Differentiation Arrest and Stromal Cell-Independent Growth of Murine Erythroleukemia Cells Are Associated with Elevated Expression of *ets*-Related Genes but Not with Mutation of *p53*

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The ELM erythroleukemia is novel in that long-term survival of leukemic cells in culture (ELM-D cells) is dependent on contact with a bone marrow-derived stromal feeder cell layer. However, a number of stroma-independent (ELM-I) mutants that vary in their ability to differentiate *in vitro* in response to erythropoietin and interleukin-3 have been derived. We have attempted to define the genetic changes responsible for these different phenotypes. At the *p53* locus in the primary leukemic cells, one copy of the gene has been lost whereas the other contains an 18-bp deletion, implicating its mutation as an early step in the development of the leukemia. Changes in *ets* gene expression have also been found. The *Fli-1* gene region is rearranged in the primary tumor because of the insertion of a retrovirus inserted upstream of one *Fli-1* allele, but this does not result in *Fli-1* gene activation in any of the ELM-D or ELM-I cell lines except one. It seems significant that this line is the only one to have lost the ability to differentiate in response to erythropoietin. In addition, up-regulation of *erg* is associated with stromal cell-independent growth, since all ELM-I mutants have moderate levels of *erg* mRNA, whereas only low or undetectable levels are found in primary leukemic cells *in vivo* or in ELM-D cells *in vitro*. This up-regulation of *erg* mRNA seems to be important for stromal cell-independent growth, since ELM-D cells show elevated expression of the *erg* gene after separation from stromal cells. This seems to be made permanent in ELM-I mutants, since they do not down-regulate *erg* mRNA when grown in contact with stromal cells. We therefore propose that *ets* family members regulate both the survival and differentiation of erythroid cells.

Direct contact with stromal cells plays a crucial part in the development, differentiation, and maintenance of many cell types. For instance, erythropoiesis occurs in specific zones, or microenvironments, within the hematopoietic tissues, and the proliferation of erythroid progenitors and their response to erythropoietin and other growth factors are dependent on cell-cell interactions with neighboring hematopoietic and stromal cells and components of the extracellular matrix, both *in vivo* (6, 8) and *in vitro* (reviewed in references 13 and 45). The molecular aspects of these contacts have not been well defined, although recently the importance of the binding of the c-Kit receptor with stromal cell-produced stem cell factor (SCF) has been identified, with the *Steel* and *W* mutant mice, which are defective for SCF and c-Kit, respectively, demonstrating the pleiotropic effects loss of this interaction can cause (59 and references therein). These mice are often severely anemic, implicating SCF/c-Kit in the normal development of erythroid cells, and this has been confirmed in colony-forming assays with purified SCF and neutralizing antibodies to c-Kit (9, 40, 41).

Erythroleukemias are rare in humans, but several murine models in which erythroid progenitors lose their normal growth factor requirements, differentiation capacity, and their dependence for the stromal microenvironment (for a recent review, see reference 4) exist. Infection of susceptible mice with the Friend virus causes a rapid increase in the numbers of erythroid progenitors, from which arise rare

leukemic clones capable of tumor formation when transplanted into other mice, including the SCF-defective *Steel* mutants (30). These cells are able to grow in semisolid or liquid medium *in vitro* and have also lost their ability to differentiate in response to physiological inducers, such as erythropoietin (EPO). Friend virus is a complex of two viral particles: a replication-defective spleen focus-forming virus (SFFV), responsible for the initial expansion of erythroid progenitors (27), and a replication-competent Friend murine leukemia virus (F-MuLV) (31). F-MuLV alone can induce similar neoplasms, but only in newborn mice from susceptible strains.

Extensive studies of these erythroleukemias have identified three cellular genes, namely, *p53* and the *ets*-related genes *Fli-1* and *PU-1*, which in addition to the constitutive activation of the EPO receptor by virus-encoded proteins, are central to progression to the leukemic state (for a review, see reference 4). Loss of function mutations in the *p53* tumor suppressor gene are present in most, if not all, such malignancies (37). The role of wild-type *p53* is not fully understood, but it is now thought to be a transcription factor that is able to exert a negative effect on cell growth in many different cell types (1, 10, 14) and induce apoptosis (24, 49, 62) in response to DNA damage (26). The normal *p53* gene can also induce the differentiation of K562 and Friend cells along the erythroid pathway (17, 24). Thus, mutation of *p53* in erythroid progenitors may affect their normal development in a number of ways. *Fli-1* and *PU-1* are both members of the *Ets* family of transcription factors, which includes

