotein, CD38, (Reinherz et al., 1980). Low levels of CD38 expression correlated with cells giving rise to undifferentiated colonies in liquid cultures (Terstappen et al., 1991) and also with cells which

preferentially responded to a combination of hematopoietic growth factors (Rusten et al., 1994). As shown in the results section of chapter 3, $CD34^{+}lin^{-}$ $CD38^{lo}$ bone marrow cells are also highly enriched in LTC-IC and relatively depleted in clonogenic progenitors. Other surface antigens such as c-kit^{lo} (Gunji et al., 1993), HLA-DR^{lo} (Brandt et al., 1990; Sutherland et al., 1989), Thy-1^{+} (Baum et al., 1992; Craig et al., 1993) and $CD4^{lo}$ (Louache et al., 1994) have been shown to be present on primitive human bone marrow cells and useful for their purification.

1.2.3 Regulation of hematopoiesis

As discussed above, steady state hematopoiesis requires the daily production of an enormous number of blood elements (~5×10^{8} and 5×10^{11} respectively for adult mouse and human, (Abkowitz et al., 1995)) which originate from a very small population of bone marrow cells. Regulation of this complex process can be simplistically pictured as the combined effects of external influences (cell-cell, cell receptor-growth factor ligand and cell-matrix interactions), and intracellular signaling events and consequent transcription factors regulation which can initiate or repress transcription of multiple genes whose products are ultimately the effectors of proliferation and differentiation (Orkin, 1995). The relative contribution of each of these various levels of control is not clear. For example, to what extent do external factors determine versus permit differentiation? Conversely, are there key events only determined by intrinsic processes? Is the role of external factors only to promote survival and trigger proliferation? Likely central to these regulatory mechanisms however are the molecules that control gene expression, the transcription factors. The following sections provide an overview of some of these regulators and examines in

detail a group of transcription factors belonging to the homeobox gene family that are strong candidates for being hematopoietic regulators.

1.2.3.1 Regulation of hematopoiesis by external factors

Hematopoietic growth factors (HGF) currently represent the most extensively described external regulators of the hematopoietic system (see Metcalf, 1993 for a detailed review). HGF are necessary for proliferation, survival and possibly differentiation of hematopoietic cells (Metcalf, 1993). The number of characterized HGF now exceed 20 and the list is still growing (Eaves, 1995; Metcalf, 1993). Besides erythropoietin (Krantz, 1991; Spivak, 1986) and also possibly thrombopoietin (Bartley et al., 1994; Foster et al., 1994; Gurney et al., 1995; Lok et al., 1994), the majority of these factors appear to have overlapping functions with the potential to act on progenitors of different committed lineages (Metcalf, 1993). For some of these factors, the nature of this "redundancy" may be due to the sharing of a common receptor subunit (e.g. IL-3, IL-5, GM-CSF and IL-6, IL-11, LIF, OSM respectively share a unique common β-subunit: (Miyajima et al., 1993; Taga and Kishimoto, 1992)) which appears to be involved in intracellular signaling.

An interesting notion that has come out of the numerous studies done on growth factors is that primitive hematopoietic cells only proliferate in the presence of two (or more) growth factors (recruitment) (Migliaccio et al., 1991; Miura et al., 1993) whereas more mature progenitors can respond to a single growth factor (Metcalf, 1993). Although primitive hematopoietic cells can be recruited into proliferation by the combination of various cytokines, net expansion of HSC has not yet been documented (Bodine et al., 1992; Li and Johnson, 1994; Muench et al., 1993; Sutherland et al., 1993). This may imply the existence of as yet unidentified HGF, but also raises the possibility that HSC

self-renewal may be predominantly regulated by other mechanisms such as signaling through adhesion molecules and / or by poorly understood "internal regulators".

Molecules with the potential to inhibit primitive hematopoietic cell proliferation have also recently emerged as key regulators (Graham et al., 1990). TGF-β1 and MIP-1α have been best characterized and shown to have potent inhibitory effects on CFU-S cycling in vivo (Lord et al., 1992), in vitro (Bodine et al., 1992), and on primitive hematopoietic progenitors (Cashman et al., 1990; Eaves, 1991; Hatzfeld et al., 1991; Jacobsen et al., 1994).

Therefore through both positive and negative regulators, proliferation of hematopoietic cells can be externally controlled by various cytokines. These cytokines exert their proliferative / inhibitory effects through cellular receptors which upon activation transmit the appropriate signals to activate nuclear factors via a complex signaling system (signal transduction). The nuclear factors that activate "effector" genes involved in proliferation and differentiation of hematopoietic cells can therefore be "influenced" by external regulators but, in some instances, may also independently regulate these processes. After a brief overview of the role these nuclear factors play in the initiation of transcription, the next section will concentrate on some of the transcription factors that have been implicated in the regulation of proliferation and differentiation of mammalian hematopoietic cells.

1.2.3.2 Regulation of hematopoiesis by intracellular factors

Unlike procaryotic organisms, eukaryotic RNA polymerase II - the enzyme responsible for transcription of messenger RNA - cannot initiate transcription by itself. It requires several general transcription factors (TFIIA to TFIIJ, see Figure

1.2) for DNA binding and transcription initiation. For most genes, the core promotor includes a TATA box (TATAAA) which is located approximately 25 bp upstream of the transcriptional start site (Breathnach and Chambon, 1981). Thus, the TATA box represents the minimal promotor to allow transcription. Additional levels of regulation are the result of other nuclear factors such as transcriptional activators that bind to a multiplicity of regulatory regions some of which are found in the proximity of the TATA box while others can be very distant, and together recruit the RNA polymerase II complex to initiate transcription as illustrated in Figure 1.2 (see Ernst and Smale, 1995 for a detailed review). Other nuclear factors termed transcriptional repressors can negatively regulate gene expression via several mechanisms. For example, transcriptional repressor of the Id gene family can repress transcription by dimerization with known activators such as E2A (Kreider et al., 1992).

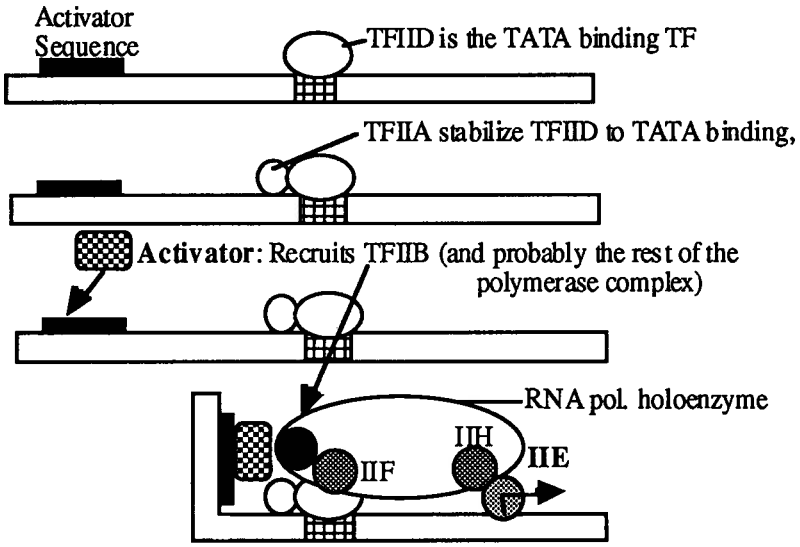

Figure 1.2. Schematic representation of initiation of transcription by the RNA polymerase II.

The activator sequence (black box) binds transcription activators (rectangle) which, in its most simple form contains a DNA-binding domain and an "activator" domain that recruits the transcriptional complex through TFIIB (black circle) to the TATA box. TFIIE is involved in initiation of transcription. Abbreviations: pol., polymerase; TFIIA to IIH, transcription factor IIA to IIH; TFIID is the TATA-binding protein (TBP). Adapted from Molecular Biology of the Cell 3rd ed., Chapter 9, Garland publishing N.Y.

The transcriptional activators (and also some repressors) characteristically contain distinct DNA-binding domains which have been used to group them into various families. Some of the well characterized families include the zinc finger proteins which includes members such as *Krox-20*, Ikaros, etc., (see later in text); basic leucine zipper proteins (e.g. Fos, Jun, etc); the helix-loop-helix proteins such as *SCL* (see later); the helix-turn-helix proteins (homeobox genes, see later); and many more (reviewed in Ernst and Smale, 1995; Harrison, 1991; Nichols and Nimer, 1992)

The recognition of the involvement of some of these nuclear factors in various leukemias has provided strong arguments implicating them in hematopoietic cell growth and differentiation (reviewed in Nichols and Nimer, 1992). In several hematopoietic malignancies for example, the products from these genes were either found at increased and / or persistent levels due to their transcriptional activation by various mechanisms (chromosomal translocations e.g. c-myc under the control of an immunoglobulin promotor in Burkitt lymphoma: t(8;14) (Cesarman et al., 1987); retroviral insertion e.g. *Spi-1* (Moreau-Gachelin et al., 1988)) or found in the form of a fusion proteins (e.g. t (1;19): *E2A-PBX*, (Kamps et al., 1990)) resulting from chromosomal translocations that unite two normally distant genes (reviewed in Nichols and Nimer, 1992). Recently, more direct strategies aimed at detecting proteins that display DNA-binding activity to specific regulatory sequences of erythroid-specific genes previously identified as being mutated in a hereditary hemoglobin regulation disease (Martin et al., 1989) have been successfully used to identify hematopoietic "active" transcription factors (e.g. GATA-1, (Tsai et al., 1989)).

Further insights into the roles such transcription factors may play in hematopoiesis have been obtained from the modulation of their expression using gene disruption or overexpression strategies. For example GATA-1 deficient cells could not contribute to erythropoiesis in chimeric mice (Pevny et al., 1991) and studies of in vitro differentiation of GATA-1 deficient (X-linked) embryonic stem (ES) cells have demonstrated the absolute requirement for this factor in early hematopoiesis. Interestingly these same studies showed that definitive hematopoiesis could be partially rescued by other GATA-like containing proteins (Weiss et al., 1994). Similar in vivo experiments have demonstrated the absolute requirement for the transcription factor NF-E2 in megakaryocytic development (Shivdasani, 1995). Genetic disruption experiments have also demonstrated the necessity for rbtn2 in primitive erythropoiesis (Warren, 1994) and of SCL / Tal-1 in primitive and definitive erythropoiesis (Shivdasani et al., 1995). Genetic disruption of another GATA protein, GATA-2 proved to be essential for definitive multilineage development suggesting a role for GATA-2 in early hematopoietic cells (Figure 1.3) (Tsai et al., 1994). The possible function(s) of many other transcription factors such as Ikaros (see later), PU.1 (see later), Pax-5 and E2A, (Bain et al., 1994); c-myb, (Mucenski et al., 1991); Id1, (Sun, 1994) have now been analyzed using similar systems (reviewed in Orkin, 1995).

Although studies of transcription factors active in hematopoietic tissues have only begun recently, no single factor that shows strict specificity for the hematopoietic system has yet emerged (Georgopoulos et al., 1992; Yomogida et al., 1994). Thus, as for most other mammalian differentiation systems, the concept has emerged that cellular identity is defined by the combinatorial action of regulatory molecules expressed in a time and site specific manner and also by the accessibility of the DNA targets they regulate.

Results obtained from these studies however point to the existence of a hierarchy of transcription factors in the hematopoietic system. Two factors potentially highly positioned in this hypothetical hierarchy are Ikaros and PU.1. Ikaros is a member of the zinc-finger family of transcription factors and has protein sequence similarity (57 amino-acids) to Hunchback– the product of one of Drosophila's segmentation genes (see below; Georgopoulos et al., 1992). The Ikaros gene gives rise to five or six alternate transcripts differentially expressed in lymphoid cells (Georgopoulos et al., 1994; Hahm et al., 1994; Molnar and Georgopoulos, 1994) and can activate transcription of lymphoid specific genes such as CD3δ chain and terminal deoxynucleotidyltransferase (TdT) (Georgopoulos et al., 1992; Hahm et al., 1994). Genetic disruption of Ikaros by gene targeting in embryonic stem cells has resulted in complete disappearance of T, B and NK cells in the homozygous Ikaros deficient mice suggesting that this gene is obligatory for the generation of early lymphoid-repopulating cells (Georgopoulos et al., 1994).

PU.1 is the product of the proto-oncogene Spi-1 (mutagenesis site for Friend virus) and is a member of the Ets family of transcription factors (Paul et al., 1991). It is expressed in monocytic, B lymphoid and erythroid cells (Galson et al., 1993; Klemsz et al., 1990). PU.1 has been implicated in the control of expression of lineage-specific genes such CD11b and the CSF-1 receptor (c-fms) (Pahl et al., 1993; Zhang et al., 1994) and in immunoglobin gene activation in B cells (Pongubala et al., 1992). It may also be involved in the regulation of β-globin gene expression since binding sites for this factor are present in intron 2 of this gene (Galson et al., 1993) and, PU.1 overexpression can transform erythroblasts (Schuetze et al., 1993). This gene thus appears to regulate targets found in more than three lineages. In accordance with these findings, embryos with genetic disruption of PU.1 have severely impaired hematopoiesis in all

lineages (except megakarypoiesis) and die at ~day 17 post conception (Scott et al., 1994). The exact nature of death is uncertain but is likely caused by hydrops (severe anemia) in a significant proportion of the homozygous mice.

Therefore loss of function of Ikaros or PU.1 causes major perturbations in multiple lineages. This is strikingly different from the case of GATA-1 or NFE-2 whose effects appear to be more limited (see Figure 1.3).

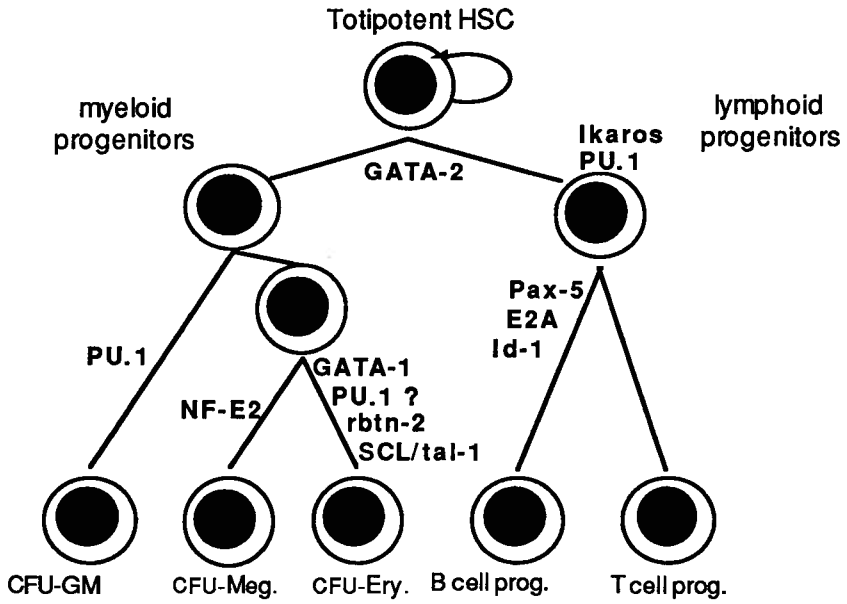

Figure 1.3. Possible hierarchical organization of some transcription factors involved in hematopoietic development.
Abbreviations: prog., progenitors; CFU, colony-forming-unit; GM, granulocytes-macrophages; Meg., megakaryocytes; Ery., erythrocytes.

This picture however is likely far from complete, particularly from the point of view of understanding those factors involved in the earliest stages of hematopoiesis. Several investigators have now speculated that strong candidates would be those transcription factors conserved through evolution and implicated in embryonic development (Kreider et al., 1992; Krumlauf, 1994; Tanigawa et al., 1993). Such genes include members of the zinc finger family such as Krox genes (Swiatek and Gridley, 1993), Trithorax, some segmentation genes and, of the helix-turn-helix family such as the homeobox genes. The

homeobox gene family has recently come under scrutiny and initial analysis of hematopoietic cell lines has revealed the expression of more than 40 distinct members of this family (reviewed in Lawrence and Largman, 1992). Furthermore, some of these genes have now been clearly implicated in human leukemias (Lawrence and Largman, 1992). The following section reviews some of the current knowledge about the classification, function, regulation and targets of these homeobox genes and evidence implicating them in hematopoiesis.

1.3 Homeodomain-containing transcription factors

1.3.1 Definition, classification and chromosomal organization of the homeobox genes

For years geneticists working with fruit flies suspected that a special phenotype described as "something that has changed into the likeness of something else" or homeotic mutation (Bateson, 1894) was the result of a single gene mutation (Bridges and Morgan, 1923). One of the most famous of these, initially called Nasobemia, (later called Antennapedia: leg transformation of an antenna (Duboule, 1994)) lead Gehring and others on the challenging path of identifying the mutations responsible for these phenotypes. Many years later, Lewis (Lewis, 1978) identified a gene complex (called bithorax) on Drosophila chromosome 3 which encoded for "substances controlling levels of thoracic and abdominal development". Chromosome walk studies performed on this chromosome (Garber et al., 1983) yielded the first isolation of a homeotic gene later called Antennapedia. cDNA clones that hybridized to this new gene also cross-hybridized to genomic DNA outside the Antennapedia gene region suggesting the presence of other related genes perhaps constituting a new

gene family (Garber et al., 1983; McGinnis et al., 1984). Subsequently, it was found that this cross-hybridization was caused by a shared highly-conserved 180 bp sequence in these genes that encoded a 60 amino acid domain referred to as the homeodomain (McGinnis et al., 1984). Thus the discovery of the first Drosophila homeobox gene is barely 10 years old (McGinnis et al., 1984; Scott and Weiner, 1984). The 60 amino acid homeodomain forms a helix-turn-helix structure that is believed to bind DNA (Hoey and Levine, 1988; Laughon and Scott, 1984; Otting et al., 1990). In Drosophila, these genes are clustered in 2 groups (Antennapedia and Bithorax) which together form a large complex termed HOM-C (Figure 1.4).

Homeobox genes were also found in vertebrates including mice and man (McGinnis et al., 1984). The homeodomain of the first cloned mouse homeobox gene was found to be very similar to that of the Drosophila (Carrasco et al., 1984). This discovery was among the first to suggest that the genetic controls implicated in development are evolutionary conserved. Interestingly, multiple mammalian homeobox genes were found to be clustered (Rabin et al., 1985) similar to that of the fruit fly and further their relative chromosomal positions are preserved ((Boncinelli et al., 1989); see also Figure 1.4). A distinguishing feature of the mammalian homeobox genes is their organization as four clusters each containing 9 to 11 different genes located on separate chromosomes (7, 17, 12 and 2 in man). It is believe that each cluster has originated by duplication of a common yet unidentified ancestral cluster (Martin, 1987). The relative position of individual genes in each cluster has been conserved and members located at the same relative position show the highest similarity constituting a so-called paralog group ((Boncinelli et al., 1989); Figure 1.4).

The name "Hox" was given to the 38 mammalian homeobox genes found in the four clusters. The nomenclature of the Hox genes was initially confusing since each gene was labeled according to its discovery (Martin, 1987). A new nomenclature based on the relative position of each gene was coined at the third Homeobox workshop (Scott, 1992). The old and new nomenclature of each Hox gene has been reported elsewhere (Scott, 1992) and only the new nomenclature is shown in Figure 1.4 and used throughout this thesis.

Within the homeobox gene complex of the fruit fly, there are a series of genes which also include a homeodomain but fail to cause homeotic transformation (DeRobertis, 1994). These genes have divergent homeodomains. Divergent Homeobox genes are also present in the mammalian genome but they are not found within any of the four Hox clusters (Boncinelli et al., 1989). These divergent genes are numerous and have been classified into more than 12 different "classes" and "families" some of which are described here: the *Pax / Prd* genes are distinguished by a 182 bp DNA-binding domain which flanks the homeodomain, (Gruss, 1992); the POU genes contain a distinctive domain of ~150 amino acids just before the divergent homeodomain and include the Pit-1 gene responsible for murine dwarfism, (Rosenfeld, 1991); the LIM proteins contain two tandemly arranged cysteine and histidine-rich regions, (Karlsson et al., 1990); the Msx family, for muscle-specific homeobox, are recognized for their role in limb bud formation, (Robert et al., 1991); the HNF-1-like factors, for hepatocyte-derived nuclear factors, are distinguished by an extra-long homeodomain and an ability to dimerize and control the transcription of albumin and other liver-specific genes (Frain et al., 1989); the endobox genes are known for their unique endodermal expression (Wright et al., 1988); the *NK* homeobox genes are highly conserved and clustered but with still unknown functions (Kim and Niremberg, 1989); the PBX

family was identified by the involvement of one of its members in a translocation associated with a human lymphoblastic leukemia (Kamps et al., 1990); and, the *Hlx* class was identified by their expression in hematopoietic cells (Deguchi and Kehrl, 1991; Deguchi et al., 1991). Further there are a series of "orphan" homeobox genes some of which will likely form new classes (DeRobertis, 1994).

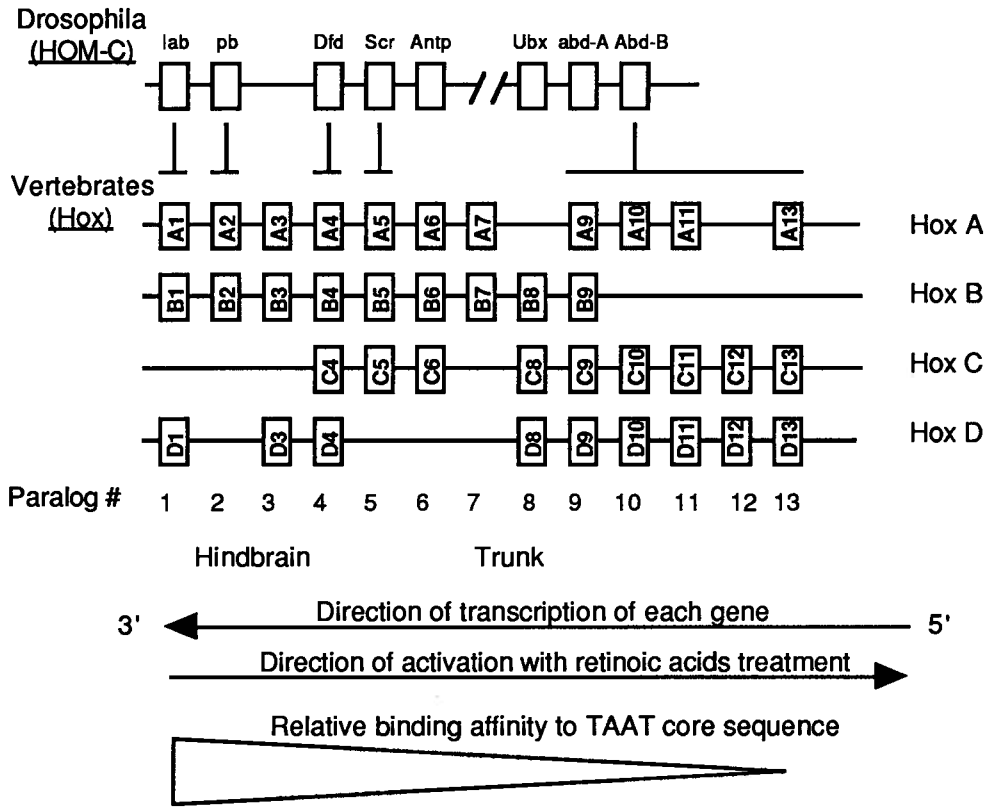

Figure 1.4. Relationship between the HOM-C Drosophila Homeobox genes of the Antennapedia and Bithorax cluster complex and the various mammalian Hox genes.

From left to right the Drosophila members of the HOM-C complex are *labial (lab)* which is similar to the group 1 paralog in vertebrate; *proboscipedia (pb)* with group 2; *Deformed (Dfd)* with group 4; *Sex combs related (Scr)* with group 5; *Antennapedia (Ant), Ultrabithorax (Ubx), Abdominal-A (abd-A)* which have no mammalian homologs; and *abdominal-B (abd-B)* with group 9 to 13. Only the recent nomenclature (Ascona Workshop, Scott, 1992) is shown. As discussed later in the text, genes located at the 3' end of the Hox clusters are expressed earlier and in more anterior structures of the embryo than those found at the 5' end of the clusters. Individual Hox genes have the same direction of transcription as indicated by one of the two arrows. Genes located at the 3' end of the cluster respond early to retinoic acid and at a lower concentration than that required to activate 5' genes. The ability of Hox genes to bind to the core DNA sequence C/GTAATTG also decreases 3' to 5' (Pellerin et al., 1994).

