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Conditional immortalization of mouse myelomonocytic, megakaryocytic and mast cell progenitors by the *Hox*-2.4 homeobox gene

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The murine myelomonocytic cell line WEHI-3B exhibits ectopic expression of the genes encoding the homeobox protein, *Hox*-2.4, and the myeloid growth factor, interleukin-3 (IL-3). We showed previously that concomitant expression of IL-3 and *Hox*-2.4 in bone marrow cells induced the development of transplantable growth factor-independent tumours resembling the WEHI-3B tumour. We have now investigated the effect of enforced expression of *Hox*-2.4 alone. Bone marrow cells were infected with *Hox*-2.4 retrovirus and then either cultured in agar or transplanted into irradiated mice. *In vitro*, colonies derived from virus-infected cells readily yielded IL-3-dependent, non-tumorigenic cell lines of the myelomonocytic, megakaryocytic and mast cell lineages. Surprisingly, both the establishment and maintenance of these lines required very high concentrations of IL-3 and reduced levels promoted differentiation. Transplanted mice analysed after 3 months appeared normal but their spleen and bone marrow contained abundant provirus-bearing progenitor cells, from which IL-3-dependent long-term cell lines could readily be established *in vitro*. Four of 18 animals monitored for up to 12 months eventually developed clonal leukaemia, associated in three cases with IL-3 production. Thus ectopic expression of *Hox*-2.4 enhances self-renewal of immature myeloid progenitors and progression to a fully malignant state is favoured by somatic mutations conferring autocrine production of IL-3.

Key words: *Hox*-2.4/immortalization/interleukin-3/myeloid leukemia/retroviral vector

Introduction

Homeobox genes encode transcription factors which appear to perform managerial roles not only in embryonic development but also in many subsequent differentiation processes (Wright *et al.*, 1989). In mammals, a number of homeobox genes are expressed by haemopoietic cells (Lawrence and Largman, 1992), in which they may participate in the control of lineage commitment and/or differentiation (Kongsuwan *et al.*, 1988). Circumstantial evidence implicates certain homeobox genes in the onset of human leukaemia. One quarter of childhood pre-B acute lymphoblastic leukaemias harbour a 1;19 (q23;p13.3) translocation that generates a chimeric gene encoding novel proteins in which the transactivational domain of the E2A helix-loop-helix protein is fused to the DNA-binding homeobox domain of the PBX1 homeoprotein (Kamps *et al.*, 1990; Nourse *et al.*, 1990). In addition, a small subset of acute T-cell lymphoblastic leukaemias which have 10;14 (q24;q11) or 7;10 (q35;q24) translocations exhibit ectopic expression of the *HOX*-11 gene as a result of its juxtaposition to the T-cell receptor δ or β locus (Dube *et al.*, 1991; Hatano *et al.*, 1991; Kennedy *et al.*, 1991; Lu *et al.*, 1991).

The *Hox*-2.4 gene (now *Hox*-B8; Scott, 1992) is expressed along the vertebral column during early murine development (Graham *et al.*, 1989) but, with the possible exception of certain erythroid lines (Mathews *et al.*, 1991), appears to be silent in haemopoietic cells (Kongsuwan *et al.*, 1989; Petrini *et al.*, 1992). In the murine myelomonocytic cell line, WEHI-3B, however, *Hox*-2.4 expression has been induced by transposition of an endogenous intracisternal A-particle (IAP) retrovirus-like element (Blatt *et al.*, 1988; Kongsuwan *et al.*, 1989). An additional IAP insertion has activated the interleukin 3 (IL-3) gene in these cells, long used as a source of this myeloid growth factor (Ymer *et al.*, 1985). Autocrine growth factor production did not account for the transformed phenotype since, although factor-dependent myeloid cell lines are rendered malignant by enforced IL-3 expression (Lang *et al.*, 1985), normal haemopoietic cells are not (Wong *et al.*, 1987; Chang *et al.*, 1989).

Collectively, these observations suggested that concomitant expression of *Hox*-2.4 and IL-3 genes may have provoked the onset of the WEHI-3B tumour. To address this hypothesis, we previously utilized a retroviral vector bearing both the *Hox*-2.4 and IL-3 genes to induce their expression in bone marrow cells. Transplantable factor-dependent cell lines closely resembling WEHI-3B were readily generated *in vitro* from infected cultures. Furthermore, mice engrafted with infected cells rapidly succumbed to a polyclonal transplantable myelomonocytic leukaemia (Perkins *et al.*, 1990). This disease differed markedly from the non-malignant myeloproliferative syndrome induced with an IL-3 retrovirus, which is characterized by excess production of mature myeloid cells (Wong *et al.*, 1987; Chang *et al.*, 1989; Perkins *et al.*, 1990).

To clarify the role of the *Hox*-2.4 gene in leukaemogenesis, we have now studied the behaviour of bone marrow cells infected with a virus expressing *Hox*-2.4 but not IL-3. The results indicate that ectopic expression of *Hox*-2.4 in early myeloid cells potentiates their self-renewal capacity but is not fully transforming. Establishment of long-term lines from *Hox*-2.4-expressing myeloid progenitor cells required very high concentrations of IL-3, suggesting that a synergistic signal for self-renewal may be triggered in these cells via the IL-3 receptor.

Results

Hox-2.4 expression does not transform immortalized fibroblasts or myeloid cells

In order to facilitate selection of infected cells, the *Hox*-2.4 retroviral vectors were designed to express the *Neo*^{R} gene

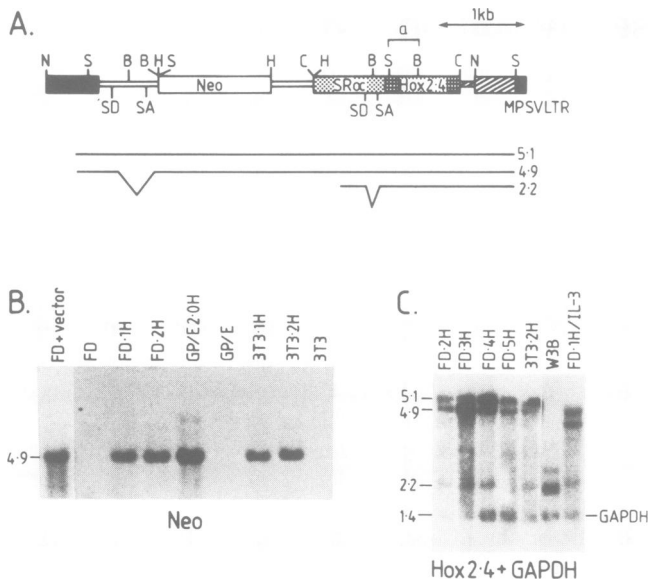

Fig. 1. Structure and expression of MPZen*Neo*/*Hox*-2.4 retrovirus. (**A**) Schematic representation of the structure of the retroviral plasmid and expected proviral transcripts. The *Neo*^{R} gene is expressed via the spliced subgenomic viral RNA and *Hox*-2.4 is expressed via the internal cassette driven by the SRα promoter/enhancer (Takabe *et al.*, 1988). Restriction sites indicated are *Nhe*I (N), *Sac*I (S), *Bam*HI (B), *Hin*dIII (H) and *Cla*I (C). SD, splice donor; SA, splice acceptor. The 5′ long terminal repeat (LTR) derives from Moloney leukaemia virus. The 3′ LTR derives largely from myeloproliferative sarcoma virus (Seliger *et al.*, 1986) (hatched sequences). (**B**) Southern blot analysis of DNA from FDC-P1, NIH-3T3 and GP+E parental lines and derived clones infected with the MPZen*Neo*/*Hox*-2.4 virus (FD.1H, 3T3.1H etc). DNA was digested with *Nhe*I, which cuts within each LTR, and hybridized with a *Neo*^{R} probe (see Materials and methods). (**C**) Northern blot analysis of polyadenylated RNA (2 μg) from FDC-P1 and NIH-3T3 clones infected with the MPZen*Neo*/*Hox*-2.4 virus, WEHI-3BD$^+$ (lane 6), and an FDC-P1 clone infected with MPZenIL-3/SRα*Hox*-2.4 virus (Perkins *et al.*, 1990) (lane 7). The filter was hybridized simultaneously with *Hox*-2.4 and GAPDH probes.

as well as *Hox*-2.4. Figure 1A shows the principal vector, MPZen*Neo*^{R}SRα*Hox*-2.4 (hereafter called *Neo*/*Hox*-2.4), in which the *Neo*^{R} gene is expressed via the promoter in the retroviral 5′ long terminal repeat (LTR) and the *Hox*-2.4 gene is expressed via an independent cassette bearing a modified SV40 promoter (Takabe *et al.*, 1988). This expression strategy was used previously for co-expression of IL-3 and *Hox*-2.4 (Perkins *et al.*, 1990). Virus stocks, generated by transfection of Ψ-2 packaging cells (Mann *et al.*, 1983), were free of helper virus. To check that the producer lines secreted virus of the correct structure, NIH-3T3 fibroblasts and FDC-P1 myeloid cells (Dexter *et al.*, 1980) were infected and G418-resistant clones isolated for DNA and RNA analysis. As expected, *Nhe*I digestion released a 4.9 kb proviral fragment (Figure 1B) and three transcripts were detected with a *Hox*-2.4 probe: the 5.1 kb genomic and 4.9 kb spliced viral RNAs initiating within the retroviral LTR and the 2.2 kb transcript directed by the SRα promoter (Figure 1C). The level of *Hox*-2.4 RNA expression from the internal cassette was comparable to that in control FDC-P1 clones infected by the IL-3/*Hox*-2.4 retrovirus (e.g. Figure 1C, lane 7) and somewhat lower than that in WEHI-3B cells (lane 6).