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c-Ets-1, c-Ets-2, Elk, Elf, and Erg, all of which show considerable homology over their DNA-binding domains (reviewed in reference 29). *PU-1* is activated by retroviral insertion in almost all erythroleukemias arising after SFFV or Rauscher virus infection (33–35), whereas *Fli-1* is similarly deregulated in 75%, or more, of the erythroleukemias induced by F-MuLV (5, 52). Most (67%) of the leukemic clones induced with another MuLV (Cas-Br-E) also contain retroviral insertions at the *Fli-1* locus (7). Furthermore, the human homolog of *Fli-1* has been implicated in the progression of Ewing's sarcoma, since a chromosomal translocation results in the DNA-binding domain of *Fli-1* substituting a putative RNA-binding domain in a ubiquitously expressed protein called EWS (12). However, the role of these genetic changes in tumor progression is unclear, although there is some evidence which implies that *PU-1* is involved in blocking erythroid differentiation (51).

Recently, a novel murine erythroleukemia, called ELM, that differs from the Friend virus-induced malignancies in a number of respects has been reported (20). The original tumor can be passaged intrasplenically in vivo but will only grow in vitro in contact with primary bone marrow-derived stromal cells (or bone marrow-derived stromal cell lines, such as MS-5) (21, 22). Moreover, such stroma-dependent cell lines still differentiate in response to EPO. We have recently shown that when these cells are separated from the stroma they undergo induced cell death, an event that can be delayed, but not prevented, by soluble growth factors such as granulocyte macrophage colony-stimulating factor (GM-CSF), interleukin-3 (IL-3), and SCF, presented either alone or in combination. Long-term growth and viability require intimate contact with the stroma. An important constituent of the stromal microenvironment in these cocultures is the transmembrane (TM) form of SCF, since anti-SCF or anti-Kit antibodies dramatically reduce the growth of ELM cells on a stromal cell layer (19). Furthermore, *ST*^d stromal cells which produce SCF in the soluble, but not TM, form (18) are unable to support the long-term growth of ELM cells in vitro (19). These results may reflect different physiological roles for the two forms of SCF, a conclusion which has also been drawn from a number of other studies (15, 18, 53, 55).

We have shown that ELM-D cells can progress to stromal cell independence in culture at a rate of about 10^{-5} , implying that a further mutation(s) is required (19). We demonstrate in this report that such stroma-independent clones (ELM-I) vary in their abilities to differentiate in response to EPO and IL-3. We have attempted to identify the mutations responsible for the ELM leukemia and in particular those involved in the progression to stromal cell independence and differentiation arrest. This should prove interesting for two reasons. Firstly, it may help to define intracellular signalling pathways involved in the stromal cell dependence of erythroid progenitors, and secondly, it should further our understanding of the roles of specific genes in preventing differentiation and induced cell death during the progression of erythroleukemias.

MATERIALS AND METHODS

Cell culture and differentiation induction. All ELM cell lines were maintained in α minimal essential medium (α -MEM) (GIBCO) supplemented with 20% donor horse serum (Sigma), 4 mM glutamine, 100 μ g of streptomycin per ml and 37.5 μ g of penicillin per ml in 5% CO₂ at 37°C. ELM-D cells, and the three clones (ELM-D2, ELM-D3, and ELM-D6) were established on MS-5 feeders (22). On passage, 10% of

the cells from a confluent culture of MS-5 cells was added to 10% of the cells from a confluent ELM-D/MS-5 culture. Visual counting of the two cell types showed that cultures used for RNA and DNA extraction were growing at a ratio of approximately six ELM-D cells per MS-5 cells. F4-12 cells (43) were grown in Eagle's minimal essential medium (GIBCO) plus 10% fetal calf serum (GIBCO) and the same concentrations of glutamine, streptomycin, and penicillin as used above in 5% CO₂ at 37°C.

For differentiation inductions, cells were grown to 90% confluence, harvested, washed in phosphate-buffered saline (PBS), and then seeded at 2.5×10^5 cells per ml in Ham's F12 medium supplemented with the same components added to α -MEM above, plus either no additional growth factors or 2 U of recombinant human EPO (Cilag) per ml and/or 10 U of murine IL-3 (generous gift of J. N. Ihle) per ml. Cultures were left for 3, 5, or 7 days, and the RNA was extracted as described below.