1.3.2 Regulation by homeobox proteins of embryonic cell growth and differentiation.

In Drosophila embryogenesis the HOM-C genes are major determinants of segment identity (Lewis, 1978). However genes of the HOM-C complex only become active after two important sets of genes have pre-established some essential embryonic structures called parasegments[2]. Before and after fertilization, products derived from more than 12 different genes are active in establishing dorso-ventral and antero-posterior axis. These genes are called maternal-effect genes because the phenotype of the embryo is only dependent on the maternal alleles (Anderson et al., 1993; St. Johnston and Nusslein-Volhard, 1992). These genes are true morphogens since they regulate gene expression in a gradient-dependent manner that is established by various mechanisms such as mRNA localization (e.g. *bicoid* for anterior boundary; *oskar* for posterior boundary (Ephrussi and Lehmann, 1992)) or transmembrane receptors ("*Toll–dorsal–cactus–decapentaplegic*" sequential activation for ventral boundary which is involved in determination of the embryonic layers (Roth et al., 1989; Rushlow et al., 1989)).

Once body polarity is established, a second set of at least 25 different genes regulated by the maternal-derived genes described above becomes activated. These genes of "second order" are termed segmentation genes and fall into three groups (Kornberg, 1993; Nusslein-Volhard and Wieschaus, 1980): i) the gap genes which include the well studied zinc-finger-containing transcription factors hunchback (which as said before shares similarity to *Ikaros*) and Krüppel that, in a cross-regulatory manner, delineates anterior segments from more posterior ones (Hülskamp and Tautz, 1991); ii) the pair-rule genes

[2] In this text, segment and parasegment have been used loosely. A parasegment is the basic unit of organization of the Drosophila body and does not correspond exactly to the segments observed on the adult's body.

such as *even-skipped*, *paired*, *hairy* and *fushi tarazu*, deletion of which induces the loss of every other segment (Akam, 1987); and iii) the segment-polarity genes involved as indicated by their name in the polarity of individual segments. Together these genes establish the various parasegments wherein the HOM-C homeobox genes become active and lead to differentiation of each parasegment (Figure 1.5).

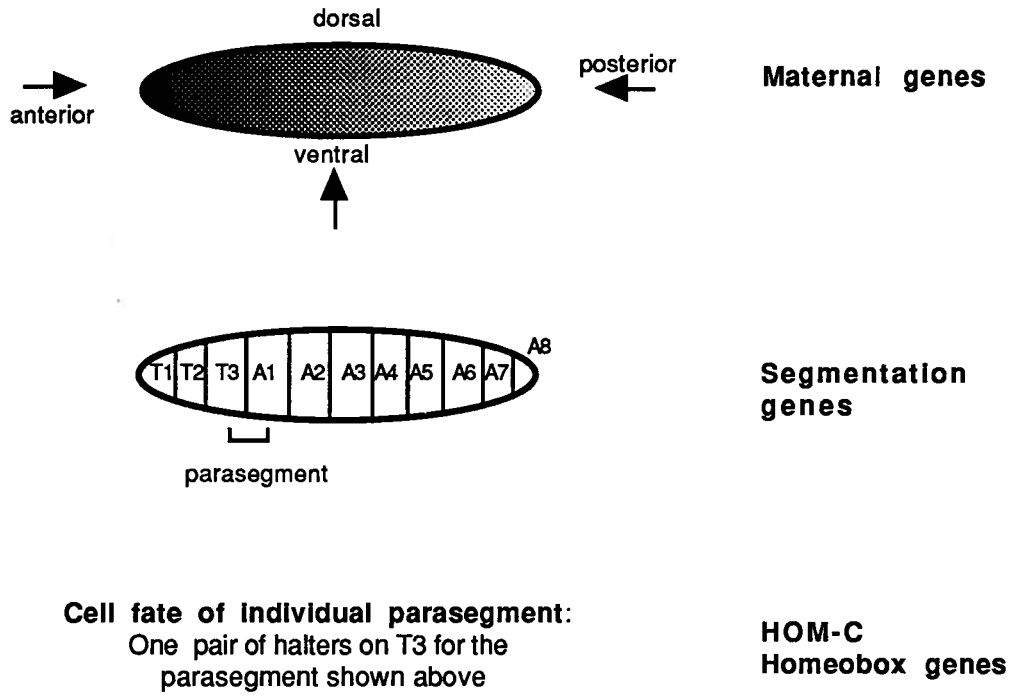

Figure 1.5. Three sets of genes must be sequentially activated for proper differentiation of a specific body part.
First maternal genes are establishing the antero-posterior and dorso-ventral axis. This occurs through external influences on the oocyte by the maternal follicular cells (see arrows). A concentration gradient of a gene (*bicoid*) involved in the setting of the anterior pole is shown. Then segmentation genes become activated. The cell fate of individual parasegments is determined by the HOM-C homeobox gene.

Thus HOM-C genes appear to be master regulators of differentiation. However, they need to be activated in a time and site specific manner. Failure to do so generally results in a homeotic transformation. Contrary to the Drosophila HOM-C complex, very little is known about embryonic activation of mammalian Hox genes besides these three characteristics. First there is a strict correlation

between the position each Hox gene occupies on the chromosome and its site of expression. Genes found at the 3' region of the Hox cluster (e.g. first paralog group: A1, B1, D1, see Figure 1.4) are expressed in the anterior region of the embryo whereas genes found at the 5' end are expressed in more posterior regions. The expression "spatial colinearity" has been coined for this phenomenon (Duboule and Dolle, 1989; Graham et al., 1989). Second, Hox genes from the different paralog groups are sequentially transcribed from the 3' to the 5' region of the chromosome, a pattern reminiscent of globin activation. This phenomenon has been referred to as "temporal colinearity" (Izpisua-Belmonte et al., 1991). The third interesting feature about Hox gene expression is termed posterior dominance. Hox genes expressed in the anterior region of the embryo will also be expressed, at lesser levels, in the more posterior regions so that a complex array of Hox genes are expressed in more posterior regions. The gene that has the greatest influence on cell fate of any posterior region is normally the one which shows no anterior overlap in expression (the latest to be expressed). Based on this pattern of expression, loss of expression of a Hox gene will generally result in "anteriorisation" of the structures normally found at this position and gain of function causes "posteriorisation" due to the same phenomenon (see Krumlauf (1994) and McGinnis and Krumlauf, (1992)) for a summary of loss- and gain- of function phenotypes of Drosophila and mouse HOM-C / Hox genes).

During development Hox gene expression is not ubiquitous but rather these genes are expressed in paraxial mesoderm, neural tube, neural crest, hindbrain segments, surface ectoderm, branchial arches, gut, gonadal tissues and limbs (reviewed in Krumlauf, 1994). In limb budding the expression of Hox gene forms a two-dimensional network which again follows spatial and temporal co-linearity patterns (Duboule, 1992).

1.3.3 Upstream regulation of homeobox (HOM-C/Hox) gene expression.

Upstream cis-regulators of Hox gene expression are still poorly defined. Retinoic acids clearly upregulate Hox gene expression (Langston and Gudas, 1992; Popperl and Featherstone, 1993). Hox genes themselves appear to cross- and auto-regulate their expression (Zappavigna et al., 1991). Segmentation genes also appear to regulate Hox gene expression (Kennison, 1993). Zinc-finger-containing genes some of which are believed to be analogous to gap segmentation genes (*Krox-20*) can directly regulate the expression of specific Hox genes (*Krox-20*: (Schneider-Maunoury et al., 1993; Swiatek and Gridley, 1993); kreisler: (Frohman et al., 1993; McKay et al., 1994)). Recently *GATA-1* was shown to possibly regulate *HoxB2* (Vieille-Grosjean and Huber, 1995) a gene apparently involved in globin regulation (Sengupta et al., 1994) .

A number of "trans-regulators" that function in chromatin structure patterning have also been identified. The polycomb group are negative regulators of HOM-C expression (Paro, 1990; Paro, 1993). They appear to function by inducing heterochromatin formation thus stabilizing suppression of HOM-C genes expression (Kraumlauf, 1994). Interestingly, genetic disruption of a recently characterized vertebrate polycomb gene, *bmi-1*, causes posterior skeletal transformation and hematopoietic defects (van der Lugt et al., 1994).

Another group of trans-regulators of HOM-C / Hox genes is the Trithorax group (Kennison, 1993). These genes are involved in chromatin structure regulation[3] but unlike the polycomb group, they appear to positively regulate HOM-C gene expression (Krumlauf, 1994). In Drosophila, loss-of-function

[3] The Trithorax genes are capable of transvection i.e. they can allow interaction of elements found on two homologous chromosome

mutations of Trithorax result in phenotypes that mimic the loss of function of multiple ANT-C and BX-C genes (Kennison, 1993). Interestingly, one vertebrate member of the Trithorax group–*HRX*–is rearranged in a type of human acute lymphocytic leukemia involving chromosome 11 (Gu et al., 1992; Tkachuk et al., 1992). A summary of some of the known positive and negative regulators of the HOM-C / Hox gene complex is shown in Figure 1.6.

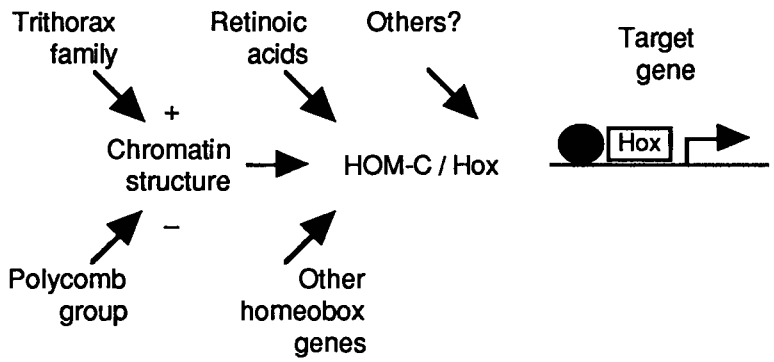

Figure 1.6. Regulation of HOM-C / Hox gene expression.
Both the Trithorax and polycomb group of genes affect chromatin structure. Cis-regulators of the complex (retinoic acids, *Krox-20* and other homeobox genes) are shown. As presented later in the text, specificity of Hox gene to their targets appears to be mediated by co-binding of another factor (dark circle) which can be another Hox gene (Zappavigna et al., 1991), or another homeodomain-containing molecules such as *extradenticle* (van Dijk and Murre, 1994) or its vertebrate homolog *PBX-1* (Chang et al., 1995).

1.3.4 Downstream targets of HOM-C / Hox genes.

Very little is yet known about the target genes regulated by genes of the HOM-C / Hox clusters. Both in Drosophila and vertebrates, three HOM-C / Hox targets have been identified by immunoprecipitation strategies involving Hox protein binding to genomic DNA targets (Gould et al., 1990; Graba et al., 1992; Tomotsune et al., 1993). Interestingly two of these three identified targets fall into the category of adhesion molecules (Gould et al., 1990; Tomotsune et al., 1993). Other studies have demonstrated the interaction of various Hox proteins with regulatory regions of some adhesion molecules (Edelman and Jones, 1993; Hirsch et al., 1990; Hirsch et al., 1991; Jones, 1993; Jones et al., 1992;

Jones et al., 1992). Recently a growth factor member of the TGF-β family (*decapentaplegic: dpp*) was shown to be directly regulated by *Ultrabithorax*, a member of the HOM-C complex (Sun et al., 1995). However these authors also showed that at least one other factor (possibly *extradenticle*) was required for specific and full activation of *dpp* by *Ultrabithorax* (Sun et al., 1995).

Through their homeodomain, Hox genes bind to a tetranucleotide core (ATTA or ATAA; (Desplan et al., 1988)). Since there is such high similarity in the different Hox gene homeodomains, questions have been raised about the ability of specific Hox genes to activate unique and different targets. Clues to these important questions have been found again in the Drosophila model where it was recently shown that *extradenticle* (a non HOM-C homeodomain-containing gene closely related to *PBX-1*) dramatically raises the DNA-binding specificity of *Ultrabithorax* (a HOM-C gene) to an oligonucleotide derived from the enhancer region of *dpp* (Chan et al., 1994; van Dijk and Murre, 1994). Similar findings were recently obtained with *PBX-1* and multiple Hox proteins (Chang et al., 1995). Other studies have shown that Hox gene binding to target DNA fragments can be modulated by Hox-Hox interaction (Zappavigna et al., 1994) and / or by different affinities exhibited by the various Hox proteins towards the same target (see Figure 1.4, Pellerin et al., 1994). It remains to be proven whether these results will be confirmed in studies with native chromatin.

1.3.5 Hox gene expression in post-developmental normal and neoplastic tissues

There is accumulating evidence to suggest that Hox genes are also post-developmentally expressed in germinal and somatic adult mammalian tissues (Barba et al., 1993; Friedmann et al., 1994; Ko et al., 1988; Kongsuwan et al., 1988; Watrin and Wolgemuth, 1993).

Most (n=30) of the 38 vertebrate Hox genes are expressed in normal human adult kidney in a tissue-specific pattern (Barba et al., 1993; Cillo et al., 1993). A difference in the relative levels of Hox expression and transcript size is observed between medullary and cortical kidney tissues. Renal cell carcinomas also express Hox genes but with a more variable pattern (Barba et al., 1993).

Cells from normal and neoplastic human colonic mucosa express most Hox genes (n=29, (De Vita et al., 1993)). Expression of various Hox genes can also be found in other tissues such as liver, lungs (Barba et al., 1993) breast (Friedmann et al., 1994) and testis (Watrin and Wolgemuth, 1993). Although each tissue appears to have its own "Hox fingerprint" (Barba et al., 1993) the nature of this difference is less clear. It may, for example, simply reflect a difference in mature to primitive cell content of each specific tissue.

1.3.6 Homeobox gene expression in normal and leukemic hematopoietic cells

1.3.6.1 Hox gene expression in leukemic cells

The idea that Hox genes may be expressed and therefore regulate hematopoiesis originated from studies which analyzed the expression of a limited number of Hox genes in various leukemic cell lines (Kongsuwan et al., 1988; Lonai et al., 1987). Subsequent extensive studies of the expression of all 38 Hox genes in four hematopoietic cell lines led to the identification of a peculiar pattern of Hox gene expression-activation which was not reminiscent of that found in embryonic development (Magli et al., 1991). In this study, the authors found that cell lines with erythroid features (K562 and OCIM2) coordinately expressed most of the Hox B and C cluster genes and that cell lines with myeloid features (HL-60 and U937) expressed predominantly A and C cluster genes. Therefore the "paralogous" Hox gene expression patterns as

found in embryonic development was not seen in these hematopoietic cell lines but rather whole clusters (or large regions of a cluster) were turned on. It was also found that cluster D Hox genes were almost completely silent in every cell line analyzed (Magli et al., 1991). Subsequent studies confirmed this cluster-associated pattern of expression where cells with erythroid (K562, HEL, MB02) but not myeloid (KG1a, KG1, HL-60, U937, THP1, PLB985, ML3, TMM) features expressed all of the B cluster genes but one (HOXB1: Mathews et al., 1991; Vieille-Grosjean et al., 1992) and also that D cluster genes were poorly expressed (reviewed in Lawrence and Largman, 1992).

Hox genes are also expressed in lymphoid cell lines (Lawrence et al., 1993; Petrini et al., 1992) but apparently not in resting B or T lymphocytes (Carè et al., 1994; Petrini et al., 1992). Observed exceptions to this rule include *HOXB7* expression in $CD8^+$ human T cells (Inamori et al., 1993) and *HOXC4* in total T lymphocytes (Lawrence et al., 1993; Meazza et al., 1995). *HOXC4* expression is unusual because it appears to be restricted to lymphoid but not myeloid cells lines (Lawrence et al., 1993) and further it was recently shown that the *HOXC4* protein migrates from the cytoplasm to the nucleus in newly activated lymphocytes (Meazza et al., 1995).

Analysis of Hox gene expression in primary human leukemias (Celetti et al., 1993; Lawrence et al., 1995) has revealed expression patterns that appear to be specific for leukemia subtypes as described by the French-American-British (FAB) classification. For example, B cell chronic lymphocytic leukemias (CLL) express *HOXB4* and *B7* whereas T cell CLL also express *HOXB2* (Celetti et al., 1993). One striking example is that of *HOXA10* which was found expressed in all acute myeloid leukemias examined but not in the promyelocytic subtype (FAB M3) (Lawrence et al., 1995). Although expressed in leukemic cells, Hox genes have not yet been directly implicated in human leukemia. The

only report implicating a Hox gene in leukemic transformation is that of *HoxB8* which together with IL-3 is overexpressed in the murine leukemic cell line WEHI-3B due to activation by insertion of an endogenous intracisternal A particle (IAP) sequence upstream of both genes (Blatt and Sachs, 1988). HoxB8 and IL-3 when co-transfected into murine bone marrow cells was also found to be highly leukemogenic (Perkins et al., 1990) whereas alone neither caused leukemia (Perkins and Cory, 1993; Wong et al., 1989).

While Hox genes have not yet been shown to be involved in human leukemias, non-clustered homeobox genes with a divergent homeodomain are major participants. *PBX1*, like *extradenticle*, appears to cooperate with Hox / HOM-C genes for DNA binding (see above). Fusion of the *PBX* DNA-binding domain, as a result of a chromosomal translocation t(1;19), with the transactivation domain of another transcription factor (*E2A*) is associated with an aggressive human pre-B leukemia (Kamps et al., 1990). Another example is *TCL3* (former *HOX-11*) a gene involved in T cell acute lymphoblastic leukemia by virtue of translocation t(10;14) which juxtaposes the T-cell receptor δ gene promotor to a region upstream of *TCL-3* resulting in ectopic expression of this gene (Hatano et al., 1991). *TCL-3* function is still unknown but interestingly, mice with genetic disruption of this gene were found to be asplenic (Roberts et al., 1994).

Numerous other divergent homeobox genes are expressed in hematopoietic cells. These include genes such as *HLX* (previously *HB24*), first identified as expressed in mitogen-stimulated human B lymphocytes and later in $CD34^+$ bone marrow cells (Deguchi and Kehrl, 1991a; Deguchi and Kehrl, 1991b; Deguchi et al., 1991); the *HOP* gene found in HL-60 cells (Hromas, 1992); the *Prh* gene isolated from AMV-transformed chicken monoblasts

(Crompton et al., 1992); and the *HEX* gene whose expression seems to be restricted to the hemopoietic system (Bedford et al., 1993).

1.3.6.2 Hox gene expression and function in normal hematopoietic cells.

At the time the research presented in this thesis was initiated, Hox gene expression in normal hematopoietic cells had not been systematically studied. The reasons for this included the recent recognition of Hox genes as possible regulators of hematopoietic cells, the technical difficulties in obtaining high numbers of "pure" functionally and phenotypically-defined bone marrow subpopulations, the low level of expression of these genes and the absence of available full sequences of most Hox cDNA. Direct evidence for Hox protein function in hematopoiesis has also only recently become available. Indeed only one report was published when this project was initiated (see later, (Shen et al., 1992)).

There are now however several lines of evidence that implicate Hox genes in the regulation of hematopoiesis. Three studies have described the use of antisense oligonucleotides to down-regulate mRNA levels of specific Hox genes (Carè et al., 1994; Takeshita et al., 1993; Wu et al., 1992). In the first of these studies (Wu et al., 1992) murine bone marrow cells were first exposed for four hours to *HoxB7* antisense oligonucleotides and then plated in semi-solid cultures supplemented with serum and growth factors. Granulocyte-macrophage colony-forming-cells were found to be reduced by approximately four-fold in the cultures initiated with the cells treated with the antisense oligonucleotide. Erythroid and megakaryocytic progenitors were unaffected by this oligo. Effects of antisense oligonucleotides directed against *HOXC6* were examined and shown to suppress the formation of colony-forming unit-erythroid (CFU-E) without affecting earlier erythroid progenitors (BFU-E) or myeloid-

derived colonies (Takeshita et al., 1993). Evidence for Hox gene involvement in human T cell proliferation has also been provided recently (Carè et al., 1994). Upon phytohemagglutinin (PHA) stimulation, mature human T lymphocytes sequentially express Hox B cluster genes (3' to 5') (Carè et al., 1994). Antisense treatment of PHA-stimulated T cells with *HOXB2* and *HOXB4* (two 3' genes) markedly inhibited proliferation.

Several studies have now been reported in which the effect of overexpression of a Hox gene was examined in cell lines or in primary cells. Cells of the HL-60 line can be induced to differentiate into morphologically identifiable granulocytes or monocytes by treatment with differentiating agents such as retinoic acids or dimethyl sulfoxide for granulocytic differentiation or with phorbol ester or vitamin D3 for monocytic differentiation (Lill et al., 1995). *HOXB7* is not expressed in undifferentiated HL-60 cells (Magli et al., 1992) but is readily detectable following monocytic differentiation with vitamin D3 and remains undetectable in cells undergoing granulocytic differentiation (Lill et al., 1995). Overexpression of *HOXB7* in this cell line inhibited granulocytic but not monocytic differentiation. Together with the antisense study of Wu et al. described above, these findings implicate *HOXB7* as an important regulator of myeloid differentiation of human (Lill et al., 1995) and murine (Wu et al., 1992) hematopoietic cells. In another study, *HOXB6* was overexpressed in K562 cells and resulted in the reduction of erythroid features of this cell line characterized by decrease globin synthesis, heme content and surface glycophorin expression (Shen et al., 1992).

HoxB8, the immediate neighbor of HoxB7 was overexpressed in murine bone marrow cells (Perkins and Cory, 1993). In this study the authors used a replication-defective retroviral vector to infect murine bone marrow cells

(Perkins and Cory, 1993). Generation of non-tumorigenic IL-3-dependent myelomonocytic cell lines was readily obtained by simply growing cells in high concentration of IL-3. The cell lines would however differentiate upon reduction of IL-3 concentration. Most mice reconstituted with these transduced cells were free of leukemia for at least seven months but approximately 20% eventually developed a myeloid leukemia which in some cases was associated with rearrangement of the IL-3 gene.

Studies involving the divergent homeobox gene *HLX* (murine hlx: (Allen and Adams, 1993; Allen et al., 1995) human *HLX*: (Deguchi et al., 1992)) showed that it may have important roles in both myeloid (Deguchi et al., 1992) and lymphoid (Allen et al., 1995) cell proliferation and differentiation. Recently *TCL-3* was shown to also generate IL-3-dependent immortal cell lines when overexpressed in murine bone marrow cells (Hawley et al., 1994). These cell lines were not leukemogenic when reinjected into syngenic recipients suggesting that a secondary "hit" may be necessary in order to allow full transformation as found in the t(10;14) human T cell leukemia.

1.4 Thesis Objectives

As reviewed in the previous sections, several lines of evidence point to Hox homeobox genes as important regulators of hematopoiesis. These include the observed expression of multiple Hox genes in <u>all</u> primary leukemias and leukemic cell lines analyzed so far; the perturbation in hematopoietic cell growth and / or differentiation upon induced alteration in specific Hox gene expression; and, finally by their clear involvement in cell growth and differentiation in embryonic development and hence the likelihood the play similar roles in the continuous process of hematopoietic development. Nevertheless at the time this project was initiated, there was extremely limited

direct evidence of Hox gene expression and function in normal primitive hematopoietic cells. Three major questions were thus address during the course of this research:

1- Are Hox homeobox genes expressed in CD34+ human bone marrow cells?

2- If so, are they differentially expressed in functionally and phenotypically "distinct" subpopulations of CD34+ cells?

3- What role do Hox genes play in the regulation of proliferation and differentiation of early hematopoietic cells?

To address these questions, work was carried out towards three specific aims as presented in chapters 2 to 4.

1- Generation of representative and extended-length cDNA from purified subpopulations of human hematopoietic cells to enable characterization of Hox gene expression patterns (Chapter 2).

2- Characterization of Hox gene expression in purified human bone marrow CD34+ subpopulations and identification of novel and / or differentially expressed Hox genes in these purified subpopulations (Chapter 3).

3- Examination of the functional role of candidate Hox genes in the regulation of proliferation and differentiation of primitive hematopoietic cells by modulating their expression in primary bone marrow cells (Chapter 4).

CHAPTER 2.

A RT/PCR method to generate representative cDNA from small number of hematopoietic cells[4]

2.1 Summary

The study of Hox gene expression in rare hematopoietic populations (<0.01% of bone marrow cells: less than 5,000 cells available) is a formidable challenge that requires the use of PCR-based techniques. Available methods for amplification of total cDNA from small cell numbers remain however suboptimal with respect to yields due to frequent manipulations, representation of rare transcripts and generation of products spanning longer mRNA. This chapter describes the optimization of an RT-PCR method that overcomes some of these limitations. The resulting methodology is demonstrated to maintain relative levels of total cDNA and yield products that extend at least 1.75 kb 5' of the polyadenylation site of even rare transcripts from cell numbers ranging from hundreds to thousands.

