MOLECULAR AND CELLULAR BIOLOGY, May 1996, p. 2453–2463 Vol. 16, No. 5
0270-7306/96/$04.00+0

Spi-1/PU.1 Transgenic Mice Develop Multistep Erythroleukemias

F. MOREAU-GACHELIN,[1]* F. WENDLING,[2] T. MOLINA,[3] N. DENIS,[1] M. TITEUX,[2] G. GRIMBER,[4] P. BRIAND,[4] W. VAINCHENKER,[2] AND A. TAVITIAN[1]

INSERM U248, Institut Curie, 75231 Paris Cedex 05,[1] INSERM U362, Institut Gustave Roussy, 94800 Villejuif,[2] Laboratoire Universitaire de Recherche en Histopathologie, 75006 Paris,[3] and INSERM U380, Institut Cochin de Génétique Moléculaire, 75014 Paris,[4] France

Received 6 November 1995/Returned for modification 28 December 1995/Accepted 8 February 1996

Insertional mutagenesis of the *spi-1* gene is associated with the emergence of malignant proerythroblasts during Friend virus-induced acute erythroleukemia. To determine the role of *spi-1*/PU.1 in the genesis of leukemia, we generated *spi-1* transgenic mice. In one founder line the transgene was overexpressed as an unexpected-size transcript in various mouse tissues. Homozygous transgenic animals gave rise to live-born offspring, but 50% of the animals developed a multistep erythroleukemia within 1.5 to 6 months of birth whereas the remainder survived without evidence of disease. At the onset of the disease, mice became severely anemic. Their hematopoietic tissues were massively invaded with nontumorigenic proerythroblasts that express a high level of Spi-1 protein. These transgenic proerythroblasts are partially blocked in differentiation and strictly dependent on erythropoietin for their proliferation both in vivo and in vitro. A complete but transient regression of the disease was observed after erythrocyte transfusion, suggesting that the constitutive expression of *spi-1* is related to the block of the differentiation of erythroid precursors. At relapse, erythropoietin-independent malignant proerythroblasts arose. Growth factor autonomy could be partially explained by the autocrine secretion of erythropoietin; however, other genetic events appear to be necessary to confer the full malignant phenotype. These results reveal that overexpression of *spi-1* is essential for malignant erythropoiesis and does not alter other hematopoietic lineages.

The *spi-1* gene was first identified as the target for insertional mutagenesis in Friend erythroblastic tumors that developed in spleen focus-forming virus (SFFV)-infected mice (32, 35, 42). As a result of the SFFV provirus integration, the normal *spi-1* gene is strongly overexpressed. This is probably due to the transcriptional enhancer elements supplied by the SFFV long terminal repeat (LTR) (33, 34). Thus, *spi-1* may be considered as a putative oncogene implicated in the malignant transformation of proerythroblasts.

The *spi-1* gene encodes the transcription factor PU.1 (also called Spi-1), a member of the Ets family (19, 24, 32) physiologically expressed in all hematopoietic cell lineages except the T-cell lineage (48). Spi-1/PU.1 recognizes a purine-rich sequence motif around a minimal core consensus, 5′-G/AGAA-3′, in transcriptional promoters of various genes expressed in myelomonocytic cell lineages such as the scavenger receptor (36), the macrophage colony-stimulating factor receptor (64), the high-affinity FcγRI (43) and FcγRIIIA (14) receptors for immunoglobulin G, the integrin cell surface receptor CD11b (40), the c-Fes/c-Fps tyrosine kinase (49), and its own promoter (8). Spi-1/PU.1 binding sites also exist in the enhancer of the interleukin-4 (IL-4) gene in mast cells (20) and in the enhancer-promoter of genes expressed during maturation and differentiation of B-lymphoid cells such as genes encoding the heavy (39, 50), joining (58), and light (12, 46, 56) chains of immunoglobulins. In addition, Spi-1/PU.1-responsive elements have been identified in enhancer regions of viruses exhibiting a tropism for B cells like Epstein-Barr virus (27) or for macrophagic cells like equine infectious anemia virus (7). The phenotype of PU.1-negative homozygous embryos is consistent with these pleiotropic functions. Indeed, disruption of the *spi-1*/PU.1 gene by homologous recombination in mouse embryonic stem cells is lethal for homozygous PU.1 mutant progeny. Embryos present a multilineage defect characterized by defective development of progenitors in monocytes, granulocytes, and B- and T-lymphoid lineages and variable impairment of erythroid maturation (57). Together these data indicate that Spi-1/PU.1 may participate to the hematopoietic development as a basal transcriptional regulator.

During the Friend disease, the multistep progression of SFFV-infected proerythroblasts towards malignancy results from interactive processes between the viral protein gp55 encoded by the SFFV *env* gene and at least two cellular proteins: p53 and Spi-1/PU.1 (3). To ascertain the effective role of *spi-1* in erythroid cell transformation, various strategies were designed. Infection of murine long-term bone marrow cultures with a *spi-1*-transducing retrovirus caused the proliferation of proerythroblast-like cells that differentiated at a low frequency into hemoglobinized cells. Transformed cells remained dependent on both erythropoietin (EPO) and an adherent bone marrow stroma cell layer for growth, allowing the authors to conclude that *spi-1* overexpression was involved in erythroblast self-renewal (55). Alternatively, antisense oligonucleotides were used to reduce the *spi-1* expression level in Friend tumor cells (11). Antisense-treated cells presented a reduced proliferative capacity, again suggesting a role for *spi-1* in the self-renewal of transformed erythroblastic cells.

To define the in vivo roles of *spi-1*, we generated transgenic mice. Transcription of the *spi-1* transgene was driven by the SFFV LTR which is naturally involved in *spi-1* deregulation (35) and contains transcriptional enhancer elements active in hematopoietic cells (6). In this paper we show that *spi-1* transgenic mice develop solely erythroleukemia. At the onset of the disease, we observed a leukemic state characterized by a large expansion of proerythroblastic cells partially blocked in differentiation and totally dependent on EPO for proliferation. Erythrocyte transfusions led to a transient regression of the

* Corresponding author. Mailing address: INSERM U248, Institut Curie, 26, rue d'Ulm, 75231 Paris Cedex 05, France. Phone: 33 1 42 34 66 52. Fax: 33 1 42 34 66 50.

disease. At relapse, tumorigenic erythroblastic cells which had acquired the capability to grow autonomously arose. Thus, *spi-1* transgenic mice develop a multistep erythroleukemia that resembles Friend virus-induced pathology.

MATERIALS AND METHODS

Construction of the LTR–*spi-1* minigene. The *spi-1* first exon with its 3′ 600-bp intronic border (*Sac*I-*Pst*I fragment) and the second exon included in its 5′ 500-bp and 3′ 150-bp intronic borders (*Pst*I-*Eco*RI fragment) have been subcloned from phages containing the *spi-1* genomic locus (34). The 5′ part of the third exon up to the *Pst*I site and its 5′ 100-bp intronic border (*Eco*RI-*Pst*I fragment) was amplified by PCR from the genomic locus. The minigene was generated by ligating these three fragments to the 3′ region of the cDNA (985-bp *Pst*I-*Eco*RI fragment) containing the 3′ part of the exon 3 and exons 4 and 5 (34). The LTR fragment (535 bp) was cloned from the SFFV molecular clone of the polycythemic Lilly and Steeves strain (29) by PCR amplification between a 5′ primer located downstream of the gp55 open reading frame and a 3′ primer complementary to the first 18 nucleotides of the U5 region of the LTR. The *Bam*HI and *Hin*dIII sites were added during PCR amplification. The final construct resulted from the ligation of the *Eco*RI-*Bam*HI minigene insert (2.6 kb) to the *Bam*HI-*Hin*dIII SFFV LTR.

Expression of the *spi-1* minigene. COS-1 cells (2×10^7) were transfected by electroporation (250 V, 125 μF) with 10 μg of an expression vector (pSG5; Stratagene) containing either the normal cDNA or the minigene construct downstream of the simian virus 40 promoter. Cells were harvested 72 h after transfection and lysed in sodium dodecyl sulfate (SDS)-loading buffer for analysis of Spi-1 expression by Western blotting (immunoblotting). For electrophoretic mobility shift assays, COS-1 extracts were prepared from cell pellets in a mixture of *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES; 1.5 mM; pH 7.9), NaCl (140 mM), $MgCl_2$ (1.5 mM), 0.5% Nonidet P-40, dithiothreitol (1 mM), 1% aprotinin, paramethylsulfonyl fluoride (100 μg/ml), and leupeptin (10 μg/ml). Electrophoretic mobility shift assays were performed by using a ^{32}P-labeled 34-bp oligonucleotide containing the simian virus 40 Spi-1/PU.1-responsive element as previously described (48).

Production and screening of transgenic mice. Microinjection and oviduct transfer procedures have been previously described (21). Briefly, fertilized eggs were recovered from matings between B6D2F1 hybrids and microinjected with approximately 200 copies of the SFFV LTR–*spi-1* minigene DNA fragment purified on agarose gels. Eggs were transferred in oviducts of pseudopregnant females. Founder transgenic mice were bred to DBA2/J mice. Homozygous transgenic mice were produced by setting up heterozygous mice intercrosses.

DNAs from potential transgenic mice (10 to 12 days old) were extracted from tail tips, incubated overnight at 37°C in 1 mM Tris-HCl (pH 7.5)–1 mM EDTA–0.25 M NaCl containing 0.2% SDS and 100 μg of proteinase K per ml, purified by repeated extractions with phenol and chloroform, and ethanol precipitated. DNAs (10 μg) were digested with *Eco*RI and analyzed on Southern blots by hybridization with a *spi-1* cDNA as previously described (35).