D6 cells were separated from MS-5 stromal cells on the basis of their size difference by using a Beckman Elutriator Rotor system operated according to the manufacturer's instructions. The centrifuge was calibrated by using pure populations of MS-5 or ELM-I-2 cells. D6 cells were then collected from a trypsinized coculture at a flow rate of between 0.3 and 0.4 ml/s, rates at which ELM-I-2, but not MS-5, cells could be collected. Cells were then seeded into fresh medium for 2 to 3 h to allow any stromal cells in the preparation to adhere to the flask; the suspension cells were then harvested, and RNA was extracted as described below.

Animal experimentation. Cells from the ELM tumor were passaged in vivo in C3H mice as described previously (20). Anemia was induced in C3H mice as described elsewhere (51).

Reverse transcription-polymerase chain reaction (RT-PCR). RT was performed as previously described (2). The following oligonucleotides were used to prime the reaction for the specified mRNA: *p53* RT, 5'-CAGCAGAAGGGAC CGGGAGGATTG-3', and *Fli-1* RT, 5'-TCCAGTTTCT TATCTCTTC-3'. The RT reaction for *erg* was primed with oligo(dT) (15-mer) (Promega). A total of 5 μ l from the RT reaction mixture was amplified by PCR with the following primers (sense-strand primers are denoted by U, and antisense primers are denoted by D): *p53* U1, 5'-ACCTCAC TGCATGGACGATCTGTTGCT-3'; *p53* D1, 5'-CTATTA CACATGTACTTGTAGTGGATG-3'; *Fli-1* U1, 5'-GAGAG AAGCTTCGGGTCAATGTGTGGAATATTG-3'; *Fli-1* D1, 5'-GAGAGAGTCGACAGAGTATCCAGTAAAGTGAGA-3'; *erg* U1, 5'-GTGGCCAGATCCAGCTTTGG-3'; and *erg* D1, 5'-CTTGGTCATGATGTTCTTGTC-3'. The reaction mixtures contained 10 μ l of 10 $\times$ reaction buffer (Promega), 8 μ l of 2.5 μ M deoxynucleoside triphosphates, 500 ng of each primer, and 10 U of *Taq* polymerase (Bethesda Research Laboratories [BRL]) in a total volume of 100 μ l, overlaid with 100 μ l of paraffin oil. The reaction mixtures were incubated for 30 cycles at 93°C for 1 min, 55 to 60°C for 1 min, and 72°C for 1 to 2 min. The products were purified on NACS columns (BRL).

The *p53* PCR products were further purified by phenol-chloroform extraction and ethanol precipitation prior to direct sequencing. The *Fli-1* product was cloned by cleavage of the *Hind*III and *Sal*I sites incorporated into the primers, ligation into *Hind*III-*Sal*I-cut, alkaline phosphatase-treated pBluescript II KS+ (Stratagene), and selection on ampicillin-X-Gal plates. The *erg* PCR product was cloned by using the TA cloning kit (Invitrogen) according to the manufacturer's instructions.

PCR from genomic DNA. Amplification of *p53* used 0.5 μ g of genomic DNA as a template and the following two primers: *p53* U2, 5'-TCAGGCTTATGGAACTGTGAGTGGAT-3', and *p53* D2, 5'-CTTCCAGTGTGATGATGGTAAGGATAG-3'. Primer *p53* U2 will not prime the *p53* pseudogene on account of its 3' end terminating in intron 2, thus allowing preferential amplification of the correct region.

For amplification of the non-coding region of *Fli-1*, 1 μ g of C3H spleen genomic DNA was used, and so were the following two primers: *Fli-1* U2, 5'-TGAAGTCACTTCCCAAAATTAG-3', and *Fli-1* D2, 5'-ACCCGGTTACAGCCTGACCTCG-3'. Both PCRs were carried out under the same conditions used in the RT-PCR protocol. Products were purified on NACS columns.

