Expression of c-MYC under the Control of GATA-1 Regulatory Sequences Causes Erythroleukemia in Transgenic Mice

By Radek C. Skoda,* Shih-Feng Tsai,‡ Stuart H. Orkin,‡ and Philip Leder*

From the *Howard Hughes Medical Institute, Department of Genetics, Harvard Medical School, and ‡Division of Hematology-Oncology, Children's Hospital, Howard Hughes Medical Institute, and the Dana Farber Cancer Institute, Department of Pediatrics, Harvard Medical School, Boston, Massachusetts 02115

Summary

To study oncogenesis in the erythroid lineage, we have generated transgenic mice carrying the human c-MYC proto-oncogene under the control of mouse GATA-1 regulatory sequences. Six transgenic lines expressed the transgene and displayed a clear oncogenic phenotype. Of these, five developed an early onset, rapidly progressive erythroleukemia that resulted in death of the founder animals 30–50 d after birth. Transgenic progeny of the sixth founder, while also expressing the transgene, remained asymptomatic for more than 8 mo, whereupon members of this line began to develop late onset erythroleukemia. The primary leukemic cells were transplantable into nude mice and syngeneic hosts. Cell lines were established from five of the six leukemic animals and these lines, designated erythroleukemia/c-MYC (EMY), displayed proerythroblast morphology and expressed markers characteristic of the erythroid lineage, including the erythropoietin receptor and β-globin. Moreover, they also manifested a limited potential to differentiate in response to erythropoietin. Studies in the surviving transgenic line indicated that, contrary to our expectations, the transgene was not expressed in the mast cell lineage. That, coupled with the exclusive occurrence of erythroleukemia in all the transgenic lines, suggests that the GATA-1 promoter construct we have used includes regulatory sequences necessary for in vivo erythroid expression only. Additional sequences would appear to be required for expression in mast cells. Further, our results show that c-MYC can efficiently transform erythroid precursors if expressed at a vulnerable stage of their development.

O ne of the genes controlling the differentiation of immature hematopoietic progenitor cells encodes a zinc finger transcription factor called GATA-1 (1). Disruption of the GATA-1 gene through homologous recombination in embryonic stem cells prevents normal erythroid development (2, 3). Consistent with its proposed role as a key regulator in erythropoiesis, GATA-1 mRNA is present in the earliest identifiable blood island cells in the yolk sac and is later found in fetal liver and in adult erythroid cells (4). In addition, GATA-1 mRNA is also expressed in adult hematopoietic cells of megakaryocytic, eosinophil, and mast cell origin (5–8). This expression pattern suggests that GATA-1 might also be present in progenitor cells committed to the erythroid, megakaryocytic, eosinophil, and mast cell lineage. Therefore, the regulatory sequences that control expression of the GATA-1 gene might provide the opportunity to direct expression of transgenes to hematopoietic progenitors with multilineage potential. Nonhematopoietic cells, with the exception of the testis, do not express GATA-1. The testis form is tran-

scribed from a separate promoter located 5′ to the erythroid first exon (9).

Deregulated expression of the c-myc proto-oncogene in vivo can immortalize a variety of hematopoietic lineages including lymphoid (10, 11) and myelo-monocytic cells (12, 13). c-myc is ubiquitously expressed and plays a central role in the control of cell proliferation (14). For oncogenic activity the myc protein must form a heterodimer with max (15), a ubiquitously expressed member of the helix-loop-helix family (16). Despite its promiscuous properties as an oncogene, its potential in the transformation of erythroid cells has yet to be defined.

To derive an in vivo model and to immortalize cell lines that represent stages in hematopoietic development dependent on GATA-1, we used regulatory sequences of the murine GATA-1 gene to express the human c-MYC proto-oncogene in transgenic mice. Several such independently derived transgenic mice developed early onset erythroleukemia and although these mice died before reaching sexual maturity, we were able to derive and characterize cell lines from these animals. One

transgenic line, by contrast, developed late onset erythro-leukemia and survived to provide an in vivo model of this disease. This surviving line also allowed us to assess the expression pattern of the transgene directed by the GATA-1 regulatory sequences used in these studies.

Materials and Methods

Construction of the GATA-1/c-MYC Transgene and Generation of Transgenic Mice. A 4.3-kb XbaI-SmaI genomic fragment, including 2.7 kb of 5' flanking region, the nontranslated exon 1 (80 bp), and 1.5 kb of intron 1 of the mouse GATA-1 gene, was ligated to a 3.8-kb SmaI-XbaI genomic fragment, comprising the 3' part of the first intron as well as exon 2, intron 2, and exon 3 of the human c-MYC gene (17). The resulting 8.1-kb GATA-1/c-MYC fragment, which contains no vector sequences, was used for oocyte microinjection. We have generated 13 transgenic founder mice in the FVB/N inbred strain (Taconic Farms, Inc., Germantown, NY) by standard oocyte injection methods (18). The animals were maintained under specific pathogen-free conditions in microisolator cages.

RNA Isolation, RNase Protection Assay, and Northern Analysis. RNA samples were prepared in 4 M guanidium isothiocyanate, followed by ultracentrifugation on a 5.7 M CsCl cushion (19). For RNase protection analysis, T3 or T7 antisense probes were synthesized and hybridized to total RNA samples as described (20). Protected fragments were separated on 6% polyacrylamide/8 M urea sequencing gels, which were then dried and exposed for autoradiography using Kodak XAR-5 film and an intensifying screen.

Human c-MYC mRNA was detected with a riboprobe transcribed from the plasmid pFcRVS (a gift from David Beier, Harvard Medical School, Boston, MA). pFcRVS consists of a 360-bp EcoRV-SstII fragment from exon 2 of the human c-MYC gene cloned into pBluescriptII vector.

Antisense RNA transcribed from pFcRVS protects a 360-bp fragment of human c-MYC and fragments of 220 and 160 bp of mouse c-myc corresponding to conserved regions between human and mouse. We synthesized ribosomal protein L32 riboprobes (gift from Michael M. Shen, Harvard Medical School) at one-tenth the specific activity of MYC probes (21) as an internal standard. The fragment protected by this riboprobe comigrates with the 220-bp mouse c-myc band protected by pFcRVS. Mouse GATA-1 mRNA was probed with a riboprobe which protects a 240-nucleotide (nt) region extending from a Hinfl site in exon 2 to an EcoRI site in exon 3 of the GATA-1 gene. Mouse α-globin riboprobe (gift from Aya Leder, Harvard Medical School) consisted of a 210-bp PstI-BamHI fragment from the mouse α-globin gene (22).

For Northern analysis, total RNA (10 μg) was electrophoresed on a 1% agarose gel with formaldehyde and transferred to a positively charged nylon membrane (Nytran; Schleicher & Schuell, Inc., Keene, NH). The filters were sequentially hybridized and stripped to the following ^{32}P-labeled double stranded probes: XhoI fragment of the murine EPO-R cDNA (gift from Alan D'Andrea, Dana Farber Cancer Institute, Boston, MA) (23); mouse β-globin (gift from Frederick Lee, Harvard Medical School) (24); SacI fragment of the Spi-1 cDNA (25, 26), Fli-1 cDNA (27); mouse GATA-1 cDNA (28); human c-MYC cDNA (17); and ribosomal protein L32 pseudogene (21, 29)

Blood and Tissue Analysis. Blood was obtained by phlebotomy of the tail vein or by cardiac puncture. Blood smears were stained with May-Grünwald-Giemsa. Bone marrow or spleen cells were resuspended in 50–100 μl PBS and concentrated on microscopic slides using a Cytospin 3 centrifuge (Shandon, Ostmore, UK).
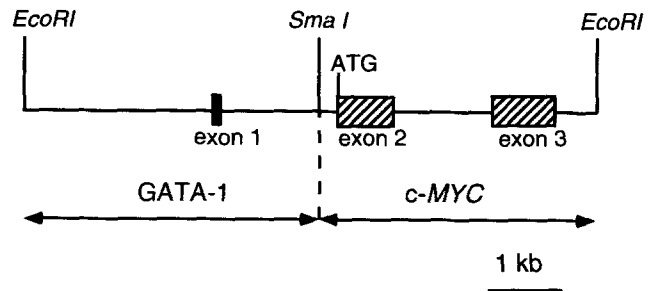

Figure 1. The GATA-1/c-MYC transgene. A 4.3-kb EcoRI-SmaI fragment including 2.7 kb of the 5' flanking region, the nontranslated first exon (*solid box*), and 1.5 kb of the GATA-1 first intron of the mouse GATA-1 gene was ligated to a SmaI-EcoRI genomic human c-MYC fragment. This fragment includes the 3' part of the first intron, the two translated exons (*hatched box*) and the 3' RNA processing signal from the human c-MYC gene. The position of the initiator ATG is indicated.