Analysis of transgene expression in transgenic tissues and cell lines. (i) RNA. Total cellular RNAs were prepared from tissues homogenized in 4 M guanidium thiocyanate solution by the method of Chomczynski and Sacchi (9). Total RNAs (20 μg) were fractionated by electrophoresis in agarose-formaldehyde-containing gels, transferred onto GeneScreen nylon membranes, and hybridized sequentially with various probes labeled with [^{32}P]dCTP by using random-primed labeling kit (Amersham, Little Chalfont, United Kingdom). After overnight hybridization, blots were washed twice with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 sodium citrate)–0.1% SDS at room temperature and twice for 1 h in 0.1× SSC–0.1% SDS at 55°C and then exposed for autoradiography. Northern (RNA) blots were hybridized with cDNA probes representative of Spi-1 (34), SFFV LTR (described above), EPO receptor (EPO-R) (26), mGATA-1 (60), and β-globin (2). Finally, a rat glyceraldehyde-3-phosphate dehydrogenase cDNA probe (15) was used to monitor RNA loading.

(ii) Protein. Tissues or cells were homogenized in radioimmunoprecipitation assay buffer (NaCl, 150 mM; Tris-HCl, 50 mM; Nonidet P-40, 1%; deoxycholate, 0.5%; SDS, 0.1%). Proteins (25 μg) were resolved by SDS–12% polyacrylamide gel electrophoresis and electrotransferred onto nitrocellulose (Schleicher & Schuell). Blots were probed for 1 h with the polyclonal Spi-1 antibodies previously described (11). Immune complexes were visualized by the enhanced chemiluminescence Western blotting detection system (Amersham).

Cytological and histological examinations. Peripheral blood was drawn from the retro-orbital sinus in heparinized microhematocrit tubes. Blood was diluted in culture medium, and smears were prepared in duplicate with a cytospin. Cells in permanent cultures were adjusted at 10^6 cells per ml, and 100 μl was used to prepare cytocentrifuged smears. Smears were stained with May-Grünwald-Giemsa stain or benzidine-hematoxylin. Organs were fixed in Bouin's solution. Thin sections (5 mm) of paraffin-embedded tissues were stained with hematoxylin-eosin.

Flow cytometry analysis. Monocellular cell suspensions were immunolabeled (30 min at 4°C) with the following fluorescein isothiocyanate-conjugated monoclonal antibodies, all purchased from Pharmingen (Clinisciences, Montrouge, France) and used at a 10^{-2} dilution: B220 (clone RA3-6B2), CD4 (L3T4, clone RM4-5), CD8 (Ly-2, clone 53-6.7), Mac-1 (CD11b, clone M1/70), Sca-1 (Ly-6A.2, clone E13 161-7), TER-119 (anti-mouse erythroid cells), and Gr-1 (clone RB6-8C5). After two washings with phosphate-buffered saline, cells were examined with a FACsort cytometer (Becton Dickinson). As controls, fluorescein isothiocyanate-conjugated rat anti-mouse immunoglobulin of matched isotypes were used.

Derivation of permanent cell lines. Cell suspensions from enlarged spleens (1×10^6 cells per ml) and femoral bone marrow cells (5×10^5 cells per ml) were seeded in tissue culture flasks in alpha minimal essential medium (GIBCO, Paisley, United Kingdom) supplemented with 10% fetal calf serum (GIBCO), 2 mM L-glutamine, 100 U of streptomycin and penicillin per ml, and 2 U of recombinant human EPO (r hu-EPO) per ml. After 3 to 4 days in culture, cells were transferred into new flasks containing fresh complete medium. When cells in suspension reached a density of 10^6 cells per ml, cultures were diluted and seeded at 10^5 cells per ml. Thereafter, cultures were passaged (1/20 diluted) twice a week. To ascertain that cell lines remained EPO dependent, cells were washed three times in 40 ml of medium before being seeded at 10^5 cells per ml in medium without EPO. This assay was repeated once every month. Spleen and bone marrow cells from mice treated with erythrocyte transfusions were seeded and grown in culture in the conditions described above, with the exception that EPO was not added to the medium.

Cell transplantation in nude recipient mice. Cells from enlarged spleens or bone marrow were dispersed in alpha minimal essential medium containing 2% fetal calf serum by mechanical shearing. Cells (10^7) were injected by the subcutaneous or intraperitoneal route into 6-week-old female nude mice bred under sterile conditions (five mice per assay). Mice were regularly examined. Tumor nodules were usually visible 3 weeks after the graft when the tumor mass reached a diameter of 0.5 cm.

Proliferation and clonogenic assays. Cells were extensively washed, deprived of growth factor for 4 h, and seeded at a final concentration of 2×10^4 cells per 100 ml in 96-well plates. r hu-EPO or 48-h conditioned media (CM) from the tumor cell lines were added at the concentrations indicated in Fig. 5. The Ba/F3 cells (41) transfected with the murine EPO-R cDNA served as controls. After 48 h in culture, cells were pulsed for 4 h with 0.5 mCi of [^{3}H]thymidine. Cells were harvested onto glass fiber filters, and radioactivity incorporated in DNA was measured by liquid scintillation counting.

For clonogenic assays, cells were seeded at 500 cells per ml in methylcellulose medium (1% final concentration) supplemented with 10% fetal calf serum, and, when indicated, in the presence of 2 U of r hu-EPO or an anti-EPO monospecific polyclonal antibody (1/500 final dilution) (kindly provided by P. Mayeux [Paris, France]) per ml. Colonies were counted after 5 to 6 days of growth.

RESULTS

Generation of transgenic mice carrying a *spi-1* minigene construct. To construct a minigene encoding the murine Spi-1 protein, the genomic DNA fragments containing exons 1 and 2 and the 5′ part of exon 3 with their respective intronic borders were joined to the cDNA part encoding exons 4 and 5 (Fig. 1A). This minigene contains its own translational start and stop codons and polyadenylation signal. It was inserted in the pSG5 vector and transfected into monkey kidney COS cells to verify that it was correctly translated. Western blotting of COS extracts (Fig. 1B) showed that the protein expressed from the minigene presented properties identical to the protein expressed from a normal cDNA. Moreover, in a band shift assay, the minigene product showed a DNA binding activity indistinguishable from that of the normal protein (Fig. 1C).

To generate transgenic mice, the minigene was set under the transcriptional control of an SFFV LTR fragment containing U3, R, and 20 nucleotides from U5 (Fig. 1A). Transgenic animals were produced by the injection of a 3.0-kb *Hin*dIII-*Eco*RI DNA fragment into fertilized oocytes. Potentially transgenic mice were screened by Southern blot analysis of tail DNAs using a *spi-1* cDNA probe. Twelve independent founder animals were obtained and mated to generate homozygous transgenic lines. A comparison of the intensity of the transgene bands with that of the endogenous *spi-1* gene indicated that each founder carried one or two copies of the transgene integrated at one or two genomic sites (data not shown).

To assess transgene expression, total RNAs from different organs were analyzed by Northern blotting and, if necessary, by the reverse transcription (RT)-PCR technique. Amplification of the transgene open reading frame was made between a

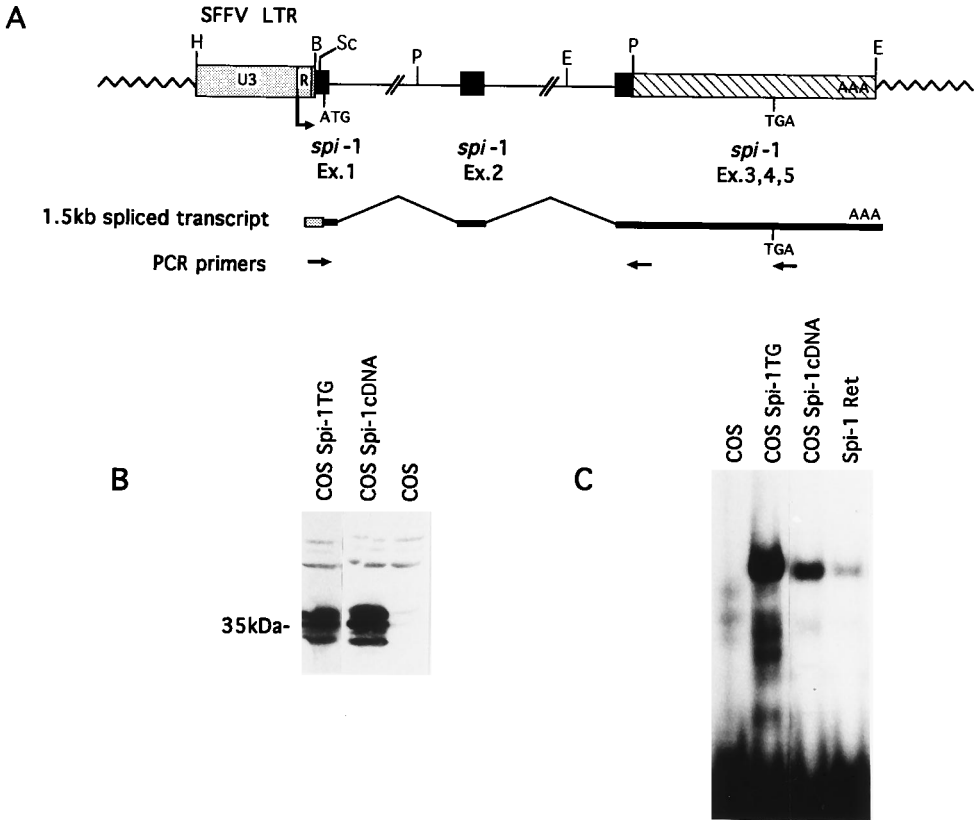


FIG. 1. Structure and expression of the SFFV LTR–*spi-1* minigene. (A) Structure of the construct used to generate transgenic mice. The five exons of the *spi-1* gene are indicated as numbered boxes. The black boxes represent the entire exons 1 and 2 and the part of exon 3 issued from the genomic locus. The hatched area is the cDNA fragment containing the other part of exon 3, as well as exons 4 and 5. The grey area is the SFFV LTR. The heavy line beneath the gene map represents the mRNA expected to be produced from the retroviral promoter. Restriction endonucleases sites used in construction are as follows: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; and Sc, *Sac*I. The arrow indicates the transcriptional start. (B) Western blotting analysis of the Spi-1 protein in cell extracts from COS-1 cells transfected with expression vectors for the *spi-1* cDNA (COS Spi-1 cDNA) or the *spi-1* minigene (COS Spi-1 TG). The 35-kDa Spi-1 protein is indicated. The multiple bands detected by antibodies are classically observed and most probably represent different phosphorylated forms of Spi-1. Lane COS, untransfected cell extracts. (C) Binding of the protein encoded by the minigene to a Spi-1-responsive element. Proteins were from COS Spi-1 TG and COS *spi-1* cDNA cells or produced by in vitro translation of the cDNA (Spi-1 Ret). The PU.1 simian virus 40 oligonucleotide was used in a gel mobility shift assay.