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[4] The method validated in this chapter has been used and described in these two published manuscripts:

1-Sauvageau G. et al., (1994). Differential expression of homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells. Proc. Natl. Acad. Sci. USA 91: 12223-12227 (see next chapter).

2-Lawrence H.J., Sauvageau G., Ahmadi N., Lau T., Lopez A.R., LeBeau M.M., Link M., Humphries R.K. and Largman C. (1995). Stage and lineage-specific expression of the *HOXA10* homeobox gene in normal and leukemic hematopoietic cells. In press, Experimental hematology.

It was also extensively used in the two following manuscripts recently submitted:

1- Helgason C., Sauvageau G., Lawrence H.J., Largman C. and Humphries, R.K. (1995). Overexpression of *HOXB4* enhances the hematopoietic potential of embryonic stem cells differentiated in vitro. Manuscript Submitted

2-Hough M.R., Chappel M.S., Sauvageau, G., Takei F., Kay R. and Humphries, R.K. (1995). Dramatic reduction in IL-7 responsive clonogenic progenitors in mice over-expressing the Heat-Stable antigen. Manuscript Submitted.

A RT/PCR method to generate representative cDNA from small number of hematopoietic cells

2.2 Introduction

The continuing evolution of cell purification strategies and PCR have greatly enhanced the accessibility of molecular analysis of populations of cells found at a low frequency. As described in the introduction, primitive hemopoietic cells found at frequencies of less than one in ten thousand bone marrow cells can now be enriched by cell sorting techniques to near homogeneity. However the current low yields of these highly purified cells (hundreds to thousands) continue to pose significant hurdles to the quantitative analysis of gene expression.

Several strategies have now been described for using PCR methods to amplify cDNA from a low number of cells (Belyavsky et al., 1989; Brady et al., 1990; Doherty et al., 1989; Domec et al., 1990; Don et al., 1993; Dumas et al., 1991; Froussard, 1992; Lambert and Williamson, 1993; Rappolee et al., 1989). Losses of nucleic acids during multiple manipulations and difficulties in obtaining amplified cDNA that adequately represents the initial mRNA remain major concerns (Lambert and Williamson, 1993). One difficulty encountered has been that of striking a balance between obtaining cDNA that preserves relative levels of even rare transcripts versus generating the longest possible cDNA. Using a procedure which minimizes precipitation steps and employs suboptimal reverse transcriptase conditions, Brady *et al.* have reported generation of representative cDNA from as few as one hematopoietic cell (Brady et al., 1990). However, this method, by design, limits the transcript size to approximately 700 bp or less, which can hamper identification of coding regions or conserved domains (such as homeodomains) that may be distant from the polyadenylation

site. Previous efforts to obtain cDNAs encompassing 5' mRNA sequences have generally increased the difficulty of the technique mainly because of incompatible buffer components. This has resulted in complicated protocols that can affect reproducibility and yield (Belyavsky et al., 1989). Single tube conditions (Don et al., 1993) have recently been described but these can be complicated by the necessity of polylinker addition to the double stranded cDNA. Procedures have been described for obtaining more extended amplified cDNA (Belyavsky et al., 1989) but their applicability to limited cell numbers is compromised by frequent manipulations and precipitation steps which can lead to significant loss of the nucleic acids.

Most recently, random primers have been used during first strand synthesis of cDNA and in subsequent amplification by PCR (Froussard, 1992). By eliminating tailing or linker addition steps, this approach decreases the complexity of the technique and should generate products spanning 5' and 3' region cDNA sequences. However, the random primers have the potential to amplify genomic DNA and ribosomal RNA which may prove problematic in the study of gene expression.

This chapter describes a reproducible and relatively straight-forward method for the generation of amplified cDNA from a low number of cells that provides requisite sensitivity, representativeness and the capacity for generating cDNA that encompasses the more 5' region of mRNA. The resulting protocol has been tested using limiting cell numbers and mixtures of hemopoietic cell lines known to differ in expression of specific genes. These results demonstrate that this method enables the generation of cDNA that retains adequate relative message abundance of even rare transcripts as exemplified by Hox genes, and includes sequences <u>at least</u> 1.75 kb 5' to the

end of mRNA. By its simplicity and reliability, this optimized method should enhance the opportunities for comparative gene expression studies between different subpopulations of cells in instances where cell number are limiting and representation of long-length mRNAs is required.

2.3 Materials and Methods

2.3.1 RNA extraction and first strand cDNA synthesis.

The choice of RNA extraction was made on comparison of published methods (Chomczynski and Sacchi, 1987; Cumano et al., 1992; Domec et al., 1990) and a commercially available kit (Pharmacia QuickPrep Micro mRNA Purification Kit). Two of these methods, one based on affinity purification (Pharmacia QuickPrep mRNA micro purification), the other as described by Cumano et al. (1992) gave consistent recovery of the RNA starting with 1,000 to 10,000 cells (data not shown). Because of its simplicity the latter method was chosen. In brief, 1,000 to 10,000 hematopoietic cells were pelleted and the supernatant removed carefully. Cells were lysed in 50 µl of a guanidium isothiocyanate solution (5 M GIT, 20 mM 1,4-dithioerythritol (DTT), 25 mM sodium citrate pH 7.0, 0.5% Sarcosyl) and the nucleic acids precipitated by adding 25 µl of 7.5 M ammonium acetate containing 40 mg of glycogen as carrier and 2 volumes of 95% ethanol. After two 70% ethanol washes, the pellet was dried at room temperature and resuspended in 3 µl of DEPC-treated water, heated at 65°C for 5 minutes and left at 22°C for 3 minutes. Reverse transcription conditions were scaled down and modified from those previously described (Ausubel et al., 1991). To the 3 µl of RNA, the reverse transcription mixture consisting of 3 µl of DEPC treated water, 2 µl of 5X RT buffer (GIBCO, BRL), 1 µl of 0.1M DTT, 0.2 µl of 25 mM dNTPs (500 mM), 0.2 µl of the oligo dT primer at 1mg/ml (60mers: 5'CATGTCGTCCAGGCCGCTCTGGAC-AAAATATGAATTCT$_{(24)}$-3' as described

by Brady *et al.* (Brady et al., 1990), Oligos Etc., Portland, Oregon) and 0.1 µl of placental RNAase inhibitor (10 U/µl, GIBCO, BRL) was added. This mixture was incubated at 37°C for 5 minutes and 0.5 µl of MMLV Superscript reverse transcriptase (200 U/l, GIBCO, BRL) with or without 0.1 ml of AMV (20 U/l, Pharmacia) was added. The reaction was left at 40 to 42°C for 1 hour and heat inactivated at 70°C for 10 minutes.

2.3.2 Homopolymeric dA Tailing.

After heat inactivation of the reverse transcriptase(s), the cDNA was precipitated with 5 µl of 7.5 M ammonium acetate and 30 µl of ethanol as described above with the help of a linear polyacrylamide carrier (Gaillard and Strauss, 1990). This carrier was chosen because when compared to tRNA, or to the absence of carrier, more consistent results were obtained. This is in accordance with results already published (Gaillard and Strauss, 1990). When compared to mock controls, it was also found that this carrier did not interfere with the enzymatic activity of all enzymes (including Taq polymerase, terminal deoxynucletidyl-transferase (TdT) and the reverse transcriptases) used in this protocol (data not shown). The pellet was washed once with 70% ethanol, dried at room temperature and resuspended in 5 µl of a tailing solution consisting of 1 µl of 5X tailing buffer (GIBCO, BRL), 0.5 µl of 100mM dATP (10 mM final, Pharmacia) and 3.5 µl of distilled water. After the pellet was resuspended, 0.5 µl of TdT (15 U/µl, GIBCO, BRL) was added to the solution and incubated at 37°C for 15 minutes followed by a 10 minutes heat inactivation of the enzyme at 70°C.

2.3.3 Amplification of the cDNA.

PCR conditions for amplification of cDNA were optimized as described in results. Final conditions were as follows: To the 5 µl of tailed cDNA, 36.75 µl of a PCR solution were added. This solution consisted of 25 µl of a 2X buffer (20

mM Tris pH 8.8, 100 mM KCl, 10 mM $MgCl_2$), 4 µl of the oligo dT primer described earlier (1 mg/ml in Tris pH 7.5), 0.5 µl of nuclease free BSA (10mg/ml, Sigma), 0.25 µl of triton X-100, 5 µl of water and 2 µl of a nucleotide mix consisting of dCTP, dGTP and dTTP each at 25mM to provide 1.0 mM final of each nucleotide including the dATP previously added in the tailing reaction. The tubes were incubated at 92°C for 2 minutes before the addition of 1.0 µl of gene 32 protein (Pharmacia), cooled on ice for 2 minutes and 1 µl of Taq polymerase (5U/µl, GIBCO, BRL) was added to the reaction. The cDNA was amplified using an Ericomp thermal cycler (Ericomp, San Diego, CA) with the following parameters: 94°C for 1 min., 55°C for 2 min. and 72°C for 10 min. for 40 cycles except for the first cycle which was performed at an annealing temperature of 37°C. The ramp temperatures were found to be critical in order to get good amplification and for this cycler were: 72°C-94°C, 0.32°C/sec; 94°C-55°C, 0.44°C/sec; 55°C-72C, 0.28°C/sec.

2.3.4 Southern Analysis of Amplified cDNA.

A 10 µl aliquot of the total amplified cDNA was electrophoresed in a 1% agarose gel and passively transferred for 6 hours onto an ionic nylon membrane (Zeta-probe, BioRad). Probes were labeled with ^{32}P-dCTP (3000 Ci/mmol; Amersham) by random priming and purified on a Sephadex-G50 column before hybridization. Blots were hybridized for 20 hours at 60°C in 4.4 X SSC, 7.5% formamide, 0.75% SDS, 1.5mM EDTA, 0.75% skim milk, 370 mg/ml of salmon sperm DNA and 7.5% Dextran sulfate. Membranes were then washed twice at 60°C for 30 min. each in 0.3 X SSC, 0.1% SDS and 1 mg/ml of sodium pyrophosphate. When necessary, greater stringency washes were performed (0.1 X SSC, 0.1% SDS and 1 mg/ml of sodium pyrophosphate). For reprobing, membranes were stripped in a 1% SDS solution at 95°C for 20 to 30

minutes and tested for the absence of a signal by overnight exposure to Kodak X-OMAT XAR5 film. Probes were as follow: HOX B4 an EcoRI/ XbaI 0.75 kb fragment of the genomic sequence obtained from the American Type Culture Collection (ATCC, Rockville, MD); HB24, a full-length cDNA including the divergent homeodomain kindly provided by Dr. J. Kehrl; neomycin phosphoribosyl-transferase (neo^r), a 1 kb fragment derived from pMC1Neo (Thomas and Capecchi, 1987); and β-actin, a 2.0 kb chicken cDNA probe. For the detection of c-myc expression 2 probes subcloned from a c-myc genomic sequence (ar-kFkushdi et al., 1983) were used: a 5' probe spanning nucleotides 67 to 455 of c-myc cDNA is a Sac-1/ Xho1 773bp fragment and the second is a 1.9kb PvuII 3' probe spanning the last 563 nucleotides (exon 3) of c-myc cDNA.

2.4 Results and Discussion

In an effort to develop a reliable method for obtaining representative and extended-length amplified cDNA from small cell numbers, I have focused both on optimizing PCR conditions in order to reduce the number of PCR cycles and on reducing the manipulation of the nucleic acids to avoid potential loss. Key considerations for the choice of a final protocol were simplicity and reproducibility together with the generation of products that included rare messages that spanned as much 5' mRNA sequence as possible.

2.4.1 Condition Adjustments.

Several known critical variables affecting PCR amplification including pH, magnesium, deoxynucleotide and primer concentrations were first optimized. First strand cDNA was prepared from 0.1μg of total RNA previously extracted from HL-60 cells by cesium chloride gradient and amplified under conditions initially based on these as described by Brady et al. (1990) and subsequently modified as described below. Amplified cDNA was evaluated from quadruplicate preparations for each condition by Southern blot analysis using a β-actin probe. Optimal cDNA amplification was found with pH8.8 and with dNTP and magnesium concentrations of 1 and 5mM respectively. Primer concentration as tested was not found to be an important determinant of amplification (data not shown). Even under these optimized conditions it was still necessary to use at least 100 PCR cycles (Taq DNA polymerase was re-added at cycle no. 50) to obtain sufficient material for Southern analysis of rare transcripts (data not shown).

To make this method readily usable for the analysis of rare transcripts, it was necessary to seek to further improve the efficiency of the amplification by

studying the effect of other factors such as the addition of gene 32 protein (Schwarz et al., 1990) and the use of a low KCl buffer (Ponce and Micol, 1992). Although gene 32 protein of bacteriophage T4 was shown to improve the efficiency of Taq polymerase in its ability to generate longer PCR fragments using specific primers, its value in amplification of total cDNA using oligo dT primers has not been reported. As shown in Figure 2.1B to 2.1D, the addition of 1.0 μl of gene 32 protein to PCR reactions from 25,000 total human bone marrow and RNA from 3,000 HL-60 cells resulted in an appreciable increase in the amount (approx. 1 log) and average size range (approx. 2 fold) of the cDNA as assessed by ethidium bromide stain (Figure 2.1B) and, as discussed in greater detail below by Southern blot analysis using probes for the 5' region (Figure 2.1C) or 3'region of c-myc (Figure 2.1D). Variations of KCl and Tris concentrations in the PCR buffer were also assessed. The low KCl buffer described by Ponce and Micol (Ponce and Micol, 1992) was less efficient in our setting. However, using a buffer with a modest increase in Tris and KCl concentrations (respectively 12 and 62 mM instead of 10 and 50 mM) noticeably and reproducibly improved the yield of the amplified cDNA (data not shown).

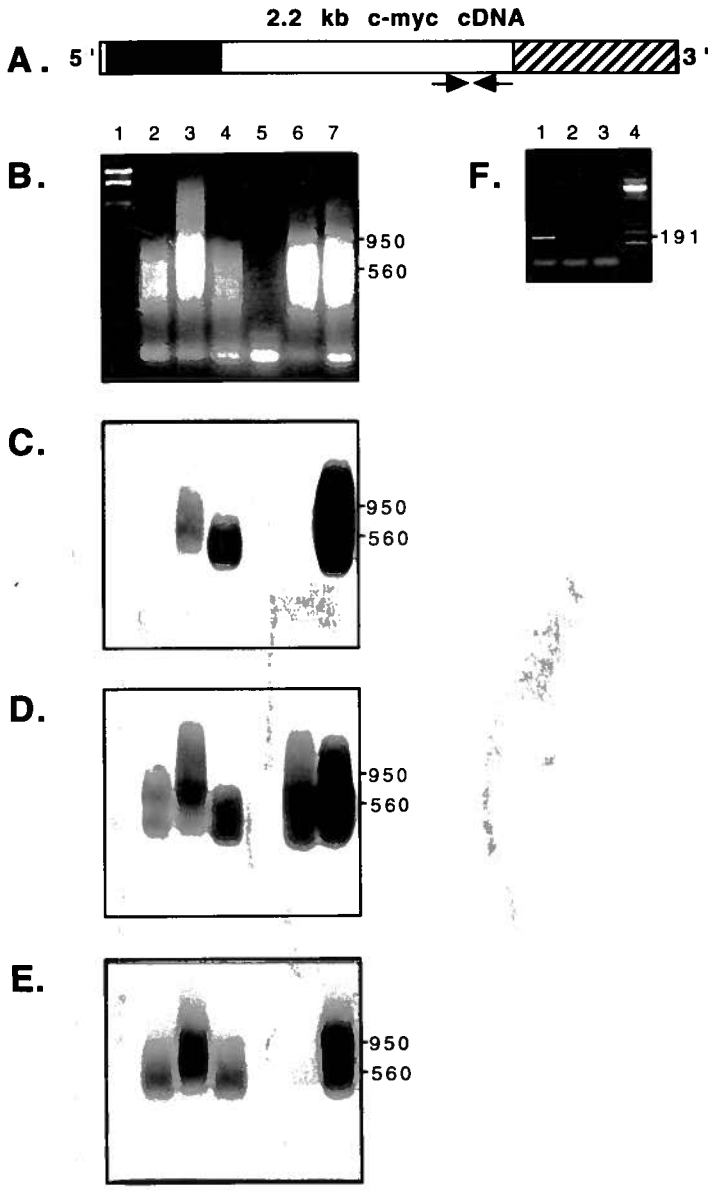

Figure 2.1. Evaluation of PCR amplified total cDNA obtained from limited numbers of HL-60 or low density human bone marrow cells.

(A) c-myc 2.2 kb cDNA schematic showing the respective location of the 5' (black box) and 3' probes (hatched box). The facing arrows indicate relative location of the c-myc specific primers. (B) Agarose gel electrophoresis and ethidium bromide staining of aliquot (10 µl) of the cDNA prepared from 3,000 HL-60 or 25,000 unseparated low-density human bone marrow cells using the amplification strategy described in the Methods section. (C) Southern blot analysis of the amplified cDNA with a probe to the 5' region of c-myc (autoradiogram exposed for 1 day at -70°C). (D) Membrane of panel C rehybridized to a probe to the 3' region of c-myc (exposed for 35 minutes). (E) Membrane of panel D rehybridized to a full-length homeobox gene *HB24* probe (exposure for 6 hours at room temperature). (F) Product of amplified HL-60 cDNA reamplified using c-myc specific primers flanking an intron. Panels B,C,D,E; lane 1, lambda marker cut with HindIII + EcoRI; lane 2, human bone marrow cells amplified without gene 32 protein; lane 3, human bone marrow cells amplified with gene 32 protein; lane 4, HL-60 cells; lane 5, HL-60 cell reaction without reverse transcriptase; lane 6, HL-60 cell reaction without tailing; lane 7, HL-60 amplified with gene 32 protein. Panel F: lane 1, HL-60 cells; lane 2, HL-60 cell reaction without reverse transcriptase; lane 3, HL-60 cells without tailing; lane 4, 123 bp ladder.

Further modifications, after RNA purification, included reduction in the number of precipitation steps to one using a very reliable linear polyacrylamide carrier. A precipitation step between the tailing reaction and the PCR amplification has been omitted by: i) performing a precipitation with ammonium acetate after the reverse transcription to resolve buffer incompatibility and, ii) minimizing the volume of the tailing solution thus allowing adjustment of the tailing deoxynucleotide to high concentration that assures an homopolymeric deoxyadenosine tail of the first cDNA strand. The final concentration of the dNTP in the PCR reaction was adjusted by taking into account the high concentration of the dATP in the tailing reaction (which is theoretically only marginally consumed in the tailing process) explaining the absence of this nucleotide in the PCR mix.

Together, the improvements made to minimize nucleic acid loss and to optimize the amplification efficiency resulted in high yield of amplified cDNA thereby allowing a decrease in the number of PCR cycles to 40 from 100 or more.

2.4.2 Presence in Amplified cDNA of Sequences Distant from the 3' end of mRNA.

Gel electrophoresis and ethidium bromide staining of amplified cDNA prepared as above revealed an average size of approximately 800-900 bp (Figure 2.1B). To better assess if sequence distant from the 3' end of mRNA was represented, Southern blot analysis was performed on amplified cDNA prepared from HL-60 cells and normal human bone marrow cells using probes for 5' versus 3' c-myc sequences (Figure 2.1C and D respectively), and, second by attempts to reamplify cDNAs with primers directed at a 191 bp region located 712 bp from the c-myc polyadenylation site (Figure 2.1F). As shown in Figure 2.1C and

2.1D, both the 5' c-myc region probe (which spans nucleotides 67 to 455 of the 2.2 kb c-myc cDNA), and a 3' c-myc region probe (which spans the last 563 nucleotides (exon 3) of the 2.2 kb c-myc cDNA) hybridized to amplified cDNA from HL-60 or bone marrow. Significantly, higher signals were observed for HL-60 cells consistent with the known overexpression of c-myc in these cells as detectable by standard Northern analyses of total cellular RNA (Figure 2.2) and suggestive of preservation of relative mRNA levels by this procedure.

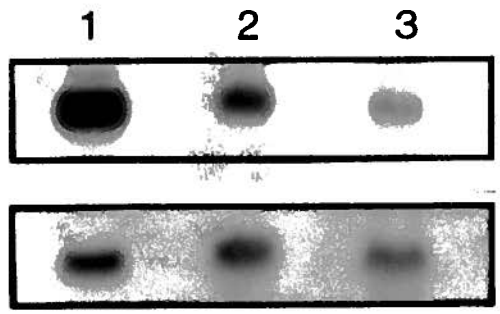

Figure 2.2. Comparative expression of c-myc in normal bone marrow, K562 and HL-60 cells by Northern blot analysis.
5 μg of total cellular RNA was loaded in each lane. HL-60 (lane 1), K562 (lane 2), or total human bone marrow (lane 3) cells. Probes were to the 5' region of c-myc (upper panel) as described in Figure 2.1, or to β-actin (lower panel) .

In both bone marrow and HL-60, signal intensities for 5' region sequence were very strong indicating significant levels of amplified cDNA extending a minimum of 1.75 kb 5' of the polyadenylation site. Further amplification of cDNA using c-myc specific primers, resulted in the expected 191 bp product (Figure 2.1F), again confirming the presence of amplified cDNA sequence at least 700 bp from the polyadenylation site. It is noteworthy that amplified cDNA obtained from reverse transcription reactions in which homopolymeric tailing with deoxyadenosine was omitted did hybridize to the 3' but not 5' c-myc region

probe (lane 6 of Figure 2.1C versus 2.1D); this material also did not give rise to a PCR product with specific c-myc primers (Figure 2.1F, lane 3), suggesting that tailing is important to obtain maximal length products but also that some degree of amplification can occur in the absence of tailing.

Based on melting temperature, I hypothesized that hybridization of the oligo dT-based primer to poly-adenosine (polyA) stretches of greater than 4-5 in length occuring in the second strand synthesis (37°C) was responsible for this result. This hypothesis was based on the fact that c-myc has a long poly-thymidine stretch in the 3' region of its mRNA and that this region is spanned by the 3' probe used here. After reverse transcription of the mRNA, the thymidine stretch is converted to an adenosine one which could become a potential target for internal priming by the oligo dT primer. This is most likely during the second strand synthesis which is done at low annealing temperature (37°C). To test this hypothesis, this same blot shown in Figure 1 was rehybridized to a full-length cDNA probe for HB24, a homeobox gene which is devoid of such thymidine tracks (Deguchi et al., 1991a). As predicted, no signal was obtained with this probe in amplified cDNA prepared without tailing (Figure 2.1E, lane 6) but a strong signal was found in every instance in which samples were subjected to reverse transcripton and deoxyadenosine tailing. This result was further confirmed by attempts to reamplify cDNA using c-myc specific primers located 5' to the polythymidine stretch; an amplified product was readily apparent for cDNA prepared with tailing (lane 1, Figure 2.1F) but was not observed in the absence of tailing (lane 3, Figure 2.1F).

As illustrated by the Southern blot analyses in Figures 2.1 smears but not bands were obtained following hybridization. Similar results were observed with all probes tested so far. This phenomenon is common to other PCR

methods amplifying total cDNA published so far (Belyavsky et al., 1989; Brady et al., 1990; Domec et al., 1990; Don et al., 1993; Dumas et al., 1991; Froussard, 1992; Lambert and Williamson, 1993) and likely represents premature termination of the first strand synthesis and of later PCR amplification products. Internal priming during first and second strand synthesis may also contribute to the range of sizes.

2.4.2 Sensitivity and Representativeness of the cDNA.

To test the sensitivity of this method for detecting moderate to rare abundance transcripts, 10,000 HL-60 cells were mixed with murine hemopoietic Ba/F3 cells that were previously transfected with a neomycin resistance gene. The level of neo^r gene expression in these latter cells has been evaluated by Northern analysis to be about 1% of the total messages (data not shown). As shown in Figure 2.3, amplified neo^r specific cDNA sequence was detected when as few as 100 neo transfected cells were present in 10,000 HL-60 cells (1%) thus suggesting this method enabled detection of messages with a frequency of about 0.01%. Moreover, the neo^r signal intensities correlated well with the proportion of neo-resistant positive Ba/F3 cells mixed with HL-60.

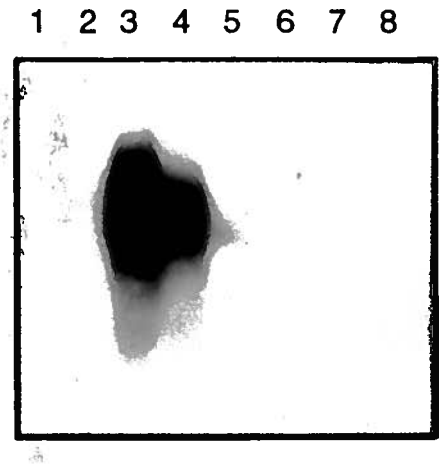

Figure 2.3. Southern blot analysis using a neo^r specific probe to test the sensitivity of PCR cDNA amplification.