FDC-P1 cells are immortal but non-malignant and their proliferation and survival *in vitro* requires an exogenous growth factor, either IL-3 or granulocyte-macrophage colony stimulating factor (GM-CSF) (Dexter *et al.*, 1980). Enforced *Hox*-2.4 expression did not perceptibly change their growth characteristics. Each of eight independent G418-resistant clones remained dependent on IL-3 for both survival and proliferation and neither their doubling time in liquid culture nor their plating efficiency in agar was altered. Like uninfected FDC-P1 cells, these clones were unable to differentiate in agar, since the colonies were compact and none contained a halo of migrating differentiated cells, even when granulocyte CSF (G-CSF), macrophage CSF (M-CSF) or GM-CSF was also included in the medium. Transplantation tests were performed by injecting 2×10^6 cells intraperitoneally and subcutaneously into pairs of unirradiated syngeneic (DBA/2) mice. All recipients of six clones remained healthy and were tumour-free when autopsied 12 months later. Both mice transplanted with each of the other two clones eventually developed a leukaemia bearing the same proviral insertion pattern as the injected clone, but the long latency (10−43 weeks) suggested that additional oncogenic mutation(s) acquired *in vivo* and/or *in vitro* had contributed to the malignancy.

NIH-3T3 cells expressing *Hox*-2.4 also remained non-malignant, irrespective of which of three different *Neo*/*Hox*-2.4 retroviral constructs (see Materials and methods) was used. The G418-resistant NIH-3T3 clones all displayed normal contact inhibition and none of eight clones tested produced tumours when injected into BALB/c nude mice.

Immortalization of bone marrow cells by Hox-2.4

To investigate the consequences of ectopic *Hox*-2.4 expression in primary haemopoietic cells, bone marrow cells harvested from DBA/2 or C57BL/6 mice treated 4 days previously with 5-fluorouracil (5-FU), and thus enriched in progenitor and stem cells (Hodgson and Bradley, 1979), were co-cultured for 5 days with virus-producing fibroblasts and then plated in soft agar. No colonies developed in the absence of exogenous growth factors, provided initially by addition of medium conditioned by pokeweed mitogen-stimulated spleen cells (SCM). The efficiency of infection of progenitor cells varied between experiments but 20−30% of colonies were usually G418-resistant. Cell counts performed on pools of G418-resistant colonies indicated that, on average, those infected with *Neo*/*Hox*-2.4 virus were 2- to 3-fold larger than those infected with the virus expressing only *Neo*.

In addition to the usual macrophage, granulocyte, granulocyte-macrophage, eosinophil, megakaryocyte and mixed colonies, a significant proportion (10−33%) of the *Neo*/*Hox*-2.4 virus-infected colonies had a relatively compact centre and a diffuse halo of differentiating cells. In our previous study, compact colonies arising after infection of bone marrow cells with IL-3/*Hox*-2.4 virus readily yielded long-term lines (Perkins *et al.*, 1990). We therefore anticipated that the compact colonies infected with *Neo*/*Hox*-2.4 virus would yield immortal but factor-dependent lines. However, using concentrations of SCM well above those required to maintain conventional IL-3-dependent myeloid cell lines such as FDC-P1, cells from only two of 91 compact colonies continued to proliferate when transferred to liquid medium and both cultures terminally differentiated a few weeks later.

Table I. Infection of bone marrow colony-forming cells[a]

Experiment	Virus	Low IL-3		High IL-3		
		Total[b]	% compact	Total[b]	% compact	Lines[c]
4	*Neo*	19 ± 17	<2	25 ± 7	4	0/24
	Neo/*Hox*-2.4	9 ± 1	3	10 ± 12	57	17/66
	IL3/*Hox*-2.4	146 ± 33	22	41 ± 6	51	9/24
5	*Neo*	31 ± 6	<2	177 ± 5	33	0/11
	Neo/*Hox*-2.4	87 ± 4	30	122 ± 9	34	14/78
6	*Neo*	174 ± 10	17	145 ± 14	31	0/24
	Neo/*Hox*-2.4	873 ± 63	33	892 ± 57	30	11/24
7a	*Neo*			3 ± 1	<2	0/24
	Neo/*Hox*-2.4			18 ± 2	29	10/24
7b	*Neo*	0/24			2 ± 1	<2
	Neo/*Hox*-2.4			9 ± 1	22	7/21

[a]Bone marrow cells from 5-FU-treated mice were infected with *Neo*, *Neo*/*Hox*-2.4 or IL-3/*Hox*-2.4 virus as described in Materials and methods. Low concentrations of IL-3 (300 U/ml) were provided as 10% (v/v) pokeweed mitogen stimulated spleen conditioned medium (SCM) except in experiment 5, where 1000 U/ml were provided as 0.3% (v/v) conditioned medium from X63Ag8-653mIL-3 cells (CMX63). High IL-3 concentrations (10^4 U/ml) were supplied either as 3.3% (v/v) CMX63 or, in experiment 7b, as partially purified recombinant IL-3 produced via a baculovirus vector. Recombinant human erythropoietin was included at 5 U/ml in cultures containing SCM in experiment 6. After 5 days, non-adherent cells were harvested and plated at 10^3, 5×10^3 and 2.5×10^4 cells/ml in agar in the same medium ± 1 mg/ml G418 or, in the case of IL-3/*Hox*-2.4 virus-infected cells, ± growth factor.

[b]Total number of virus-infected progenitors (G418-resistant or IL-3-independent colonies) per 10^4 bone marrow cells. Colony frequency was determined from plates containing 30–150 colonies.

[c]The ratio indicates the number of relatively compact colonies which could be established as lines in liquid medium containing the same growth factor(s) used for agar culture. Most of those selected from control plates were the largest granulocyte-macrophage colonies which often have somewhat compact centres. In experiment 6, 24 clones infected with *Neo* virus and 20 clones infected with *Neo*/*Hox*-2.4 virus selected from agar plates containing 300 U/ml IL-3 were subsequently cultured in liquid medium containing 300 U/ml IL-3; the only line obtained was a *Hox*-2.4-expressing mast cell line.

Since WEHI-3B and analogous lines produced by infection with IL-3/*Hox*-2.4 virus produce very high levels of IL-3 (typically 5×10^3 to 2×10^4 units per ml at stationary phase), the experiments were repeated using much higher concentrations of IL-3 (10^4 units/ml) than those afforded by SCM (300 units/ml). Diffuse G418-resistant colonies again terminally differentiated, whether derived from *Neo*/*Hox*-2.4 or *Neo* virus-infected cultures. However, the growth potential of the relatively compact colonies expressing *Hox*-2.4 was strikingly elevated: 58 of 213 (27%) colonies tested from four independent experiments were readily propagated in liquid culture (Table I). Most lines were cryopreserved after 3 months but four were maintained continuously for 22 months and two others for more than 6 months. The source of IL-3 used for most of these experiments was a cell line (X63Ag8-653mIL-3) engineered to secrete IL-3 (Karasuyama and Melchers, 1988), but comparable results were obtained with partially purified recombinant IL-3 derived from a baculovirus expression vector (experiment 7 in Table I), arguing against the possibility that another factor within the conditioned medium contributed to the immortalization.

When deprived of high concentrations of IL-3, the lines differentiated, as illustrated in Table II for a myelomonocytic line. Both the total number of viable cells and the proportion of blasts and promyelocytes were directly related to the IL-3 concentration. Thus self-renewal of *Hox*-2.4-expressing cells appears to be augmented by a signal conveyed by high concentrations of IL-3. This observation explains why cell lines were rarely established from *Neo*/*Hox*-2.4 virus-infected bone marrow cultured in conventional amounts of IL-3.