Sequencing. All sequencing was performed with the Sequenase version 2.0 kit according to the recommended protocol (U.S. Biochemicals). To anneal the primer, 2 μ g of the template was boiled for 5 min with 10 ng of primer in 1 $\times$ reaction buffer from the kit, frozen on dry ice, and then slowly thawed to room temperature. The primer used to sequence *p53* PCR product was as follows: *p53* U3, 5'-CTCCTCCCCAGCATCTTATCC-3'. The *erg* plasmids were sequenced by using either the primers used for the PCR or the reverse or -40 primer available from U.S. Biochemicals.

Northern (RNA) blot analysis. Total cellular RNA was isolated by using RNazol B according to the manufacturer's (Cinna/Biotech) instructions. Diethylpyrocarbonate was used to remove RNases from all tubes and water used in the extraction. RNA (20 μ g) was denatured in 1 $\times$ morpholinepropanesulfonic acid (MOPS)-EDTA buffer (20 mM MOPS, 5 mM sodium acetate, 1 mM EDTA [pH 7]), 10% (wt/vol) formaldehyde, and 50% (vol/vol) formamide plus 0.5% bromophenol blue at 65°C for 10 min. Samples were separated on a 1% agarose gel containing 6.5% (wt/vol) formaldehyde in a 1 $\times$ MOPS-EDTA buffer. Gels were washed twice for 20 min (each) in sterile water and then once for 15 min in 20 $\times$ SSC (1 $\times$ SSC is 150 mM NaCl plus 15 mM sodium citrate) prior to being vacuum blotted onto Hybond-N membrane (Amersham) with 20 $\times$ SSC as the transfer buffer. Filters were fixed by being baked for 2 h at 80°C. Prehybridization was performed at 42°C for between 6 and 18 h in 5 $\times$ SSPE (1 $\times$ SSPE is 180 mM NaCl, 10 mM sodium dihydrogen phosphate, and 1 mM EDTA), 5 $\times$ Denhardt's solution (0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone), 0.5% sodium dodecyl sulfate (SDS), 50% (vol/vol) formamide, and 50 ng of denatured herring testis DNA per ml. Hybridization was performed for 18 to 24 h in the same solution containing probes radiolabelled with α -³²P by using a random priming kit (Boehringer Mannheim) and purified from unincorporated nucleotides by gel filtration on a Sephadex G-50 column. Filters were washed twice at room temperature in 2 $\times$ SSPE-0.1% SDS for 10 min, once for 15 min at 65°C in 1 $\times$ SSPE-0.1% SDS, and finally once for 10 min at 65°C in 0.1 $\times$ SSPE-0.1% SDS. Some autoradiographs were scanned by using a Molecular Dynamics ImageQuant version 2.0 computing densitometer operated according to the manufacturer's instructions. The value of the optical density multiplied by the band area (in square millimeters) was derived for each band on the autoradiograph and used for comparative analysis of gene expression between RNA samples.

Probes used in this study include the *Fli-1* and *erg* cDNAs described above, a 1.3-kb *Pst*I fragment of mouse muscle actin from pAM91 (32), a 377-bp *Pst*I fragment spanning the first two exons of the α -globin gene (39), and a 720-bp glyceraldehyde 3-phosphate dehydrogenase (GAPDH)

cDNA fragment generated by RT-PCR by M. Walker (Beatson Institute). Filters were stripped in boiling 0.1% SDS prior to reprobing. Sizes of RNA species were determined by comparison with an RNA ladder (BRL) run next to the samples, which was blotted and fixed as described above, cut off the blot and treated for 15 min in 5% acetic acid, 10 min in 0.5 M sodium acetate-0.04% (wt/vol) methylene blue, and then washed in water to visualize the bands.