Automated blood counts were performed with a cell counter (model H-1; Tecnicon Co., Tarrytown, NY). For histology, freshly dissected tissues were fixed in Optimal*Fix (American Histology Reagent Co., Stockton, CA). Fixed specimens were embedded in paraffin, sectioned, and stained by the Transgenic Pathology Laboratory at the University of California at Davis.

Tumor Transplants. Spleen cells from transgenic erythroleukemia/c-MYC 1 (TG.EMY-1)[1] and TG.EMY-2 founder or lymph node cells from TG.EMY-1 were suspended in PBS and 3 × 10^6 cells were each injected either intravenously or subcutaneously into syngeneic FVB (n = 7 for EMY-1; n = 4 for EMY-2) or nu/nu mice (n = 7 for EMY-1; n = 3 for EMY-2). Only one FVB injected with EMY-1 and one FVB injected with EMY-2 and two nu/nu injected with EMY-1 remained tumor free. All other animals (n = 14) developed leukemia by day 28 or were found dead in the cage (n = 3). Histological examination confirmed the presence of tumor infiltrates.

Cell Culture and Colony Assays. Cell lines were established by plating suspensions of cells from bone marrow or spleen in RPMI media supplemented with 10% bovine calf serum, glutamine, and antibiotics. After a few days, cells were growing rapidly in suspension and after a few weeks the cells appeared morphologically homogeneous. The cell lines required fresh media every 2 d. Subclones were derived by placing single cells into 96-well plates lined with NIH 3T3 cells that had been treated with mitomycin C to irreversibly prevent cell division.

We tested the differentiative potential of EMY-1 and EMY-2 cells by growing them in media containing mouse erythropoietin (sp act: 350,000 U/mg) (a gift from the Genetics Institute, Cambridge, MA) at a final concentration of 0.1 μg/ml (~35 U/ml). After 10 d, total RNA was prepared for Northern analysis.

Methylcellulose cultures of bone marrow cells were plated as triplicates at two densities (5 × 10^4 and 1 × 10^5 cells/ml) in IMDM containing 4% FCS, erythropoietin (EPO) (0.08 U/ml), hIL-1 (2 ng/ml), mIL-3 (100 ng/ml), kit ligand (2% COS cell supernatant), BSA fraction V, transferrin, and lipids according to Iscove (30). Colony assays of EMY cell lines were performed in IMDM/methylcellulose supplemented with 10% FCS without addition of growth factors.

Table 1. *Summary of GATA-1/c-MYC Transgenic Mice*

Transgenic mouse	Transgene expression	Life span	Spleen weight	Cell line established	Fertility
		d	*mg*		
TG.EMY-1	+	45	ND*	+	−
TG.EMY-2	+	50	770	+	−
TG.EMY-3	+	35	830	+	−
TG.EMY-4	+	30	700	+	−
TG.EMY-5 #1	+	240	1,600	+	+
#2		260	1,300	−	+
TG.EMY-6	+	30	520	−	−
TG.EMY-7	+	Normal	110	−	+
TG.EMY-8	+	Normal	90	−	+
TG.EMY-9	+	Normal	105	−	−
TG.EMY-10	−	Normal	90	−	+
TG.EMY-11	−	Normal	95	−	+
TG.EMY-12	−	Normal	100	−	−
TG.EMY-13	−	Normal	95	−	−

* Spleen enlarged but weight not determined. Transgenic F_1 offspring or infertile transgenic founder animals were analyzed. Spleen weights in age-matched wild-type controls ranged between 80 and 110 mg (not shown).
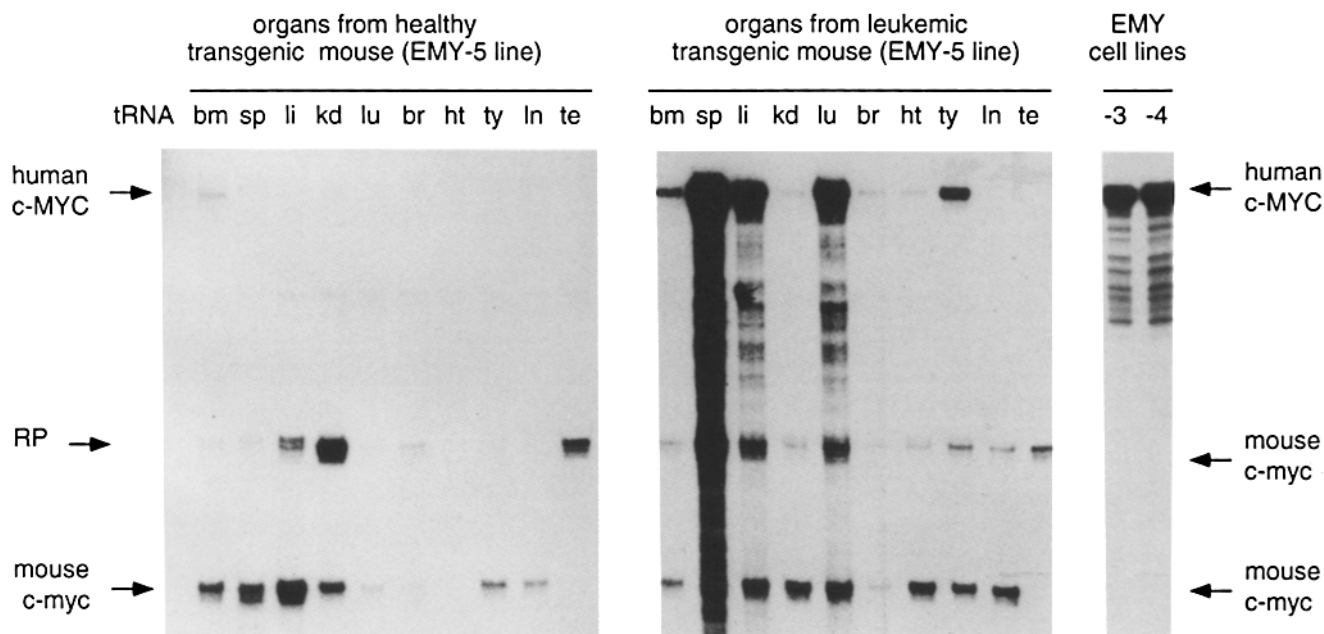