Table II. Reduction in IL-3 concentration enhances differentiation of myelomonocytic cells immortalized by *Hox*-2.4

IL-3 (U/ml)	Total viable cells ($\times 10^{-4}$)	Differential counts (%)			
		Blast/pro	Myl/meta	Band/neut	Mono
10^4	200	51	18	12	19
10^3	200	47	18	12	23
10^2	62	20	33	13	34
10	6	1	18	21	60
1	2	2	8	19	71

4-220.1 cells were washed three times by centrifugation through 1 ml fetal calf serum and 5×10^4 cells were plated in duplicate 2 ml cultures containing the indicated concentrations of IL-3 (supplied as X63CM). After 7 days, the mean number of viable cells per well was determined by counting cells excluding eosin. Cytocentrifuge specimens were stained with a modified Wright's stain (Diff-Quick, Lab Aids, Narrabeen, Australia) and counts performed on 100 cells to identify the relative proportions of blasts and promyelocytes (blast/pro), myelocytes and metamyelocytes (myl/meta), bands and segmented neutrophils (band/neut), and monocytes and macrophages (mono).

Multiple haemopoietic lineages are immortalized by Hox-2.4

Twenty-one IL-3-dependent lines established from *Neo*/*Hox*-2.4 virus-infected colonies were characterized in more detail (as illustrated in Table III and Figure 2). Eleven were myelomonocytic and stained positive for the promyelocytic enzymes myeloperoxidase (Figure 2B), but also contained a variable proportion of granulocytes and monocytes (Table III and Figure 2A and C). Four mast cell lines (Figure 2D), which stained strongly with Astra blue, and five megakaryocytic lines (Figure 2E), which stained

Table III. Characterization of *Hox*-2.4 cell lines

Cell line[a]	Cytochemistry[b]				Differential counts (%)[c]				Transplantation[d]	Cobblestone growth[e]	CFU-S frequency[f]
	MGG	Ace	AstraB	Perox	M/Blst	E.myl	L.myl	Mono			
In vitro											
4-220.1	myl	−	−	+	68	18	14	0	0/4	+	2×10^{-4}
4-218.2	myl	−	−	+	5	22	58	15	0/4	+	$<2 \times 10^{-5}$
4-190.2	myl	−	−	+/−	10	10	32	48	0/4	nd	nd
4-E1	myl	−	−	+	22	32	32	14	0/4	+	nd
4-F1	myl	−	−	+	72	16	6	6	0/4	+	$\sim 2 \times 10^{-4}$
5-72-4	mst	−	+	−	0	0	0	0	nd	nd	nd
4-C5	meg	±	−	−	0	0	0	0	0/4	+	$\sim 2 \times 10^{-4}$
4-B2	meg	+	−	−	0	0	0	0	0/4	+	8×10^{-5}
In vivo											
C1-12.8	myl	−	−	+	28	30	42	0	nd	nd	nd
C4-52.6	myl	−	−	+	40	42	12	6	nd	nd	nd
C4-60.5	myl	−	−	+	16	30	54	0	nd	nd	nd
C5-78.11	meg	+	−	−	0	0	0	0	nd	nd	nd

[a]Cell lines derived from G418-resistant colonies generated from bone marrow cells cultured *in vitro* immediately after virus infection or from spleen or bone marrow cells of irradiated mice transplanted with virus-infected marrow cells. All *in vitro*-derived lines are DBA/2 in origin, except for 5-72-4 which is C57BL/6. All lines derived from mice are C57BL/6.
[b]MGG, May−Grunwald−Giemsa staining; Ace, acetylcholinesterase activity; AstraB, Astra Blue staining; Perox, myeloperoxidase activity.
[c]Differential counts were performed on myelomonocytic lines as described in Table II. M/Blst, myeloblasts; E.myl, early myeloid cells (promyelocytes and myelocytes); L.myl, late myeloid cells (bands, neutrophils); Mono, monocytes.
[d]The proportion indicates the number of tumour-bearing mice amongst those injected.
[e]5×10^4 cells were plated on monolayers of S17 stromal cells in T25 flasks and cultures were scored 7 days later for evidence of cobblestone colony formation.
[f]Cells were injected in graded doses (5×10^3, 5×10^4 and 5×10^5 cells/mouse) into pairs of lethally irradiated (two doses of 5.5 Gy separated by 3 h) syngeneic mice and spleen colonies counted 13 days later. The number of spleen colonies was proportional to the number of cells injected. The frequency of CFU-S was calculated from the cell number required to generate 5−12 colonies per spleen.

positive for acetylcholinesterase, were also identified. Electron microscopy performed on one megakaryocytic line (4-B2) revealed the nuclear hypersegmentation, multiple centrioles (arrowed in Figure 2G), α-granules and demarcation membranes (Figure 2H) typical of megakaryocytes. Moreover, flow cytometry of propidium iodide-stained 4-B2 cells indicated that 4% of the nuclei were hyperdiploid (8N, 16N or 32N).

Both the degree and pattern of differentiation of the *Hox*-2.4 lines remained relatively unchanged after prolonged culture. For example, when cells recovered from stocks cryopreserved after 3 and 12 months were compared under identical culture conditions, both cultures of the 4-218.2 line displayed substantial monocytic (52%, 32%) and granulocytic (26%, 38%) differentiation, and both C1-12.8 cultures exhibited predominantly granulocytic (64%, 78%) differentiation. Marked differentiation was also still apparent in the two megakaryocytic lines (4-B2 and C5-78.11) cultured for 22 months.

Flow cytometric analysis of two of the IL-3-dependent myelomonocytic lines (4-218.2 and 4-F1) (not shown) indicated that, like WEHI-3B D^+, both express Mac-1. Some cells also express the granulocytic marker Gr-1, which WEHI-3B cells express only weakly. A moderate level of Thy-1 was apparent on 50% of 4F1 cells and ~10% of 4-218.2 cells. About 20% of the cells in both lines displayed B220 (the B-lymphoid specific epitope of CD45R) but other B lymphoid markers (BP-1, ThB, surface immunoglobulin) were not detectable.

Northern blot analysis (Figure 3) verified that the cell lines expressed the expected retroviral transcripts. The relative concentration of the LTR- and SRα-driven transcripts varied in different lines but not in a cell-type specific manner. As expected, the two mast cell lines expressed the α chain of the IgE receptor, while the myelomonocytic and megakaryocytic lines did not. Both the mast and megakaryocytic lines expressed the transcription factor GATA-1. Although the megakaryocytic line also contained transcripts of the β-globin and erythropoietin receptor genes (not shown), haemoglobin was not detected by benzidine staining, even after culture for 5 days in the presence of 15 U/ml of erythropoietin.

Primitive haemopoietic cells can be targets for immortalization by Hox-2.4

Certain features of the IL-3-dependent *Hox*-2.4 cell lines suggested that at least some were derived from a relatively primitive haemopoietic cell. Of four myelomonocytic and two megakaryocytic lines tested, all readily produced 'cobblestone' colonies on bone marrow stromal cell lines such as S17 (Collins and Dorshkind, 1987) (Figure 2F and Table III). Furthermore, four of five lines tested (two megakaryocytic and two myelomonocytic) contained detectable numbers of spleen colony-forming cells (d13 CFU-S) (Table III). Like those produced by normal bone marrow CFU-S, these spleen colonies consisted primarily of maturing erythroid cells. Unfortunately, the CFU-S activity of the lines was lost with continued passage *in vitro*.

Direct evidence that primitive haemopoietic cells are susceptible to immortalization by *Hox*-2.4 was obtained by infecting a population of sorted 5-FU-treated bone marrow cells substantially enriched for long-term lymphomyeloid reconstituting ability (Szilvassy and Cory, 1993). Sorted stem cells, kindly provided by Dr S.J.Szilvassy, were co-cultured at limiting dilution (3, 10 and 25 cells/well) in 96 well plates containing a mixture of irradiated virus-producing fibroblasts (7×10^3 cells/well) and unirradiated stromal cells (3×10^3 cells/well) in medium containing 10^4 units/ml of IL-3. After 7 days, cobblestone areas appeared, followed a few days