primer overlapping the *spi-1* stop codon and various primers localized in the U5 and R regions of the SFFV LTR (Fig. 1A). This allowed a specific amplification of transgene transcripts which were distinguishable from endogenous *spi-1* mRNA as well as from contaminating genomic DNA. Among the 12 transgenic lines, transgene expression was detectable in 4 (F13, F19, G53, and R36). In the F13, F19, and G53 lines, transgene transcripts could not be seen on Northern blots but were detected after RT-PCR amplification of spleen RNAs at a level below the endogenous *spi-1* expression level (data not shown). In contrast, two mRNAs migrating at 1.4 and 3 kb were revealed by a *spi-1* cDNA probe on Northern blots of various tissues from 3-week-old asymptomatic transgenic mice from the R36 line (exemplified by mouse R494 in Fig. 2). The 1.4-kb mRNA detected only by the *spi-1* probe represents most likely the endogenous *spi-1* transcript. The additional 3-kb mRNA, seen in all organs of transgenic animals, hybridized with both the *spi-1* probe and an SFFV LTR probe (see Fig. 4A), suggesting that it could correspond to the minigene transcripts. However, because the size was larger than expected, we used RT-PCR to check whether the transgene was correctly processed. Amplification of mRNAs with primers located in the SFFV LTR U5 and in exon 3 yielded a 240-bp fragment, demonstrating that the minigene was correctly spliced (data not shown). When RT-PCR was performed with the same U5 primer and a primer located on the *spi-1* gene stop codon, an 870-bp fragment that included the normal Spi-1 open reading frame as assessed by sequencing was amplified. The nature of the additional transcribed sequences is not yet clarified, but this transcript might result from a read-through of the natural polyadenylation. Since in Friend erythroleukemia, enhancers of the SFFV LTR play a crucial role in the transcriptional deregulation of *spi-1*, we analyzed the structure of the endogenous *spi-1* locus in the R36 line. By Southern blotting, the *spi-1* genomic locus appears normal, suggesting that the transgene was not integrated near the endogenous *spi-1* locus (data not shown). Strikingly, the 3.0-kb mRNA was not detected in all the R36 animals (five of eight mice tested).

Spi-1 transgenic mice develop leukemia. Gross examination of tissues in the G53 homozygous transgenic mice was unremarkable during 24 months of observation, and F13 and F19 animals were healthy after 12 months. Consequently, these animals were not extensively studied.

Heterozygous and homozygous mice from the R36 transgenic line had normal development and size, and no mortality occurred until 6 weeks of age. At a later age, about 50% of the mice (91 of 187), observed over a 12-month period, developed a dramatic enlargement of the spleen (1,200 ± 300 mg; control, 95 ± 10 mg) and the liver (2,300 ± 900 mg; control, 950 ± 75 mg) associated with severe anemia (hematocrit below 25%).

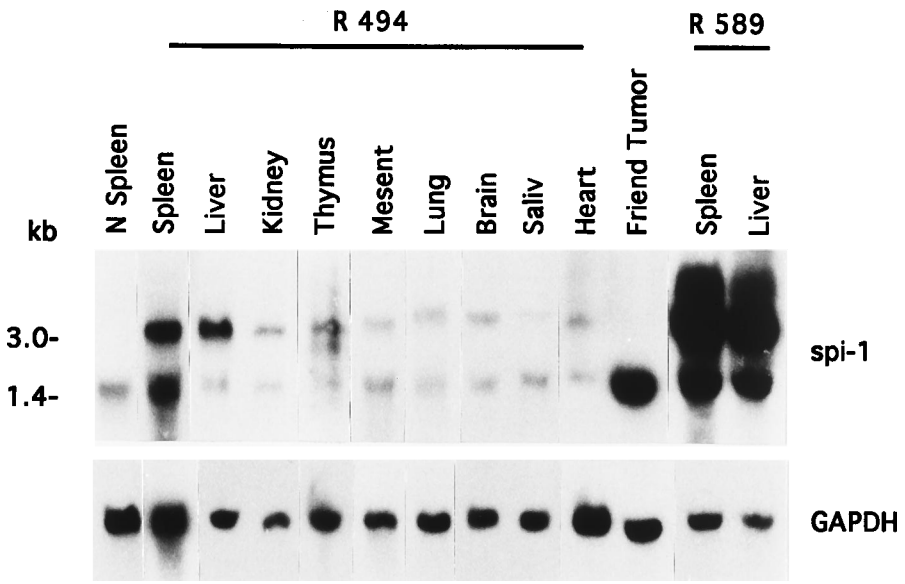


FIG. 2. In vivo expression of the transgene. Total RNAs (20 μg) were isolated from the indicated tissues from an asymptomatic transgenic mouse (R494) and from the spleen (1.3 g) and the liver (3.9 g) from mouse R589 in the ultimate phase of the disease. Friend tumor RNAs were isolated from 745-A Friend cell line (35). RNAs were hybridized with a *spi-1* cDNA probe. As a control of RNA loading, the same filter was rehybridized with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe.

The frequency of diseased mice was similar in males and females, and the average survival was 3.6 ± 1.5 months.

Spleens, livers, and sometimes kidneys were massively infiltrated by a predominant population of hyperbasophilic immature blastic cells with a highly proliferative state reflected by numerous mitotic manifestations. Rare lymphoid follicules were seen in the white pulp of spleens, while the livers presented a typical leukemic infiltration of the hepatic sinusoids (Fig. 3a to d). Occasionally, intraperitoneal lymph nodes showed extramedullary hematopoiesis. Thymuses were significantly reduced in size compared with those in normal littermates.

Hematological studies of severely sick animals revealed a high number of circulating nucleated cells in peripheral blood. Absolute numbers of polymorphonuclear cells, lymphocytes, and monocytes were increased 5- to 10-fold compared with those in healthy transgenic mice (Table 1). In addition, numerous undifferentiated blasts (5%) and poorly hemoglobinized erythroblasts (28%) were seen (Table 1 and Fig. 3e to h). Quantification of CFU-spleen in femoral marrows showed that their numbers were decreased, while their absolute number in enlarged spleens was augmented, a phenomenon often reported in murine leukemias (52).

Spi-1 transgenic mice develop erythroleukemia. Because *spi-1* is expressed in all hematopoietic cell lineages except the T-cell lineage, we tried to identify the nature of the blastic cells by immunolabeling and flow cytometry analyses. Various monoclonal antibodies directed against cell surface differentiation markers were used. In enlarged spleens (around 800 mg), the percentages of cells positive for B220 (B marker), CD4 and CD8 (T markers), Mac-1 (monocytic marker), Gr-1 (granulocytic marker), and Sca-1 (primitive hematopoietic cell marker) were reduced compared with those in normal spleens. In addition, the immunoglobulin heavy-chain and the T-cell receptor β loci were in a germ line configuration on Southern blots, confirming that the major cell population in these organs was not of the lymphoid type. In contrast, cells positive for the erythroid marker Ter-119 were significantly augmented in number (data not shown).

Bone marrow and spleen cells from six transgenic mice (R482, R483, R589, R633, R718, and R799) at the ultimate phase of the disease were examined for their ability to grow in vitro in response to various hematopoietic growth factors. Cells were grown in culture conditions that potentiate the growth of B-lymphoid (IL-7), myelomonocytic (granulocyte colony-stimulating factor plus granulocyte-macrophage colony-stimulating factor), megakaryocytic (SCF plus TPO), or progenitor cells of all lineages (pokeweed mitogen-stimulated spleen cell CM). The requirement for a hematopoietic microenvironment was also tested by cocultures on the murine MS5 stromal layer, which supports primitive hematopoiesis (22). None of these conditions were able to sustain cell growth for more than a week. In contrast, when cells were cultured with EPO (2 U/ml), nonadherent continuous cell lines were easily obtained from each diseased transgenic mouse tested. It is noteworthy that these cell lines retained their total EPO dependency even after 12 months in culture. Cells in permanent cultures were undifferentiated proerythroblast-like cells (Fig. 3e to h) with a morphology very similar to that of cells invading the organs of diseased mice. Benzidine staining of cells in culture revealed a low percentage (below 3%) of hemoglobinized erythroblasts. In contrast, when spleen or bone marrow cells from six asymptomatic transgenic mice of the same age were cultured in the presence of 2 U of EPO per ml, no cell lines were derived.