Electrophoretic mobility shift assays (EMSAs). Blunt-end 5'-end-labelled double-stranded oligonucleotides were prepared as previously described (44). Nuclear proteins were extracted by a rapid extraction protocol as follows. Cells (5×10^7) were homogenized in hypotonic buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, 1.6 mM MgCl₂, 1 mM CaCl₂) and then spun at 2,000 $\times$ g in an IEC Centra-8R centrifuge at 4°C for 10 min. The nuclear pellet was washed with 10 ml of TMS (0.25 M sucrose, 5 mM MgCl₂, 10 mM Tris-HCl [pH 7.4]) then resuspended gently in 100 μ l of storage buffer (50 mM NaCl, 20 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [HEPES; pH 7.9], 5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, and 20% glycerol). A 1/10 volume of 4 M NaCl was added, mixed, incubated on ice for 20 min, and then spun at 200,000 $\times$ g at 4°C for 1 h in a Beckman benchtop ultracentrifuge, and the supernatant stored at -70°C. Protein extract (5 μ g) was incubated for 10 min on ice with 6 μ g of poly(dI-dC) and any appropriate unlabelled competitor oligonucleotides, made up to a final volume of 15 μ l with storage buffer. A total of 100 pg of labelled oligonucleotide was added to each sample, made up to 20 μ l with storage buffer, and incubated for 20 min at room temperature. Samples were electrophoresed in a 5% polyacrylamide gel in 0.25 $\times$ TBE (prerun for 1 h at 150 V) for 2 h at 150 V at 4°C; the gel was then dried for autoradiography. The oligonucleotides used were E74, GATCTCTAGCTGAATAACCGGAAGTAACTCATCCTAG, and E74(mut), GATCTCTAGCTGAATAACCCAAAGTAACTCATCCTAG (47); PEA3, GATCCTCGAGCAGG AAGTTCGA (58); and PU-1, GATCCATAACCTCTGAAA GAGGAAGTTGGTTAGGT (25).

Southern blot analysis. Genomic DNA was extracted from cells and tissues as previously described (50) and then dialyzed in 1 $\times$ TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA) for 18 h at 4°C. DNA (20 μ g) was digested for 16 h in the appropriate buffer with 50 U of enzyme in a final volume of 100 μ l and then for a further 6 h after addition of a further 20 U of the enzyme. Samples were separated on a 0.9% agarose gel which was then depurinated with a 15-min treatment with 0.2 M HCl, denatured in 1.5 M NaCl-0.5 M NaOH for two spells of 15 min, and then neutralized for 1 h in 3 M NaCl-0.5 M Tris-HCl (pH 7.5), changing the solution twice. Blotting, hybridization, and washing were carried out as for the Northern blots, except formamide was omitted from the prehybridization and hybridization stages which were performed at 65°C. Probes used in this study include the 800-bp *Hinc*II-*Eco*RI fragment; SU-9, a generous gift from E. Rassart (7); and the 5' noncoding region of *Fli-1*, generated by PCR (see above). Radiolabelling was performed as for the Northern blots.

Isolation of lambda phage clones. A genomic library was prepared by ligating ELM-I-1 DNA partially digested with *Sau*3AI into *Bam*HI-digested λ GEM11 as previously described (3). Duplicate lifts were taken from plated phage onto Hybond-C membranes (Amersham) and treated according to the manufacturer's instructions. Filters were hybridized in the same way as Southern blots. Positive clones were picked, replated, and screened again. Single positive clones

FIG. 1. Derivation of the cell lines within the ELM system (see text). RNA and DNA samples for use in this study were taken from all the ELM cell lines, and from early and late passages of the in vivo-passaged ELM tumor cells at the stages labelled IVP ELM Early and IVP ELM Late.

were picked and used to generate large-scale phage lysates from which phage particles were purified by a glycerol step gradient, DNA was prepared, and restriction maps were developed (50). Relevant fragments were excised from the DNA, purified on NACS columns (BRL) and subcloned into pBluescript II KS+ (Stratagene) for sequencing.

RESULTS

Generation of stroma-independent cell lines. The origin of the ELM cell lines is depicted in Fig. 1. The ELM leukemia originated after a long latency in a C3H mouse and was found to consist of erythroid, rather than myeloid, cells (20). Initially, inoculation of 10^6 primary leukemic spleen cells produced splenomegaly and death after about 2 months, whereas subsequent passage in vivo produced the same degree of splenomegaly within 2 weeks, indicating selection for more rapidly growing leukemic clones. The ELM-D cell line was derived by culturing cells from the ELM tumor initially on primary bone marrow-derived stromal cells and then on a bone marrow-derived stromal cell line, MS-5 (21). This cell line is a fairly heterogeneous population of ELM cells, containing cells that have lost stromal cell dependence (42). Thus, several stromal cell-dependent clones were derived from this original ELM-D cell line, called ELM-D (clones 2, 3, and 6) and are referred to here as D2, D3, and D6. ELM-I-1 was established from the nonadherent cell population from a dense culture of ELM-D cells grown on feeders for 7 weeks (21). We have derived 14 other independent cell lines. ELM-I-2 arose after ELM tumor cells were cultured directly in the absence of feeders. The remaining ELM-I cell lines were independently derived from D6 by selection for growth in suspension in the presence (11 cell lines) or absence (I-5 and I-6) of GM-CSF. However, con-

tinued growth proved not to be dependent on GM-CSF for any of the cell lines once feeder dependence was lost (19).