Ten thousand HL-60 cells were mixed with different numbers of neo^r -expressing Ba/F3 cells and amplified cDNA prepared: lane 1, 10,000 HL-60 cells alone with no reverse transcriptase; lane 2, HL-60 cells alone with no tailing; lanes 3 to 8, 10,000 HL-60 cells mixed with 10^4, 10^3, 100, 10, 1, or no neo^r positive Ba/F3 cells. Autoradiographic exposure was 16 hours at -70°C.

To further evaluate this method for the detection of rare messages, a probe for the homeobox *HOXB4* gene was hybridized to a nylon membrane containing different amplified cDNA derived from a mixture of HL-60 (negative for *HOXB4* expression (Magli et al., 1991)) and K-562 cells (positive for low level expression of *HOXB4* (Magli et al., 1991)). As shown by Southern blot analysis (Figure 2.4A), the intensity of the *HOXB4* signal was directly correlated with the number of K-562 cells added and was absent in HL-60 cells alone. *HOXB4* signal was detected with as few as 100 K-562 cells suggesting this method has the potential to detect rare messages present in a small proportion of the cells.

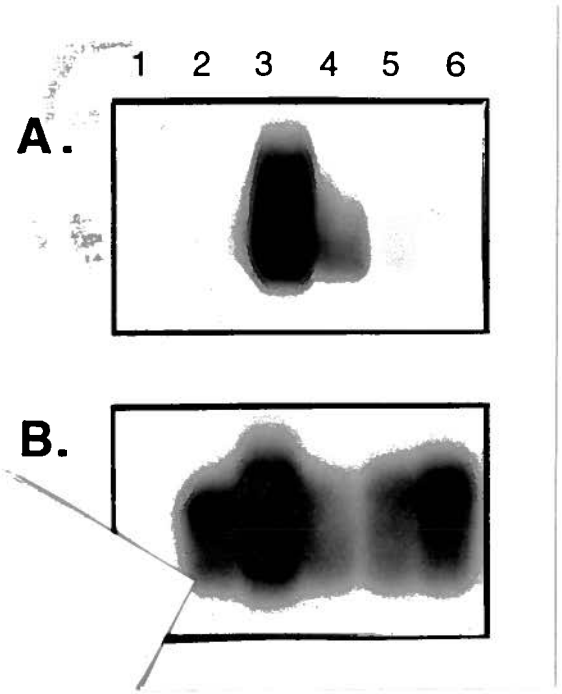

Figure 2.4. Southern blot analysis of PCR amplified cDNA to detect expression of *HOXB4* in HL-60 versus K562 cells.

Panel A, hybridization to *HOXB4* specific probe (exposure for 96 hours at -70°C); panel B, hybridization to 5' c-myc specific probe (exposure for 72 hours at -70°C). Lane 1, 10,000 HL-60 cells no tailing; lane2, 10,000 HL-60 cells alone; lanes 3-6, 10,000 HL-60 cells mixed with 10^4, 10^3, 100 or 10 K562 cells respectively.

Together these results suggest that this method provides a simplified approach to obtain amplified cDNA from small cell number that enables the detection of even low abundance message and the quantitative comparison of expression level of a given gene in different cell populations by Southern analysis.

It should be noted that internal priming on poly-adenosine tracts during the first strand cDNA synthesis would likely affect representation of a message since such cDNA would subsequently be "tailed" and PCR-amplified. Thus, for

those transcripts with poly-adenosine stretches, there is a likelihood that they will be over-represented as various partial cDNAs. This problem is likely to be common to any oligo dT primer-based cDNA amplification procedure. Moreover, the chances of occurrence of internal priming are high during the generation of the second cDNA strand since it is performed at a low temperature (37°C). This is likely to result in loss of full-length product for mRNA that have very long poly-thymidine tracts since the amplification of the smaller fragments would be more effective. For these two reasons, extreme care should be used in comparing amplified cDNA signal intensities between two different genes or for the same gene when using two different probes. Nevertheless, these concerns should not apply when using the same probe to study the relative level of expression of a given gene in different cell populations.

In conclusion, this simple method generates amplified cDNA that encompasses sequence at least 1.75 kb distant from the polyadenylation site of mRNA and preserves relative amplification of individual messages by optimization of PCR amplification which includes the use of gene 32 protein and by minimization of internal priming by using only one primer and high annealing temperature. Such a procedure was successfully used in the studies described in the next chapter to characterize Hox gene expression in purified $CD34^+$ human bone cell subpopulations.

CHAPTER 3

Differential expression of Hox homeobox genes in functionally distinct CD34+ subpopulations of human bone marrrow cells[5]

[5] The material presented in this chapter is essentially as described in :
Sauvageau G., Lansdorp, P.M., Eaves C.J., Hogge D., Dragowska W.H., Reid D.S., Largman C., Lawrence H.J. and Humphries, R.K. (1994). Differential expression of Hox homeobox genes in functionally distinct CD34+ subpopulations of human bone marrrow cells. Proc. Natl. Acad. Sci. USA 91: 12223-12227.
Ms Vishia Dragowska and Dianne Reid are gratefully acknowledged for their help in cell purification and culture, respectively.

Differential expression of Hox homeobox genes in functionally distinct CD34+ subpopulations of human bone marrow cells

3.1 Introduction

Studies of Hox gene expression during the differentiation of primitive normal hematopoietic cells have been hampered by technical difficulties not only in obtaining the relevant subpopulations of appropriate phenotypic and functionally defined purity but also in the low numbers of such cells that would be available ($<10^4$) given their known frequencies, making standard mRNA analyses impossible. The recent characterization of the pattern of CD45RA and CD71 expression on CD34+ cells in normal human bone marrow now allows the reproducible isolation of relatively homogeneous progenitor populations of lineage-restricted erythroid, granulopoietic and more primitive cells (Lansdorp et al., 1990; Lansdorp and Thomas, 1991). Such populations might therefore be expected to provide suitable starting material for investigating possible changes in Hox gene expression during early stages of hematopoiesis. Using a modified RT-PCR procedure that allows representative amplification of extended-length cDNAs from a few thousand cells (chapter 2), evidence for the expression of multiple Hox genes in primitive normal hematopoietic cells in now presented together with the description of striking differences in the patterns of expression of certain Hox genes within functionally distinct subpopulations of these cells.

3.2 Materials and Methods

3.2.1 Cell Purification.

Low density cells (<1.077g/cm^3) of 5 different heparinized cadaveric human bone marrows (CAD3, CAD6, CAD7, CAD9, CAD10) were isolated by centrifugation on Ficoll-Paque (Pharmacia LKB, Uppsala, Sweden) and kept frozen in Iscove's medium containing 2.5% human serum albumin and 7.5% DMSO. Cells from CAD3, CAD6 and CAD9 were thawed and stained with directly conjugated fluorescent antibodies to CD34 (8G12-Cy5), CD45RA (8d2-R-phycoerythrin, R-PE) and CD71 (OKT9-fluorescein isothiocyanate) washed twice and resuspended in 2 mg/ml of Propidium Iodide (P-5264; Sigma Chemical Co.) prior to sorting on a FACStarPlus® (Becton-Dickinson Immunocytometry, San Jose, CA) as previously described (Lansdorp and Dragowska, 1992). After initial separation of CD34$^+$ cells on the basis of CD45RA and CD71 expression (Figure 3.1A and 3.1C), CD34$^+$ CD45RA^{lo} CD71^{lo} cells were stained with the CD38 (Leu17-PE, BD)-conjugated antibody and further separated into subpopulations expressing different levels of CD38 (Figure 3.1B and 3.1D). Each subpopulation was subjected to two rounds of sorting and cell purity was assessed after the first sort. Cells from each of the 5 marrows were also stained with 8G12-Cy5 alone and separated into total CD34$^+$ and CD34$^-$ subpopulations.

3.2.2 Clonogenic Progenitor Assays.

Aliquots from purified CD34$^+$ subpopulations were assayed for colonies derived from CFU-E, mature BFU-E, primitive BFU-E, CFU-GM or CFU-GEMM in methylcellulose cultures containing fetal calf serum, 3 U/ml of highly purified human erythropoietin (100,000 units/mg, Stem Cell Technologies Inc, Vancouver, B.C.), 50 ng/ml of human Steel factor (Amgen), 20 ng/ml each of

human IL-6 (Immunex), human GM-CSF (Sandoz), human G-CSF (Amgen) and human IL-3 (Sandoz) (Eaves, 1985).

3.2.3 Long-term Culture.

The long term culture-initiating cells (LTC-IC) content of each CD34+ subpopulation was evaluated essentially as described previously by placing 2,400 cells on irradiated (8000 cGy) murine fibroblast feeder layers consisting of 1.5×10^5 cells per 35 mm dish of both M2-10B4 cells (Lemoine et al., 1988) and Sl/Sl cells (Sutherland et al., 1990) engineered by retroviral gene transfer to produce 10 ng/ml of human IL-3, 130 ng/ml of human G-CSF and 10 ng/ml of human Steel factor. After 6 weeks, the clonogenic progenitor content of the LTC was determined and the number of LTC-IC calculated by dividing this number by 4 based on previous studies (Sutherland et al., 1990) and also confirmed here by limiting dilution analysis (data not shown).

3.2.4 cDNA Generation and Amplification.

The generation and amplification of total cDNA obtained from each bone marrow subpopulations was performed exactly as described in chapter 2.

3.2.5 PCR Amplification of Hox Homeodomains.

The amplified total cDNA derived from each different bone marrow subpopulation or cell line was subjected to a second round of PCR amplification using degenerate primer sets designed to match all of the different sequences represented at the conserved α-1 helix (5'(TC)(TCGA)GA(AG)(TC)T(CG)GA-(AG)AA(AG)GA(AG)TT 3') and the α-3 helix (5' C(GT)(ACGT)C(GT)(AG)TTC-TG(AG)AACCA(ACGT)A 3') regions of the human Hox gene homeodomains (Vieille-Grosjean et al., 1992). One percent of the total cDNA (0.5 μl) of each subpopulation was amplified and the resulting 118 bp products were subcloned and sequenced. Control preparations from original RNA not reverse transcribed

failed to yield a band indicating that contamination by genomic DNA was not encountered.

3.2.6 Southern Blot Analysis of Total Amplified cDNA.

One fifth of the total amplified cDNA prepared from each purified subpopulation or from cell lines was analyzed by Southern blot (see Chapter 2). Homeodomain free probes for all Hox genes but *HOXB3* and *B4* were prepared from subcloned sequences of cDNA or genomic clones initially kindly provided by Dr. E. Boncinelli. Probes for *HOXB3*, *HOXB4*, CD71 and MDR1 were derived from clones obtained from the American Type Culture Collection (ATCC, Rockville, MD). Full length *HB24* cDNA and CD34 cDNA sequences were kindly provided by Drs J. Kehrl and B. Seed respectively. Densitometric analysis was performed by using two different autoradiogram exposure times for each blot to ensure linearity of the results.

3.3 Results

3.3.1 Characterization of Purified CD34+ Bone Marrow Cell Subpopulations.

Antibodies directed against CD34, CD45RA and CD71 were used to isolate by FACS 3 phenotypically and functionally distinct subpopulations (I, IIM and IIIE) from the low density fraction of 3 different normal human bone marrow samples (CAD3, CAD6 and CAD9). Figure 3.1A and 3.1C shows the FACS profile of 2 of these 3 marrows; CAD3 and CAD6 (CAD9 profiles were virtually superimposable to CAD6, not shown). In each case, subpopulation I was further subdivided into two (CAD3, Figure 3.1B) or three (CAD6, Figure 3.1D; CAD9, data not shown) subpopulations by sorting the cells based on their surface expression of the CD38 antigen. After the first found of sorting, each of these subpopulations was analyzed and found to be over > 98% pure.

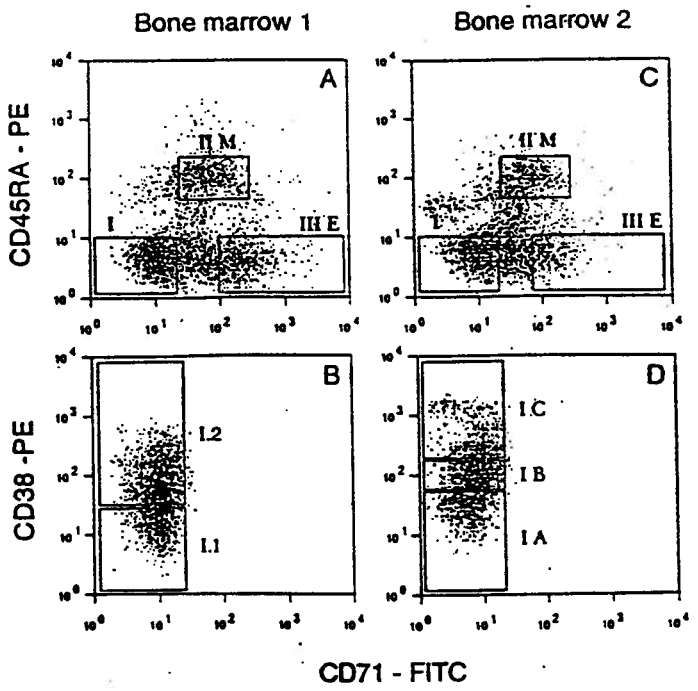

Figure 3.1. FACS profiles of the CD34+ subpopulations isolated from bone marrows no.1 (CAD3) and no.2 (CAD6).

For both marrows, the cells in subpopulation I (CD34+ CD45RA- CD71-) shown in Panels A and C were further fractionated according to their expression of CD38 into 2 fractions for marrow no.1 (I.1 and I.2) (Panel B) and into 3 fractions for marrow no. 2 (IA, IB, IC) (Panel D). Each of these subpopulations represent between 0.4 and 3% of the low density bone marrow cells and are > 99% pure.

For CAD3 and CAD6, functional assays were performed on aliquots from each of the purified subpopulations (Table 3.1). For both marrows, long term culture-initiating cells (LTC-IC) were detected exclusively in subpopulation I and subdivision of these cells according to their expression of CD38 revealed further segregation of the LTC-IC to the $CD38^{low-med}$ cells. In contrast, cells of subpopulation I capable of proliferating in semi-solid assays in response to a potent cocktail of soluble growth factors (Steel factor + G-CSF + GM-CSF + IL-3 + IL-6) were more concentrated in the $CD38^{med-high}$ fraction. As a result, for both marrows it was possible to demonstrate that in the $CD38^{low}$ fractions, LTC-IC constituted the major class of detectable hematopoietic cells and at least 50% (CAD3; population I.1) and up to 98% (CAD6; population IA) of these

could not be detected as clonogenic cells in a 4 week direct assay. Those few clonogenic cells that were identified as co-purifying with the LTC-IC in the CD38^{low} fraction, although classified as CFU-GM, all showed delayed initiation and generated very large colonies of cells of a morphology *in situ* (i.e. without staining) of granulocytes and macrophages. Subpopulations IIM and IIIE were highly and differentially enriched in granulopoietic and erythroid clonogenic cells, respectively, in each case to a purity of 20% with $\leq$ 5% of clonogenic cells of the opposite lineage still present.

Table 3.1. Clonogenic progenitor[a] and LTC-IC[b] content of the different purified CD34$^+$ bone marrow subpopulations.

Fractions Sorted	CFU-E		BFU-E		CFU-GM		CFU-GEMM		LTC-IC	
	(%purity)	Enrich-ment	(%purity)	Enrich-ment	(%purity)	Enrich-ment	(%purity)	Enrich-ment	(%purity)	Enrich-ment
CAD3:										
I.1	0	0	0	0	2.5	2	0	0	4.3	> 2100
I.2	0	0	8.4	9	26	24	5.1	113	5.3	> 2600
IIM	0	0	0	0	26	24	0	0	0	< 20
IIIE	4.3	13	13	14	6.5	6	5.6	124	0	< 20
Total Marrow[c]	0.3	1	0.95	1	1.1	1	0.05	1	< 0.002[d]	1
CAD6:										
IA	0	0	0	0	0.5	1	0	0	29	1450
IB	0	0	1.5	3	5.8	8	0.5	167	29	1450
IC	0.8	1	4	7	17	24	0.5	167	0	0
IIM	0	0	0	0	24	34	0	0	0	0
IIIE	14	18	22	37	2.8	4	0	0	0	0
Total Marrow	0.8	1	0.6	1	0.7	1	0.003	1	0.02	1

[a]Based on analysis of 400 cells.
[b]Based on analysis of 2400 and 1000 cells for marrow 1 and 2 respectively.
[c]This part of the experiment was performed on a different aliquot of the same marrow thawed and assayed at a later time.
[d]Based on the analysis of 6.6 x 10^4 cells.

3.3.2 Hox Gene Expression in CD34$^+$ Subpopulations.

To assess Hox gene expression in purified bone marrow cell subpopulations, total amplified cDNA were generated from 1,000 to 5,000 cell aliquots using the RT-PCR procedure described in chapter 2. Amplified total cDNA from the purified CD34$^+$ subpopulations were analyzed for Hox gene expression using two different approaches. The first of these was aimed at obtaining an initial

indication of the range and potential pattern of Hox gene expression in the CD34+ purified subpopulations. It was performed on one marrow (CAD3) and consisted of reamplifying the cDNA derived from each subpopulation with a set of Hox gene specific degenerate primers that spanned a 118 bp region of the homeodomain. Resulting products were subcloned and a total of 92 clones were sequenced. As summarized in Table 3.2, this analysis revealed the expression of 12 different Hox genes in the various CD34+ subpopulations: seven from cluster A, three from cluster B and two from cluster C. No expression of any cluster D genes was detected in these cells. In all, 82 of the 92 clones sequenced were found to belong to cluster A. This difference probably reflects a higher expression of cluster A genes in these cells since, as reinforced by the analysis of the cell line data described below, it is unlikely that the degenerate primers used preferentially amplified this particular group of Hox genes. Interestingly, most of the cluster A sequences detected (34/46; 76%) in the most primitive subpopulations (I.1 and I.2) were found to be within the 3' region (A1 to A6) of this cluster, whereas 27 of the 38 (71%) cluster A sequences found in the more differentiated subpopulations (IIM and IIIE) were concentrated at the 5' region of this cluster (A7 to A13). Also, of the 8 sequences belonging to cluster B, 6 were limited to subpopulation I.1 and I.2 suggesting a more restricted expression of this Hox cluster to the most primitive hematopoietic cells.

For comparison, and to assess both the sensitivity and specificity of this approach, a similar analysis was carried out using equivalent numbers of cells from the K562 (erythroleukemic) and HL-60 (promyelocytic) cell lines. Homeodomain sequences were obtained from 27 clones derived from K562 cells and 22 clones derived from HL-60 cells. As also shown in Table 3.2, five of the seven expressed Hox genes detected in HL-60 were Hox A genes, while six of the eight genes detected in K562 were Hox B genes. These results are in

agreement with previous reports of Hox gene expression in these 2 cell lines (Magli et al., 1991). Moreover, they are consistent with previous observations of preferential expression of Hox A genes in myeloid cell leukemias and Hox B genes in leukemic cell lines with erythroid phenotypes (Lawrence and Largman, 1992).

Table 3.2. Expression of Hox genes in purified bone marrow subpopulations and in hemopoietic cell lines determined by sequence analyses of amplified homeobox containing cDNA

Hox genes[a]	Purified subpopulations[b] (CAD3)				Cell lines	
	I.1	I.2	IIM	IIIE	HL-60	K562
A1					+(1)[c]	+(2)
A2		+(1)				
A3						
A4	+(3)	+(6)	+(1)			
A5	+(5)	+(11)	+(7)	+(2)	+(5)	
A6	+(4)	+(3)		+(1)	+(1)	
A7			+(6)	+(5)	+(8)	
A9	+(5)	+(6)	+(8)	+(4)		
A10			+(1)	+(3)	+(2)	
A11						
A13						
B1						
B2						+(2)
B3	+(1)					+(3)
B4						
B5						+(2)
B6						
B7		+(4)	+(1)	+(1)		+(2)
B8						+(2)
B9		+(1)				+(3)
C4-6						
C8				+(1)	+(1)	
C9	+(1)				+(4)	+(12)
C10-13						
D1-13						
Total:	19	32	24	17	22	27

[a]For each cluster, the genes are listed as found 3' to 5' on their respective chromosome.
[b]Functional characteristics of each of these subpopulations are presented in Table 3.1.
[c]"+" indicates detection of one or more cDNA clones containing Hox gene specific homeobox sequence. The total number of independent clones identified for a specific gene is shown between parenthesis.

3.3.3 cDNA Southern Blot Analysis of Hox Gene Expression in CD34+ Subpopulations.

To characterize further the extent and possible differential expression of Hox genes in early hematopoietic cells, the initial amplified total cDNA prepared from the various CD34+ subpopulations was also analyzed by Southern blot using homeodomain-free Hox gene specific probes. For marrow CAD6 the five

CD34+ subpopulations were analysed using 11 different Hox gene probes (Figure 3.2A): 7 of the 9 known cluster B genes (B2, B3, B4, B5, B6, B8, B9), 4 of the 11 cluster A genes (A4, A5, A9, A10) and one non-cluster homeodomain-containing gene (*HB24*: Figure 3.2B) whose expression was recently described as restricted to CD34+ cells (Deguchi et al., 1992). Expression of 10 of these 11 Hox genes was detected in all subpopulations with the exception of B6 which was not detected in any of the primitive subpopulations nor in HL-60 or K562 cells (Figure 3.2A). On the other hand, *HB24* appeared to be equally expressed in all subpopulations paralleling that of actin (Figure 3.2B). Densitometric analysis of these blots showed that *HOXB9*, which is located towards the 5' end of the B cluster, was expressed at similar levels in all CD34+ subpopulations, whereas B3 and B4 which are more 3' cluster B genes were expressed at much higher levels (17 and 6-fold respectively) in subpopulation IA, which is highly enriched in LTC-IC, than in IIM, which is enriched in myeloid progenitors and devoid of LTC-IC. This differential expression of 3' cluster B genes was similarly observed when subpopulation IA was compared with the erythroid cell enriched subpopulation IIIE. These results suggest that the 3' genes are preferentially expressed in the most primitive hemopoietic cells in contrast to more 5' genes which appear to be more uniformly expressed at least in the early stages of differentiation. This finding was reinforced by the findings for Hox genes located in the middle of the B cluster (B5 and B7) whose expression was only slightly more restricted to the more primitive subpopulations IA and IB.

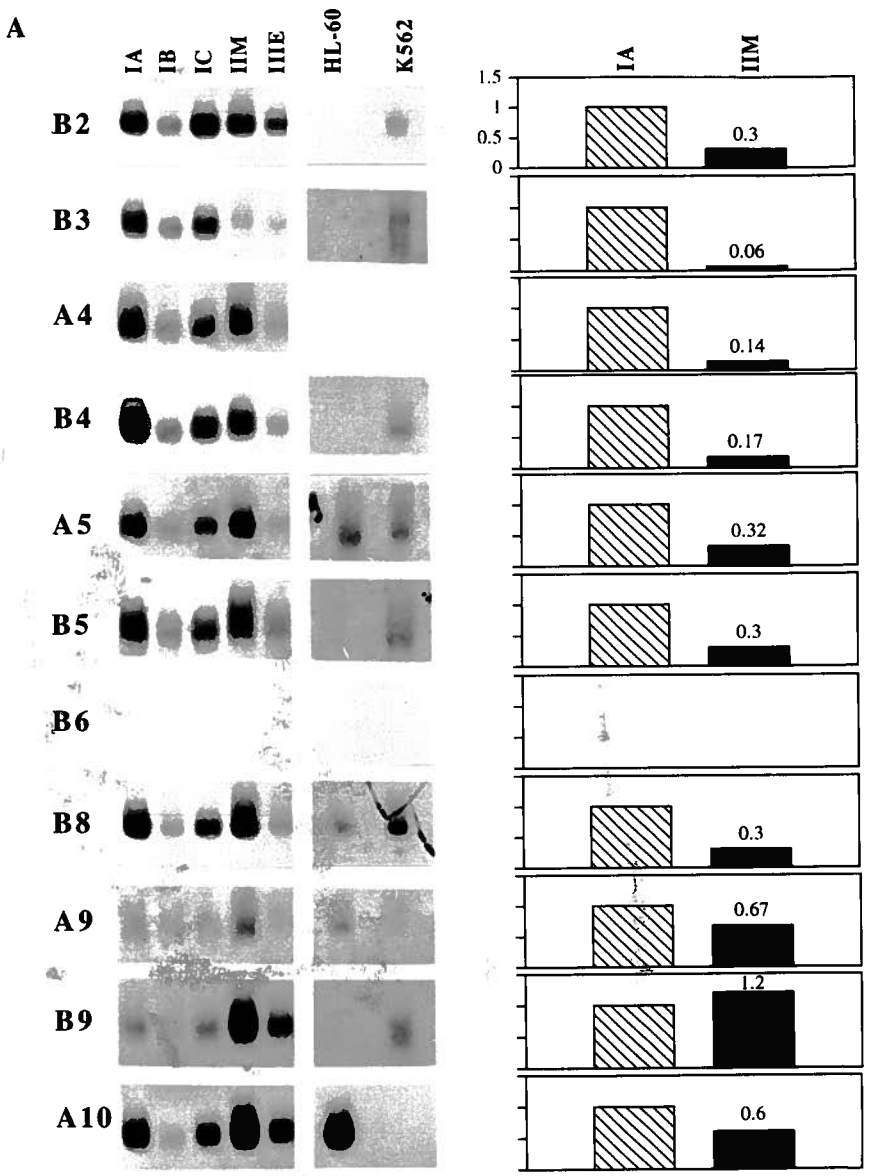

Figure 3.2A. Southern blot analysis of the total amplified cDNA derived from each CD34+ subpopulation.