Because cells grew in vitro in response to EPO stimulation, we checked whether their in vivo proliferation was dependent on the endogenous EPO level which increased in response to anemia (25). Four anemic animals (R601, R606, R620, and R622) with a marked splenomegaly on palpation (spleen weight over 1 g) were intraperitoneally transfused with isogenic washed erythrocytes every 3 days in order to maintain the hematocrit value around 60%. Mice were regularly examined for spleen size by palpation, and the number of circulating nucleated blood cells was determined. As soon as day 4 after

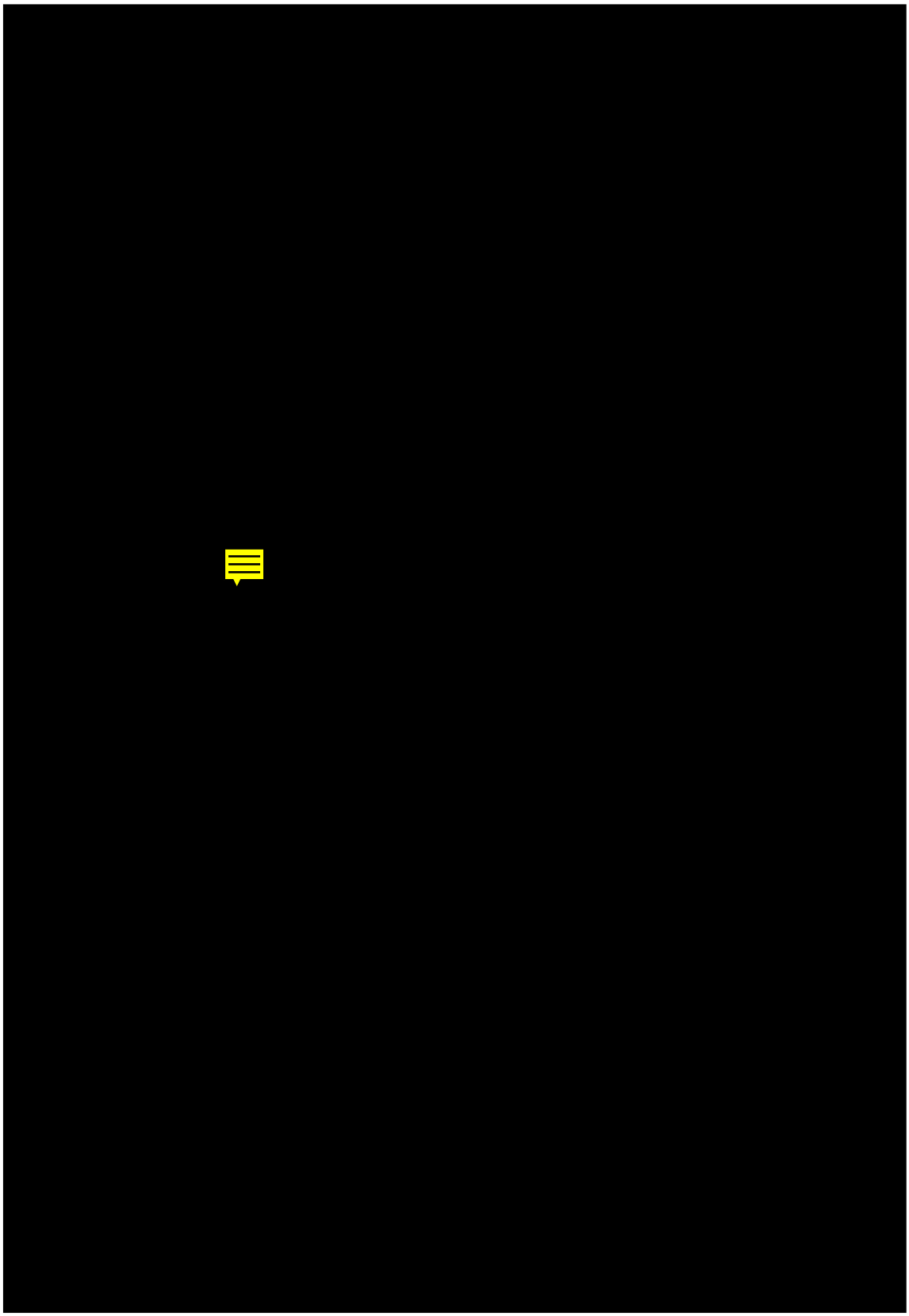


FIG. 3. (a to d) Histology of spleen and liver from normal and diseased *spi-1* transgenic mice. Cross sections were stained with hematoxylin and eosin (magnification, ×171). (a) Spleen of a normal mouse in which a lymphoid follicule (LF) and the red pulp (RP) can be seen. (b) Enlarged spleen of a diseased *spi-1* transgenic mouse (R589). The RP is massively infiltrated by a morphologically homogenous population of blastic cells. A persistent LF is evident. (c) Normal liver. (d) Enlarged liver of mouse R589. The hepatic sinusoids are invaded by blastic cells. (e to h) Blood smear of a diseased transgenic mouse (R589) stained with May-Grünwald-Giemsa stain (e) or benzidine and hematoxylin (f) (magnification, ×620). Arrows indicate polynuclear neutrophils (PN) and erythroblasts at various maturation stages: basophilic (BE) and acidophilic (AE) erythroblasts and reticulocytes (R). Smears of R589 EPO-dependent (g) and R601 EPO-independent (h) cell lines are also shown. Both smears are stained with May-Grünwald-Giemsa stain (magnification, ×620).

TABLE 1. Cellularity in peripheral blood and CFU-spleen content in bone marrow and spleens of transgenic mice

Transgenic mouse type	Mean no. of cells/mm^3 ± SD (%)[a]						Mean no. of platelets/mm^3 ± SD	Mean no. of CFU-spleen ± SD[b] in:	
	Nucleated	Undifferentiated blast	Polynuclear	Lymphocyte	Monocyte	Erythroblast		Bone marrow	Spleen
Asymptomatic, 5 wk old	6,900 ± 3,100	0	3,200 ± 1,400 (47)	3,200 ± 2,000 (46)	600 ± 250 (8)	0	590,000 ± 5,400	3,000 ± 600	3,700 ± 1,600
Diseased	61,500 ± 15,000	3,100 ± 1,600 (5)	18,000 ± 6,400 (29)	20,300 ± 5,100 (33)	6,100 ± 3,200 (10)	17,000 ± 8,700 (28)	221,000 ± 132,000	1,800 ± 700	14,400 ± 7,600

[a] From seven animals. Undifferentiated blasts are immature precursor cells presenting no clear characteristics supporting their identification. Erythroblasts are the sum of basophilic, polychromatophilic, and acidophilic erythroblasts. Values in asymptomatic mice were identical to those from nontransgenic mice with the same genetic background (data not shown).

[b] Bone marrow, one femur. Values were determined by the mean number of day 9 spleen colonies obtained from 10 irradiated recipients injected with cells from one donor and are the means from three donors.

transfusion, spleens became hardly palpable and peripheral nucleated cell counts returned to control values.

In addition, we checked whether cells in large spleens from moribund animals or from permanent cultures were tumorigenic by subcutaneous or intraperitoneal grafts in nude recipient mice. None of the transplantation assays were successful (12 diseased mice tested).

To definitively ascertain the lineage origin of the transformed cells, we examined the expression of several molecular markers. GATA-1 is a transcriptional factor (45) implicated in the regulation of erythroid genes such as those for the EPO-R and β-globin (44, 63). Enlarged spleens and cell lines expressed GATA-1, EPO-R, and β-globin transcripts (R590 mouse in Fig. 4A). However, since GATA-1 and EPO-R are also expressed in megakaryocytic cells (51), we looked for the presence of transcripts of two specific markers of megakaryocytopoiesis: the platelet factor 4 and the platelet integrin α-subunit IIb. No expression could be detected. Thus, the coexpression of GATA-1, EPO-R, and β-globin strongly argued for the erythroid nature of the cells proliferating in diseased transgenic mice. Moreover, the expression levels of these three genes in the spleen, liver, and kidney of the same animal was correlated with the level of transgene expression (R590 in Fig. 4A), suggesting that GATA-1, EPO-R, and β-globin genes and the *spi-1* transgene are coexpressed in a cell population that invaded first the spleen and gradually the liver and kidney.

Overall, these results show that *spi-1* transgenic mice develop an erythroleukemia characterized by a dramatic proliferation of nontumorigenic, immortalized erythroblastic cells partially blocked in differentiation that remain strictly EPO dependent for proliferation.

Malignant erythroleukemic cells arise in hypertransfused transgenic mice. Despite continuous erythrocyte transfusions, all treated animals relapsed after 12 to 25 days. Their spleen and liver volumes quickly increased, and again their blood was massively invaded by blastic cells. All died 3 to 4 weeks after the beginning of treatment. To determine whether tumorigenic cells had arisen, spleen and bone marrow cells from seven transfused mice were injected subcutaneously and intraperitoneally into nude recipients and grown in liquid cultures. Five weeks after in vivo graft, all recipients exhibited large subcutaneous tumors (2 cm across) at the injection site, as well as a massively infiltrated omentum. Spleens and livers from tumor-bearing nude mice were not macroscopically altered. Genomic analysis of cells taken from either subcutaneous tumors or infiltrated omentum revealed the presence of the *spi-1* transgene, demonstrating that cells in tumors were of donor origin (data not shown). All tumors were serially transplantable into nude recipients. When tested for their ability to proliferate in vitro, spleen and bone marrow cells from primary donors grew as nonadherent permanent cell lines. In contrast to cells isolated during the initial hepatosplenomegaly, their in vitro establishment was not dependent upon EPO or any other growth factors. Morphologically, these cells were undistinguishable from the EPO-dependent cells (Fig. 3e to h), with the exception that benzidine-positive cells were not seen. In addition, molecular analysis of the expression of erythroid markers confirmed their erythroid nature (R601 in Fig. 4A).