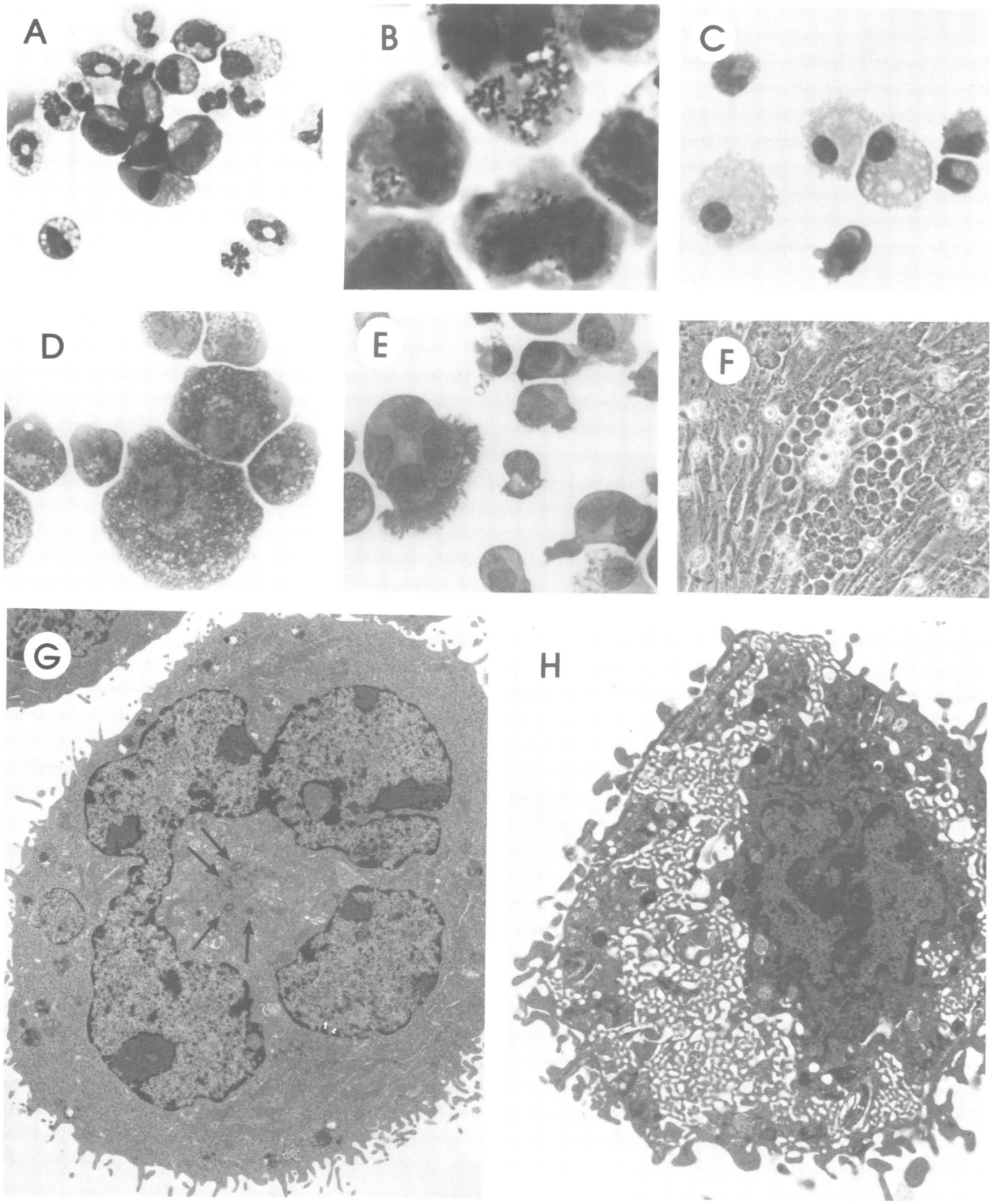

Fig. 2. Morphology of *Hox*-2.4 cell lines. Panels A and C–E show cytocentrifuge preparations of clones stained with May–Grunwald–Giemsa. (**A**) 4-218.2 (myelomonocytic); (**C**) 4-190.2 (myelomonocytic); (**D**) 7-75-10 (mast); (**E**) 4-B2 (megakaryocytic). Panel **B** is a myeloperoxidase stain of 4-220.1 (promyelocytic). Panel **F** demonstrates the cobblestone growth of 4-220.1 on S17 stromal cells. Panels **G** and **H** are electron micrographs of the megakaryocytic cell line, 4-B2. Magnification: ×3600 for A and C–E, ×9000 for B, ×400 for F and ×36 000 for G and H.

later by a burst of proliferation of non-adherent cells. Most such wells contained only one cobblestone colony, and the frequency of cobblestone-positive wells in cultures exposed to *Neo*/*Hox*-2.4 virus (24 of 48 wells seeded at 25 cells/ml) was comparable to that in control cultures with *Neo* virus (17 of 48 wells). However, whereas all but one of the control cultures terminally differentiated within three weeks, 18 of the *Neo*/*Hox*-2.4 cultures (75%) continued to proliferate vigorously. The adherent population was readily passaged by plating trypsinized cells on to fresh semiconfluent stromal cells and continued to produce cobblestone cells and non-adherent cells until cryopreserved at 6 months.

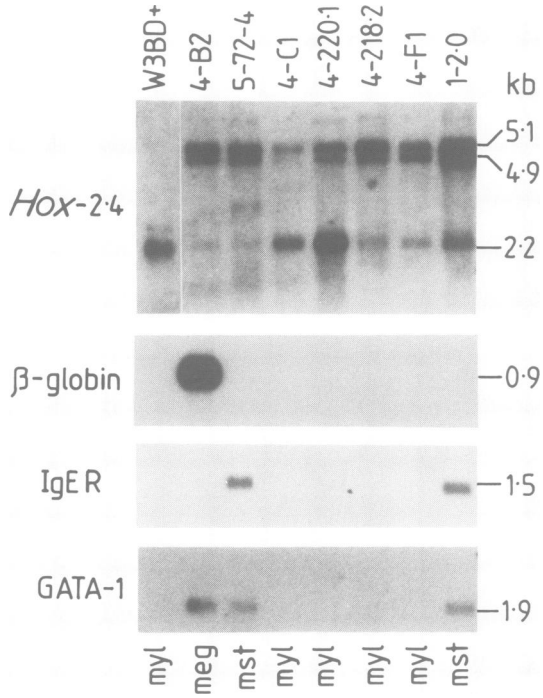

Fig. 3. RNA analysis of *Hox*-2.4 cell lines. The cytochemical classification of each line is indicated: myl, myelomonocytic; mst, mast cell; meg, megakaryocytic. Polyadenylated RNA (2 μg) was size fractionated and Northern blots were hybridized with the indicated probes. Ethidium bromide-stained agarose gels demonstrated comparable loading (not shown). Other experiments showed somewhat higher levels of the 2.2 kb RNA in 4-B2 and 5-72-4.

Long-term lines were also readily established from the non-adherent population and Southern blot analysis confirmed that each of 12 lines analysed was clonal, harbouring two or three *Neo*/*Hox*-2.4 proviruses in equimolar amounts. Each of seven non-adherent lines tested at 6 weeks contained CFU-S at a frequency of 1−8 per 10^5 cells. CFU-S were also readily detected in the corresponding adherent cultures (typically 20 per cm^2). Three C57BL/6 lines (Ly-5.2) were tested for long-term repopulating stem cell activity by transplantation into lethally irradiated congenic Ly-5.1 mice (Spangrude *et al.*, 1988), but no Ly-5.2 cells were detectable in the blood of recipients analysed after 3 months.

Enhanced self-renewal of Hox-2.4-expressing progenitor cells in vivo

To determine the consequences of ectopic *Hox*-2.4 expression *in vivo*, unfractionated 5-FU-treated bone marrow cells were co-cultivated with *Neo*/*Hox*-2.4 virus-producing fibroblasts in medium containing high concentrations of IL-3 and injected into lethally irradiated syngeneic mice. Control mice received marrow cells infected with viruses expressing both IL-3 and *Hox*-2.4 or only the IL-3 or Neo^R gene. After recovering from irradiation, most mice transplanted with cells infected with the *Neo*/*Hox*-2.4 virus (subsequently denoted *Hox*-2.4 animals) remained healthy for many months (Figure 4), like control mice transplanted with cells infected with the *Neo* virus (*Neo* mice). In contrast, as in previous studies, all mice engrafted with cells infected with the IL-3/*Hox*-2.4 virus died within 3 weeks of a fulminant, transplantable leukaemia (Perkins *et al.*, 1990) and those

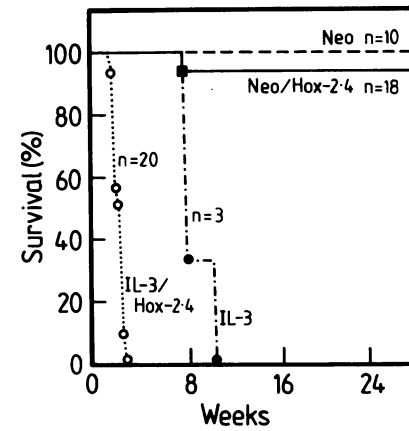

Fig. 4. Survival of mice transplanted with virus-infected bone marrow cells. 5-Fluorouracil-treated bone marrow cells were infected with *Neo*/*Hox*-2.4, IL-3/*Hox*-2.4, IL-3 or *Neo* virus and transplanted into lethally irradiated syngeneic mice. The number of mice in each cohort monitored for 12 months, or until they became ill and were killed, is indicated. There were no significant differences between C57BL/6 and DBA/2 mice, so the survival data have been pooled.

receiving cells infected with the IL-3 virus died from the effects of excess production of mature myeloid cells induced by high circulating levels of IL-3 (Wong *et al.*, 1987; Chang *et al.*, 1989; Perkins *et al.*, 1990).