Figure 2. Expression of the transgene in tissues and cell lines from transgenic mice. RNase protection assay with the pFcRVS riboprobe for exon 2 of human c-*MYC*. As indicated by arrows, this riboprobe generates a protected fragment corresponding to the human c-*MYC* transcripts, as well as smaller fragments that represent mouse c-*myc* transcripts and correspond to conserved regions between man and mouse. (*RP*) Ribosomal protein L32 mRNA as an internal control (*left* and *middle* only). (*Left*) 6-wk-old healthy TG.EMY-5 animal; (*middle*) 34-wk-old leukemic TG.EMY-5 mouse; (*right*) cell lines EMY-3 and EMY-4. Bone marrow (*bm*), spleen (*sp*), liver (*li*), kidney (*kd*), lung (*lu*), brain (*br*), heart (*ht*), thymus (*ty*), lymph node (*ln*), and testes (*te*).
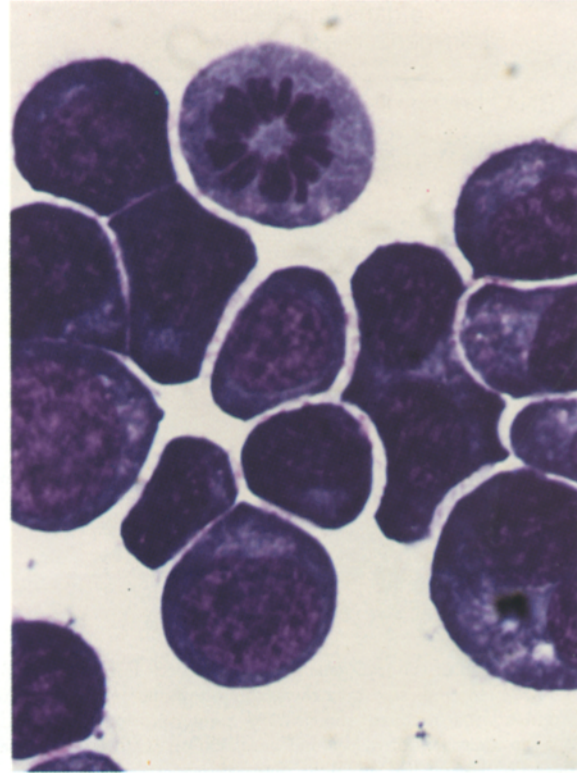
1605   Skoda et al.
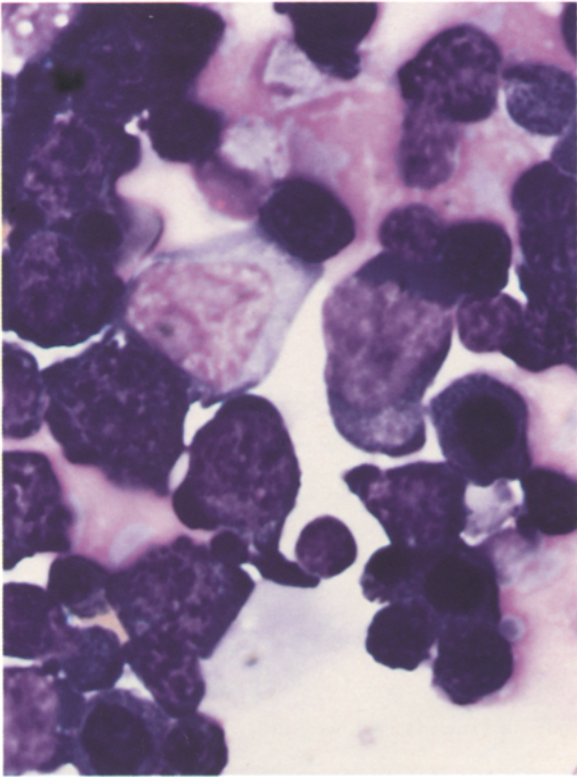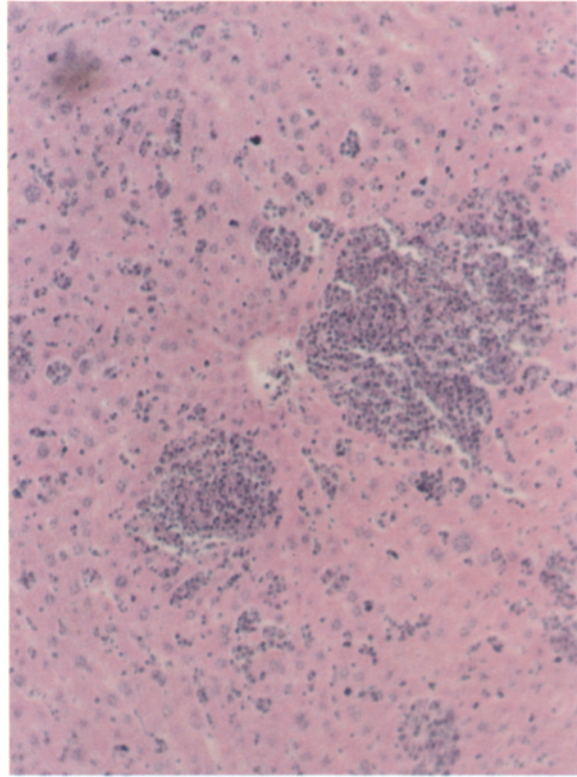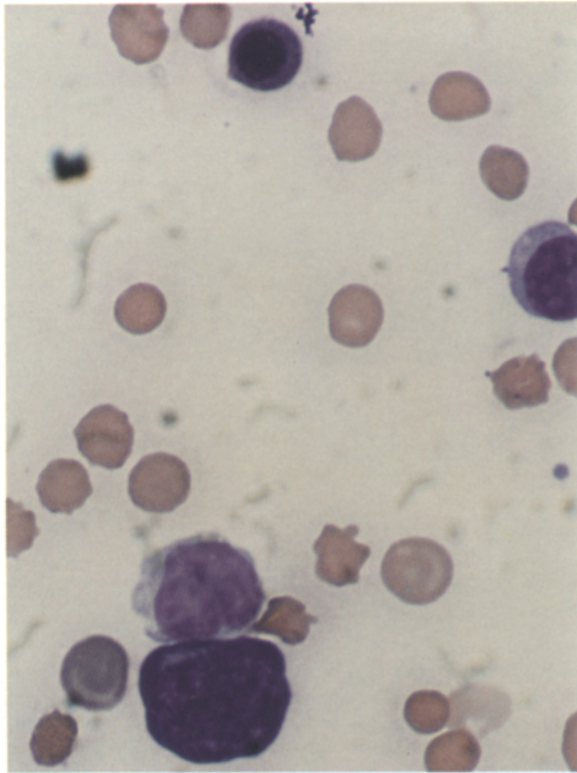
Mast cells from TG.EMY-2 and TG.EMY-5 were derived by first culturing bone marrow cells for 3 d in Dexter media and then in RPMI media with 10% bovine calf serum and 10% WEHI-3 conditioned media as a source for IL-3. Leukemic cells died in Dexter media and a homogeneous population of IL-3–dependent cells with the typical morphology of mast cells emerged. After 3 wk of culture, cells were analyzed morphologically using Cytospin preparations stained with May-Grünwald-Giemsa or toluidine blue.

Immunoprecipitations. Cell lines (10^7 cells each) were labeled with 0.25 mCi/ml [^{35}S]-methionine for 6 h at 37°C. The cells were lysed on ice in 0.9 ml TBS (150 mM NaCl, 50 mM Tris, pH 8) with 1% Triton X-100 and protease inhibitors. The lysates were spun for 30 min at 10,000 g. Supernatants were adjusted to 0.2% SDS, 0.5% NP40, and 0.5% deoxycholate final concentration and incubated with a goat anti-Rauscher MuLV gp70 polyclonal antiserum that cross-reacts with SFFV gp55 (31) at a 1:200 dilution for 12 h at 4°C. Protein A-Sepharose beads (Pharmacia, Piscataway, NJ) were added, incubated for 2 h and washed four times in RIPA buffer (0.1% SDS, 0.5% NP40, and 0.5% deoxycholate in TBS) and twice with TBS. The immune complexes were eluted with SDS-PAGE loading buffer.