Bone marrow no.2 (CAD6) and from HL-60 and K562 cells. Exposure times were adjusted to facilitate comparison of the expression of individual Hox genes between the 5 subpopulations studied. For *HOXA4*, hybridization to HL-60 or K562 was not done. Vertical alignment of the blots follows the relative positions 3' to 5' of each Hox gene in its cluster. Each blot is accompanied by a display (right panel) that shows the normalized expression (to actin and to subpopulation IA) of the different Hox genes found in subpopulations IA and IIM as determined by densitometric analysis. (B) The same membrane was also hybridized to probes for actin, CD34, β-globin, P-glycoprotein (MDR1), CD71 and *HB24* (see next page).

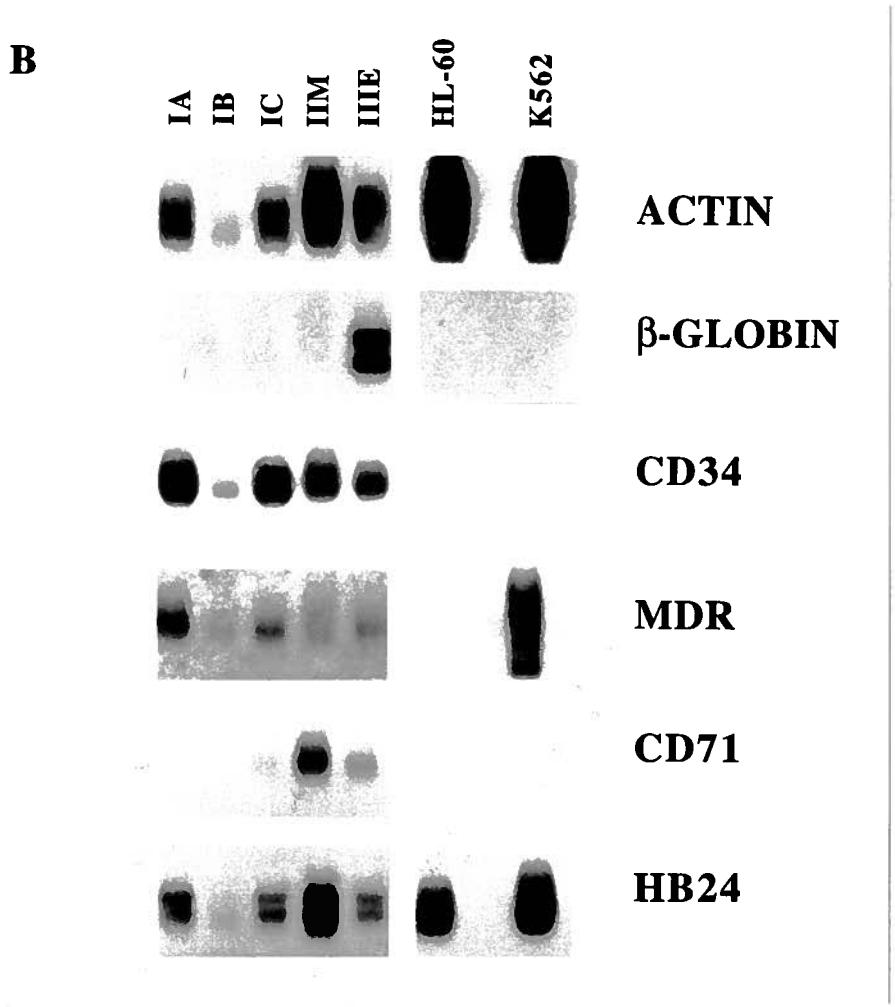

Figure 3.2B. Southern blot analysis of the total amplified cDNA derived from each CD34+ subpopulation.
See legend of Figure 3.2A for abbreviations.

This pattern also extended to the cluster A genes. As shown in the right panel of Figure 3.2A, expression of the 3' gene *HOXA4* was 7-fold higher in subpopulation IA than in IIM and, a relatively uniform expression for the 5' genes A9 and A10 was found. Again, for a gene from the middle of the cluster, *HOXA5*, expression was moderately elevated in subpopulation IA (3-fold higher than in subpopulation IIM). Moreover, the relative expression of the six paralogs

analyzed (i.e., A4 and B4, A5 and B5 and, A9 and B9) was strikingly similar in each of the five subpopulations suggesting that, as in embryonic development, a simultaneous expression of the Hox paralogs may occur during hematopoietic cell maturation.

In order to examine the reproducibility of this apparent trend, Southern blot analyses were similarly carried out on the four subpopulations of CAD3 and the 5 subpopulations of a third marrow (CAD9) fractionated exactly as for CAD6 (Figure 3.1). Two selected Hox probes (one 3'- *HOXB3*, and one 5'- *HOXA10*) were used for this analysis (data not shown). Densitometric analysis of Hox gene expression normalized to actin revealed again that *HOXB3* expression was mainly limited to the most primitive subpopulations (15 and 28 fold higher in the $CD34^+$ $CD38^-$ cells of subpopulation IA than in subpopulation IIM respectively for CAD9 and CAD3) and that *HOXA10* expression was invariant among all of the subpopulations.

The significance of these patterns of Hox gene expression in the various $CD34^+$ populations analyzed was reinforced by several controls. First, the level of CD34 expression in these subpopulations as detected by cDNA Southern analysis was found to correlate with the surface expression of this antigen as studied by FACS analysis with a progressive decline: IA>IB>IC>IIM=IIIE (see Figure 3.2B for cDNA Southern results). MDR1 expression was also higher in the more primitive cell subpopulations, was not found in HL-60 and was present at high level in K562 consistent with the expected expression pattern for this gene (Chaudhary and Roninson, 1991). Similarly, CD71 expression determined by cDNA analysis correlated with its surface expression (i.e., more expressed in the IIM and IIIE subfractions, Figure 3.2B) and only the

subpopulation enriched for late erythroid progenitors (CFU-E, subpopulation IIIE) was found to express significant levels of ß-globin mRNA.

3.3.4 cDNA Southern Analysis of Hox Gene Expression in CD34⁻ Cells.

To explore the possibility of additional changes in Hox gene expression with further hematopoietic differentiation, expression of two selected Hox genes, *HOXB3* and *HOXA10*, was analyzed in the $CD34^-$ fraction of mononuclear cells from five different marrows compared to the total or $CD34^+$ fractions. Results of Southern blot analysis of total amplified cDNA generated from the various fractions of 3 representative marows are shown in Figure 3.3 (CAD7, CAD9 and CAD10). As expected from the analysis of $CD34^+$ subpopulations, expression of both *HOXB3* and *HOXA10* was readily detected in the total $CD34^+$ mononuclear cell fraction of the three bone marrows. In sharp contrast however, expression of both genes was virtually extinguished in the $CD34^-$ fraction. Consistent with these observations, the signal intensity from total mononuclear cells was lower in proportion to the percentage of $CD34^+$ cells.

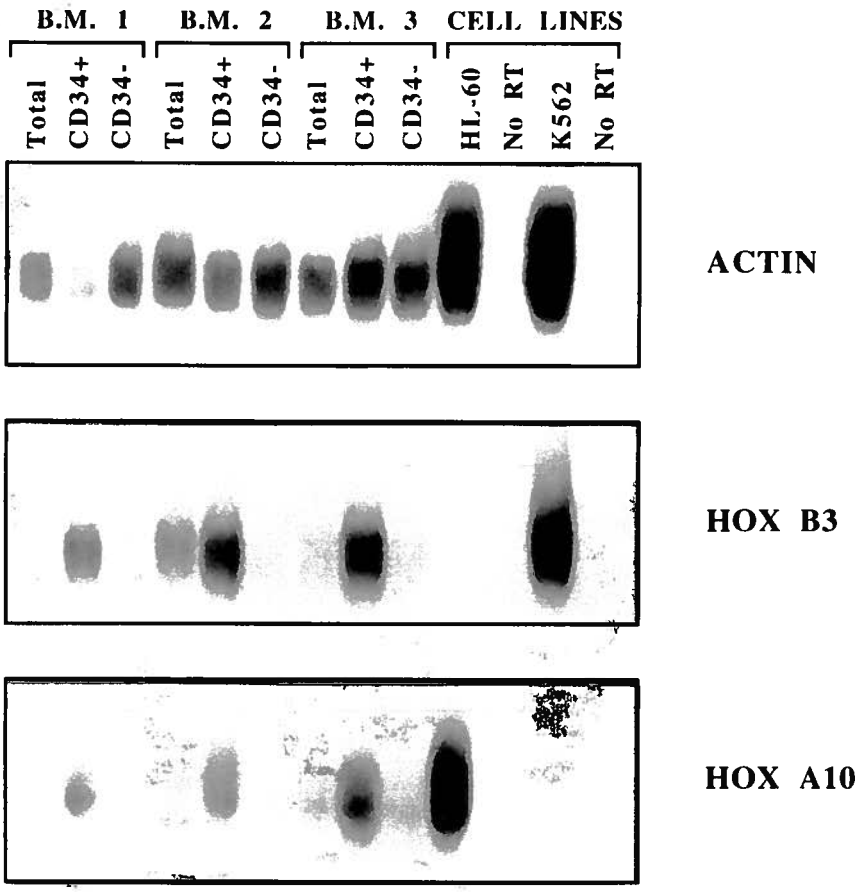

Figure 3.3. Southern blot analysis of total amplified cDNA derived from the low density cells fraction of three different marrows.

Cells were sorted in unfractionated, CD34-enriched and CD34-depleted subpopulations. $CD34^+$ content of the unfractionated, $CD34^+$ and $CD34^-$ fractions were 5.5% / 95.8% / 0.4% for marrow 1 (CAD7); 16.1% / 95.2% / 1.8% for marrow 2 (CAD9) and 7.3%; / 95.5% / 0.7% for marrow 3 (CAD10). Viability was over 95% in each fraction. No RT, no reverse transcriptase.

3.4 Discussion

These studies document expression of at least 16 different Hox genes in highly purified subpopulations of primitive CD34+ human marrow cells. Moreover, these data suggest that the lineage- subfractions of these primitive cells are characterized by markedly higher levels of expression of certain Hox genes in comparison to their levels in lineage+ subfractions. Detailed functional characterization of these cell subpopulations indicates that the preferential expression is associated with a very primitive CD34+ subpopulation containing all LTC-IC and relatively few lineage-restricted progenitors detectable in direct colony assays. This enhanced expression appears to involve genes located in the 3' regions of the A and B clusters. On the other hand, 5' located genes were expressed at relatively equal levels in each of the primitive CD34+ subpopulations analyzed. Our results suggest that early differentiation events involve or are accompanied by a down-regulation (potentially 3' to 5') of class I Hox genes. For some, such as *HOXB3*, this down regulation appears to coincide with the earliest stages of hematopoietic differentiation whereas for others, such as *HOXA10*, expression may persist to later stages of development. Interestingly, however, for at least two Hox genes examined here, expression was virtually extinguished with further progression to the CD34- stage.

Using various cell lines as prototypes of cells at different stages of hemopoietic maturation, it has been suggested that expression of some Hox genes may be lineage-restricted (Lawrence and Largman, 1992; Magli et al., 1991; Mathews et al., 1991). For example, *HOXB3* has been described as erythroid-specific because it is expressed in both OCIM2 and K562 cells, and both of these cell lines exhibit some erythroid features (Magli et al., 1991;

Mathews et al., 1991). Our results failed to demonstrate preferential expression of *HOXB3* in normal human erythroid progenitors (subpopulation IIIE). In fact, this gene was expressed at about 20-fold higher levels in more primitive subpopulations in which no CFU-E or BFU-E were detectable by comparison to the only subpopulation in which these erythroid progenitors were found. Another Hox gene, *HOXA10* has been similarly described as myeloid-restricted (Magli et al., 1991; Shen et al., 1989). However, in the present study, we found this gene to be equally expressed in all of the CD34+ subpopulations.

Using a growth factor-stimulated peripheral blood-derived CD34+ purified subpopulation, Giampaolo et al. (1994) concomitantly reported that *HOXB3* is the only Hox gene expressed in this "quiescent hemopoietic progenitor cell population" and that there is a sequential activation (3' to 5') of the B cluster genes which follows growth factor stimulation of this purified population (Giampaolo et al., 1994). These results differs from those described in our study in that we have observed down-regulation of Hox gene expression with maturation of bone marrow cells. It thus appear that Hox gene expression differs in peripheral blood progenitors and that growth factor stimulation may sequentially recruit the clustered homeobox genes.

Taken together our results combined with that of Giampaolo point to the existence of a highly regulated program of Hox gene expression at the earliest stages of hemopoietic cell differentiation. The exact relationship between these patterns of mRNA expression and actual proteins levels will need to be clarified. Nevertheless, such findings set the stage for future identification of mechanisms regulating hematopoietic cell proliferation and lineage determination.

CHAPTER 4

Overexpression of *HOXB4* in Hematopoietic Cells Causes the Selective Expansion of More Primitive Populations in Vitro and in Vivo[6] .

[6] The material presented in this Chapter is essentially as descirbed:
Sauvageau G., Thorsteinsdottir U., Eaves C.J., Lawrence H.J., Largman C., Lansdorp P.M. and. Humphries R.K. (1995). Overexpression of *HOXB4* in Hematopoietic Cells Causes the Selective Expansion of More Primitive Populations in Vitro and in Vivo. Genes and Dev. 9; 1753-1765.
Unnur Thorsteinsdottir significant contribution to the work involving the CRU evaluation of the mice transplanted with *HOXB4*-transduced cells is gratefully acknowledged.

Overexpression of *HOXB4* in Hematopoietic Cells Causes the Selective Expansion of More Primitive Populations in Vitro and in Vivo

4.1 Introduction

In the previous chapter, it was shown that most Hox A and B cluster genes are expressed in $CD34^+$ normal human bone marrow cells (Sauvageau et al., 1994). Purification of this $CD34^+$ bone marrow fraction into functionally distinct subpopulations and RT-PCR-based analysis of Hox gene expression in these cells has revealed two patterns of expression: one in which the level of expression of a given Hox gene (e.g. *HOXA10*, *B9, C8*) was essentially invariant in the different subpopulations and the other in which the expression level was much higher (up to 40-fold) in subpopulations containing the most primitive hematopoietic cells (e.g. *HOXB3, B4*). No gene was found to be up-regulated in the more mature $CD34^+$ cell subpopulations. Comparison of the levels of expression of selected Hox genes in $CD34^+$ and $CD34^-$ cells showed that Hox gene expression was higher in the $CD34^+$ cells and lower or undetectable in the $CD34^-$ cells. Together, these data suggest that Hox genes undergo down-regulation of expression with hematopoietic differentiation and further that some, such as *HOXB3* and *HOXB4*, are almost exclusively expressed in the most primitive bone marrow cells. Interestingly, this apparently highly regulated program of Hox gene expression in hematopoiesis has striking parallels with changes in expression associated with embryonic development (3' to 5') and is different from that reported in cell lines (i.e. paralogs, see general introduction). Based on these results, the hypothesis that the patterns and levels of Hox gene expression play critical roles in determining primitive hematopoietic cell properties was generated.

To test this hypothesis, *HOXB4* overexpression was engineered in murine bone marrow cells by retroviral-mediated gene transfer. The effects produced by this manipulation on the subsequent behaviour of the transduced cells and their progeny in vitro and in vivo was then analyzed. The results of these experiments show that the enhanced expression of *HOXB4* can profoundly and selectively increase the proliferative potential of primitive hematopoietic cells without detectable effects on the relative or absolute numbers of mature end cells they generate in vivo.

4.2 Materials And Methods

4.2.1 Animals

Mice used as recipients were 7 to 12 week old (C57Bl/6J x C3H/HeJ) F1 ((B6C3)F1) and those used as bone marrow donors (C57Bl/6Ly-Pep3b x C3H/HeJ) F1 ((PepC3)F1) were male and female mice bred and maintained in the animal facility of British Columbia Cancer Research Center from parental strain breeders originally obtained from The Jackson Laboratories (Bar Harbor, MA). (B6C3)F1 and (PepC3)F1 mice are phenotypically distinguishable on the basis of allelic differences at the Ly5 locus: (B6C3)F1 mice are Ly5.2 homozygotes and (PepC3)F1 mice are Ly5.1/Ly5.2 heterozygotes. All animals were housed in microisolator cages and provided with sterilized food and acidified water.

4.2.2 Retroviral Generation

The MSCV 2.1 retroviral vector (kindly provided by Dr. R. Hawley; Sunnybrook Research Institute, Toronto, Ontario) contains a polylinker cloning site immediately 5' to a murine pgk promoter-neo cassette (Hawley et al., 1992). The *HOXB4* cDNA region encompassing the complete coding sequence, was isolated as a BamHI fragment from a plasmid (kindly provided by Dr. E. Boncinelli, Ospedale S. Faffaele, Milan, Italy) and cloned upstream of the pgk-neo cassette at the XbaI site of MSCV 2.1 by blunt-end ligation using standard procedures (Davis et al., 1994b). To produce helper-free recombinant retroviruses, 10 μg of MSCV 2.1 and MSCV 2.1-*HOXB4* plasmid vector DNA were first transfected using calcium phosphate precipitation into both the ecotropic GP+E-86 (Markowitz et al., 1988b) and the amphotropic GP+envAM12 (Markowitz et al., 1988a) packaging cell lines. Virus containing supernatant were harvested 24-48 hours after the transfection and used to cross-infect the amphotropic and ecotropic packaging cells in the presence of 6

μg/ml polybrene (Sigma Chemical Co., St. Louis MO). Infected cells were then selected in 1 mg/ml G418 (Gibco/BRL Canada; Burlington, Ontario) to obtain a polyclonal population of amphotropic and ecotropic viral producing cells. To increase the viral titer of these cells, filtered supernatant from ecotropic and amphotropic virus producing cells harboring the same retroviral construct were used to cross-infect these same cells 4 times. The viral titers of the GP+E-86-MSCV2.1-pgk-neo and GP+E-86-MSCV2.1-*HOXB4*-pgk-neo virus (hereafter called neo and *HOXB4*-neo) producer cells were 3-5 X 10^6 CFU/ml and 3-5 X 10^5 CFU/ml respectively as assessed by transfer of G418 resistance to NIH 3T3 cells (Cone and Mulligan, 1984). Absence of helper virus generation in the *HOXB4*-neo viral producer cells was verified by failure to serially transfer virus conferring G418 resistance to NIH 3T3 cells (Cone and Mulligan, 1984). The absence of helper virus in serum of 5 mice transplanted with *HOXB4*-neo-transduced bone marrow (5 mice analyzed) was also confirmed using a rescue assay.

4.2.3 Cell Lines

The ecotropic packaging cell lines, GP+E-86 and the amphotropic cell line, GP+envAM12, used to generate the recombinant retroviruses were maintained in HXM medium which consists of Dulbecco´s Modified Eagles Medium (DMEM), 10% heat-inactivated (55°C for 30 minutes) newborn calf serum (NCS) (Gibco/BRL), 15 μg/ml hypoxanthine (Sigma), 250 μg/ml xanthine (Sigma) and 25 μg/ml mycophenolic acid (Sigma). Virus producing cells were maintained in HXM medium supplemented with 1 mg/ml of neomycin analog G418 and for the amphotropic cell line in addition 200 μg/ml hygromycin (Sigma). Twenty-four hours prior to harvest of viral supernatant or co-cultivation with bone marrow cells, virus producer cells were cultured in RPMI with 10% fetal calf serum (FCS) or DMEM with 10% NCS respectively. The murine

hematopoietic cell line FDC-P1 (Dexter et al., 1980) was maintained in RPMI with 10% fetal calf serum and supplemented with 5% mouse pokeweed-stimulated spleen cell conditioned medium (SCCM). The human hematopoietic cell line K562 (Lozzio and Lozzio, 1975) was maintained in RPMI with 10% FCS. Unless specified otherwise all cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. All media, serum and growth factors unless otherwise specified were obtained from StemCell Technologies Inc., Vancouver, B.C., Canada.

4.2.4 Retroviral Infection of Primary Bone Marrow Cells and Hematopoietic Cell Lines

Bone marrow cells were obtained from (PepC3)F1 (Ly5.1/Ly5.2) mice injected intravenously 4 days previously with 150 mg/kg body weight of 5-fluorouracil (5-FU) in phosphate-buffered saline, by flushing femurs and tibias with DMEM 2% FCS using a 21 gauge needle. Single cell suspensions of 1-5 x 10^5 cells/ml were then cultured on a petri dish for 48 hours in DMEM containing 15% FCS, 10 ng/ml human interleukin-6 (IL-6), 6 ng/ml murine IL-3 and 100 ng/ml murine Steel factor. All cells were then harvested and plated at 1-2 x 10^5 cells/ml in the above medium supplemented with 6 μg/ml polybrene on viral producer cell monolayers irradiated (1500 cGy X-ray) the same day at 80-100% confluence. Cells were co-cultured for 48 hours with a medium change after 24 hours. Loosely adherent and non-adherent cells were recovered from the co-cultures by agitation and repeated washing of dishes with Hank´s balanced salt solution containing 2% FCS. Recovered bone marrow cells were washed once and then counted using a hemocytometer. All growth factors were used as diluted supernatant from transfected COS cells prepared in the Terry Fox Laboratory.

Murine and human hematopoietic cell lines FDC-P1 and K562 were infected by exposure to filtered (0.22 μm, low protein binding filter, Millipore, Bedford,

MA) viral supernatant from the ecotropic or amphotropic virus producer cells respectively. The viral supernatants were supplemented with 6 µg/ml polybrene and FCS to give final concentrations of 20%. For FDC-P1 cell infection, virus supernatant was additionally supplemented with 5% SCCM. Transduced cells were selected and maintained in 1 mg/ml of G418 .

4.2.5 Transplantation of Retrovirally Transduced Bone Marrow

Lethally irradiated 7-10 week old (B6C3)F1 (Ly5.2) mice (950 cGy, 110cGy/min, ^{137}Cs gamma- rays) were injected intravenously with 2 x 10^5 bone marrow cells derived from (PepC3)F1 (Ly5.1/Ly5.2) immediately after co-cultivation of these cells with *HOXB4*-neo or neo viral producer cells. The levels of Ly5.1 donor-derived repopulation in recipients were assessed 12, 20 and 34 weeks post transplantation by flow cytometric analysis of peripheral blood samples obtained by tail vein puncture (Rebel et al., 1994). In all animals > 86% of the peripheral blood leukocytes were of donor Ly5.1 origin. At these same time points peripheral blood cell counts and hematocrit were determined for some of these animals.

4.2.6 In Vitro Clonogenic Progenitor Assays

For myeloid clonogenic progenitor assays, cells were plated on 35mm petri dishes (Greiner, Germany) in a 1.1 ml culture mixture containing 0.8% methylcellulose in alpha medium supplemented with 30% FCS, 1% bovine serum albumin (BSA),10^{-4}M ß-mercaptoethanol (ß-ME), 3 U/ml human urinary erythropoietin (Epo) and 2% SCCM in the presence or absence of 1.4 mg/ml of G418. To ensure random colony selection in single colony replating experiments, cultures were also supplemented with 500 ng/ml murine IL-3, which abrogates the size difference between colonies. Bone marrow cells harvested from the co-cultivation with virus producer cells or from reconstituted transplant animals were plated at a concentration of 2 x 10^3 cells/dish or 2-4 x

10^4 cells/dish respectively. Spleen cells from animals transplanted with *HOXB4*-neo- or neo-transduced cells were plated 3 x 10^5 cells/dish or 3 x 10^6 cells/dish respectively. Colonies were scored on day 12-14 of incubation as derived from CFU-GM, BFU-E or CFU-GEMM according to standard criteria (Humphries et al., 1981). In two experiments identification of colony types was confirmed by Wright staining of cytospin preparations of colonies. For pre-B clonogenic progenitor assays, cells were plated in 0.8% methylcellulose in alpha medium supplemented with 30% FSC, 10^{-4} M β-ME and 0.2 ng/ml of IL-7. Pre-B colonies were scored on day 7 of incubation.