Ten healthy *Hox*-2.4 mice autopsied after 3 months exhibited no pathology and could not be distinguished from control *Neo* mice by histology or haematology (see Materials and methods). *Hox*-2.4 provirus DNA was readily detected in the spleen and bone marrow of all five C57BL/6 mice and one of five DBA/2 mice. The concentration of proviral DNA was approximately equal to that of the endogenous *Hox*-2.4 gene, as judged by Southern blot analysis of DNA digested with *Nhe*I, which cuts within each LTR (Figure 5A). Clonality was assessed using a probe/enzyme combination (*Hox*-2.4/*Hin*dIII; see Figure 1A) which detects provirus−chromosome junction fragments and is thus integration site-specific. No bands were detected (not shown), indicating that the infected population was polyclonal.

Although these observations suggested that haemopoiesis was unaffected by *Hox*-2.4 expression, a difference emerged when spleen and bone marrow cells of the reconstituted mice were assayed for *in vitro* colony-forming cells (CFC) (Table IV). Despite the comparable titres of the viruses used to infect the transplanted cells, substantially higher numbers of G418-resistant colonies were obtained from the *Hox*-2.4 mice than from the *Neo* mice. Indeed, the majority of CFC in three of the five C57BL/6 *Neo*/*Hox*-2.4 animals were G418-resistant and their frequency per spleen was comparable to, or higher than, the total number of CFC of control mice (~1000/spleen). These observations suggested that *Hox*-2.4 expression had enhanced the self-renewal capacity of progenitor cells *in vivo*.

Direct evidence for enhanced self-renewal was sought by determining the number of cells per primary colony able to generate secondary colonies upon replating. Large G418-resistant colonies derived from spleen cells grown in high concentrations of IL-3 were individually replated in secondary agar cultures containing 10^4 units/ml of IL-3. The primary colonies selected were predominantly either multicentric or relatively compact. Half of the colonies

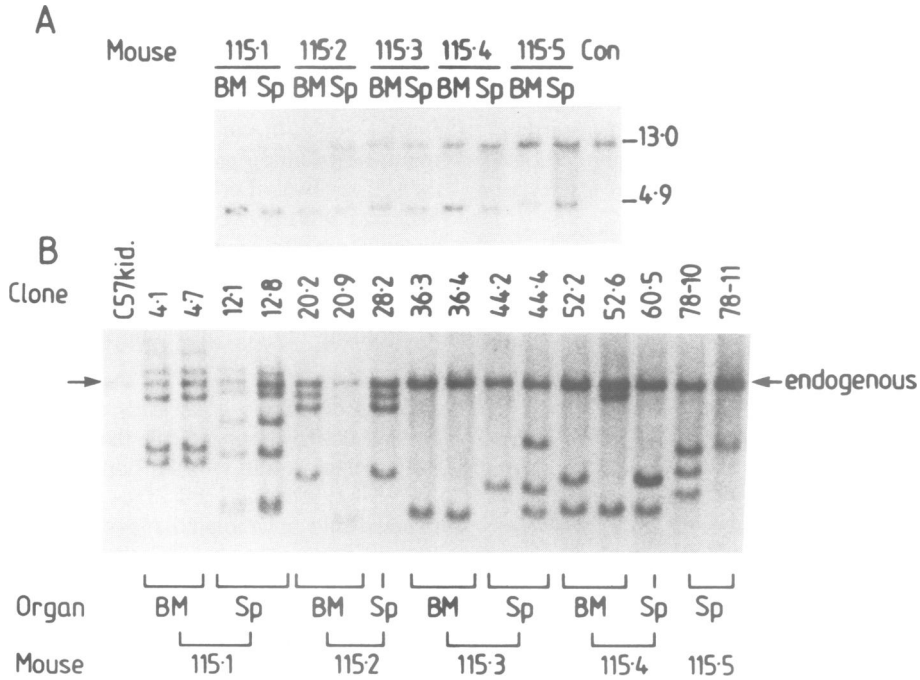

Fig. 5. DNA analysis of proviral insertions in mice transplanted with *Neo*/*Hox*-2.4 virus-infected bone marrow cells. (**A**) Spleen and bone marrow DNA from the indicated C57BL/6 mice was digested with *Nhe*I and hybridized with a *Hox*-2.4 probe (see Figure 1A). The control is a mouse transplanted with cells infected with *Neo* virus. The 13.0 and 4.9 kb bands correspond to the endogenous and proviral genes respectively. (**B**) DNA from cell lines established from primary colonies was digested with *Hin*dIII and hybridized with a *Hox*-2.4 probe. The fragment bearing the endogenous *Hox*-2.4 gene is indicated. The other fragments are provirus−chromosome junction fragments.

Table IV. Mice transplanted with *Hox*-2.4 virus-infected bone marrow cells have elevated progenitor cells

Mouse	G418^R CFC/spleen[a]		G418^R CFC/femur[b]	
	SCM[c]	IL-3[d]	SCM[c]	IL-3[d]
Neo/*Hox*-2.4				
115.1	7070 (93%)	9150 (64%)	9050 (68%)	>20 000 (100%)
115.2	220 (17%)	310 (31%)	1390 (17%)	1090 (15%)
115.3	850 (34%)	640 (90%)	830 (10%)	860 (9%)
115.4	220 (7%)	190 (30%)	590 (9%)	>5500 (>50%)
115.5	>6830 (100%)	>6830 (100%)	980 (11%)	>4900 (100%)
Neo				
116.1	<21 (<1%)	<21 (<1%)	<20 (<1%)	110 (1%)
116.2	39 (2%)	39 (5%)	440 (5%)	470 (1%)
116.3	<21 (<1%)	42 (7%)	<10 (<1%)	50 (<1%)
116.4	37 (1%)	56 (7%)	40 (<1%)	<10 (<1%)

C57BL/6 mice were sacrificed 3 months after irradiation and transplantation with bone marrow cells infected with *Neo*/*Hox*-2.4 or *Neo* virus and their spleen and bone marrow cells were cultured at 2×10^5 cells/ml and 5×10^4 cells/ml respectively in soft agar containing the indicated growth stimulant and at 2×10^6 cells/ml and 5×10^5 cells/ml respectively in the same medium plus G418 (1 mg/ml). Colonies were counted on day 7.
[a]The number of G418-resistant colony forming cells (CFC) per spleen is indicated and, in brackets, the percentage of total CFC they represent.
[b]The number of G418-resistant CFC per femur is indicated and, in brackets, the percentage of total CFC they represent.
[c]SCM at a final concentration containing 300 U/ml of IL-3.
[d]CMX63 at a final concentration containing 10^4 U/ml of IL-3.

obtained from two *Neo*/*Hox*-2.4 mice each produced at least 100 secondary colonies (Figure 6), a significant proportion of which were similar in morphology to the primary colonies. Smaller macrophage and granulocyte-macrophage colonies were also observed. In contrast, despite the high concentrations of IL-3, only 11% of the colonies from a control *Neo* mouse contained clonogenic cells; their frequency per colony was low and most of the secondary colonies were small and diffuse.

As anticipated from the *in vitro* results described above, many colonies expressing *Hox*-2.4 displayed long-term proliferative potential. Forty-four of 96 G418-resistant primary colonies (46%) from *Neo*/*Hox*-2.4 mice could be expanded into lines when picked into liquid medium containing 10^4 units per ml of IL-3. None of 83 colonies from *Neo* mice could be similarly expanded. Once again, high concentrations of IL-3 were essential, since only three of 74 (4%) *Hox*-2.4 colonies maintained in SCM continued to proliferate. Both myelomonocytic and megakaryocytic cell lines were established (e.g. Table III).

To search for further evidence of enhanced self-renewal capacity of progenitor cells expressing *Hox*-2.4, we

investigated the clonal relationship of lines established from multiple primary colonies from individual mice (Figure 5B). For each mouse, at least two different insertion patterns were detected by Southern blot analysis. Strikingly, however, multiple independent lines had the same proviral insertion pattern. For example, seven clones obtained from the bone marrow of mouse 115.1 had the same pattern of inserts as that shown for clones 4.1 and 4.7, and five of six clones derived from the bone marrow of mouse 115.4 had the same pattern as clone 52.2, as did the only spleen clone analysed. Each mouse thus contained multiple progenitor cells derived from a single infected cell. The most plausible explanation for these results is that *Hox*-2.4 expression had promoted the self-renewal of progenitor cells. In no case was the same pattern shared between lines derived from different mice, even though all five mice had been transplanted with aliquots of the same pool of infected cells. Amplification of progenitor cells thus appeared to have occurred *in vivo* rather than during the co-cultivation period *in vitro*.