Results

Generation of Transgenic Mice. To direct expression of an immortalizing oncogene to erythroid or perhaps to multipotent hematopoietic precursors, we placed the human c-*MYC* gene under the control of mouse GATA-1 regulatory sequences (Fig. 1). To preserve the configuration of the GATA-1 regulatory region (32), we chose to use 2.7 kb of the 5' flanking region, the noncoding first exon, and 1.5 kb of intron 1 from the GATA-1 gene. The first exon of c-*MYC* was excluded in this construct, as it represents a noncoding sequence that is not required for transformation (17). Thus, this fusion gene is composed of GATA-1 sequences at the 5' portion of the first intron and of c-*MYC* sequences at the 3' portion (Fig. 1).

13 founder mice carrying the GATA-1/c-*MYC* transgene were generated (Table 1). We have observed a phenotype in 6 of the 13 transgenics. Five transgenic founders (TG.EMY-1, TG.EMY-2, TG.EMY-3, TG.EMY-4, and TG.EMY-6) developed a rapidly progressive disease with signs of respiratory distress and anemia, and died between days 30 and 50 (Table 1). These founders were unable to generate offspring. A sixth founder, TG.EMY-5, remained healthy, allowing us to establish a breeding transgenic line. Thus far, two transgenic offspring from the TG.EMY-5 founder displayed a similar phenotype, but with a late onset, resulting in death at days 240 and 260 (Table 1). The disease is variably penetrant in this line since several transgenic mice have remained healthy past the age of 18 mo.

Analysis of the Tissue Specificity of Transgene Expression Conferred by the GATA-1 Regulatory Sequences. To assess the transgene expression we performed an RNase protection assay with a human c-*MYC* riboprobe that can distinguish between transcripts originating from the transgene and from the endogenous

mouse c-*myc* gene. We examined the surviving TG.EMY-5 line and compared the tissue distribution of transgene expression in a healthy young TG.EMY-5 animal to that in a leukemic TG.EMY-5 mouse (Fig. 2). The transgene in the healthy animal was expressed in bone marrow and, at low levels, in the spleen. This distribution agrees with that of the endogenous GATA-1 gene. In the leukemic TG.EMY-5 animal the transgene RNA was expressed at much higher levels and in several additional organs, including spleen, liver, lung, and thymus, and at low levels also in kidney, brain, and heart. Histology of the tissues from this mouse revealed leukemic infiltration of the spleen, liver (Fig. 3 *D*), lung, and thymus. The kidney, brain, heart, lymph node, and testis were histologically less affected or normal (not shown). This suggests that the transgene expression in these organs originated from the tumor cells. The endogenous mouse c-*myc* transcripts detected in the organs of the leukemic mouse seem to be derived from RNA from nonleukemic tissue, since endogenous mouse c-*myc* RNA was not detectable in cell lines derived from leukemic TG.EMY mice (Fig. 2). Suppression of endogenous c-*myc* transcripts by expression of an exogenous *myc* gene is observed regularly and appears to occur at the transcriptional level (33, 34).

In the five founders with early onset of leukemia, TG.EMY-1, TG.EMY-2, TG.EMY-3, TG.EMY-4, and TG.EMY-6, we observed a similar expression pattern of the transgene as in the leukemic TG.EMY-5 mouse and the same histopathological findings (not shown). In some cases, the lymph nodes and brain were affected as well. Three additional lines (TG.EMY-7, TG.EMY-8, and TG.EMY-9) showed expression of the transgene in bone marrow and spleen (not shown), similar to that of the healthy TG.EMY-5. We have not studied these lines in detail, although in the transgenic offspring of one of these strains a mouse was found dead that displayed splenomegaly. Offspring of the other two strains remained healthy for up to 20 mo.

As the endogenous GATA-1 gene is expressed in mast cells (5, 6), and regulates the promoters of some mast cell–specific proteases (7), we assessed expression of the GATA-1/c-*MYC* transgene in a homogeneous population of mast cells derived from a leukemic TG.EMY-5 animal (Fig. 4). Had the regulatory sequences used in our transgene construct been complete, we would have expected the transgene to be expressed in all cells that express the endogenous GATA-1 gene. Surprisingly, the c-*MYC* transgene was not expressed in mast cells from this TG.EMY-5 animal, despite expression of the endogenous GATA-1 gene. Although there was less RNA loaded in the lanes from both mast cells samples (as assessed by ribosomal protein L32 probe), mRNA for GATA-1 and Fli-1 was easily detectable. Southern analysis of DNA from these mast cells confirmed that they had not lost the transgene (not shown). Similarly, mast cells derived from the TG.EMY-1 transgenic founder did not express the transgene

Figure 3. Morphology of EMY-5 tumor cells. May-Grünwald-Giemsa stain of blood smear (*A*). Cytospin preparation from spleen (*B*) and from the EMY-5 leukemia cell line (*C*). Hematoxilin-eosin–stained section of the liver (*D*) from the leukemic TG.EMY-5 mouse.

EMY-5   WT
tu   mast mast
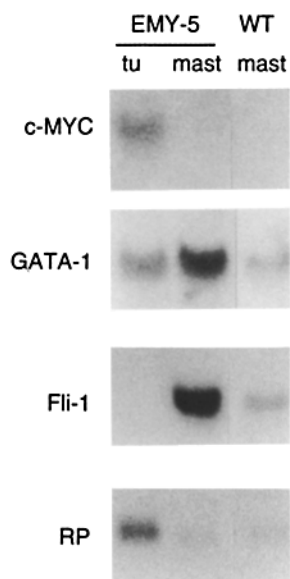
c-MYC

GATA-1

Fli-1

RP

Figure 4. Northern analysis of total RNA from mast cells and leukemic cells derived from the TG.EMY-5 mouse and from mast cells derived from a wild type control. The same blot was sequentially probed with specific ^{32}P-labeled cDNAs as indicated.

by the RNase protection assay (not shown). Thus, the GATA-1 sequences used in this construct are insufficient to direct expression of the transgene to the mast cell lineage.

Characterization of Primary Tumors and Derivation of Erythroleukemia Cell Lines. The peripheral blood of all leukemic animals contained proerythroblasts and erythroblasts, as shown for a leukemic TG.EMY-5 mouse (Fig. 3 *A*). Automated blood counts were performed on two leukemic animals and compared to healthy transgenic and wild type mice (Table 2). Leukocytosis was found in the leukemic TG.EMY-5 mouse, but not in TG.EMY-1, reflecting variability in the numbers of circulating leukemic cells. Both animals displayed severe anemia and moderate thrombocytopenia. The spleens of all leukemic mice were found to be largely replaced with leukemic cells (Fig. 3 *B*). Primary tumor cells from spleens of TG.EMY-1 and TG.EMY-2 animals were clonogenic in methylcellulose without addition of growth factors and were also

transplantable into *nu/nu* mice or syngeneic FVB. Transplanted animals developed the same pathology within 3–4 wk and the leukemic cells displayed the same proerythroblast morphology (not shown).

We were able to derive cell lines from the bone marrow or spleen of leukemic animals TG.EMY-1, TG.EMY-2, TG.EMY-3, TG.EMY-4, and TG.EMY-5. All cell lines grew rapidly in suspension culture without the addition of growth factors. After a few weeks in culture they appeared homogeneous and displayed predominantly a proerythroblast morphology (Fig. 3 *C*). These cell lines formed colonies in methylcellulose without the addition of growth factors and 5–15% of EMY cells were clonogenic (Table 3).

The EMY cells were negative for the megakaryocytic marker acetylcholinesterase (35, 36) and the specific toluidine blue staining of mast cell granules (37). To confirm the erythroid origin of the cell lines, we performed Northern analyses with probes for erythroid-specific markers (Fig. 5 *A*). Both β-globin and erythropoietin receptor (*EPO-R*) mRNAs were expressed in all EMY cell lines, consistent with representing cells of the erythroid lineage. Treatment with recombinant mouse EPO led to upregulation of β-globin RNA (Fig. 5 *B*) but did not induce any morphological changes associated with maturation. We were also unable to induce the appearance of benzidine-positive cells in EMY cell lines treated with 1.2% DMSO, indicating that no significant amounts of hemoglobin were formed. Thus, erythroleukemia cells immortalized by the c-*MYC* proto-oncogene are partially blocked in their differentiation, similar to MEL cells transfected with c-*myc* expression vectors (38–40).