4.2.7 CFU-S Assay

Day 4 5-FU bone marrow cells were harvested after infection by co-cultivation with viral producers and injected immediately into lethally irradiated (B6C3)F1 mice or after 1 week culture at an initial density of 1-5 x 10^5 cells/ml in 30% FCS, 1% BSA, 10^{-4} M ß-ME, 3 U/ml of Epo, 2% SCCM with or without 1.4 mg/ml of G418. The number of cells that each mouse received was adjusted to give 10-15 macroscopic spleen colonies (2-4 x 10^3 bone marrow cells harvested from co-cultivation with virus producer cells or a proportion of the 1 week old liquid cultures, described above, corresponding to 2 x 10^3 or 1 x 10^5 *HOXB4*- or neo-transduced input cells, respectively). CFU-S content of bone marrow cells obtained from mice transplanted 20 weeks earlier with neo- or *HOXB4*-infected cells was also evaluated by intravenous injection of 2 x 10^5 or 2 x 10^4 bone marrow cells/mouse respectively. Untransplanted lethally irradiated mice were tested in each experiment for endogenous CFU-S surviving irradiation and consistently gave no spleen colonies. Twelve days after injection, animals were sacrificed by neck dislocation and the number of macroscopic colonies on the spleen were evaluated after fixation in Telleyesniczky's solution .

4.2.8 Marrow Repopulating Ability (MRA) Assay

Lethally irradiated (B6C3)F1 mice were injected intravenously with 2×10^5 day 4 5-FU bone marrow cells directly after they were harvested from the co-cultivation with viral producer cells or with a proportion of these cells kept for 7 days in the liquid culture described above, corresponding to 1.5×10^5 neo- or *HOXB4*-infected input cells. Thirteen days later 3 mice per group were sacrificed and femoral cells harvested, counted and pooled. Dilutions corresponding to various proportions of a femur were then injected intravenously into lethally irradiated recipients for macroscopic spleen colony evaluation as described above. As a control to determine endogenous MRA surviving irradiation in primary recipients, half a femur, pooled from two untransplanted lethally irradiated mice was assayed in three secondary recipients.

4.2.9 Competitive Repopulating Unit (CRU) Assay

Bone marrow cells pooled from 3-4 mice transplanted 12, 16 or 20 weeks earlier with neo- or *HOXB4*-transduced cells derived from (PepC3)F1 (Ly5.1/Ly5.2) mice were injected at different dilutions into lethally irradiated (B6C3)F1 (Ly5.2) mice together with a lifesparing dose of 1×10^5 competitor bone marrow cells from (B6C3)F1 (Ly5.2) mice. The level of lympho-myeloid repopulation with Ly5.1 donor derived cells in these secondary recipients was evaluated >13 weeks later by flow cytometric analysis of peripheral blood as described (Rebel et al., 1994) in all experiments but for the secondary recipients where it was evaluated at 5 weeks post reconstitution. Recipients with $\geq 1\%$ donor (Ly5.1) derived peripheral blood lymphoid and myeloid leukocytes as determined by the side scatter distribution of $Ly5.1^+$ cells, were considered to be repopulated by at least one CRU. CRU frequency in the test cell population

was calculated by applying Poisson statistics to the proportion of negative recipients at different dilutions as described before (Szilvassy et al., 1990).

4.2.10 DNA and RNA Analyses

Southern blot analyses to assess proviral integration were performed as previously reported (Pawliuk et al., 1994) using standard techniques. High-molecular weight DNA was digested with SstI which cuts in the LTRs to releases the proviral genome or with EcoRI which cuts the provirus once to release DNA fragments specific to the proviral integration site(s). Total cellular RNA was isolated using TRIzol (Gibco/BRL) and separated using formaldehyde/agarose gel electrophoresis. The RNA was transferred to nylon membrane (Zeta-Probe; Bio-Rad) prehybridized, hybridized and washed as described (Davis et al., 1994a). Probes used were a XhoI/SalI fragment of pMC1neo (Thomas and Capecchi, 1987), KpnI/MseI fragment of pXM(ER)-190 which releases the full-length erythropoietin receptor cDNA, (kindly provided by A. D'Andrea) and full-length *HOXB4* cDNA labeled as described (Lawrence et al., 1995).

4.2.11 Western Analysis

To detect HOXB4 protein, FDC-P1 and K562 transfected cells were harvested and lysed in cracking buffer (1% SDS, 6 M urea, 1% ß-ME, 0.01 M sodium phosphate, pH 7.2). Proteins (5 μg) were subjected to SDS-PAGE in a 12.5% gel and transferred to nitrocellulose membrane. Membranes were incubated with a mixture of two polyclonal antisera (1/5,000 dilution for each) raised against peptides deduced from the N-terminal and C-terminal regions flanking the homeodomain of the HOXB4 protein, respectively (BAbCo, Richmond, CA), and incubation with secondary antibody coupled to alkaline phosphatase. Each peptide antisera was previously shown to specifically detect HOXB4 expressed as a bacterial fusion protein.

4.3 Results

4.3.1 Retroviral-Mediated Transduction of HOXB4 to Murine Bone Marrow Cells

In an effort to achieve increased and persistent expression of *HOXB4* in primitive hematopoietic cells, the human *HOXB4* cDNA was introduced into murine bone marrow cells by retroviral-mediated gene transfer. This *HOXB4* cDNA was chosen based on its availability and its derivation from a hematopoietic tissue (Piverali et al., 1990). Of the 361 amino acids found in the HOXB4 protein, only 9 are divergent between human and mouse and none of these occur within the homeodomain. This high degree of similarity (97%) made it very likely that murine and human *HOXB4* would be interchangeable (Bachiller et al., 1994).

The *HOXB4* cDNA (Piverali et al., 1990) was inserted into the MSCV retroviral vector 5-prime to a phosphoglycerate kinase promoter (pgk)-driven neo gene such that *HOXB4* expression was driven from the promotor-enhancer sequences contained within the viral long terminal repeat (LTR) (Figure 4.1A). The LTR sequences of MSCV were derived from a myeloproliferative sarcoma virus modified to show enhanced activity in embryonic stem cell lines and therefore likely to have similar activities in primitive hematopoieitic cells (Grez et al., 1990; Hawley et al., 1992). High titer polyclonal viral producer cells were generated from the GP+E-86 ecotropic packaging cell line using standard methods. Integrity of the *HOXB4*-neo retrovirus was verified by Northern blot analysis to detect the expected mRNA in viral producer cells (Figure 4.1B) and by Western blot analysis to detect HOXB4 protein in transduced murine (FDC-P1) and human (K562) hematopoietic cell lines (Figure 4.1C).

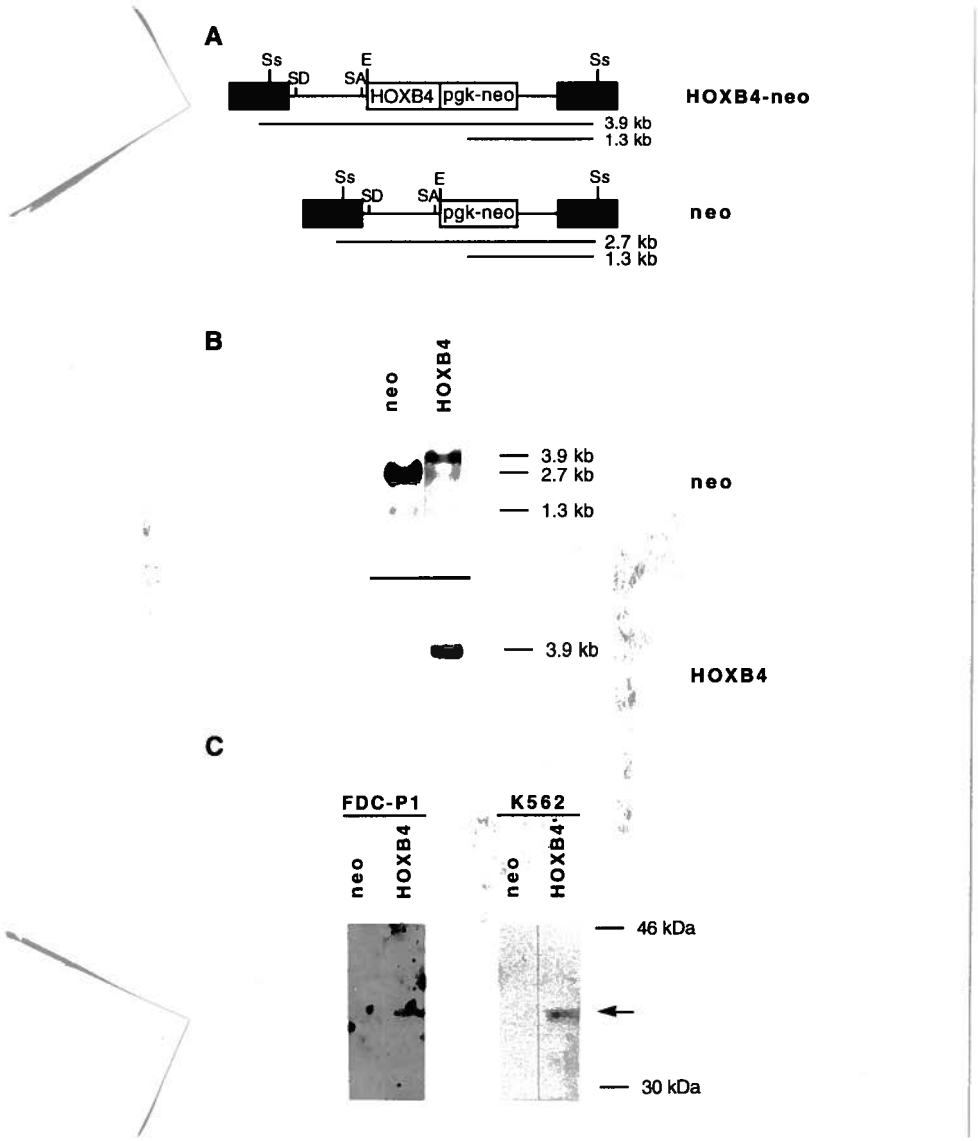

Figure 4.1. Structure and expression of *HOXB4* and control neo retroviruses used in this study.

(A) Diagrammatic representation of the integrated *HOXB4*-neo and the neo proviruses. Constructions were based on the MSCV2.1 vector in which the neo gene is linked to an internal murine pgk promotor. Expected size of full-length viral transcripts and also those initiated from the pgk promoter are shown. SD and SA denote splice donor and splice acceptor sites. Alternate transcripts derived from these sites are not shown. Restriction sites indicated are EcoRI (E), and SstI (Ss). (B) Northern blot analysis of the neo and *HOXB4*-neo viral producer cell lines. The membrane was sequentially hybridized to a probe specific for neo or *HOXB4*. (C) Western blot analysis of K562 and FDC-P1 cell lines transduced with the *HOXB4*-neo or the neo virus and probed with a polyclonal antibody directed against a *HOXB4* synthetic oligopeptide.

The experimental strategy used to study the effects of *HOXB4* overexpression on the behaviour of primitive hematopoietic cells and their progeny is depicted schematically in Figure 4.2.

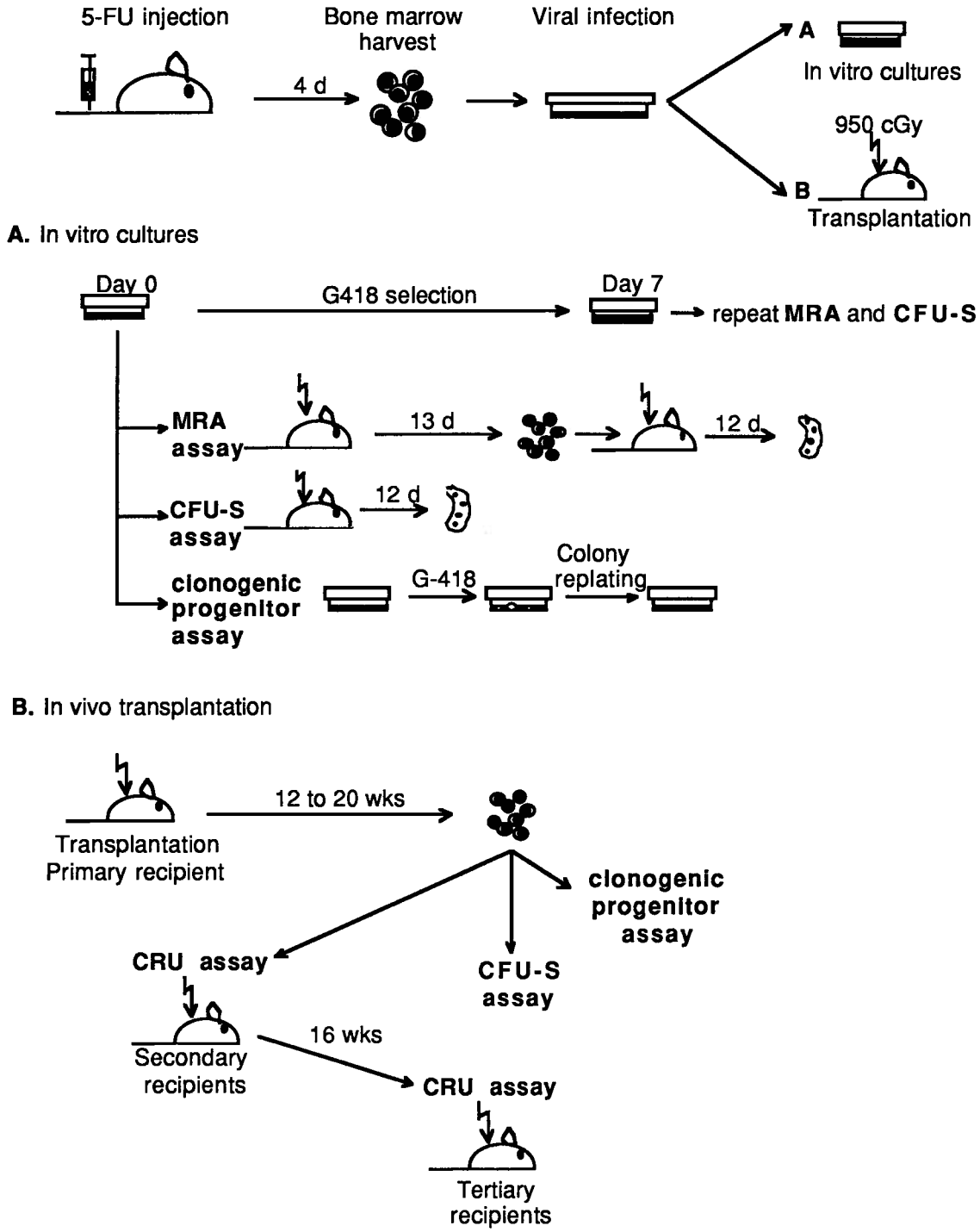

Figure 4.2. Overview of the experiments and assays used in these studies.

4.3.2 Increased Proliferative Activity in Vitro of Clonogenic Progenitors Overexpressing HOXB4

Initial studies examined possible effects of *HOXB4* overexpression in committed clonogenic progenitor cells detected by their ability to give rise to myeloid, erythroid or myeloid/erythroid colonies in semi-solid culture. Bone marrow cells from mice treated 4 days previously with 5-fluorouracil (5-FU) were co-cultivated with *HOXB4* or control neo viral producer cells and 48 hours post infection were plated in methylcellulose cultures. In three independent experiments, the gene transfer efficiency to clonogenic progenitors was similar for *HOXB4* and control neo viruses, with 58-70% of colonies showing G418 resistance (Table 4.1). Neo- or *HOXB4*-transduced bone marrow cells did not give rise to colonies in the absence of exogenous growth factors showing that *HOXB4* overexpression did not render clonogenic cells growth factor-independent. Neither were there any differences detected in the proportions of different colony types generated by *HOXB4*- or neo-infected cells (assessed by both in situ scoring and by Wright staining of cytospin preparations of individually plucked colonies, Table 4.1). This suggests that *HOXB4* overexpression also does not alter the ability of committed clonogenic progenitors to complete their differentiation into different types of mature blood cells. However, *HOXB4*-infected cells did give rise to significantly more large colonies (containing >1000 granulocytes and macrophages) than what was observed in control cultures containing neo-transduced cells. The majority of these large colonies had a diffuse morphology. Only rarely could the type of dense colonies with a diffuse halo described by Perkins and Cory (1993) who studied bone marrow cells overexpressing HoxB8, be identified in our experiments.

Table 4.1. Clonogenic progenitor frequencies in bone marrow cells immediately post infection with *HOXB4*-neo or neo retroviruses[a]

Virus	Total CFC[b] per 10^3 cells	G-418[r] (%± SD)	Distribution of Progenitor Classes (%± SD)			
			CFU-GM small	CFU-GM large	BFU-E	CFU-GEMM
neo	55 ± 47	70 ± 6.7	72 ± 11	16 ± 14	2.5 ± 2.5	9.0 ± 1.5
HOXB4-neo	50 ± 31	58 ± 7.6	51 ± 8.5	41 ± 14[c]	1.4 ± 2.4	6.9 ± 4.5

[a] Results are expressed as mean ± S.D. from 3 experiments. In situ colony scoring was confirmed by microscopic examination of Wright stained cytopsin preparations of a representative sampling of individually plucked colonies.

[b] Abbreviations: CFC, colony-forming cells; CFU-GM, colony-forming unit granulocytes-macrophages; CFU-GM large= more than 1000 cells per colony; BFU-E, burst forming unit erythroid; CFU-GEMM, mixed myeloid,erythroid colony forming unit

[c] Significantly different from the neo control (Student t test, $p<0.05$)

To further characterize the proliferative capacity of the *HOXB4*-infected cells, whole methylcellulose cultures were harvested seven days after plating and various proportions assayed in secondary methylcellulose cultures. The results obtained from two separate experiments revealed that the cells harvested from the primary assays of *HOXB4*-infected cells were able to generate two to three-fold more secondary colonies than neo-transduced control cells obtained from primary assays initiated with the same number of original input cells. Furthermore, one third (32 ± 8%) of the colonies obtained in the secondary cultures of *HOXB4*-transduced cells were large in size (>10^3 cells/colony) whereas the proportion of such colonies in the assays of the replated neo-transduced cells was much lower (3.5 ± 0.5%). To assess whether this increase in proliferation reflected a generalized effect of *HOXB4* on the majority of clonogenic progenitors, well isolated *HOXB4*- and neo-transduced G418-

resistant colonies were picked at random either 7 (Figure 4.3A) or 11 days (Figure 4.3B) after initiation of the primary cultures and one third of each colony then individually replated into secondary cultures. Under these conditions, ~60% of neo-transduced colonies did not replate whereas 80% of the individually analyzed *HOXB4*-transduced colonies replated generating a median of 160 and 450 secondary colonies per clone, respectively, in these 2 experiments.

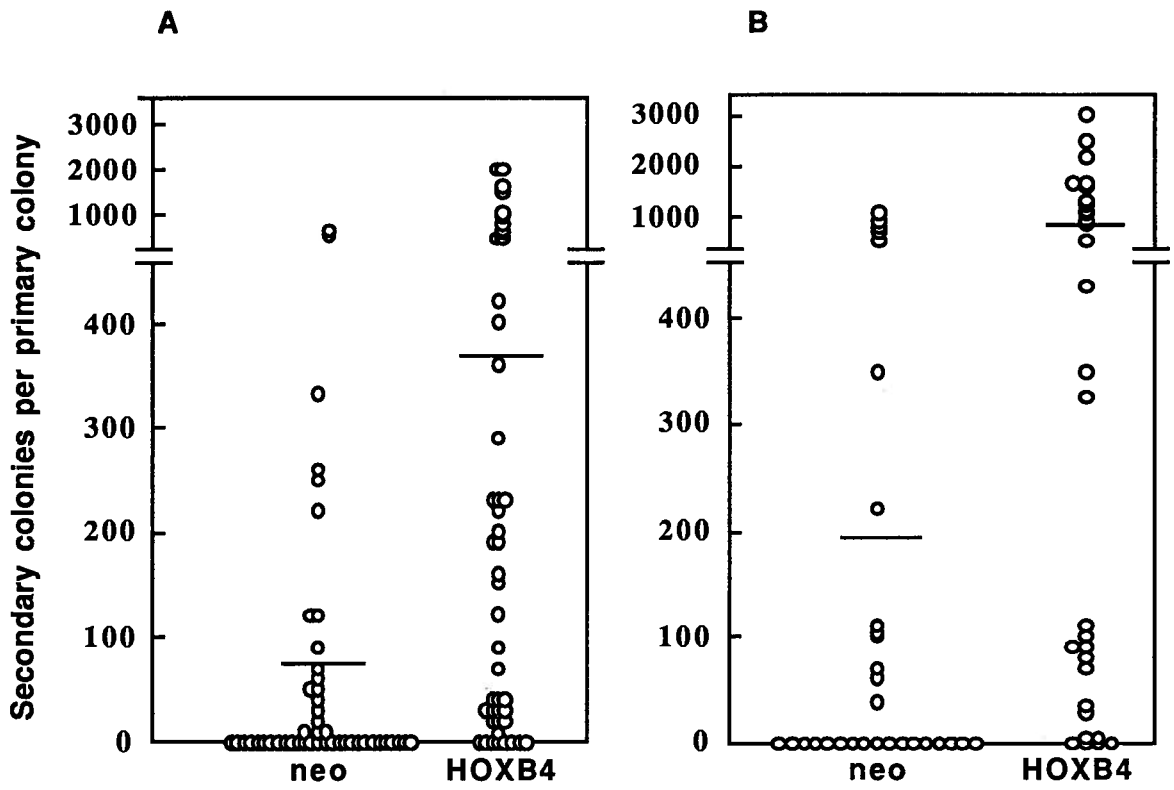

Figure 4.3. The effect of *HOXB4* overexpression on the ability of individual methylcellulose colonies to form secondary colonies upon replating.
In 2 independent experiments, well isolated *HOXB4*- or neo-transduced colonies from primary cultures were randomly picked after seven (A) or eleven days (B) of G418 selection in methylcellulose cultures. Each dot represents the number of secondary colonies generated from each primary colony that was picked and replated in the same culture conditions described above. The calculated mean number of secondary colonies obtained per primary colony is indicated by the broad dash. The difference in means was significant to $p<0.005$ for A and B (Two tailed Student t test).

4.3.3 HOXB4 Effects on the Maintenance in Vitro of Day 12 CFU-S and Cells with Marrow Repopulating Ability (MRA)

Based on the observed increase in the proliferative ability of progenitors with in vitro clonogenic potential following their transduction with *HOXB4*, additional experiments were performed to determine whether similar effects on the behaviour of earlier cells detectable as day 12 CFU-S or as cells with marrow repopulating ability (MRA) could be seen. For these studies, infected bone marrow cells were assayed for CFU-S and MRA content immediately after the period of co-cultivation with viral producer cells and again after an additional seven days in liquid culture in the presence of 1.4 mg/ml of G418.

Day 12 CFU-S frequencies of cells harvested immediately after co-cultivation with *HOXB4* viral producers were similar to those obtained for the neo control (Figure 4.4, day 0). However, after maintaining the *HOXB4*- or the neo-transduced cells in liquid culture for 1 week, the CFU-S content of the cultures initiated with the neo-transduced cells decreased to less than 1% of input (Figure 4.4, day 7). In contrast, the day 12 CFU-S content of the cultures initiated with *HOXB4*-transduced cells increased to 200 - 500% of input (day 0) values (Figure 4.4). As a result, there were ~200 fold more day 12 CFU-S in cultures initiated with the *HOXB4*-transduced bone marrow cells as compared to neo-transduced controls at the end of a seven day period of incubation. Southern blot analysis of DNA extracted from 23 well isolated spleen colonies produced in recipients of *HOXB4*-transduced cells showed each of the 23 colonies to be uniquely retrovirally marked indicating that the 2 to 5-fold net expansion of day 12 CFU-S observed in these cultures was due to a polyclonal expansion of *HOXB4*-transduced day 12 CFU-S or even an earlier cell type (data not shown). Wright staining of cell preparations obtained from these spleen colonies showed a similar spectrum of late erythroid and myeloid

elements compared to neo control colonies (data not shown), indicating that *HOXB4* overexpression does not affect the pattern of differentiation that day 12 CFU-S undergo during spleen colony formation in vivo.