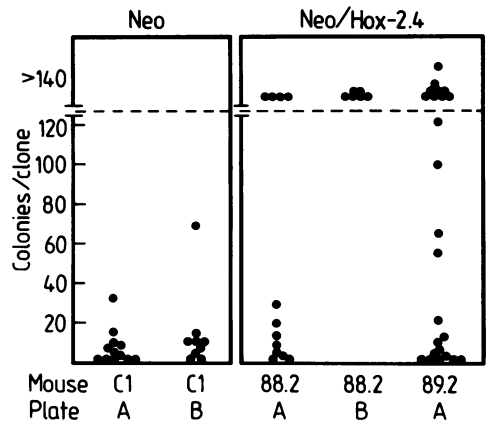

Fig. 6. Self-renewal of colony-forming cells expressing *Hox*-2.4. Single G418-resistant colonies were picked from agar cultures of *Neo* and *Neo/Hox*-2.4 virus-infected DBA/2 spleen cells, resuspended in 100 μl of medium, replated in agar and cultured for a further 7 days. Each dot represents the number of secondary colonies generated from a single primary colony. Both the primary and secondary cultures contained 10^4 U/ml of IL-3. A and B refer to separate primary culture dishes. The frequency of G418-resistant colonies per spleen was 8400 (3% of total) and 275 000 (32% of total) for mouse 88.2 and 89.2 respectively, and <1200 (<1% of total) for the control mouse.

Acute leukaemias arise after further somatic mutation

Several observations indicate that *Hox*-2.4 expression is not fully transforming. First, none of 10 factor-dependent *Hox*-2.4-expressing cell lines produced tumours when injected into unirradiated syngeneic hosts (e.g. Table III). Second, no tumorigenic cells could be detected upon transplantation of spleen and bone marrow cells from nine of 10 *Hox*-2.4 mice killed at 12–14 weeks. Finally, 17 of the remaining 18 *Hox*-2.4 mice stayed healthy for at least 7 months (Figure 4).

Four *Hox*-2.4 animals eventually developed acute leukaemia (three myeloid and one erythroid). These animals had elevated white cell levels in the blood (Figure 7A, panel A and Table V) and were anaemic and thrombocytopaenic. Histologic and cytologic examination revealed extensive infiltration of the bone marrow, spleen, lymph nodes (panel B) and many non-haemopoietic organs by blast cells (panels C and D). The tumours were readily transplantable

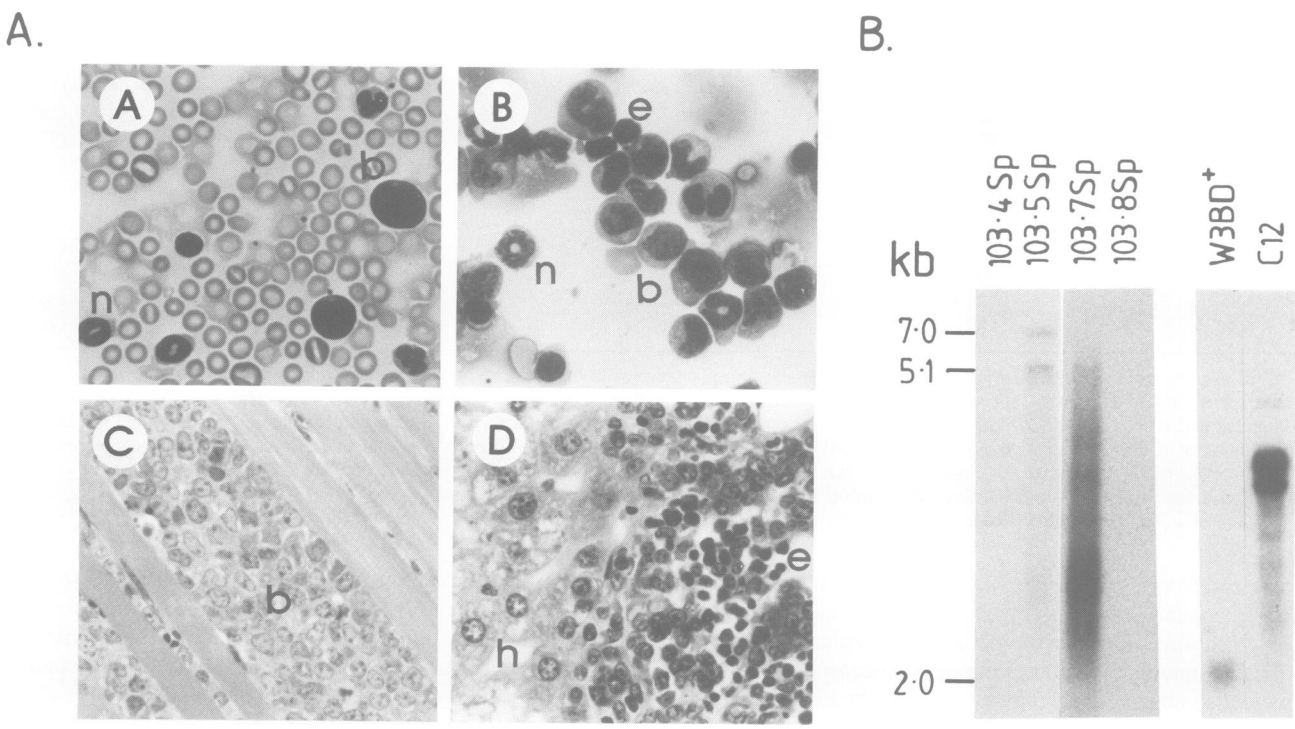

Fig. 7. Haematology and IL-3 expression in leukemic mice. **(A)** Photomicrographs of blood and haemopoietic tissues. Panel A: blood film from mouse 103.4 stained with May–Grunwald–Giemsa (MGG). Panel B: MGG-stained cytocentrifuge preparation of spleen cells from mouse 103.5. Panel C: histological section of parasternal muscle from mouse 103.7 stained with haematoxylin and eosin (H&E). Panel D: H&E-stained histological section of liver from mouse 103.8. Magnification: ×3600 (A and B); ×1800 (C and D). n, neutrophil; b, blast; e, nucleated erythroblast; h, hepatic parenchyma. **(B)** Aberrant IL-3 transcripts in spleens of leukaemic mice detected by Northern blot analysis of poly(A)$^+$ RNA with an IL-3 cDNA probe. Positive controls are from C12, a line derived with IL-3/*Hox*-2.4 virus, and WEHI-3B D$^+$. The size of normal IL-3 mRNA is 2.1 kb.

(Table V) and Southern blot analysis (not shown) established that they contained *Neo*/*Hox*-2.4 proviral DNA and were clonal. The long latency and monoclonality of the primary tumours implied that they originated from a single virus-infected cell following somatic mutation *in vivo*.

The tumours were screened for evidence of the obvious candidate for a synergistic mutation: activation of the IL-3 gene. IL-3 was detected in the sera of two mice (103.5 and 103.7) (Table V) and aberrant IL-3 transcripts were apparent in their tumour-infiltrated spleens (Figure 7B). An IL-3-secreting cell line which displayed rearrangement of the IL-3 gene was isolated from mouse 103.5. The tumour in mouse 103.4 also displayed a rearranged IL-3 gene. Although these cells were not factor-independent and no IL-3 was detectable in the serum, autocrine IL-3 production may nevertheless have contributed to its onset. None of the tumours showed rearrangement or expression of the GM-CSF gene.

Discussion

Hox-2.4 was the first vertebrate homeobox gene directly demonstrated by gene transfer to have oncogenic potential (Perkins *et al.*, 1990). Following up observations that the murine myelomonocytic leukaemia, WEHI-3B, exhibits ectopic expression of both the *Hox*-2.4 and IL-3 genes (Ymer *et al.*, 1985; Blatt *et al.*, 1988; Kongsuwan *et al.*, 1989), we showed that infection of bone marrow cells with a retrovirus expressing both of these genes recapitulated the disease (Perkins *et al.*, 1990). Since autocrine IL-3 production is insufficient to transform myeloid cells (Wong *et al.*, 1987; Chang *et al.*, 1989; Perkins *et al.*, 1990), presumably because terminal differentiation continues, it seemed likely that *Hox*-2.4 had played a critical role in the malignant transformation. This study assessed the nature of that contribution.

The results established that *Hox*-2.4 readily immortalized myeloid progenitor cells: 5-FU-treated bone marrow cells infected with *Neo*/*Hox*-2.4 retrovirus yielded continuously proliferating clones of IL-3-dependent myelomonocytic, megakaryocytic and mast cells. However, surprisingly, long-term proliferation of the lines *in vitro* was dependent on extremely high concentrations of IL-3 and reduction to conventional levels promoted differentiation. These results imply that signalling through the IL-3 receptor enhances self-renewal of *Hox*-2.4 expressing cells. Experiments performed with recombinant IL-3 verified that IL-3 was required rather than another factor present at limiting concentrations in the conditioned media. Even with very high levels of IL-3, no long-term lines could be established from cultures of 5-FU-treated bone marrow cells infected with the control *Neo* virus, indicating that both *Hox*-2.4 and IL-3 participated in the immortalization process.

Despite their capacity for unlimited growth *in vitro*, the IL-3-dependent *Hox*-2.4 cell lines were not transplantable and therefore not fully malignant. Furthermore, most irradiated mice transplanted with *Neo*/*Hox*-2.4 virus-infected cells remained healthy for many months and their only notable haemopoietic abnormality was an increased frequency of myeloid progenitor cells. Since many progenitor cells within an individual animal displayed the same proviral insertion pattern, we infer that *Hox*-2.4 expression had enhanced their self-renewal potential. The enhanced replating capacity of primary colonies generated from such cells *in vitro* corroborated this conclusion.