Since leukemia in TG.EMY-5 mice occurs stochastically after a latency of more than 30 wk, we examined whether expression of the c-*MYC* transgene has an effect on erythropoiesis in younger healthy transgenics. We found no difference in blood counts or red blood cell parameters between healthy 6–8-wk-old TG.EMY-5 mice and the wild type controls (Table 2). However, a subtle effect on erythroid differentiation was detectable when colony assays in methylcellulose with bone

Table 2. *Blood Counts of Transgenic Mice and Wild-type Controls*

	TG.EMY-1 leukemic	TG.EMY-5 leukemic	TG.EMY-5 healthy	Wild-type controls
	$n = 1$	$n = 1$	$n = 6$	$n = 6$
WBC × $10^3/\mu$l	3.3	41.1	5.7 ± 4.2	4.9 ± 2
RBC × $10^6/\mu$l	0.7	3.4	8.8 ± 0.6	8.6 ± 0.5
HCT%	4	16	46 ± 4	47 ± 7
HGB g/100 ml	1.4	5.2	14 ± 0	14 ± 1
MCV fl	66	47	53 ± 4.5	55 ± 7.8
MCH pg	21	15	16 ± 0.3	16 ± 0.6
PLT × $10^3/\mu$l	215	248	1,170 ± 278	1,022 ± 144

Blood counts were performed on a Technicon H1.
HCT, hematocrit; *Hgb*, hemoglobin; *MCH*, mean corpuscular hemoglobin (*pg*); *MCV*, mean corpuscular volume (*fl*); *PLT*, platelets; *RBC*, red blood cells; *WBC*, white blood cells.

Table 3. *Clonogenicity Assay*

Cell line	CFU/10³ cells
EMY-1	56/33
EMY-2	150/115
EMY-3	144/215
EMY-4	115/58

Numbers of CFU from duplicate plates are given.

marrow cells from 10-wk-old healthy EMY-5 mice and littermate controls were performed (Table 4). On day 7, we observed an about twofold increase in blast-forming unit–erythroid (*BFU-E*) colonies in the transgenic group. When we analyzed the colonies on day 10, the number of pure erythroid colonies (*E*) was again increased in the transgenic group. However, no difference in GM colonies, bi-lineage colonies consisting of erythroid and megakaryocytic cells (*E/Mg*), or multilineage colonies containing erythroid cells (*E/multi*), was observed. Thus, this effect is confined to relatively late progenitor cells committed to the erythroid lineage, whereas

Table 4. *Frequency of Colony-forming Cells in Bone Marrow from Healthy EMY-5 and Wild-type Mice*

	EMY-5	Wild-type
Cells per femur × 10^{-6}	19.6 ± 2.4	19.2 ± 5.6
BFU-E per 10^5 cells		
(day 7)	10.8 ± 3	5.0 ± 2
Total CFU per 10^5 cells		
(day 10)	143 ± 21	141 ± 28
GM	105 ± 13	108 ± 20
E	9.3 ± 3.7	4.6 ± 0.8
E/Mg	25 ± 5.3	25 ± 7.6
E/multi	2.5 ± 1.4	3.3 ± 1

Bone marrow cells from 10-wk old EMY-5 and wild-type littermates were assayed in methylcellulose in the presence of kit ligand, IL-1, IL-3, and EPO. BFU-E were counted after 7 d, total CFU and the CFU subtypes after 10 d. Results are expressed as mean ± standard deviation (*n* = three mice per group). For each mouse, colonies from six plates were counted. *GM*, granulocyte and/or macrophage colonies, *E*, erythroid; *E/Mg*, erythroid and megakaryocytic; *E/multi*, large (>1 mm) multilineage colonies consisting of erythroid, megakaryocytic, and macrophage or granulocytic cells.
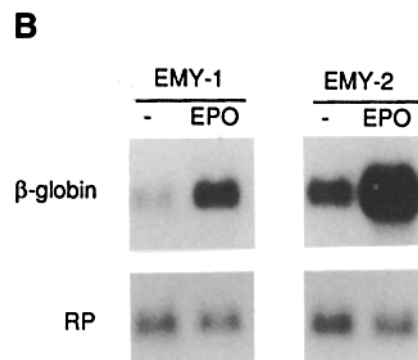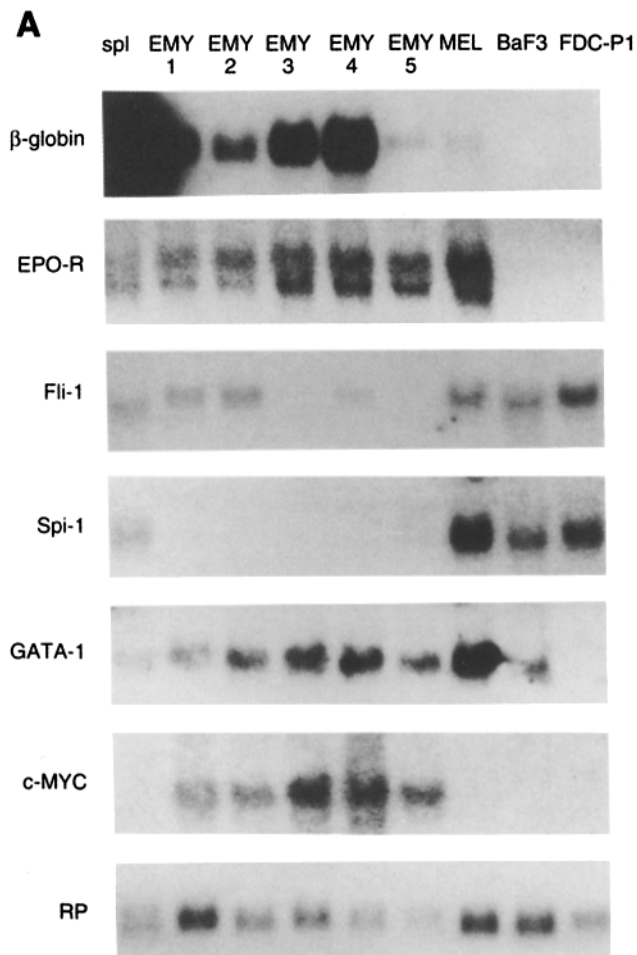