Furthermore, spleen and bone marrow cells taken from mice on day 4 (R683), day 6 (R749), or day 21 (R744) after the beginning of the hypertransfusion treatment, when no relapse had yet occurred (normal spleen size), failed to produce tumors in vivo and to grow without EPO in vitro. These data indicate that the late splenic enlargement is due to the emergence of a population of immature erythroid cells which differ

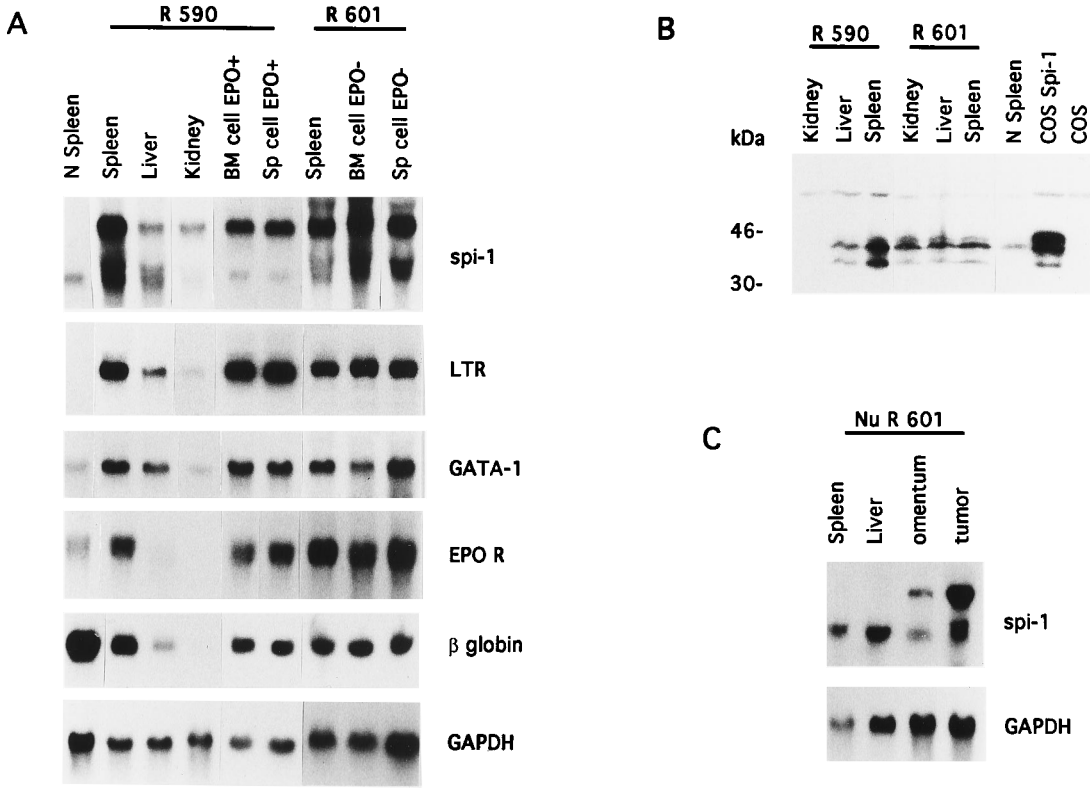


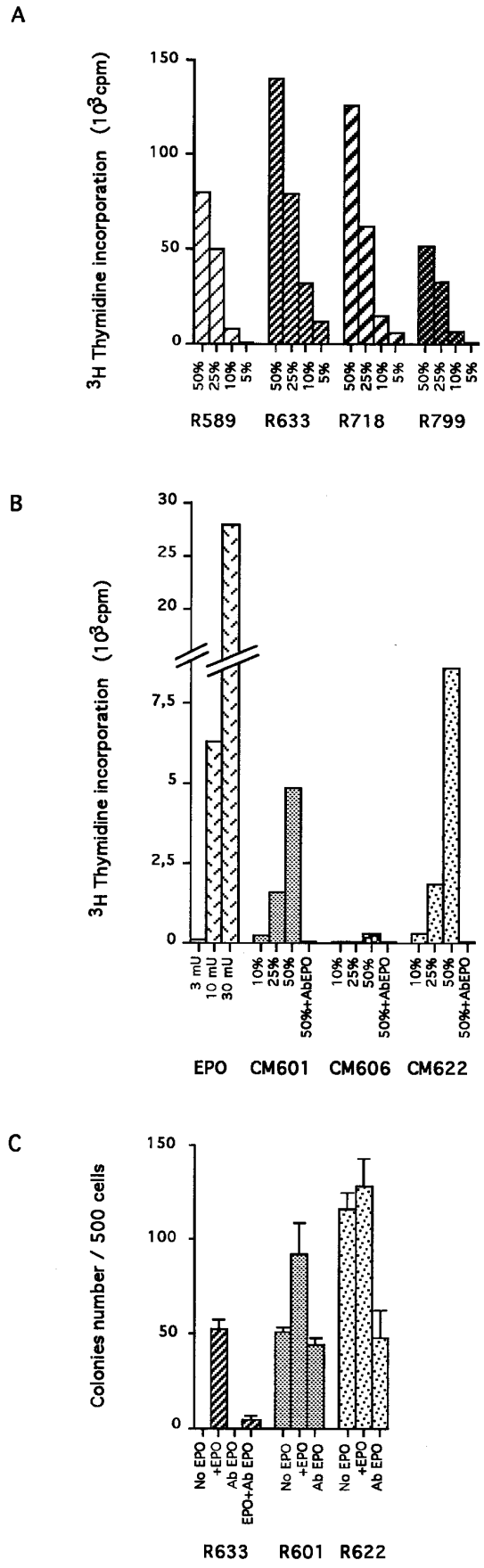
FIG. 4. Expression of the *spi-1* transgene and erythroid markers in tissues from transgenic diseased mice. (A) Northern analysis of *spi-1* transgenic tissues and cell lines. Mouse R590 was sacrificed during the progression of the disease when it presented a spleen of 1.3 g, a liver of 1.7 g, and apparently normal kidneys. R590 BM cell EPO+ and Sp cell EPO+ are EPO-dependent cell lines established from this mouse. R601 was a diseased animal that relapsed after erythrocyte transfusions (spleen, 1.8 g; liver, 3.8 g). R601 BM cell EPO− and Sp cell EPO− are EPO-independent cell lines established from this mouse. N Spleen, RNAs from a normal mouse. Filters of total RNAs (20 μg) were hybridized sequentially with the indicated murine probes. (B) Western blot of Spi-1 protein in tissues from R590 and R601 as compared with endogenous Spi-1 expression in the spleen of a normal mouse (N Spleen). COS Spi-1, cell extracts from COS-1 cells transfected with a *spi-1* cDNA expression vector; COS, untransfected cell extracts. (C) Northern blot of *spi-1* transgene expression in the tumor, omentum, spleen, and liver from a nude mouse grafted with spleen cells from mouse R601. Total RNAs (20 μg) were hybridized successively with the *spi-1* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probes.

from cells proliferating during the early splenomegaly by their EPO independence and their tumoral nature.

Acquisition of growth factor autonomy by tumor cells. We first investigated whether the switch from EPO dependency to growth autonomy resulted from an autocrine production of EPO. CM from three tumoral cell lines (R601, R606, and R622) were prepared (48-h CM from exponentially growing cells), and their ability to sustain the growth of EPO-dependent cell lines (R589, R633, R718, and R799) was tested. As shown in Fig. 5A, supernatant from the R622 tumor induced proliferation of the four EPO-dependent cell lines in a dose-dependent manner. Similar results were obtained with R601 CM, while R606 CM was poorly effective (data not shown). To ascertain that the proliferative activity was due to an autocrine secretion of EPO, CM were tested on both the Ba/F3 cells expressing the EPO-R after transfection (Ba/F3-EPO-R, responsive to both EPO and IL-3) and parental Ba/F3 cells (responsive only to IL-3) (41). None of the CM were able to sustain Ba/F3 cell growth, eliminating the possibility that cells were producing IL-3 (data not shown). In contrast, CM from the R601 and R622 tumors induced Ba/F3-EPO-R cells to proliferate (Fig. 5B). In addition, when EPO-neutralizing antibodies were added to the CM, the proliferative activity was totally abolished (data not shown). From the dose-response curves of the Ba/F3-EPO-R cells to recombinant EPO, the EPO concentration in R601 and R622 CM might be estimated to 15 and 25 mU/ml, respectively (Fig. 5B). To understand whether EPO secretion was a mechanism involved in the autonomous growth of the tumor cells, clonogenic assays were performed in the presence of EPO-neutralizing antibodies. Addition of antibodies at a concentration that neutralized 10 U of EPO per ml reduced but did not totally inhibit the tumor cell growth (Fig. 5C). These data clearly indicate that two of three tumors secrete enough EPO to be detectable on biological assays. However, this autocrine mechanism does not fully account for the autonomous cell growth, suggesting that other additional genetic events must have occurred to explain the full malignant phenotype.

Since alterations or loss of the p53 function is often involved in tumorigenicity of Friend erythroleukemic cells, the structure of the p53 gene in *spi-1* transgenic tumorigenic cells was analyzed by Southern and Northern strategies that reveal p53 mutations in 95% of the Friend tumors (4, 37, 38). No abnormalities were seen. Immunoprecipitations of radiolabeled p53 from cell line extracts with monoclonal antibodies (Oncogene Science) which recognize preferentially the wild-type (PAb246) or mutant (PAb240) conformation of the protein were unconclusive since similar patterns were seen whether cells were derived from the early or late phase of the disease.

Transgene expression in hyperplasic tissues and cell lines. Transcription of the transgene was analyzed on Northern blots hybridized with either the *spi-1* cDNA probe or an SFFV LTR



probe. The levels of the 3.0-kb mRNA were 10- to 20-fold increased in enlarged spleens from diseased mice (R589 in Fig. 2; R590 and R601 in Fig. 4A), in EPO-dependent or EPO-independent cell lines (EPO+ or EPO− in Fig. 4A), and in tumors (Nu R601 in Fig. 4C) compared with the level in the spleen of an asymptomatic mouse (R494 in Fig. 2). However, we could not determine whether the *spi-1* transgene expression level in proerythroblasts from an asymptomatic mouse was elevated since we cannot obtain enough RNA from fluorescence-activated cell sorter-sorted proerythroblasts (Ter-119$^+$) from an individual animal. It is noteworthy that no transgene mRNA was detected in the spleens or the livers of tumor-bearing nude mice, indicating that these malignant cells had poor metastatic properties. The transgene overexpression was correlated to the synthesis of an abundant Spi-1 protein, easily detected by Western blotting (Fig. 4B). Among the different mice studied during the early and late stages of the leukemia (10 animals), the highest Spi-1 protein level was seen in hyperplasic spleens, while expression of the protein was related to the degree of leukemic infiltration in the liver or the kidney.

In addition, we tested transgenic tissues and cell lines for retrovirus gene expression by RNA hybridization with probes specific for ecotropic or dually tropic *env* genes. All were found negative (data not shown), indicating that the hepatosplenomegaly was not due to in vivo generation of replication-competent retroviruses.