Four of 18 mice monitored for up to 12 months eventually developed leukaemia. IL-3 mutations were identified in three myeloid leukaemias, suggesting that autocrine growth factor production had figured in their onset. The mechanism by which these mutations occurred has yet to be resolved but the aberrantly large IL-3 transcripts (Figure 7B) suggest deregulated expression from ectopic upstream promoters. These may be triggered by IAP transposition, as in the original WEHI-3B tumour (Ymer *et al.*, 1985) and in many tumorigenic variants of myeloid cell lines such as FDC-P1 (Stocking *et al.*, 1988; Laker *et al.*, 1989; Duhrsen *et al.*, 1990; Heberlein *et al.*, 1990). The evolution of the WEHI-3B and *Hox*-2.4 tumours has interesting parallels with that of the myeloid tumour, WEHI-274, which arose from a mouse infected with Moloney (and Abelson) leukaemia viruses and expresses an N-terminally truncated c-*myb* gene due to MoMuLV proviral insertion (but no Abelson provirus) (Gonda *et al.*, 1987). Early subclones from this tumour carry IAP transpositions well upstream of the IL-3 or GM-CSF genes (Leslie and Schrader, 1989) and it is presumed that autocrine growth factor production enhanced the expansion of the clone bearing the *myb* mutation.

Neither NIH-3T3 fibroblasts nor factor-dependent FDC-

Table V. Leukaemia in mice transplanted with bone marrow cells infected with *Neo*/*Hox*-2.4 virus

Mouse	Disease	WCC $\times 10^{-6}$/ml	Serum CSF[b] (U/ml)	IL-3 gene[c]		Transplantation[d]
				DNA	RNA	
103.4	myeloid (29 weeks)	52	<1	R	−	8/8 (4−5 weeks)
103.5	myeloid (29 weeks)	45	50	G	+	8/8 (4−5 weeks)
103.7	myeloid (39 weeks)	33	100	G	+	8/8 (4−5 weeks)
103.8	erythroid (51 weeks)	65	200	nd	−	2/2 (6 weeks)

[a]18 lethally irradiated DBA/2 or C57BL/6 mice transplanted with bone marrow cells infected with *Neo*/*Hox*-2.4 virus (see Figure 5) were monitored for disease for up to 12 months. Leukaemia developed in four mice after a latency indicated in weeks in brackets. Of the remaining mice, eight stayed healthy for the entire period; one found dead after 4 weeks had an enlarged spleen but autolysis prevented diagnosis; two killed at 33 weeks had thymoma, presumably due to irradiation; one died of hepatoma after 45 weeks and two died of unknown causes after 39 and 49 weeks.
[b]Serum from 103.5 and 103.7 was active in both FDC-P1 and 32D C13 assays, consistent with the presence of IL-3 and/or GM-CSF; serum from 103.8 contained other factor(s), since it stimulated bone marrow macrophage colonies but not FDC-P1 cells.
[c]Structure and expression of the IL-3 gene determined by Southern blot analysis of DNA (R, rearranged; G, germline configuration) and Northern blot analysis of polyadenylated RNA. A factor-independent cell line with a rearranged IL-3 gene was derived from the spleen of mouse 103.5 but the rearrangement was not detected in the primary spleen.
[d]The transplanted mice all developed leukaemia after the indicated latency (in weeks); four recipients received spleen cells and the other four received bone marrow cells, except for mouse 103.8, where recipients received only spleen cells.

P1 myeloid cells were rendered tumorigenic by infection with *Neo*/*Hox*-2.4 virus. Complete transformation usually requires complementary mutations (Weinberg, 1989), so it is perhaps not surprising that we were unable to detect any effect of *Hox*-2.4 in these cells already immortalized by unknown mutation(s). Others have reported that *Hox*-2.4-expressing fibroblasts grow as subcutaneous tumours in nude mice, although growth was extremely slow compared with that of *ras*-transformed cells (Aberdam *et al.*, 1991). The reason for the discrepancy remains unclear but may be related to some variation in sublines of NIH-3T3 cells or to a disparity in *Hox*-2.4 expression levels.

The E2A/PBX1 gene created by the 1;19 translocation associated with paediatric pre-B ALL also displays poor transforming potential when assayed on NIH-3T3 cells (Kamps *et al.*, 1991). However, using an experimental strategy similar to that employed for *Hox*-2.4, E2A/PBX1 was recently shown to have significant leukaemogenic potential (Kamps and Baltimore, 1993). Most irradiated mice injected with E2A/PBX virus-infected cells developed transplantable acute myeloid leukaemia after 3–7 months. The tumours were mono- or biclonal and some were growth-factor independent. Presumably E2A/PBX1 also has lymphomagenic activity, although this remains to be demonstrated. In view of the results reported here for *Hox*-2.4, it will be important to test whether E2A/PBX1 has the capacity to generate immortal but non-tumorigenic cell lines.

IL-3-dependent myeloid cell lines have been isolated previously, primarily from long-term bone marrow cultures (Dexter *et al.*, 1980; Greenberger *et al.*, 1983; Ihle and Askew, 1989), and most require conventional levels of growth factor. However, the FDC-Pmix lines developed from bone marrow cultures infected with Moloney sarcoma virus (Spooncer *et al.*, 1986) require high concentrations of IL-3 and, like the *Hox*-2.4 lines, differentiate into mature granulocytes and macrophages when the IL-3 level is reduced (Heyworth *et al.*, 1990). The genetic lesion responsible for immortalization of FDC-Pmix cells remains unknown. Although FDC-Pmix cells do not bear the v-*src* provirus (Wyke *et al.*, 1986), the v-*src* gene must have participated indirectly, because equivalent lines did not arise from control cultures infected with the Moloney leukaemia helper virus.

The nature of the cells susceptible to immortalization by *Hox*-2.4 requires further study. The frequency with which CFC infected with *Neo*/*Hox*-2.4 virus could be established as long-term lines (Table I) is impressive. Nevertheless, many of the G418-resistant colonies were indistinguishable in appearance from those in control cultures and died rapidly in liquid culture. Assuming these colonies expressed Hox-2.4 protein, as expected, these results imply that certain cell types are unaffected by *Hox*-2.4 expression. Perhaps only the most immature subpopulation of stem and/or progenitor cells has the capacity to be immortalized by *Hox*-2.4. The observation that cultures of bone marrow fractions enriched for multipotential stem cells (Szilvassy and Cory, 1993) contained a high frequency of immortalizable cells is consistent with this hypothesis.

IL-3 promotes both differentiation and self-renewal. Usually differentiation predominates, thus ensuring the ultimate extinction of the expanding haemopoietic clone. Why does self-renewal predominate in the *Hox*-2.4 cell lines when IL-3 levels are high? Do these cells express a distinctive form of the IL-3 receptor or signal transduction pathway, or is IL-3 cross-reacting with another receptor? Whatever the nature of the critical difference, has it been induced by *Hox*-2.4 expression, or is it intrinsic to primitive cell type(s) susceptible to immortalization by *Hox*-2.4? Although scant, the existing evidence tends to support the latter hypothesis, since *Hox*-2.4 does not alter the dose response of FDC-P1 cells to IL-3 and IL-3 delivers a primarily proliferative (rather than differentiative) stimulus to cells enriched for CFU-S activity (Ponting *et al.*, 1991). The IL-3 levels necessary for immortalization *in vitro* far exceed those secreted by bone marrow stromal cells (Kittler *et al.*, 1992). Nevertheless, IL-3 might play a role in maintaining the stem/progenitor cell compartment *in vivo* if co-stimulation by other factor(s) reduces the concentration required for the self-renewal signal.

The mechanism by which the Hox-2.4 protein fosters immortalization remains to be established. Hox-2.4 may mimic the effect of a homeobox protein whose normal function facilitates cellular self-renewal. Alternatively, it may competitively inhibit a homeobox protein which promotes differentiation, as suggested by a study showing that enforced *Hox*-2.4 expression in the mouse myeloid leukaemia, M1, inhibited the capacity of these cells to differentiate in response to interleukin 6 (Blatt *et al.*, 1992). Irrespective of the mechanism, *Hox*-2.4 should prove an invaluable tool. We have already demonstrated that it can be used to establish factor-dependent myeloid lines with granulocyte-macrophage, megakaryocytic and mast cell differentiation potential. Even after many months in culture, the *Hox*-2.4 lines have retained their capacity for spontaneous differentiation, the degree and direction of which can be controlled by modulating the IL-3 concentration and providing other growth factors (A.C.Perkins, N.Nicola and S.Cory *et al.*, in preparation). In the context of other appropriate cytokines, *Hox*-2.4 may prove able to immortalize other haemopoietic lineages. Since there are many homeobox proteins with DNA-binding domains similar to that of *Hox*-2.4, some of these may also have immortalizing potential for haemopoietic cells.