Figure 5. Northern analysis of gene expression in EMY cell lines. (*A*) The same blot was sequentially probed with specific ^{32}P-labeled cDNAs as indicated, except for EPO-R, for which a second blot with the identical RNAs was used. (*RP*) Was used to normalize for RNA loading. (*B*) Analysis of the effect of EPO on expression of β-globin in EMY cell lines.
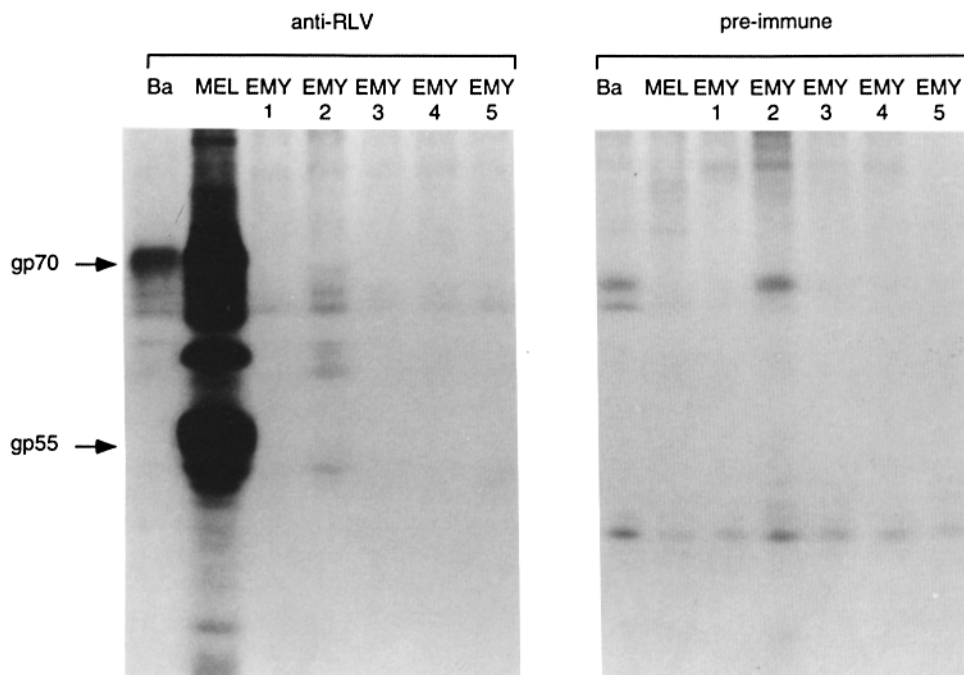
anti-RLV

Ba MEL EMY EMY EMY EMY EMY
        1   2   3   4   5

pre-immune

Ba MEL EMY EMY EMY EMY EMY
        1   2   3   4   5

gp70 →

gp55 →

Figure 6. Immunoprecipitation of cell lysates from [^{35}S]methionine-labeled BaF3 (*Ba*), MEL and EMY cell lines with polyclonal antibodies against the Friend virus envelope protein (*anti-RLV*) or preimmune serum.

more immature precursors with bi- or multilineage potential seem to be unaffected.

Search for Secondary Events Involved in Transformation of EMY Cell Lines. To assess the possibility that the secondary events frequently associated with Friend virus or spleen focus forming virus transformation might also be involved in leukemogenesis in our transgenic mice, we analyzed the expression of Fli-1 (27) and Spi-1 (25, 26) in EMY cell lines (Fig. 5 *A*). Spi-1 was not expressed in EMY erythroleukemic cell lines and therefore does not play a role. In contrast, Fli-1 mRNA was expressed in three of the five cell lines (EMY-1, EMY-2, and EMY-4). Thus, activation of Fli-1 could be one of the mechanisms in the leukemogenesis in these TG.EMY mice.

Friend helper virus can activate dormant endogenous mink cell focus viruses (MCFV) to express the oncogenic truncated envelope protein gp55 (41). To rule out the possibility that the deregulated expression of the c-*MYC* transgene could activate endogenous MCFV, we analyzed expression of gp55 protein in metabolically labeled EMY cell lines by immunoprecipitation with antienvelope antibodies (Fig. 6). In MEL cells this antiserum (31) recognizes both the wild-type envelope protein (gp70) and the mutated gp55, as well as an uncharacterized intermediate of ∼60 kd (42). In EMY cell lines we found no envelope protein. The bands visible in EMY-2 display a faster mobility than gp55 and on longer exposure appeared in all lanes, including the preimmune sera panel. Therefore, the c-*MYC* transgene does not activate dormant MCFV and does not induce expression of gp55. Thus, despite the fact that EMY cells resemble Friend virus–derived MEL cells in some respects, they have been generated by a distinct mechanism.

Discussion

We have generated transgenic mice that are prone to developing erythroleukemias because of the deregulated expression of the c-*MYC* proto-oncogene. We have derived erythroleukemia cell lines from these animals that morphologically resemble proerythroblasts (Fig. 3 *C*) and constitutively express the erythroid markers EPO-R and β-globin (Fig. 5 *A*). The occurrence of erythroleukemia infers that the regulatory elements necessary for expression in the erythroid compartment are present in our construct. Interestingly, committed hematopoietic precursors deficient for the GATA-1 gene display a block in erythropoiesis at the proerythroblast stage, as determined by an in vitro differentiation assay of GATA-1⁻ embryonic stem cells, indicating that GATA-1 is required for differentiation to later stages of erythroid development (43).

The reproducible observation of leukemias with exclusively erythroid characteristics was unexpected, since the endogenous GATA-1 gene is also expressed in other hematopoietic lineages, such as mast cells, megakaryocytes, and eosinophils (5–8). The regulatory sequences from the GATA-1 gene used in this transgenic construct might include the elements necessary for erythroid expression only. Consistent with this notion we found that the transgene was not expressed in mast cells derived from the TG.EMY-5 line (Fig. 4). This suggests that the region of the mouse GATA-1 gene comprising 2.7 kb of the 5′ upstream region and 1.5 kb of the first intron lacks element(s) required for expression in mast cells in vivo. The EMY cell lines did not display megakaryocytic markers, such as acetylcholinesterase (35, 36) or 4A5 (44). We were unable to determine expression of the transgene in megakaryo-

cytes or eosinophils from leukemic mice, because of the limiting amounts of cells. As expected, we have not detected transgene transcripts in the testis (Fig. 2), since expression of GATA-1 in testis was found in neonates only (9) and is initiated from a testis-specific promoter not included in this construct. Recently, factor-dependent cell lines with pluripotent characteristics were derived from transgenic mice that express a temperature-sensitive mutant of the SV40 large T antigen driven by GATA-1 regulatory sequences (45). This promoter–enhancer construct includes only a part of the sequences used in our transgenic mice and might differ in its pattern of expression. Alternatively, T antigen and c-MYC might preferentially immortalize different stages of development.

It is interesting that erythroleukemia arises in these transgenic strains in two distinctly different kinetic patterns. The late onset transgenic line (TG.EMY-5) develops leukemia, if at all, after 8–9 mo of age. By contrast, the five early onset animals (TG.EMY-1, TG.EMY-2, TG.EMY-3, TG.EMY-4, and TG.EMY-6) developed leukemia and died before they were 2 mo old. The basis for this difference is not clear, but we suspect that it is related to some difference in transgene expression. The levels of c-MYC transgene expression in cell lines derived from mice with early onset leukemia were similar to that detected in the late onset EMY-5 cell line (Fig. 5 A) suggesting that any difference must involve either differing numbers of individual cells expressing the transgene in each lineage or a difference in the developmental status of cells in which the transgene is expressed. Unfortunately, we cannot confirm either of these related models because the disease occurred too soon after birth in the early onset strains and breeding lines could not be established. Nonuniform expression of transgenes among strains bearing the same construct is known to occur in other systems (46).

Our experiments do, however, clarify the status of c-myc as a transforming oncogene in the erythroid lineage. Avian retroviruses expressing c-myc primarily cause myeloid leukemia and do not transform the erythroid lineage (47), whereas coexpression of both v-myc and v-raf was required to transform erythroid cells in murine fetal liver cell cultures infected with retroviruses (48). By contrast, similar experiments using two retroviruses (Zen and MPZen) that carry c-myc as their only oncogene yielded erythroid cell lines that initially displayed poor viability and grew slowly, but became fully transformed after several months in culture (49). This long latency, before fully transformed cell lines arise in vitro, kinetically resembles the long in vivo latency period seen in the TG.EMY-5 line. It remains possible that infection with the Zen or MPZen retroviruses activated expression of Friend spleen focus virus envelope protein gp55, a mechanism that has been excluded in our transgenic cell lines (Fig. 6). The early onset of erythroleukemia in the majority of our transgenic mice, however, demonstrates that c-MYC can efficiently transform erythroid progenitors. The GATA-1 regulatory sequences therefore seem to deregulate expression of c-MYC at a particularly vulnerable phase in the erythroid development.