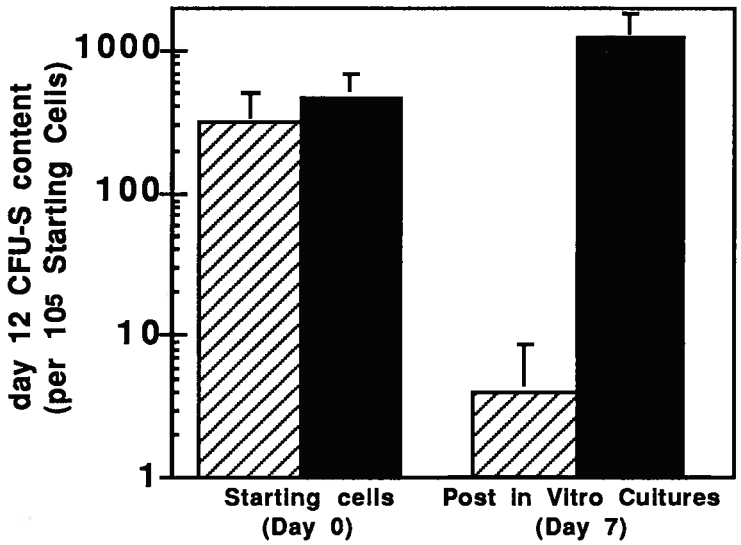

Figure 4.4. *HOXB4* effects on day 12 CFU-S in vitro.
Day 4 5-FU bone marrow cells were prestimulated and co-cultivated for a total of 4 days with the *HOXB4* or control neo viral producer cells. The CFU-S content of recovered cells was assessed immediately after co-cultivation (day 0) and also after seven days in liquid culture and is expressed as day 12 CFU-S colony numbers per 10^5 starting day 0 cells. Results shown represent the mean ± S.D. from 4 independent experiments.

The MRA is an assay that measures the ability of a test cell population to regenerate day 12 CFU-S in the bone marrow of a lethally irradiated recipients transplanted 13 days earlier (Figure 4.2). The cells thus detected share with HSC a resistance to cycle-active chemotherapeutic drugs (Hodgson and Bradley, 1984) and are thought to be precursors of most CFU-S (Mauch and Hellman, 1989). MRA measured immediately after infection of bone marrow cells with the *HOXB4*-virus (day 0 after co-cultivation) was 10-fold higher than that measured for the neo-infected cells (Figure 4.5). Following seven days of liquid culture, the MRA content of the neo-transduced cells was undetectable (i.e. less than two day 12 CFU-S per femur = background) suggesting that, as for the CFU-S, the maintenance of MRA was compromised under the culture conditions used. In contrast, the MRA of the cells present in the day 7 cultures of

HOXB4-transduced cells was maintained at readily detectable levels and although reduced to 25 % of input (day 0), this level was still >200 fold higher than that present at day 7 in control cultures of neo-transduced cells (Figure 4.5).

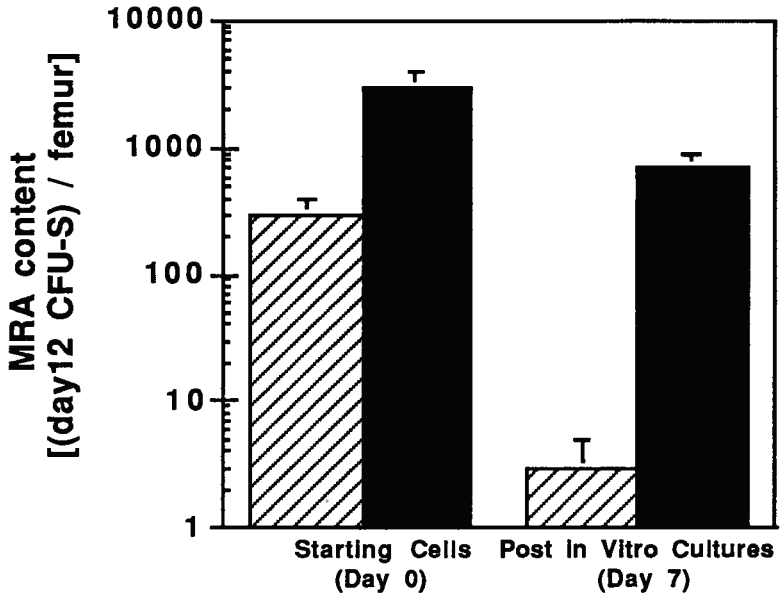

Figure 4.5. *HOXB4* effects on cells with marrow repopulating ability (MRA). MRA was assayed by the content of day 12 CFU-S per femur present in recipients transplanted 13 days previously with *HOXB4*- (black) or neo-transduced (hatched) bone marrow cells. The MRA of 2 x 10^5 cells was determined immediately after viral infections (day 0) or after their culture for 7 days. Results shown are the mean ± S.D. of day 12 CFU-S determined in a minimum of 10 recipients.

Thus, it appears that the marked (i.e. > 2 log) reduction in day 12 CFU-S numbers that occurred when neo-transduced cells were maintained in vitro for seven days was accompanied by a similar reduction in MRA. In contrast, *HOXB4* overexpression reversed this decline leading to a net increase in CFU-S content and a near maintenance of MRA.

4.3.4 HOXB4-Induced Expansion in Vivo of Clonogenic Progenitors and Day 12 CFU-S

To assess possible effects of *HOXB4* overexpression on hematopoietic cells maintained for prolonged periods in vivo, *HOXB4*- or neo-transduced bone marrow cells were transplanted immediately after infection into lethally

irradiated syngeneic recipients and reconstitution of various hematopoietic populations evaluated (Figure 4.2). Each mouse received an innoculum of 2 x 10^5 marrow cells estimated to contain ~ 30-40 competitive repopulating units (CRU), as subsequently determined (see Table 4.4). Gene transfer efficiencies in the transplant marrow as assessed by the proportion of G418-resistant in vitro clonogenic progenitors was 58±8% and 70±7% for *HOXB4*- and neo-transduced cells, respectively (Table 4.1). This suggests that less than half of the CRU in the transplant were transduced since frequencies of retroviral infection into these cells are typically lower than into in vitro clonogenic progenitors (Fraser et al., 1993). The extent of donor-derived reconstitution of peripheral blood leukocytes measured 12 or 20 weeks post-transplantation was similar (>86%) for animals transplanted with either *HOXB4*- or neo-transduced marrow cells. Reconstitution of the hematopoietic system of recipients with both types of transduced cells was confirmed by Southern blot analysis of DNA extracted from bone marrow and spleen cells of mice sacrificed 20 weeks after transplantation (Figure 4.6). Northern blot analyses of RNA obtained from these tissues showed high levels of expression of *HOXB4* and neo (Figure 4.6). Hematopoietic reconstitution by transduced cells was also confirmed by the high frequency of splenic and bone marrow G418-resistant myeloid clonogenic progenitors (75±17% and 42±18%, respectively, for recipients of *HOXB4*- and neo-infected cells).

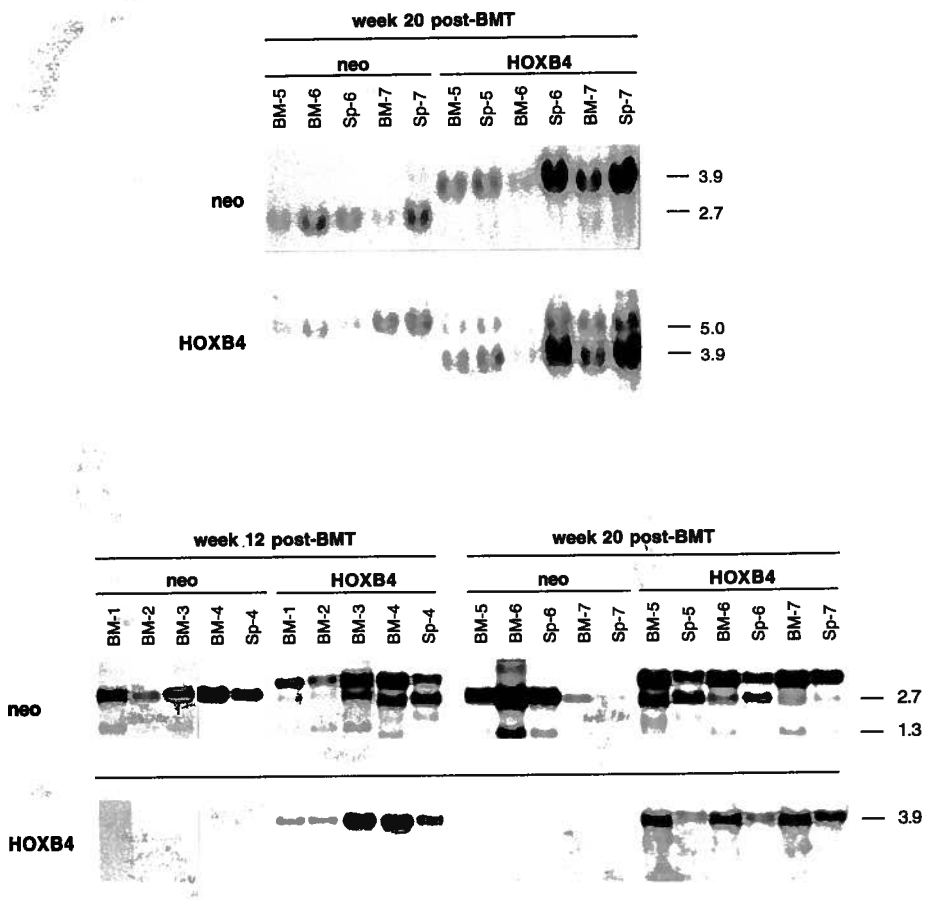

Figure 4.6. Northern and Southern blot analyses to demonstrate hematopoietic reconstitution by *HOXB4*- or neo-transduced bone marrow cells.

Upper panel: Southern blot analysis of DNA from bone marrow and spleen cells to demonstrate the presence of integrated virus. DNA was digested with SstI and membranes hybridized to probes specific for neo or *HOXB4*. SstI releases the integrated *HOXB4* (3.9 kb) and neo (2.7 kb) proviruses. A 5kb band derived from the endogenous murine *HOXB4* gene cross hybridizing to the human *HOXB4* probe provides a single gene copy control of loading. Lower panel: Northern blot analysis to detect expression of transduced *HOXB4* and neo genes. Total RNA (5 μg) isolated from bone marrow and spleen cells obtained from mice sacrificed at 12 or 20 weeks post-transplantation was sequentially hybridized to a neo and a *HOXB4* probe. In addition to full length transcripts, an expected 1.3 kb transcript corresponding to the pgk-driven neo gene is observed at lower levels in both neo- and *HOXB4*-transduced cells. An additional transcript of ~ 2.7 kb is also observed using a neo but not full length *HOXB4* probe in the cells transduced with the *HOXB4* virus (this transcript is also observed in *HOXB4*-neo viral producer cells; see Figure 4.1B); this likely represents a spliced transcipt arising from use of a splice donor site in the MSCV2.1 vector (Hawley et al., 1994). Each number assigned to the various lanes identifies a specific mouse which also corresponds to the same mice depicted in Table 4.2. Abbreviations: BMT, bone marrow transplantation; BM, bone marrow; Sp, spleen.

The pre-B and myeloid clonogenic progenitor content of bone marrow 12 weeks post-transplantation with *HOXB4*-transduced cells was on average two-fold higher than that of recipients of neo-infected cells (Table 4.2). Myeloid clonogenic progenitor numbers in the spleen were also elevated ~ten fold in the one mouse analyzed (Table 4.2). Twenty weeks post-transplantation with *HOXB4*-transduced marrow, an even greater increase in bone marrow and splenic clonogenic progenitor numbers was evident (5 and 32-fold higher, respectively, than in neo-transduced marrow recipients).

Table 4.2. Mice transplanted with *HOXB4* transduced cells have increased myeloid and pre-B lymphoid clonogenic progenitors in bone marrow and spleen.

Mouse #	Myeloid clonogenic progenitors / femur ($\times 10^3$)	Pre-B clonogenic progenitors / femur	Myeloid clonogenic progenitors / spleen	PreB clonogenic progenitors / spleen
12 weeks post transplantation.				
neo-1	22	1,500		
neo-2	45	2,300		
neo-3	60	2,000		
neo-4	38	5,100	595	210
mean±SD	**41± 16**	**2,700±1600**		
HOXB4-1	89	5,000		
HOXB4-2	55	5,400		
HOXB4-3	150	7,000		
HOXB4-4	58	5,900	6,650	420
mean±SD	**88± 44**[a]	**5,800±800**[a]		
20 weeks post transplantation.				
neo-5	45		173	
neo-6	48		250	
neo-7	29		163	
mean±SD	**41± 10**		**195± 47**	
HOXB4-5	250		5,453	
HOXB4-6	190		6,583	
HOXB4-7	230		>100,000	
mean±SD	**223± 30**[b]		**6,018± 799**[b,c]	

[a]significantly different from neo control ($p<0.05$)
[b]significantly different from neo control ($p<0.01$)
[c]mouse *HOXB4*-7 not included in this calculation

In order to evaluate whether the increase in clonogenic progenitor cell numbers was accompanied by an expansion of an earlier cell type, day 12 CFU-S frequencies were also assessed in two independent experiments. In each of these, marrow cells from three mice transplanted 16 or 20 weeks earlier with either *HOXB4*- or neo-transduced cells were pooled and then assayed. By 16 to 20 weeks the frequency of CFU-S in the recipients of control cells was back to normal (pretransplantation) levels (i.e., $1.0 \pm 0.3/10^4$ cells; (Chang and Johnson, 1991)) whereas in mice reconstituted with *HOXB4*-transduced bone

marrow cells, the frequency of CFU-S was 5.0 and 7.6-fold higher, respectively, for the two time points.

4.3.5 HOXB4-Induced Expansion in Vivo of Cells With Long-Term Lympho-Myeloid Repopulating Ability

To determine if *HOXB4* overexpression affects the expansion of the earliest hematopoietic cells, their numbers were quantified by limiting dilution analysis using the competitive repopulating unit (CRU) assay (Figure 4.2) (Szilvassy et al., 1990). For this purpose, various numbers of bone marrow cells pooled from three mice transplanted either with *HOXB4*- or neo-transduced marrow cells 12 or 20 weeks earlier were transplanted into lethally irradiated recipients. The presence or absence of lympho-myeloid repopulation (>1%) with donor-derived Ly5.1+ cells in these mice was then evaluated 13 or more weeks later and CRU frequencies calculated using Poisson statistics, from the proportion of recipients negative for donor-derived lympho-myeloid repopulation at different cell innocula (Szilvassy et al., 1990).

By 12 weeks post-transplantation, recipients of neo-transduced marrow had reconstituted CRU to a frequency of 0.6 in 10^5 bone marrow cells (Table 4.3) or only 6% that of normal (unmanipulated) mouse marrow (Szilvassy et al., 1990). This low CRU frequency is consistent with the previously well documented finding that even non-retrovirally infected marrow cells will not regenerate CRU numbers to >10% of pre-transplant values ((Harrison, 1982; Harrison et al., 1990); R. Pawliuk and R.K.H., unpublished observations). For recipients of *HOXB4*-transduced marrow a limiting dilution was not reached suggesting a CRU frequency of > 2.9 in 10^5 bone marrow cells or at least 29% of normal levels and > 4 fold that seen in the recipients of neo-infected cells (Table 4.3).

CRU frequencies in the marrow were also measured in primary recipients sacrificed 20 weeks post-transplantation. The CRU frequency of the mice

transplanted with neo-transduced marrow was similar to that obtained at 12 weeks or ~3% of normal values (Table 4.3). In contrast, the frequency of CRU in recipients of *HOXB4*-infected marrow was 14 per 10^5 cells. This represents a 1.4 fold increase above normal marrow values and a 47-fold increase above the CRU frequency measured in the marrow of recipients of control cells (Table 4.3).

Table 4.3. Evaluation by limiting dilution analysis of competitive long-term repopulating cells (CRU) in mice transplanted with *HOXB4* versus neo control transduced bone marrow cells

	CRU evaluation of primary recipients[a]			
number of cells injected into secondary recipients	12 week post-transplantation		20 week post-transplantation	
	neo	*HOXB4*	neo	*HOXB4*
6,700,000	7/7 (30±12)	5/5 (76±21)	n.d.	n.d.
670,000	8/8 (11±8)	5/5 (63±17)	4/4 (20±16)[d]	n.d.
67, 000	2/7 (3±0)	3/3 (25±3)	0/6 (<1)	n.d.
33,500	n.d.	n.d.	n.d.	7/7 (37±27)[d]
11,000	n.d.	n.d.	n.d.	5/7 (15±12)
6,600	n.d.	n.d.	n.d.	4/6 (14±20)
CRU frequency per 10^5 cells (range)[b]	0.6 (0.25-1.6)	>2.9	0.3 (0.2-0.5)	14 (10-20)
Relative to normal (%)[c]	6	>29	3	140

	CRU evaluation of secondary recipients[a]	
number of cells injected into tertiary recipients	16 week post-transplantation	
	neo	*HOXB4*
15,000,000	2/3 (3±2)	5/5 (54±5)
1,500,000	3/5 (5±3)	5/5 (31±11)
150,000	1/5 (5±6)	6/6 (8±4)
15,000	n.d.	1/6 (1±1)
1,500	n.d.	0/5 (<1)
CRU frequency per 10^5 cells (range)[b]	0.02 (0.01-0.04)	2.1 (0.8-5.3)
Relative to normal (%)[c]	0.2	21

[a]Results are expressed as number of mice repopulated with donor-derived cells ($Ly5.1^+$) over total. Numbers in parentheses represent the mean % ± S.D. of peripheral blood $Ly5.1^+$ cells found in the transplant recipients.
[b]CRU frequency was calculated using limiting dilution analysis (see Experimental Procedures).
[c]Compared to control values (Szilvassy et al., 1990).
[d] mice used for evaluaton of CRU amplification in secondary animals selected from these groups and are identifiable in Figure 4.7 as mice number 25.1, 25.2, 25.3, 29.1, 29.2, 29.3, 30.1 and 30.3.

Recipient mice used to quantitate CRU (20 week posttransplant, Table 4.3) were further assessed by Southern blot analysis to identify those repopulated by transduced CRU. Of 14 mice analyzed that received 5 or fewer CRU from mice initially transplanted with *HOXB4*-infected marrow, 12 were positive for proviral integration in thymic and / or bone marrow tissue, all of which had previously been scored as positive for donor-derived Ly5.1 lympho-myeloid repopulation in the CRU assay (Figure 4.7). Moreover, common proviral integration patterns for thymic and bone marrow tissue were clearly apparent in five of these secondary recipients confirming the lympho-myeloid repopulating potential of the regenerated CRU. The intensities of the proviral integration signals also roughly correlated with the percentages of donor-derived Ly5.1 cells in the peripheral blood (e.g., compare the intense signal for mouse 29.2 (81% Ly5.1$^+$) vs. a much reduced signal for mouse 29.1 (30% Ly-5.1$^+$)) and for two mice (31.3 and 32.1) who were scored as negative for donor-derived lymph-myeloid repopulation, proviral integrants were not detected. This correlation strongly indicates that *HOXB4*-transduced HSC can terminally differentiate in vivo as shown above for day 12 CFU-S and in vitro clonogenic progenitors. Together these results strongly suggest that the measured CRU expansion in vivo was due to the selective expansion of *HOXB4*-transduced CRU. In contrast, four recipients of marrow from primary mice initially transplanted with neo-infected bone marrow were all negative for proviral integrants (3 of 4 mice shown, Figure 4.7) but positive for donor Ly5.1 cells indicating that CRU regeneration in the primary mice included nontransduced CRU.

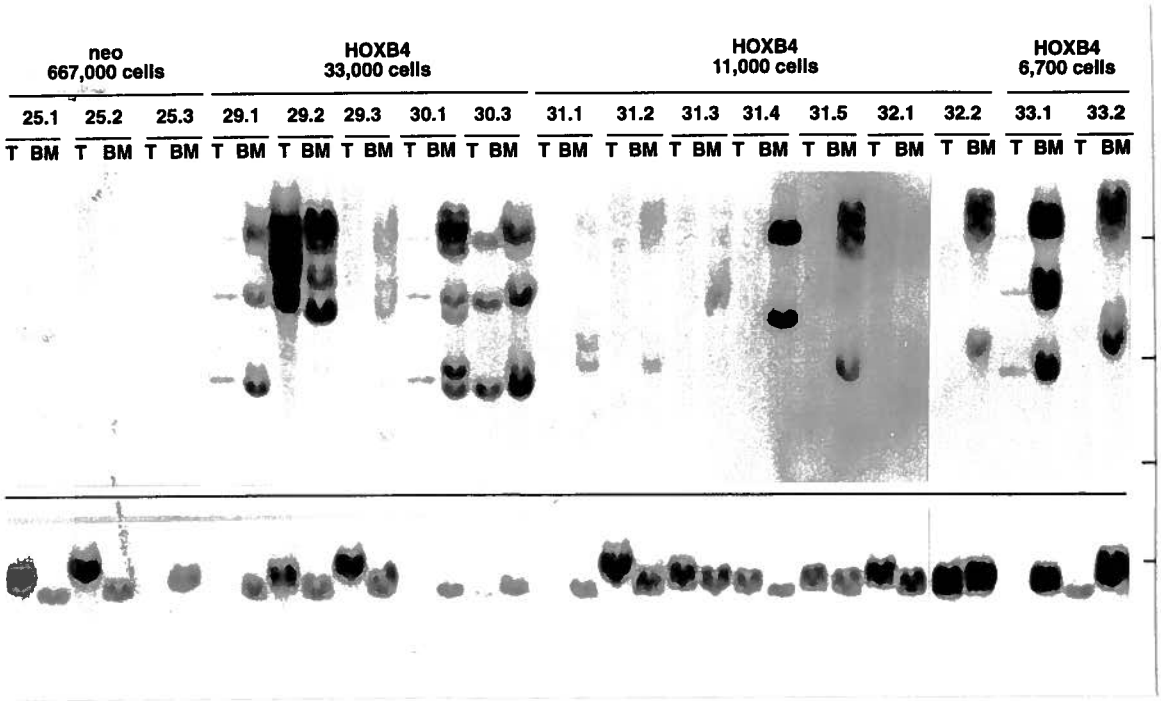

Figure 4.7. Southern blot analysis of proviral integration patterns in bone marrow and thymic DNA isolated from secondary recipients transplanted with varying numbers of *HOXB4* or neo-transduced bone marrow cells.

DNA was digested with EcoRI which cuts the integrated MSCV provirus once generating DNA fragments specific to each integration site. The membranes were first hybridized to a probe for neo (top) to identify proviral fragments. The membranes were rehybridized to a probe for the erythropoietin receptor (bottom) to provide a control for DNA loading. Exposure times were equivalent for both probes (~1 day). Primary mice used as donors were those sacrificed 20 weeks post transplantation and are described in Table 4.2 and 4.5. The secondary recipients analyzed are as presented in Table 4.3. The number of bone marrow cells injected into each secondary recipient is indicated at the top of the Figure. Each mouse is identified with a specific identification number. Percentage Ly5.1$^+$ cells in peripheral blood of the mice transplanted with *HOXB4*-transduced marrow are; 29.1(30%), 29.2(81%), 29.3(39%), 30.1(62%), 30.3(30%), 31.1(16%), 31.2(3.2%), 31.3(2%), 31.4(29%), 31.5(9%), 32.1(1.1%), 32.2(9%), 33.1(44%) and 33.2(6%). Abbreviations: T, thymus; BM, bone marrow. Fragment sizes range from over 12 kb (upper tick on the left of the Figure) to a little less than 4 kb (second tick from the top).

Self-renewal of *HOXB4*-transduced CRU was also demonstrated by detection of the same pattern of proviral DNA integration in thymus and bone marrow cells of four different secondary recipients, mice 29.1, 30.3, 33.1 and 30.1 (Figure 4.7). Another uniquely identified totipotent CRU was detected in recipient 29.2. Self-renewal of a CRU with apparent subsequent restriction to

the marrow was detected in secondary recipients 31.2, 31.5, 32.2 and 33.2. These latter mice, however, showed lympho-myeloid repopulation by FACS analysis of Ly-5.1 positive peripheral blood leukocytes suggesting that this clone had B lymphoid and myeloid potential. The degree of self-renewal detected is consistent with the marked expansion of CRU observed in primary animals (nearly 900-fold, Table 4.4) and the fact that mice were initially transplanted with small numbers of CRU (approximately 32 of which at most half would be estimated to have been transduced).