DISCUSSION

This work was undertaken to study the role of *spi-1* in the malignant transformation of hematopoietic cells. Our data establish that *spi-1* acts as an oncogene in vivo and demonstrate its key role in the development of erythroid tumors by blocking the differentiation of erythroblasts.

A *spi-1* minigene driven by the SFFV LTR was constructed to obtain transgenic mice. Of 12 founders, *spi-1* transgene expression was detectable in offsprings of 4 lines (R36, G53, F13, and F19). Fifty percent of animals from the R36 line developed a pathological erythropoiesis when aged 6 weeks to 6 months. In tissues, tumors, and cell lines derived from leukemic R36 mice, the transgene was expressed at a level higher than was the *spi-1* gene in Friend erythroleukemic cells, strengthening the idea that the overexpression of *spi-1* is involved in the development of an erythroproliferative syndrome. In the three other transgenic lines, transgene expression was detected to a level lower than that of the endogenous *spi-1* gene and no pathological disorders developed. The reasons for the differences in the transgene expression levels between the R36 and other transgenic mice lines are unclear. They might be related either to sequences surrounding the transgene integration site or to alterations in the transgene promoter-enhancer as a consequence of integration. The simplest hypothesis involves a linkage of the transgene to a con-

FIG. 5. Proliferative effects of CM prepared from tumor cell lines and clonogenic assays. (A) Proliferative response of the EPO-dependent cell lines to CM from tumor R622. Results are the means from triplicate wells from which background incorporation levels were subtracted. (B) Dose-response of the BaF3/EPO-R cells to r hu-EPO or CM from the tumor cell lines. One milliliter of CM was incubated with a polyclonal anti-EPO antibody (1/500 final dilution) for 30 min at 4°C before being used for proliferation assays at a final concentration of 50% (50%+AbEPO). Results are means from triplicate wells minus background level (BaF3/R-EPO cells without growth factors). (C) Cloning efficiency of R601 and R622 tumor cells grown in methylcellulose medium without growth factor (No EPO), with 2 U of r hu-EPO per ml (+EPO), or with anti-EPO antibodies (Ab EPO). The EPO-dependent R633 cell line was used as an internal control. Results are means ± standard deviations (triplicate cultures).

stitutively activated transcription control sequence in the R36 line. Another intriguing feature is that all homozygous R36 animals did not express the transgene. Indeed, when spleens from eight randomly selected R36 mice, less than 50 days old, were screened for transgene expression, only five were found positive by Northern analysis. Since this frequency is close to that of mice developing leukemia, we may assume that *spi-1* overexpression plays a direct role in the outgrowth of erythroleukemic cells. In agreement with this presumption is the fact that no transgene transcripts were seen in any tissues from mice that remained asymptomatic for more than 8 months (eight animals analyzed). No obvious explanation of why the transgene is not expressed in all animals of transgenic offspring is available as yet. One possibility stems from the observation that the transcriptional activity of the SFFV LTR is repressed during embryonic life. Indeed, we were unable to detect transgene expression in embryo bodies, fetal livers, and placentas analyzed on days 16 and 18 of gestation (data not shown). Thus, relaxation of this repression may not occur in all animals. More work is needed to fully understand this phenomenon.

Diseased transgenic mice exhibited simultaneously splenic hyperplasia and anemia. The erythroblastic nature of the cells responsible for the spleen enlargement was ascertained by immunological, cytological, biological, and molecular studies. We show that, in vivo, the proliferative disorder remains under a tight control of the endogenous level of EPO, as demonstrated by the rapid regression of splenomegaly associated with a total disappearance of circulating erythroblasts following erythrocyte transfusions. This in vivo observation was further strengthened by our in vitro data showing that permanent erythroblastic cell lines could be grown only when EPO was added to the culture medium. These data strongly suggest that overexpression of the Spi-1 protein impairs the differentiation of erythroid precursor cells which remain strictly dependent on EPO for their self-renewal. However, although permanent EPO-dependent cell lines were easily established from hematopoietic tissues of diseased mice, these immortalized cells (either taken directly from animals or from established cultures) were unable to produce tumor nodules when grafted into nude recipient mice.

In contrast, erythroid tumorigenic cells were readily isolated from mice treated by erythrocyte transfusions. This suggests that erythroleukemia development in *spi-1* transgenic mice involves multiple-step processes. The progression to full malignancy may depend on the occurrence of other genetic alterations, among which activation of cellular proto-oncogenes, loss of tumor suppressor genes, or autocrine mechanisms are the most common (5). Such secondary events often lead to the outgrowth of clonal or oligoclonal tumor cells. In the absence of genetic markers, such as T-cell receptor or immunoglobulin gene rearrangements in lymphomas, we could not assess the clonal status of the malignant cells. When supernatants from the autonomous cell lines were tested for autocrine production of growth factors, we found that two of three tumors produced an activity that induced the proliferation of the EPO-dependent lines. Moreover, since the proliferative activity was totally abolished by an anti-EPO neutralizing antibody, we conclude that these cells produce biologically active EPO. This mechanism is reminiscent of rare events occurring during erythroleukemogenesis in humans (31) or erythroleukemia in mice infected with the replication-competent Friend murine leukemia virus (59). However, clonogenic assays performed in the presence of an anti-EPO neutralizing antibody showed that the EPO secretion could only partially explain the autonomous growth of these tumorigenic cells, since their growth was not inhibited by the antibody. Because the cells grow in tight colonies, it is conceivable that the lack of complete inhibition reflects the failure of the antibody to penetrate into the interior of the colony. It could also be a result of either intracrine EPO production or other genetic alterations. An attractive candidate in this system is mutation of p53 since, in Friend erythroleukemic cells, overexpression of *spi-1* and genetic alterations of p53 are simultaneously observed (3). However no gross alterations of p53 could be seen in the three tumors examined, and further investigations involving RT-PCR and sequence analysis are required to exclude the possibility that point mutations had occurred in these tumor cells.

The erythroleukemic process that develops in *spi-1* transgenic mice has striking similarities to the SFFV-induced erythroleukemias. As previously demonstrated, Friend erythroleukemias progress as multistep diseases (30, 62). The early step is characterized by a massive polyclonal expansion of immature proerythroblasts capable of differentiating into erythrocytes either spontaneously or with a hypersensitivity to EPO (23). The molecular mechanism involves the SFFV *env* gene-encoded gp55 glycoprotein (1), which interferes with the EPO-signaling pathway by constitutively activating the EPO receptor (28). Highly proliferative erythroblasts appear as a cell population prone to subsequent oncogenic events leading to the emergence of tumorigenic proerythroblasts blocked in their differentiation. Friend tumor cells are clonal with respect to alterations of the two cellular genes, *spi-1* and the p53 gene. Since overexpression of *spi-1* is a marker of the malignant phenotype of the Friend tumor cells and since Spi-1 protein expression is down-regulated during hexamethylene bisacetamide- or dimethyl sulfoxide-induced differentiation of Friend cells (11, 54), Spi-1 appears to act as a transcriptional modulator involved in the control of the multiplication and differentiation of erythroid progenitors. In transgenic mice, the ectopic expression of *spi-1* maintains in an immature state the proerythroblastic cells that exhibit a strict requirement for EPO to survive and proliferate.

If it is clear that *spi-1* acts as an erythroleukemic oncogene in vivo, it remains to be determined how its up-regulation maintains proerythroblasts in an undifferentiated state. In vivo, Spi-1/PU.1 is a transcriptional activator whose function is modulated by the DNA sequence of its binding site, by posttranslational processing like phosphorylation (47), and by direct interaction with other nuclear proteins (13, 46). Spi-1/PU.1 can also be a transcriptional repressor, as illustrated by the reciprocal transcriptional interference that occurs between Spi-1 and some hormonal receptors (18) which behave like modulators of maturation of normal erythroid progenitors (53). This functional interference between Spi-1 and hormonal receptors suggests that ectopic expression of *spi-1* could interfere and/or block hormone-induced signals during determination of growth or differentiation of proerythroblasts. In this respect, the role of *spi-1* is reminiscent of the role of v-*erbA* in the acute erythroblastosis induced by the avian erythroblastosis virus. Indeed, the v-*erbA* oncogene encodes a ligand-independent form of the T3 thyroid hormone receptor (10) and arrests the differentiation of erythroid precursors (17) by acting as a constitutive repressor of the expression of erythrocyte-specific genes necessary for the development of chicken erythrocytes (16). The activity of a transcriptional regulator is believed to be modulated by the DNA sequence and other DNA-binding or non-DNA-binding proteins. It is therefore tempting to speculate that *spi-1* expression at a nonphysiological level, as observed in Friend erythroleukemic cells and in *spi-1* transgenic mice, affects erythroid differentiation either directly, by titration of target elements away from their normal regulator, or indirectly, by trapping proteins that regulate expression of ery-

throid differentiation markers and/or proliferation of proerythroblasts.

The *spi-1* transgenic model of erythroleukemia presents several points in common with human primitive erythroleukemia. In this rare malignancy, leukemic cells are also blocked at the CFU-erythroid–proerythroblast stage of differentiation. In most cases, autonomous growth which is also related to autocrine stimulation by EPO (31) is observed. The molecular defects responsible for this differentiation arrest are presently unknown. However, despite autocrine stimulation by EPO, there is no polycythemic state preceding the occurrence of erythroleukemia, suggesting as in the *spi-1* transgenic model that EPO autocrine stimulation is a secondary event that is related to acquisition of full malignancy (31, 61). Therefore, the *spi-1* transgenic model of erythroleukemia seems to have more similarities to the human disease than does the Friend model. Further investigation is required to ascertain whether *spi-1* or another member of the *ets* family is primarily involved in human primitive erythroleukemia.

ACKNOWLEDGMENTS

We thank Pascale Meneceur for her expert technical assistance, Patrice Ardouin and Annie Rouchès for helpful assistance with the animals, and Martine Douheret for histological procedures. We are grateful to J. L. Villeval for the gift of BaF3/EPO-R cells and P. Mayeux for the monospecific polyclonal anti-EPO antibody. We thank M. Hallier and D. Ray-Gallet for critical discussions.