The TG.EMY-5 mice and the EMY cell lines also allowed

us to examine the ability of c-MYC to influence terminal differentiation in the erythroid lineage. Expression of c-MYC in TG.EMY-5 mice before the manifestation of leukemia had no effect on erythropoiesis in vivo. The blood counts and red blood cell parameters in young healthy TG.EMY-5 mice were indistinguishable from the controls (Table 2). A twofold increase in BFU-E or in day 10 pure erythroid colonies was detectable in bone marrow colony assays in vitro (Table 4), whereas more immature precursors with bi- or multilineage potential were unaffected. This parallels the mature phenotype of erythroleukemic cells and the lack of transformation at the level of multipotent progenitors in TG.EMY mice and is consistent with predominant expression of the transgene at a late erythroid progenitor stage. Albeit the effect of c-MYC on erythropoiesis in TG.EMY-5 mice is relatively minor and is compensated in vivo, it sufficed as a first hit to initiate events that resulted in full transformation after a long latency. Deregulated expression of c-MYC in leukemic cell lines caused a more pronounced effect on erythroid differentiation. EMY cell lines failed to become hemoglobinized in response to 1.2% DMSO and failed to display morphological characteristics of terminal differentiation. This is in agreement with the observation that c-myc inhibits terminal differentiation in response to DMSO and other inducing agents in transfected MEL cells (38–40). Deregulated expression of c-MYC did not prevent upregulation of β-globin mRNA in response to EPO (Fig. 5 B). A similar partial block in differentiation was observed in Ba/F3 cells, an IL-3–dependent progenitor cell line. Treatment with EPO induced globin mRNA but not the appearance of hemoglobin in Ba/F3 cells transfected with EPO-R (50, 51). The effect of c-MYC on hemoglobinization and terminal differentiation in transformed cells and the apparent lack of a block in bone marrow from healthy EMY-5 mice might reflect different levels of transgene expression. Alternatively, the transformed cell lines might be more sensitive to c-MYC because of secondary changes associated with transformation.

Our search for known secondary events linked to viral leukemogenesis in the erythroid lineage revealed Fli-1 as a potential oncogenic partner of c-MYC. Fli-1 mRNA was expressed in three of the five EMY cell lines (Fig. 5 A) and is also upregulated in 95% of Friend erythroleukemias (27). In contrast, activation of Spi-1, another member of the ets family that is upregulated in spleen focus forming virus erythroleukemia (25), does not seem to be a common secondary event in c-MYC-based erythroleukemia since it was expressed in neither the EMY lines nor in the retrovirally transformed myc-erythroleukemias (49).

The TG.EMY-5 transgenic line creates a new in vivo model for erythroleukemia. Because of the slow occurrence of erythroleukemia in this transgenic strain, it should provide a useful system in which to identify the secondary genetic events that can collaborate with c-MYC to accelerate tumor formation in the erythroid lineage. The use of insertional mutagenesis with retrovirus or appropriate genetic crosses with other tumor-predisposing transgenic lines should be particularly fruitful in this regard.

References

1. Orkin, S.H. 1992. GATA-binding transcription factors in hematopoietic cells. *Blood.* 80:575–581.
2. Pevny, L., M.C. Simon, E. Robertson, W.H. Klein, S.F. Tsai, V. D'Agati, S.H. Orkin, and F. Costantini. 1991. Erythroid differentiation in chimaeric mice blocked by a targeted mutation in the gene for transcription factor GATA-1. *Nature (Lond.).* 349:257–260.
3. Simon, M.C., L. Pevny, M.V. Wiles, G. Keller, F. Costantini, and S.H. Orkin. 1992. Rescue of erythroid development in gene targeted GATA-1⁻ mouse embryonic stem cells. *Nature Genetics.* 1:92–98.
4. Whitelaw, E., S.F. Tsai, P. Hogben, and S.H. Orkin. 1990. Regulated expression of globin chains and the erythroid transcription factor GATA-1 during erythropoiesis in the developing mouse. *Mol. Cell. Biol.* 10:6596–6606.
5. Martin, D.I., L.I. Zon, G. Mutter, and S.H. Orkin. 1990. Expression of an erythroid transcription factor in megakaryocytic and mast cell lineages. *Nature (Lond.).* 344:444–447.
6. Romeo, P.H., M.H. Prandini, V. Joulin, V. Mignotte, M. Prenant, W. Vainchenker, G. Marguerie, and G. Uzan. 1990. Megakaryocytic and erythrocytic lineages share specific transcription factors. *Nature (Lond.).* 344:447–449.
7. Zon, L.I., M.F. Gurish, R.L. Stevens, C. Mather, D.S. Reynolds, K.F. Austen, and S.H. Orkin. 1991. GATA-binding transcription factors in mast cells regulate the promoter of the mast cell carboxypeptidase A gene. *J. Biol. Chem.* 266:22948–22953.
8. Zon, L.I., Y. Yamaguchi, K. Yee, E.A. Albee, A. Kimura, J.C. Bennett, S.H. Orkin, and S.J. Ackerman. 1993. Expression of mRNA for the GATA-binding proteins in human eosinophils and basophils: potential role in gene transcription. *Blood.* 81:3234–3241.
9. Ito, E., T. Toki, H. Ishihara, H. Ohtani, L. Gu, M. Yokoyama, J.D. Engel, and M. Yamamoto. 1993. Erythroid transcription factor GATA-1 is abundantly transcribed in mouse testis. *Nature (Lond.).* 362:466–468.
10. Adams, J.M., A.W. Harris, C.A. Pinkert, L.M. Corcoran, W.S. Alexander, S. Cory, R.D. Palmiter, and R.L. Brinster. 1985. The c-myc oncogene driven by immunoglobulin enhancers induces lymphoid malignancy in transgenic mice. *Nature (Lond.).* 318:533–538.
11. Schmitt, V.E., P.K. Pattengale, L. Weir, and P. Leder. 1988. transgenic mice bearing the human c-myc gene activated by an immunoglobulin enhancer: A pre-B-cell lymphoma model. *Proc. Natl. Acad. Sci. USA.* 85:6047–6051.
12. Graf, T., and H. Beug. 1978. Avian leukemia viruses: interac-

tion with their target cells in vivo and in vitro. *Biochim. Biophys. Acta.* 516:269–299.
13. Baumbach, W.R., E.R. Stanley, and M.D. Cole. 1987. Induction of clonal monocyte-macrophage tumors in vivo by a mouse c-myc retrovirus: rearrangement of the CSF-1 gene as a secondary transforming event. *Mol. Cell. Biol.* 7:664–671.
14. DePinho, R.A., A.N. Schreiber, and F.W. Alt. 1991. myc family oncogenes in the development of normal and neoplastic cells. *Adv. Cancer Res.* 57:1–46.
15. Amati, B., M.W. Brooks, N. Levy, T.D. Littlewood, G.I. Evan, and H. Land. 1993. Oncogenic activity of the c-Myc protein requires dimerization with Max. *Cell.* 72:233–245.
16. Blackwood, E.M., and R.N. Eisenman. 1991. Max: a helix-loop-helix zipper protein that forms a sequence-specific DNA-binding complex with Myc. *Science (Wash. DC).* 251:1211–1217.
17. Batey, J., C. Moulding, R. Taub, W. Murphy, H. Potter, G. Lenoir, and P. Leder. 1983. The human c-myc oncogene: structural consequences of translocation into the IgH locus in Burkitt Lymphoma. *Cell.* 34:779–787.
18. Hogan, B., F. Constantini, and E. Lacy. 1986. Manipulating the Mouse Embryo: A Laboratory Manual. Cold Spring Harbor Press, Cold Spring Harbor, NY. 153–173.
19. Chirgwin, J.M., A.E. Przybyla, R.J. Macdonald, and W.J. Rutter. 1979. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. *Biochemistry.* 18:5294–5299.
20. Krieg, P.A., and D.A. Melton. 1987. *In vitro* RNA synthesis with SP6 RNA polymerase. *Methods Enzymol.* 155:397–415.
21. Shen, M.M., and P. Leder. 1992. Leukemia inhibitory factor is expressed by the pre-implantation uterus and selectively blocks primitive ectoderm formation *in vitro. Proc. Natl. Acad. Sci. USA.* 89:8240–8244.
22. Leder, A., A. Kuo, M.M. Shen, and P. Leder. 1992. In situ hybridization reveals co-expression of embryonic and adult α-globin genes in the earliest murine erythrocyte progenitors. *Development (Camb).* 116:1041–1049.
23. D'Andrea, A.D., H.F. Lodish, and G.G. Wong. 1989. Expression cloning of the murine erythropoietin receptor. *Cell.* 57:277–285.
24. Pavlakis, G.N., R.E. Lockard, N. Vamvakopoulos, L. Rieser, U.L. RajBhandary, and J.N. Vournakis. 1980. Secondary structure of mouse and rabbit- alpha- and beta-globin mRNAs: differential accessibility of alpha and beta initiator AUG codons towards nucleases. *Cell.* 19:91–102.
25. Moreau-Gachelin, F., A. Tavitian, and P. Tambourin. 1988.