To further assess the regenerative capacity of *HOXB4*-transduced CRU, their expansion in secondary recipients was also evaluated. Bone marrow cells were harvested from secondary recipients (n=3 for neo and 5 for *HOXB4*) transplanted 16 weeks earlier with ~2 to 5 Ly5.1^{+} CRU (Table 4.3) and CRU frequencies measured by limit dilution analysis in tertiary recipients. CRU frequency in the secondary recipients of neo-transduced marrow was 1 in 4.8 X 10^{6} cells (Table 4.3) or less than 0.2% of that found in normal unmanipulated marrow and indicative of a 17 fold expansion over input (summarized in Table 4.4). In sharp contrast, secondary recipients of *HOXB4*-transduced marrow had a CRU frequency 130 times higher or ~20% of normal levels and indicative of a further 900 fold expansion over input (Table 4.4).

Table 4.4. Expansion of donor-derived CRU in primary and secondary recipients of *HOXB4*- or neo-transduced bone marrow cells

Virus	Donor-derived CRU content per mouse[a]			
	Primary recipents		Secondary recipients	
	# of CRU transplanted[b]	# of CRU 20 weeks post Tx[c]	# of CRU transplanted	# of CRU 16 weeks post Tx[c]
neo	32	600	2	35
HOXB4-neo	32	28,000	5	4,700

[a] Results are expressed as number of Ly5.1$^+$ CRU content per mouse considering that one femur represents approximately 10% of the total marrow of a mouse.
[b]CRU frequency of 5-FU-treated bone marrow cells cells after co-cultivation was measured in a subsequent experiment and was 1 in 6,000 cells (95% confidence interval: 1 in 2,000 to 1 in 12,000).
[c]Estimates based on data presented in Table 4.3.

Despite this dramatic expansion of *HOXB4*-transduced HSC, it is noteworthy that the relative numbers of the various types of in vitro myeloid clonogenic progenitors (i.e. CFU-GM, BFU-E and CFU-GEMM) in primary and secondary recipients of *HOXB4*-infected cells were the same as in recipients of control cells. In addition, the total cellularity of the bone marrow was also not different and the red blood cell, white blood cell and differential counts were also within the normal range (Table 4.5, data for secondary recipients not shown). Thus despite a marked and sustained effect of *HOXB4* overexpression on the numbers of myeloid and lymphoid clonogenic progenitors as well as day 12 CFU-S, there was no gross effect on lineage determination nor evidence of a consequent expansion of later cell types. Moreover, despite a significant expansion in the numbers of the most primitive hematopoietic cells, none of the primary recipients (n>25) of *HOXB4*-transduced marrow have developed

leukemia or any other obvious blood dyscrasia for up to 12 months post transplantation.

Table 4.5. Enumeration of bone marrow, spleen and peripheral blood cell counts of primary mice transplanted 16-34 weeks earlier with *HOXB4*- or neo-transduced cells[a].

Gene	Cells/ femur (10^7)	Cells/ spleen (10^8)	RBC[b] ($10^6/\mu l$)	Hb (g/dl)	wbc ($10^3/\mu l$)	lym.[c] %	gran. %	mo %	baso/ eosi. %
neo	1.9±0.2	3.3±0.5	7.9±0.4	14.8±1.2	7.9±2.5	70±6	22±7	7±1	1±2
HOXB4-neo	2.0±0.2	3.6±0.6	7.6±1.0	13.8±1.7	7.8±2.2	75±11	21±6	6±4	0

[a]Results are expressed as mean ± S.D. for 9 primary recipients except for bone marrow and spleen cellularity measured on 3 mice.
[b]Abbreviations, RBC, red blood cells; Hb, hemoglobin; wbc, white blood cells; lym., lymphocytes; gran., granulocytes, mo, monocyctes; baso/eosi., basophils/ eosinophils.
[c]All differential counts were done manually on 200 cells (Wright stain).
RBC, WBC and Hb were done with a CBC5 Coulter counter using 40 μl of heparinized blood.

4.4 Discussion

Our previous work established that *HOXB4* and several other Hox A and B genes are preferentially expressed in the most primitive purified subpopulations of CD34+ bone marrow cells. We now demonstrate, using an in vivo murine model, that the engineered overexpression of *HOXB4* can have profound effects on the proliferation of long-term in vivo repopulating hematopoietic stem cells and to a lesser extent on the proliferation of intermediate types of hematopoietic progenitor cells including both lymphoid (pre-B) and myeloid-restricted populations (Figure 4.8). Nevertheless, this deregulation of primitive progenitor cell amplification does not appear to lead to leukemia and is not translated into an altered output of any type of mature blood cell or an altered commitment to any specific blood cell lineage.

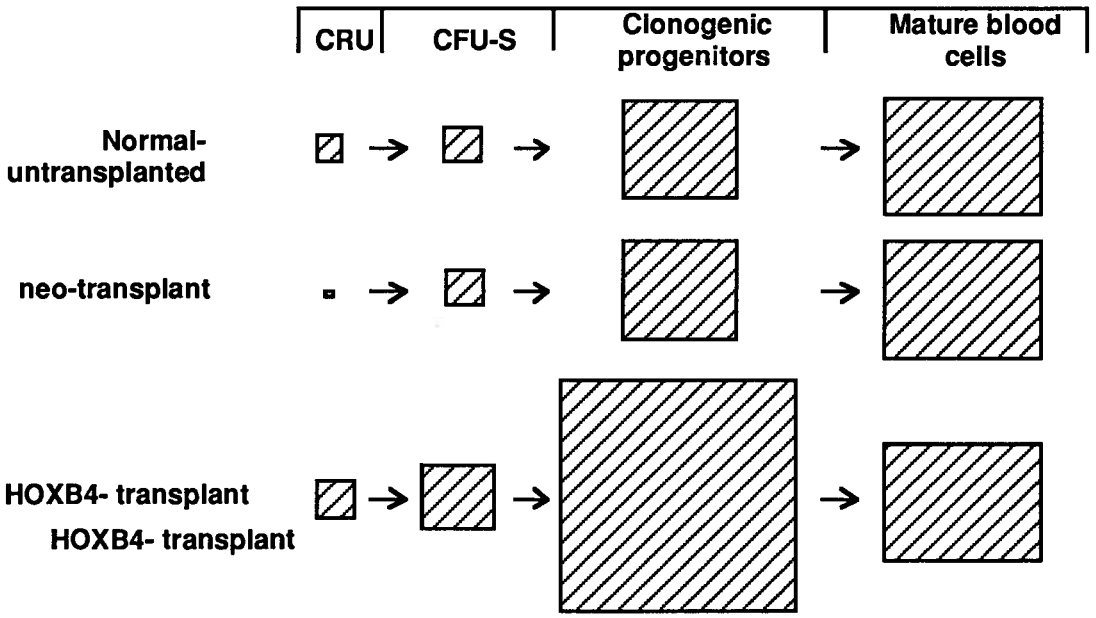

Figure 4.8. Schematic depiction of the sizes of various hematopoietic populations reconstituted in primary recipients of *HOXB4*- or neo-transduced bone marrow cells compared to normal (unmanipulated) mice.

Except for the boxes representing the peripheral blood cells which were equivalent in numbers, the surface area of each box is drawn to scale to indicate the relative frequencies of the cell populations.

Previous studies have shown that, even after a single transplantation, the repopulating competence of the regenerated bone marrow cells is reduced ~ten fold (Harrison, 1982; Harrison et al., 1990). The reasons for this change are not known, although it has been hypothesized that the sustained proliferative stress imposed upon at least some of these cells during the early phase of regeneration of the system may result in a decline in their probability of self-renewal in subsequent divisions. Consistent with these earlier studies, the pool of long-term repopulating stem cells (CRU) regenerated in recipients of neo-transduced cells did not recover beyond a level equivalent to 3-6% of that characteristic of normal mice despite the return to normal levels of bone marrow cellularity and clonogenic progenitors and frequencies.

A different picture emerged in mice transplanted with marrow overexpressing *HOXB4*. Here, CRU numbers recovered to a level that was 1.4-fold higher than the normal value or 47-fold higher than that observed in animals transplanted with neo-transduced marrow. The enhanced proliferative capacity of *HOXB4*-transduced CRU was further demonstrated by serial transplantation studies in which as few as 5 CRU transplanted into secondary recipients were shown to be capable of regenerating a significant CRU pool not demonstrably compromised in repopulating ability. In contrast serial transplantation compromised even further the ability of neo-transduced and/or non-transduced CRU to regenerate CRU in successive recipients.

Expansion of day 12 CFU-S and in vitro clonogenic progenitors was also documented in the mice transplanted with *HOXB4*-transduced marrow cells. Expansion of these later types of hematopoietic cells might simply be secondary to the expansion of CRU. However, the fact that clonogenic progenitors transduced with *HOXB4* also replated much better in vitro than those transduced with neo strongly suggests that this gene can also directly influence

the proliferative capacity of later progenitors. The absence of any perturbation in the proportions of different types of lineage-restricted clonogenic progenitors produced in vivo or the number of mature blood cells present in the circulation of mice repopulated with *HOXB4*-transduced cells strongly suggest that this gene can influence stem cell self-renewal events in the absence of effects on lineage commitment or terminal differentiation. This is consistent with the concept recently proposed by Fairbairn et al. (Fairbairn et al., 1993) based on studies with the FDCP-mix cell line that self-renewal and commitment processes may not necessarily be linked at the molecular level. Together with the results presented in chapter 3 which showed preferential expression of *HOXB4* in the most primitive bone marrow cell populations, the present data suggest that the absolute level of *HOXB4* can be a critical determinant of HSC proliferative ability. Interestingly, it was recently shown that inhibition of *HOXB4* expression using antisense oligonucleotides in peripheral blood progenitors compromised the proliferation of these cells (Carè et al., 1994; Giampaolo et al., 1994).

In addition to clear effects on earlier hematopoietic cells in vivo, overexpression of *HOXB4* had marked effects on cells cultured in vitro. Of these, the most striking effect was on the enhanced recovery of day 12 CFU-S after seven days of in vitro culture (Figure 4.4). It has been previously reported that the CFU-S content of growth factor-stimulated post-5-FU treated bone marrow cells decreases dramatically with time using similar culture conditions (Bernad et al., 1994). The results obtained here with neo-transduced cells are consistent with this report (i.e., there was a decrease of ~2 log in the number of CFU-S at the end of 7 days in culture). However, the CFU-S content of the cultures initiated with *HOXB4*-transduced cells expanded by 2- to 5- fold during this same period. Similar differences in the recovery of day 12 CFU-S after in vitro

culture were observed in comparisons of non 5-FU-treated bone marrow harvested from mice previously reconstituted with *HOXB4*- versus neo-transduced marrows (data not shown). This difference in CFU-S content after seven days of liquid culture may therefore be the result of *HOXB4* "involvement" on various cellular mechanisms including cell death or proliferation of CFU-S precursors. In order to test a possible involvement of *HOXB4* on cell death, two growth factor-dependent cell lines (FDC-P1 and BAF3) were transduced with *HOXB4*-neo (Figure 4.1C shows the Western of the FDC-P1) or neo viruses. Cell death assessement by trypan blue exclusion done every eight hours after growth factor deprivation of log phase growing FDC-P1 or BAF3 cells failed to show any effect of *HOXB4* on apoptosis (data not shown, n=2 independent experiments). Therefore the results presented in this Chapter are more suggestive of *HOXB4* playing a role in proliferation (cell division) than in the prevention of cell death but definitive proof to this statement remains to be shown. Nevertheless the magnitude of the differences observed in the "delta CFU-S" assay suggest that this system could offer a powerful experimental tool for the identification of *HOXB4* target genes.

Expansion of hematopoietic precursors without a concomitant increase in the number of peripheral blood cells has not been previously observed when the effects of overexpression of various hematopoietic growth factors have been studied in a similar model (Chang and Johnson, 1991; Fraser et al., 1993; Hawley et al., 1992; Johnson et al., 1989; Tanaka et al., 1992; Wong et al., 1991). In most of these reports, it was found that overexpression of the cytokines studied resulted in an increase in the numbers of peripheral blood cells but that the content of bone marrow clonogenic progenitors was either unchanged or in some cases, diminished. Interestingly, overexpression of the non-clustered homeodomain-containing gene, *TCL-3* (previously called *HOX11*, (Hawley et

al., 1994)) or *HoxB8* (Perkins and Cory, 1993) in murine bone marrow cells were also found to have proliferative effects. In both studies, generation of cell lines from transduced bone marrow cells was observed with high frequency in the presence of IL-3. In contrast, efforts to generate cell lines in similar conditions (i.e. high IL-3 concentration) were unsuccessful with *HOXB4* (data not shown). Interestingly and similar to our findings, mice transplanted with HoxB8-transduced marrow cells showed increased levels of bone marrow and splenic clonogenic progenitors at three months post-transplantation. A significant proportion of mice transplanted with *HoxB8*-transduced cells developed leukemic transformation at ~7 months post transplantation. In contrast, no leukemic transformation was observed in the "*HOXB4* mice" even after 1 year of observation and despite persistant high levels of expression of *HOXB4*. Thus, although some similarities are observed between these three studies, the results presented in this Chapter suggest fundamental differences in *HOXB4*-mediated effects.

Taken together, these results suggest that *HOXB4* is a key regulator of proliferation of HSC and that overexpression of this gene does not override the regulatory mechanisms involved in lineage determination or in the control of end cell output. These findings demonstrate that it is possible to reverse the decline of HSC that normally occurs during regeneration of the hematopoietic system after bone marrow transplantation resulting in a dramatic expansion of genetically modified HSC in vivo. Given the possibility of examining the function of analogous populations of primitive human hematopoietic cells in immunodeficient mice, it will be of interest to analyze such targets using this approach.

CHAPTER 5

General Discussion and Conclusions

5.1 Hox genes are expressed in normal early hematopoietic cells

The nuclear factors involved in the regulation of proliferation and differentiation of primitive hematopoietic cells are still poorly understood. The goal pursued in this thesis was to identify candidate factors and provide insight into their possible functional roles. The strategy taken was to focus on a particular class of transcription factors –the clustered homeobox genes– which as discussed in the introduction, are central to differentiation and proliferation of embryonic cells. Considerable circumstantial evidence had implicated these genes in hematopoiesis notably findings of their expression in primary leukemic cells and leukemic cell lines and of effects on proliferation and differentiation of various hematopoietic cells following modulation of their expression.

Capitalizing on the emergence of powerful reverse-transcriptase / polymerase-chain reaction strategies and on the recent availability of human CD34+ bone marrow subpopulations that are not only phenotypically pure but which also dramatically differ in their progenitor content, it was possible to document the expression of more than 16 different Hox genes in early populations of human bone marrow cells. These findings allowed the identification of genes (e.g. *HOXB3*, *HOXB4*, etc.) which showed preferential expression in the earliest subpopulations studied; and of others–generally found at the 5' region of the Hox cluster– that were equally expressed in all populations analyzed. Based on these observations, the hypothesis was put forward of the existence of a link between the position a Hox gene occupies on

the cluster and the function this gene has in hematopoiesis with 3'-positioned genes postulated to have important roles in stem cell function and 5'-positioned genes postulated to be involved in function of more mature cells.

Another provocative feature of the expression pattern observed was the absence of "upregulation" of any Hox gene with differentiation. In fact the expression of each gene analyzed to date appears to decrease during maturation of hematopoietic cells from the most primitive where all 16 genes studied were expressed ($CD34^{++}$lineage$^{-}CD38^{lo}$) to the more mature $CD34^{-}$ cells where expression was barely detectable. Another intriguing finding was the absence of expression of the D cluster genes in these cells confirming results from studies of leukemic cell lines and raising the question of why this particular cluster is not expressed.

5.2 *HOXB4* overexpression causes expansion of early hematopoietic cells

Functional studies presented in Chapter 4 provide striking new evidence implicating at least one 3' Hox gene in stem cell expansion without detectable effects on differentiation of later cells. This suggested that *HOXB4* overexpression did not override the regulation of the control of end-cell output even in the presence of large effects on earlier cells.

What cellular mechanism was altered by *HOXB4* overexpression? One can only speculate, at least at this time, four mechanisms to explain the observed expansion of HSC described in Chapter 4. *HOXB4* overexpression may inhibit cell death. Unfortunately very little is known about cell death of early hematopoietic cells in a BMT set up. Nevertheless, this possibility was explored by overexpressing *HOXB4* or neo^{r} in two growth factor-dependent cell lines. Upon growth factor deprivation, cells transduced with *HOXB4* died just as quickly as the neo-controls thus suggesting that *HOXB4* overexpression has

little or no effect on inhibition of apoptosis at least related to growth factor control. Another possibility is that *HOXB4* increased the recruitment of the normally quiescent HSC into cycle (increased turn-over with attendant self-renewal divisions). This hypothesis which would predict a gradual CRU expansion with time is currently being investigated. Interestingly clonogenic progenitors obtained from mice transplanted with *HOXB4*-transduced cells expanded with time (see Table 4.2) but it remains to be shown if earlier cells will also follow this trend. Alternatively, *HOXB4* may increase the probability of self-renewal division of CRU cells during reconstitution. Effects observed after *HOXB4* overexpression may also result from improved seeding efficiencies of *HOXB4*-transduced CRU cells at the time of transplantation. This alternative is interesting since Hox genes are known to regulate adhesion molecules. However due to the magnitude of the effect reported (~50 fold more CRU in mice transplanted with *HOXB4*-transduced cells than in neo-controls) this hypothesis unlikely explains the phenomenon by itself.

5.3 Hox genes: Master regulators of hematopoiesis?

The "functional" studies previously reported (Carè et al., 1994; Giampaolo et al., 1994; Lill et al., 1995; Perkins and Cory, 1993; Shen et al., 1992; Wu et al., 1992) combined with those described in Chapter 4 have all demonstrated that seven out of the seven Hox genes tested in various systems produced profound effects on hematopoietic differentiation or proliferation. Considering that at least 16 (see chapter 3, and likely >30) of these genes are expressed in primary hematopoietic cells, it appears that Hox genes have the potential to regulate a multiplicity of targets in hematopoietic cells. This potential is even greater if one considers the numerous alternate transcripts observed with some of these genes (*HOXB3* for example has at least 5 different transcripts in K562 cells,

data not shown). Together these results point to the existence of a complex "Hox code" that may be deterministic for differentiation and proliferation of hematopoietic cells.

5.4 Future studies

Consistent with the expression study reported in Chapter 3, it will be necessary to "test more exhaustively the hypothesis that there is a link between the position of a particular Hox gene in the complex, its pattern of expression in immature (3' and 5' Hox genes) versus more mature (5' Hox genes) bone marrow populations, and the effect of overexpression". To test this hypothesis, *HOXA10* (a 5' gene also expressed in "mature" CD34+ subpopulations, see Figure 3.2) was recently overexpressed in murine bone marrow cells and preliminary results from these studies also indicate effects on differentiation that are dramatically different from those (no effect) observed with *HOXB4*[7].

Finally *HOXB3*, which shows an even more restricted pattern of expression in early hematopoietic cells than *HOXB4*, would be of interest to study in similar experimental set ups. In an effort to pursue this goal and to obtain relevant hematopoietic *HOXB3* transcripts, a phage cDNA library was constructed with messenger RNA obtained from purified CD34+ cells and a full-length (coding) novel *HOXB3* transcript was isolated from this cDNA library (data not shown). The coding region of this cDNA was recently incorporated into a retroviral vector and functional studies are underway.

Hox genes found on the same paralog (see introduction, Figure 1.4) may have functional redundancies. A dramatic example of this is the ability of the murine *HoxA5* gene (which is highly similar to Drosophila's *Sex combs reduced*

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[7]Thorsteinsdottir U., Sauvageau G., Hough M., et al. Manuscript in preparation.

(*Scr*)) to specifically activate *Scr* targets in a *HoxA5-* transgenic fruit fly (Jiagang et al., 1993). Since paralogous genes may also likely regulate similar targets in hematopoietic cells, one may predict that disruption of a specific Hox gene would not result in dramatic hematopoietic perturbations. Indeed, hematopoietic perturbations have never been reported with "knock out studies" involving more than nine Hox genes including animals with genetic disruption of two Hox genes (*HoxD3 + HoxA3*) (Condie and Capecchi, 1994; Krumlauf, 1994). In collaboration with two other groups (Drs. H.J. Lawrence - C. Largman and Dr. M. Capecchi), we are currently studying the hematopoietic system of *HoxA9* "knock out" mice and, although no obvious hematopoietic defects were initially detected, detailed quantitative analysis of B, T and myeloid lineages are suggestive of defective B lymphopoiesis in these mice (Lawrence et al., 1995). Therefore it will be important to test other available "knock out" mice (such as *HoxB4*, *HoxB3* and *HoxA10*) for other, perhaps subtle hematopoietic defect(s) that may have been overlooked. If viable, cluster and especially paralog disruptions may generate more revealing results.

Another important question that will need to be addressed is whether Hox genes are also involved in embryonic and / or fetal hematopoiesis. This issue can be addressed by using the mouse embryonic stem (ES) cell which, under controlled conditions, can be induced to differentiate in vitro into primitive (i.e. yolk sac derived) and more definitive (i.e. fetal) hematopoietic cells. In a collaborative effort, Cheryl Helgason (a post doctoral fellow that has introduced in the laboratory the in vitro differentiation of ES cells) and myself, have recently analyzed the effects of overexpressing *HOXB4* in these cells and observed striking proliferative effects on definitive multipotential progenitors whereas no

effect was observed on primitive hematopoietic cell differentiation[8]. This system should provide a powerful approach to the analysis of Hox gene function that will complement studies done in adult bone marrow as described in this thesis. In particular, it may facilitate detailed structure / function analysis of multiple Hox genes to identify critical domains that specifify proliferative versus differentiative effects.

The recent findings that HOM-C genes and their mammalian Hox homologs may require accessory molecules such as *extradenticule* or *PBX*, respectively, (Chang et al., 1995; van Dijk and Murre, 1994) to bind and possibly fully activate their targets (Sun et al., 1995) has raised questions about the role these "accessory factors" may play in hematopoiesis. Is *PBX* required for some (all?) Hox mediated effects in hematopoietic cells?. This question has clear clinical relevance since it has been shown that the E2A-PBX fusion protein involved in the t(1, 19) human pre-B leukemia interacts as well with the Hox proteins as does PBX alone (Chang et al., 1995). Considering that Hox messenger RNA has been detected in all human leukemias and that the overexpression of certain Hox gene in murine bone marrow cells can lead to leukemic transformation (*HoxB8*, Perkin and Cory, 1993; *HOXA10*, unpublished observations), it is possible that E2A-PBX requires certain members of the Hox proteins to transform hematopoietic cells. Attempts at clarifying the necessicity of PBX-Hox interaction in hematopoietic cells can now be investigated by exploiting the recent generation of *HOXB4* mutants that cannot interact with any of the PBX proteins[9].

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[8]Helgason C., Sauvageau G., Lawrence H.J., Largman C. and Humphries, R.K. (1995). Overexpression of *HOXB4* enhances the hematopoietic potential of embryonic stem cells differentiated in vitro. Manuscript Submitted

[9]Shen W.-F., Chang C.-P., Rozenfeld S., Sauvageau G., Humphries R.K., Lu M., Lawrence H.J., Cleary M.L., Largman C. Hox homeoproteins exhibit selective complex stabilities with PBX and DNA, submitted.

In conclusion, the work presented in this thesis showed that multiple genes of the highly complex Hox family of transcription factor are expressed in early hematopoietic cells. This work also demonstrated that overexpression of the *HOXB4* gene in bone marrow cells can dramatically influence expansion of early cells. Based on these results, a hypothesis was generated which suggested the existence of a relationship between the chromosomal position of a particular Hox gene versus its function in primary hematopoiesis (see earlier). More studies will need to be done in order to gather further information about the possible functions fulfilled by other Hox genes in early hematopoiesis. These should probably initially focus on determinating the function of 3' versus 5' genes of the same cluster and also of paralogs (*HOXA4* versus *B4* and *C4*, etc.). Such studies should provide a better understanding of the possible "spectrum of Hox-mediated effects" and might allow the identification of an emerging functional pattern that may or may not be related to the chromosomal position each gene occupy.

Finally, there is accumulating evidence to suggest that molecules known to regulate Hox gene expression during embryogenesis are also active in hematopoietic cells. These, as mentioned earlier, include members of the trithorax and polycomb families and also the "segmentation-like" genes such as Ikaros, erythroid krupple-like factor (EKLF) (Perkins et al., 1995) and the runt genes (Levanon et al., 1994). Therefore, the possibility that the machinery involved in embryonic development might also be active in the regulation of hematopoiesis needs to be explored. Ultimately, it will be essential to identify the mechanisms which are being regulated by the Hox proteins. This step may involve the difficult task of identifying Hox target genes which ultimately appear to control proliferative and differentiative decisions.

CHAPTER 6

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