Materials and methods

Retroviral vectors

The MPZen vector (Hariharan *et al.*, 1988) used for construction of the *Hox*-2.4 retroviruses incorporates the U3 region of the myeloproliferative sarcoma virus (MPSV) (Seliger *et al.*, 1986) in its 3′ LTR, which is more efficient in myeloid cells than that from Moloney virus (Bowtell *et al.*, 1988). Because of the nature of retroviral replication, both LTRs of the provirus in infected cells will contain this sequence. In the principal construct used to co-express Neo^R and *Hox*-2.4 (Figure 1A), the Neo^R gene is expressed via the spliced subgenomic viral RNA, like the envelope gene (*env*) in murine leukaemia viruses, while the *Hox*-2.4 cDNA is expressed via the SRα promoter (Takabe *et al.*, 1988) within a cassette cloned into the 3′ *Cla*I site. To assemble the vector, a 1.4 kb *Hin*dIII–*Sma*I fragment containing the Neo^R gene was excised from pSV2*Neo* (Southern and Berg, 1982), cloned into the *Hin*dIII and *Sma*I sites of pIC20R (Marsh *et al.*, 1984), re-excised as an *Xho*I–*Sma*I fragment and ligated into the *Xho*I and *Hpa*I sites of an MPZen derivative, JZen1 (prepared by J.Chang), which incorporates a multicloning site (*Bam*HI-*Xho*I-*Eco*RI-*Hpa*I-*Hin*dIII-*Bgl*II) in place of the *Xho*I site of pMPZen (Hariharan *et al.*, 1988). An SRα*Hox*-2.4 expression cassette identical to that used to construct MPZenIL-3/SRα*Hox*-2.4 (Perkins *et al.*, 1990) was then ligated into the 3′ *Cla*I site. In two other vectors, one based on the MPZen vector and the other on the GD vector (Daley *et al.*, 1990), *Hox*-2.4 cDNA was inserted into the *env* position and Neo^R expressed via a 3′ cassette driven by the conventional early promoter of SV40 virus (A.Perkins, unpublished).

Virus-producing cell lines

To produce helper-free virus, 10 μg of linearized MPZen*Neo*/SRα*Hox*-2.4 plasmid was electroporated into either the Ψ-2 (Mann *et al.*, 1983) or GP+E-86 (Markowitz *et al.*, 1988) packaging cell lines, which express viral proteins encoded by *gag*, *pol* and *env* genes of Moloney virus. After selection for 10−14 days in the antibiotic G418 (Geneticin, Sigma; 600 μg/ml), 92 transfectants were ring-cloned and expanded. Clones with the highest virus titre were identified by a rapid assay (Elefanty and Cory, 1993) on FDCP-1 cells and reassayed on NIH-3T3 cells as described previously (Cory *et al.*, 1987). The highest titres were 2×10^5 G418-resistant c.f.u./ml, which in our experience is insufficient for efficient gene delivery to 5-FU-treated bone marrow cells. Infection of tunicamycin-treated Ψ-2 cells (Bowtell *et al.*, 1988) with virus from one of the transfected clones enabled the isolation of a clone (Ψ-2:MPZen*Neo*/*Hox*-2.4 clone T1-2.0) producing $1-2 \times 10^6$ G418-resistant c.f.u./ml of MPZen*Neo*/*Hox*-2.4 virus. No helper virus secretion was detectable using an XC syncytial assay (Rowe *et al.*, 1970). Other virus-producing lines used were Ψ-2:Zip*Neo* clone 3C2.3 (7.5×10^5 G418-resistant c.f.u./ml); Ψ-2:MPZenIL-3/SRα*Hox*-2.4 clone ZIL 15 (Perkins *et al.*, 1990) and Ψ-2:MPZenIL-3 clone M5 (Chang *et al.*, 1989). Virus-producing cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (GP+E-86) or calf serum (Ψ-2, NIH-3T3).

Sources of interleukin-3

IL-3 used for the bone marrow cultures was supplied as (i) conditioned medium (CMX63) from the IL-3-secreting cell line, X63Ag8-653mIL-3 (Karasuyama and Melchers, 1988), (ii) pokeweed mitogen-stimulated spleen cell conditioned medium (SCM) prepared as described by Johnson and Metcalf (1978), or (iii) semi-purified material from an insect cell line (Sf900) infected with recombinant baculovirus expressing IL-3 (T.Willson and N.Gough, unpublished). IL-3 activity was assayed by colony formation by 5×10^4 C57BL/6 bone marrow cells in 1 ml agar cultures (Metcalf, 1984) and/or by a sensitive microwell culture assay (Metcalf *et al.*, 1987) using IL-3-dependent 32D cl 3 mast cells (Greenberger *et al.*, 1983). Units of activity were calculated from the linear portion of the dose response curve, assigning 50 units to the concentration required to stimulate half maximal numbers of granulocyte-macrophage colonies in agar cultures of marrow cells (Metcalf, 1985). Purified recombinant IL-3 of known activity, generously supplied by D.Metcalf, was used as a standard.

Infection of bone marrow cells

Cells from the femurs of DBA/2 or C57BL/6 mice that had been injected intravenously 4 days previously with 150 mg/kg of 5-fluorouracil (5-FU) were co-cultivated (one femur equivalent per 10 cm dish) in DMEM containing 20% fetal calf serum (FCS), 0.05 mM 2-mercaptoethanol and either SCM or CMX63 with virus-producing Ψ-2 cells plated 24 h previously at 5×10^4 cells per 10 cm dish. After 5 days, non-adherent marrow cells were harvested from the fibroblast monolayers and plated at 10^3, 5×10^3 and 2.5×10^4 cells/ml into 35 mm Petri dishes in 1 ml of DMEM containing 0.3% Bacto-agar and the same source of growth factor(s) with or without G418 (1 mg/ml).

Alternatively, the harvested bone marrow cells were injected intravenously into lethally irradiated (9.0 Gy) syngeneic recipients (5×10^5 cells/mouse). To reduce deaths due to infection, neomycin (1.1 g of base/litre; Sigma N-1876) and polymyxin B (10^6 U/litre; Sigma P-1004) were included in the drinking water of the mice for the first 2 weeks. Mice were periodically venesected by collecting 50 μl of blood from the retro-orbital venous plexus into 450 μl of acid-citrate-dextrose as an anti-coagulant. Haematocrit, haemoglobin and platelet counts were performed on a Sysmex K-1000 machine. White cell counts were performed using a Coulter counter as described by Harris *et al.* (1988). Tissues of autopsied mice were fixed in Bouin's solution or buffer formalin, sectioned and stained with haematoxylin and eosin (H&E) or other specific stains. Bone marrow or spleen cells were washed in phosphate-buffered saline and progenitor cells quantified by their ability to produce colonies in soft agar after 7 days of culture as described above.

Cell phenotype analysis

Cytocentrifuged cells were stained with May−Grunwald−Giemsa (MGG), Astra blue or acetylcholinesterase according to standard protocols (Metcalf, 1984). The pseudoperoxidase activity of haemoglobin in agar colonies and cell lines was detected with benzidine-HCl as described by Cooper *et al.* (1974). Cell surface marker analysis was performed on cells resuspended in phosphate-buffered saline containing 2% FCS and 10 mM sodium azide and supernatant from the anti-Fcγ receptor hybridoma 2.4G2 (Unkeless, 1979) to block non-specific Fc-receptor interactions. Staining was performed by standard methods with protein G-purified, fluorescein isothiocyanate- or biotin-conjugated monoclonal antibodies to Gr-1 (RB2-8C5), Mac-1 (M1/70), Thy-1 (19F12) and CD45R (RA3-6B2). Stained cells were analysed with a FACScan instrument (Becton-Dickinson). Dead cells were excluded from analysis on the basis of low forward scatter and propidium iodide uptake.

Transplantation

Cells were injected into non-irradiated syngeneic mice both subcutaneously (10^6 cells/mouse) and intraperitoneally (10^6 cells/mouse) and recipients were monitored weekly for tumour development. Those remaining healthy at 12 months were autopsied to check that they were free of tumour.

RNA and DNA analysis

DNA and poly(A)$^+$ RNA were prepared from tissues and cell lines and Southern and Northern blots were performed as described by standard procedures. Probes used were *Neo* [*Hin*dIII−*Sma*I fragment from pSV2*neo* (Southern and Berg, 1982)], *Hox*-2.4 [350 bp *Sac*I−*Bam*HI cDNA fragment (see Figure 1A) (Kongsuwan *et al.*, 1989)] and IL-3 [cDNA fragment encompassing all of the murine coding region (N.Gough, unpublished)]. The origin of the other probes is described elsewhere (Elefanty and Cory, 1992). All probes were labelled with [α-^{32}P]dATP using a random hexamer priming kit (Bresatec Limited, Adelaide, SA, Australia). Filters were washed at a final stringency of 0.2 × SSCE for 20 min at 65°C.

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