Spi-1 is a putative oncogene in virally induced murine erythroleukaemias. *Nature (Lond.).* 331:277–280.

26. Klemsz, M.J., S.R. McKercher, A. Celada, B.C. Van, and R.A. Maki. 1990. The macrophage and B cell–specific transcription factor PU.1 is related to the ets oncogene [see comments]. *Cell.* 61:113–124.

27. Ben-David, Y., E.B. Giddens, K. Letwin, and A. Bernstein. 1991. Erythroleukemia induction by Friend murine leukemia virus: insertional activation of a new member of the ets gene family, Fli-1, closely linked to c-ets-1. *Genes & Dev.* 5:908–918.

28. Tsai, S.F., D.I. Martin, L.I. Zon, A.D. D'Andrea, G.G. Wong, and S.H. Orkin. 1989. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. *Nature (Lond.).* 339:446–451.

29. Dudov, K.P., and R.P. Perry. 1984. The gene family encoding the mouse ribosomal protein L32 contains a uniquely expressed intron-containing gene and an unmutated processed gene. *Cell.* 37:457–468.

30. Iscove, N.N., A.R. Shaw, and G. Keller. 1989. Net increase of pluripotential hematopoietic precursors in suspension culture in response to IL-1 and IL-3. *J. Immunol.* 142:2332–2337.

31. Ruta, M., S. Clarke, B. Boswell, and D. Kabat. 1982. Heterogeneous metabolism and subcellular localization of a potentially leukemogenic membrane glycoprotein encoded by Friend erythroleukemia virus. Isolation of viral and cellular processing mutants. *J. Biol. Chem.* 257:126–134.

32. Tsai, S.F., E. Strauss, and S.H. Orkin. 1991. Functional analysis and in vivo footprinting implicate the erythroid transcription factor GATA-1 as a positive regulator of its own promoter. *Genes & Dev.* 5:919–931.

33. Penn, L.J., M.W. Brooks, E.M. Laufer, and H. Land. 1990. Negative autoregulation of c-myc transcription. *EMBO (Eur. Mol. Biol. Organ.) J.* 9:1113–1121.

34. Ma, A., R.K. Smith, A. Tesfaye, P. Achacoso, R. Dildrop, N. Rosenberg, and F.W. Alt. 1991. Mechanism of endogenous myc gene down-regulation in E mu-N-myc tumors. *Mol. Cell. Biol.* 11:440–444.

35. Jackson, C.W. 1973. Cholinesterase as a possible marker for early cells of the megakaryocytic series. *Blood.* 42:413–421.

36. Long, M.W., and N. Williams. 1981. Immature megakaryocytes in the mouse: morphology and quantitation by acetylcholinesterase staining. *Blood.* 58:1032–1039.

37. Enerback, L. 1966. Mast cells in rat gastrointestinal mucosa. II. Dye binding and metachromatic properties. *Acta Pathol. Microbiol. Scand.* 66:303–304.

38. Coppola, R.G., and M.D. Cole. 1986. Constitutive c-myc oncogene expression blocks mouse erythroleukemia cell diferentiation but not commitment. *Nature (Lond.).* 320:760–763.

39. Dmitrovsky, E., W.M. Kuehl, G.F. Hollis, I.R. Kirsch, T.P. Bender, and S. Segal. 1986. Expression of a transfected human c-myc oncogene inhibits differentiation of a mouse erythroleu-

kaemia cell line. *Nature (Lond.).* 322:748–750.

40. Prochownik, E.V., and J. Kukowska. 1986. Deregulated expression of c-myc by murine erythroleukemia cells prevents differentiation. *Nature (Lond.).* 322:848–850.

41. Ruscetti, S., and L. Wolff. 1984. Spleen focus-forming virus: relationship of an altered envelope gene to the development of a rapid erythroleukemia. *Curr. Top. Microbiol. Immunol.* 12:21–44.

42. Li, J.P., A.D. D'Andrea, H.F. Lodish, and D. Baltimore. 1990. Activation of cell growth by binding of Friend spleen focus-forming virus gp55 glycoprotein to the erythropoietin receptor. *Nature (Lond.).* 343:762–764.

43. Weiss, M.J., G. Keller, and S.H. Orkin. 1994. Novel insights into erythroid development revealed through in vitro differentiation of gata-1(-) embryonic stem cells. *Genes & Dev.* 8:1184–1197.

44. Burstein, S.A., P. Friese, T. Downs, and R.L. Mei. 1992. Characteristics of a novel rat anti-mouse platelet monoclonal antibody: application to studies of megakaryocytes. *Exp. Hematol.* 20:1170–1177.

45. Cairns, L.A., S. Crotta, M. Minuzzo, E. Moroni, F. Granucci, S. Nicolis, R. Schiro, L. Pozzi, B. Giglioni, P. Ricciardi-Castagnoli, and S. Ottolenghi. 1994. Immortalization of multipotent growth-factor dependent hematopoietic progenitors from mice transgenic for GATA-1 driven SV40 tsA58 gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4577–4586.

46. Guy, C.T., M.A. Webster, M. Schaller, T.J. Parsons, R.D. Cardiff, and W.J. Muller. 1992. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc. Natl. Acad. Sci. USA.* 89:10578–10582.

47. Beug, H., and T. Graf. 1989. Co-operation between viral oncogenes in avian erythroid and myeloid leukaemia. *Eur. J. Clin. Invest.* 19:491–502.

48. Klinken, S.P., N.A. Nicola, and G.R. Johnson. 1988. In vitro-derived leukemic erythroid cell lines induced by a raf- and myc-containing retrovirus differentiate in response to erythropoietin. *Proc. Natl. Acad. Sci. USA.* 85:8506–5810.

49. Cory, S., T. Maekawa, J. McNeall, and D. Metcalf. 1991. Murine erythroid cell lines derived with c-myc retroviruses respond to leukemia-inhibitory factor, erythropoietin, and interleukin 3. *Cell Growth & Differ.* 2:165–172.

50. Liboi, E., M. Carroll, A.D. D'Andrea, and B. Mathey-Prevot. 1993. Erythropoietin receptor signals both proliferation and erythroid-specific differentiation. *Proc. Natl. Acad. Sci. USA.* 90:11351–11355.

51. Chiba, T., Y. Nagata, A. Kishi, K. Sakamaki, A. Miyajima, M. Yamamoto, J.D. Engel, and K. Todokoro. 1993. Induction of erythroid-specific gene expression in lymphoid cells. *Proc. Natl. Acad. Sci. USA.* 90:11593–11597.