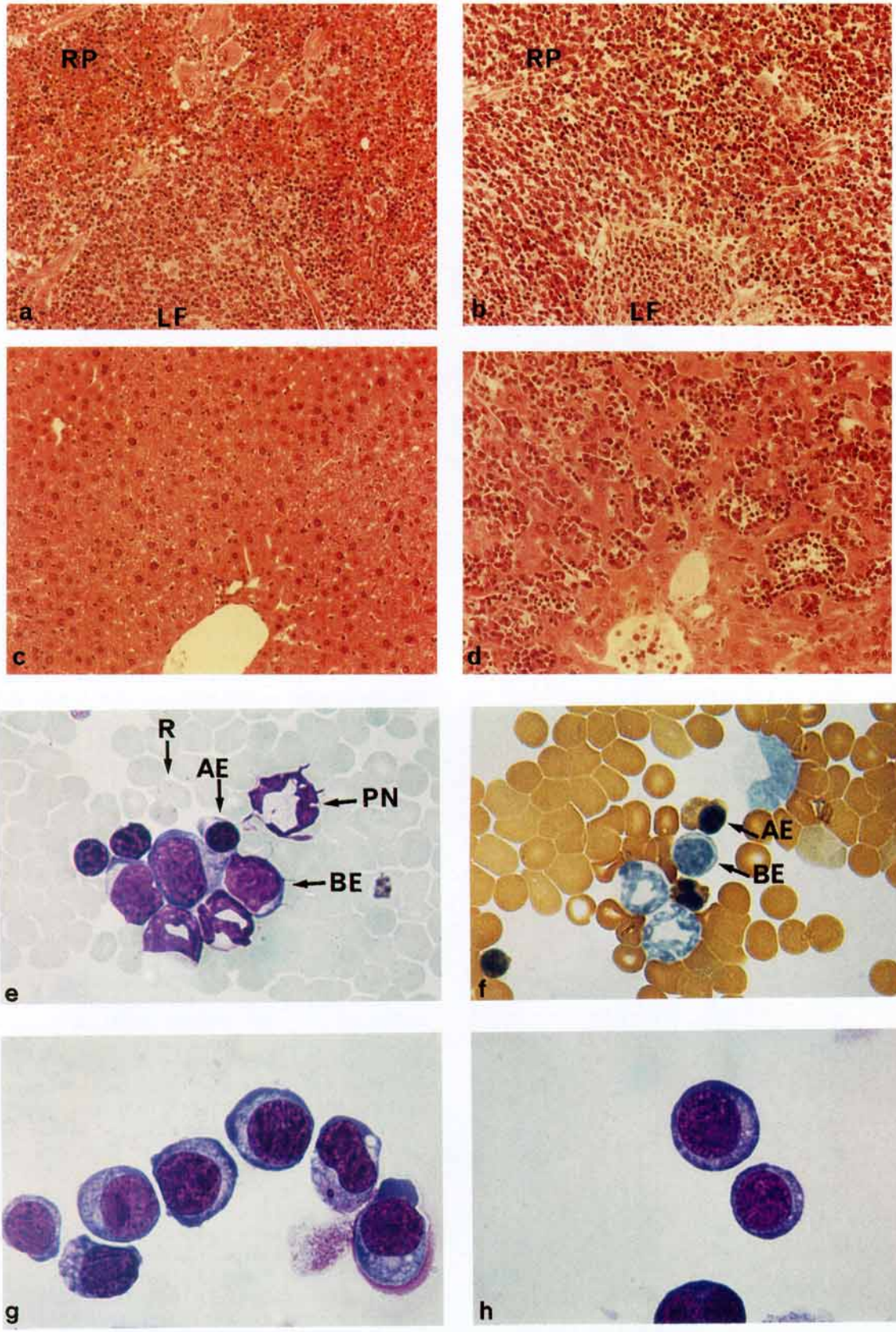
The research was supported by the Institut National de la Santé et de la Recherche Médicale, the Institut Gustave Roussy, the Comité Départemental de Paris de la Ligue Nationale Française contre le Cancer, the Fondation Saint-Louis pour la Recherche sur les Maladies du Sang, and the Association pour la Recherche sur le Cancer.

The first two authors contributed equally to this work.

REFERENCES

1. **Aizawa, S., Y. Suda, Y. Furuta, T. Yagi, N. Takeda, N. Watanabe, M. Nagayoshi, and Y. Ikawa.** 1990. *Env*-derived gp55 gene of Friend spleen focus-forming virus specifically induces neoplastic proliferation of erythroid progenitor cells. EMBO J. **7:**2107–2116.
2. **Baron, M. H., and T. Maniatis.** 1986. Rapid reprogramming of globin gene expression in transient heterokaryons. Cell **46:**591–602.
3. **Ben-David, Y., and A. Bernstein.** 1991. Friend virus-induced erythroleukemia and the multistage nature of cancer. Cell **66:**831–834.
4. **Ben-David, Y., V. R. Prideaux, V. Chow, S. Benchimol, and A. Bernstein.** 1988. Inactivation of the p53 oncogene by internal deletion or retroviral integration in erythroleukemic cell lines induced by Friend leukemia virus. Oncogene **3:**179–185.
5. **Bishop, J. M.** 1991. Molecular themes in oncogenesis. Cell **64:**235–248.
6. **Bösze, Z., H. J. Thiesen, and P. Charnay.** 1986. A transcriptional enhancer with specificity for erythroid cells is located in the long terminal repeat of the Friend murine leukemia virus. EMBO J. **5:**1615–1623.
7. **Carvalho, M., and D. Derse.** 1993. The PU.1/Spi-1 proto-oncogene is a transcriptional regulator of a lentivirus promoter. J. Virol. **67:**3885–3890.
8. **Chen, H. M., D. Ray-Gallet, P. Zhang, C. J. Hetherington, D. A. Gonzalez, D. E. Zhang, F. Moreau-Gachelin, and D. G. Tenen.** 1995. PU.1 (Spi-1) autoregulates its expression in myeloid cells. Oncogene **11:**1549–1560.
9. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
10. **Damm, K., C. C. Thompson, and R. M. Evans.** 1989. Protein encoded by *v-erbA* functions as a thyroid-hormone receptor antagonist. Nature (London) **339:**593–597.
11. **Delgado, D., M. Hallier, P. Meneceur, A. Tavitian, and F. Moreau-Gachelin.** 1994. Inhibition of Friend cells proliferation by *spi-1* antisense oligodeoxynucleotides. Oncogene **9:**1723–1727.
12. **Eisenbeis, C. F., H. Singh, and U. Storb.** 1993. PU.1 is a component of a multiprotein complex which binds an essential site in the murine immunoglobulin λ2-4 enhancer. Mol. Cell. Biol. **13:**6452–6461.
13. **Eisenbeis, C. F., H. Singh, and U. Storb.** 1995. Pip, a novel IRF family member, is a lymphoid-specific, PU.1-dependent transcriptional activator. Genes Dev. **9:**1377–1387.
14. **Feinman, R., W. Q. Qiu, R. N. Pearse, B. S. Nikolajczyk, R. Sen, M. Sheffery, and J. V. Ravetch.** 1994. PU.1 and an HLH family member contribute to the myeloid-specific transcription of the FcγRIIIA promoter. EMBO J. **13:**3852–3860.
15. **Fort, P., L. Marty, M. Piechaczyk, S. E. Sabrouty, C. Dani, P. Jeanteur, and J. M. Blanchard.** 1985. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res. **13:**1431–1441.
16. **Fuerstenberg, S., I. Leitner, C. Schroeder, H. Schwarz, B. Vennström, and H. Beug.** 1992. Transcriptional repression of band 3 and CALL in *v-erbA* transformed erythroblasts accounts for an important part of the phenotype. EMBO J. **11:**3355–3365.
17. **Gandrillon, O., P. Jurdic, B. Pain, C. Desbois, J. J. Madjar, M. G. Moscovici, C. Moscovici, and J. Samarut.** 1989. Expression of the *v-erbA* product, an altered nuclear hormone receptor, is sufficient to transform erythrocytic cells *in vitro*. Cell **58:**115–121.
18. **Gauthier, J. M., B. Bourachot, V. Doucas, M. Yaniv, and F. Moreau-Gachelin.** 1993. Functional interference between the Spi-1/PU.1 oncoprotein and steroid hormone or vitamin receptors. EMBO J. **12:**5089–5096.
19. **Goebl, M. G., F. Moreau-Gachelin, D. Ray, P. Tambourin, A. Tavitian, M. J. Klemsz, S. R. McKercher, A. Celada, C. Van Beveren, and R. A. Maki.** 1990. The PU.1 transcription factor is the product of the putative oncogene *spi-1*. Cell **61:**1165–1166.
20. **Henkel, G., and M. A. Brown.** 1994. PU.1 and GATA: components of a mast cell-specific interleukin 4 intronic enhancer. Proc. Natl. Acad. Sci. USA **91:**7737–7741.
21. **Hogan, B. L., F. Costantini, and E. Lacy.** 1986. Manipulating the mouse embryo: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
22. **Issaad, C., L. Croisille, A. Katz, J. Guichard, J. Breton-Gaurius, and W. Vainchenker.** 1993. A murine stromal cell line allows the proliferation of very primitive human $CD34^+/CD38^-$ progenitor cells in long-term cultures and semi-solid assays. Blood **81:**2916–2924.
23. **Kabat, D.** 1989. Molecular biology of Friend viral erythroleukemia. Curr. Top. Microbiol. Immunol. **148:**1–42.
24. **Klemsz, M. J., S. R. McKercher, A. Celada, C. Van Beveren, and R. A. Maki.** 1990. The macrophage and B-cell-specific transcription factor PU.1 is related to the *ets* oncogene. Cell **61:**113–124.
25. **Krantz, S. B.** 1991. Erythropoietin. Blood **77:**419–434.
26. **Lacombe, C., S. Chretien, V. Lemarchandel, P. Mayeux, P. H. Romeo, S. Gisselbrecht, and J. P. Cartron.** 1991. Spleen focus-forming virus long terminal repeat insertional activation of the murine erythropoietin receptor gene in the T3Cl-2 Friend leukemia cell line. J. Biol. Chem. **266:**6952–6956.
27. **Laux, G., B. Adam, L. J. Strobl, and F. Moreau-Gachelin.** 1994. The Spi-1/PU.1 and Spi-B ets family transcription factors and the recombination signal binding protein $RBP\text{-}J_k$ interact with an Epstein-Barr virus nuclear antigen 2 responsive cis-element. EMBO J. **13:**5624–5632.
28. **Li, J.-P., A. 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 (London) **343:**762–764.
29. **Linemeyer, D. L., S. K. Ruscetti, E. M. Scolnick, L. H. Evans, and P. H. Duesberg.** 1981. Biological activity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus DNA. Proc. Natl. Acad. Sci. USA **78:**1401–1405.
30. **Mager, D. L., T. W. Mak, and A. Bernstein.** 1981. Quantitative colony method for tumorigenic cells transformed by two distinct strains of Friend leukemia virus. Proc. Natl. Acad. Sci. USA **78:**1703–1707.
31. **Mitjavila, M. T., J. P. Le Couedic, N. Casadevall, S. Navarro, J. L. Villeval, A. Dubart, and W. Vainchenker.** 1991. Autocrine stimulation by erythropoietin and autonomous growth of human erythroid leukemic cells *in vitro*. J. Clin. Invest. **88:**789–797.
32. **Moreau-Gachelin, F.** 1994. Spi-1/PU.1: an oncogene of the Ets family. Biochim. Biophys. Acta **1198:**149–163.
33. **Moreau-Gachelin, F., D. Ray, N. J. de Both, J. M. van der Feltz, P. Tambourin, and A. Tavitian.** 1990. *Spi-1* oncogene activation in Rauscher and Friend murine virus-induced acute erythroleukemias. Leukemia **4:**20–23.
34. **Moreau-Gachelin, F., D. Ray, M. G. Mattei, P. Tambourin, and A. Tavitian.** 1989. The putative oncogene *spi-1*: murine chromosomal localization and transcriptional activation in murine acute erythroleukemias. Oncogene **4:**1449–1456.
35. **Moreau-Gachelin, F., A. Tavitian, and P. Tambourin.** 1988. Spi-1 is a putative oncogene in virally induced murine erythroleukemia. Nature (London) **331:**277–280.
36. **Moulton, K. S., K. Semple, H. Wu, and C. K. Glass.** 1994. Cell-specific expression of the macrophage scavenger receptor gene is dependent on PU.1 and a composite AP-1/ets motif. Mol. Cell. Biol. **14:**4408–4418.
37. **Munroe, D. G., J. W. Peacock, and S. Benchimol.** 1990. Inactivation of the cellular p53 gene is a common feature of Friend virus-induced erythroleukemia: relationship of inactivation to dominant transforming alleles. Mol. Cell. Biol. **10:**3307–3313.
38. **Munroe, D. G., B. Rovinski, A. Bernstein, and S. Benchimol.** 1988. Loss of a highly conserved domain on p53 as a result of gene deletion during Friend virus-induced erythroleukemia. Oncogene **2:**621–624.
39. **Nelsen, B., G. Tian, B. Erman, J. Gregoire, R. Maki, B. Graves, and R. Sen.**

1993. Regulation of lymphoid-specific immunoglobulin μ heavy chain gene enhancer by ETS-domain proteins. Science **261:**82–86.
40. **Pahl, H. L., R. J. Scheibe, D. E. Zhang, H. M. Chen, D. Galson, R. A. Maki, and D. G. Tenen.** 1993. The proto-oncogene PU.1 regulates expression of the myeloid-specific CD11b promoter. J. Biol. Chem. **268:**5014–5020.
41. **Palacios, R., and M. Steinmetz.** 1985. IL-3-dependent mouse clones that express B-220 surface antigen, contain Ig genes in germ-line configuration, and generate B lymphocytes *in vivo*. Cell **41:**727–734.
42. **Paul, R., S. Schuetze, S. L. Kozak, and D. Kabat.** 1989. A common site for immortalizing proviral integrations in Friend erythroleukemia: molecular cloning and characterization. J. Virol. **63:**4958–4961.
43. **Perez, C., E. Coeffier, F. Moreau-Gachelin, J. Wietzerbin, and P. D. Benech.** 1994. Involvement of the transcription factor PU.1/Spi-1 in myeloid cell-restricted expression of an interferon-inducible gene encoding the human high-affinity Fcγ receptor. Mol. Cell. Biol. **14:**5023–5031.
44. **Peters, L. L., N. C. Andrews, E. M. Eicher, M. B. Davidson, S. H. Orkin, and S. E. Lux.** 1993. Mouse microcytic anaemia caused by a defect in the gene encoding the globin enhancer-binding protein NF-E2. Nature (London) **362:**768–770.
45. **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 (London) **349:**257–260.
46. **Pongubala, J. M. R., S. Nagulapalli, M. J. Klemz, S. R. McKercher, R. A. Maki, and M. L. Atchinson.** 1992. PU.1 recruits a second nuclear factor to a site important for immunoglobulin κ 3′ enhancer activity. Mol. Cell. Biol. **12:**368–378.
47. **Pongubala, J. M. R., C. Van Beveren, S. Nagulapalli, M. J. Klemz, S. R. McKercher, R. A. Maki, and M. L. Atchinson.** 1993. Effect of PU.1 phosphorylation on interaction with NF-EM5 and transcriptional activation. Science **259:**1622–1625.
48. **Ray, D., R. Bosselut, J. Ghysdael, M. G. Mattei, A. Tavitian, and F. Moreau-Gachelin.** 1992. Characterization of Spi-B, a transcription factor related to the putative oncoprotein Spi-1/PU.1. Mol. Cell. Biol. **12:**4297–4304.
49. **Ray-Gallet, D., C. Mao, A. Tavitian, and F. Moreau-Gachelin.** 1995. DNA binding specificities of Spi-1/PU.1 and Spi-B transcription factors and identification of a Spi-1/Spi-B binding site in the c-fes/c-fps promoter. Oncogene **11:**303–313.
50. **Rivera, R. R., M. H. Stuiver, R. Steenbergen, and C. Murre.** 1993. Ets proteins: new factors that regulate immunoglobulin heavy-chain gene expression. Mol. Cell. Biol. **13:**7163–7169.
51. **Romeo, P. H., M. H. Prandini, V. Joulin, V. Mignotte, M. Prenant, W. Vainchenker, G. Marguerie, and G. Uzan.** 1990. Megacaryocytic and erythrocytic lineages share specific transcription factors. Nature (London) **344:** 447–449.
52. **Sawyers, C. L., C. T. Denny, and O. N. Witte.** 1991. Leukemia and the disruption of normal hematopoiesis. Cell **64:**337–350.
53. **Schroeder, C., L. Gibson, M. Zenke, and H. Beug.** 1992. Modulation of normal erythroid differentiation by the endogenous thyroid hormone and retinoic acid receptors: a possible target for v-erbA oncogene action. Oncogene **7:**217–227.
54. **Schuetze, S., R. Paul, B. C. Gliniak, and D. Kabat.** 1992. Role of the PU.1 transcription factor in controlling differentiation of Friend erythroleukemia cells. Mol. Cell. Biol. **12:**2967–2975.
55. **Schuetze, S., P. E. Stenberg, and D. Kabat.** 1993. The Ets-related transcription factor PU.1 immortalizes erythroblasts. Mol. Cell. Biol. **13:**5670–5678.
56. **Schwarzenbach, H., J. W. Newell, and P. Matthias.** 1995. Involvement of the Ets family factor PU.1 in the activation of immunoglobulin promoters. J. Biol. Chem. **270:**898–907.
57. **Scott, E. W., M. C. Simon, J. Anastasi, and H. Singh.** 1994. Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages. Science **265:**1573–1577.
58. **Shin, M. K., and M. E. Koshland.** 1993. Ets-related protein PU.1 regulates expression of the immunoglobulin J-chain gene through a novel Ets-binding element. Genes Dev. **7:**2006–2015.
59. **Tambourin, P. E., N. Casadevall, J. Choppin, C. Lacombe, J. M. Heard, S. Fichelson, F. Wendling, and B. Varet.** 1983. Production of erythropoietin-like activity by a murine erythroleukemia cell line. Proc. Natl. Acad. Sci. USA **80:**6269–6273.
60. **Tsai, S. F., D. I. K. Martin, L. I. Zon, A. D. D'Andrea, G. W. Gordon, and S. H. Orkin.** 1989. Cloning of cDNA for the major DNA-binding protein of the erythroid lineage through expression in mammalian cells. Nature (London) **339:**446–451.
61. **Villeval, J. L., M. T. Mitjavila, I. Dusanter-Fourt, F. Wendling, P. Mayeux, and W. Vainchenker.** 1994. Autocrine stimulation by erythropoietin (EPO) requires EPO secretion. Blood **84:**2649–2662.
62. **Wendling, F., F. Moreau-Gachelin, and P. Tambourin.** 1981. Emergence of tumorigenic cells during the course of Friend virus leukemias. Proc. Natl. Acad. Sci. USA **78:**3614–3618.
63. **Youssoufian, H., L. Zon, S. H. Orkin, A. D. d'Andrea, and H. F. Lodish.** 1990. Structure and transcription of the mouse erythropoietin receptor gene. Mol. Cell. Biol. **10:**3675–3682.
64. **Zhang, D., J. Hetherington, H. Chen, and D. G. Tenen.** 1994. The macrophage transcription factor PU.1 directs tissue-specific expression of the macrophage colony-stimulating factor receptor. Mol. Cell. Biol. **14:**373–381.


RP
LF
a
RP
LF
b
c
d
R
AE
PN
BE
e
AE
BE
f
g
h