Differentiation capability. During the course of testing the tumorigenicity of the various ELM cell lines in vivo, we noticed that mice injected with I-1 died more rapidly and appeared more anemic than mice injected with other ELM cell lines. We therefore decided to test their differentiation capacity in response to EPO and IL-3. Cells were grown to near confluence, harvested, and reseeded at 2.5×10^5 cells per ml in assay medium containing 2 U of EPO per ml, and/or 10 U of IL-3 per ml. After 72 h, RNA was extracted and analyzed by Northern blotting. Probing with a mouse α -globin gene fragment demonstrated that I-1 differentiated only slightly in response to EPO and undetectably in IL-3, whereas the other stroma-independent cell lines and D6 showed extensive globin induction with EPO alone or in combination with IL-3 (Fig. 2). As a control, F4-12 erythroleukemic cells, which arose from SFFV infection and are known to be blocked in differentiation (43), show the same insensitivity to EPO and IL-3 as that seen in the I-1 line. Identical results were seen with all cell lines after 5- and 7-day treatments with growth factors.

Thus, a number of cell lines have been derived from the same ELM tumor that show marked differences in (i) their stromal cell dependence and (ii) their ability to differentiate in vitro. In the case of the I-1 cell line, the differentiation block may be responsible for the severe anemia induced in the host animals. We have therefore attempted to define the mutations responsible for these varied phenotypes.

p53 status. Wild-type p53 has been implicated in the induction of apoptosis (24, 49, 62) and erythroid differentiation (17, 24), and mutation of p53 is a common event in the progression of virally induced erythroleukemias (4, 37). Since D6 cells undergo induced cell death after separation

FIG. 2. Expression of α -globin as a marker for erythroid differentiation in erythroleukemia cell lines treated with 2 U of EPO per ml and/or 10 U of IL-3 per ml for 3 days. Total cellular RNA (20 μ g) was electrophoresed and blotted as described in Materials and Methods and hybridized with a 32 P-labelled α -globin probe (39). Equal loading of the samples was checked by stripping the filters and hybridizing with a 32 P-labelled GAPDH cDNA probe.

from the stromal cell layer (19), and the ELM cell lines vary in their ability to differentiate, we reasoned that mutation of *p53* may be responsible for differences observed between ELM cell lines.

Strong nuclear staining was observed in the D6 cells by using anti-p53 antibody PAb246 (data not shown). Although this recognizes the wild-type p53 conformation, such strong staining suggested the protein is highly abundant, a characteristic of mutant p53. Direct sequencing of PCR products generated from D6 mRNA, identified an 18-bp in-frame deletion in the coding region of the *p53* mRNA that results in the loss of six amino acids, from residue 206 to 211, inclusive (Fig. 3A). Adjacent to each breakpoint is the sequence ACAG; short repeats are a common feature in deletion mutations and are probably important in the mechanism of deletion (23, 38). Since a *Dra*III restriction site is lost as a consequence of the deletion, this was exploited diagnostically to check the other ELM cells for the mutation and to assess whether a normal allele was present. Primers annealing to *p53* sequences either side of the deletion were used to generate a 2.3-kb fragment from the DNA of cells in the ELM system which was then digested with *Dra*III, electrophoresed on a 1% agarose gel, and blotted onto a nylon membrane. Hybridization with the 2.3-kb PCR product, radiolabelled with 32 P, shows that none of the products from any of the ELM cells was cleaved by *Dra*III, whilst the same fragment from normal spleen DNA digested completely to the expected 1.8- and 0.5-kb products (Fig. 3B). The small amount of D6 product that has digested is due to contaminating feeder cell DNA (~15%) in the D6 genomic DNA preparation. In a control experiment using an equimolar mixture of spleen and ELM PCR products, 50% digested as expected (data not shown). These results demonstrate that the deletion is present in the ELM tumor in vivo and the cell lines derived from it, with the normal allele being absent. This has also been confirmed by probing Southern blots of *Dra*III-digested genomic DNA with *p53* cDNA fragments.

FIG. 3. (A) Direct sequencing of *p53* PCR products demonstrating the presence of an 18 bp deletion in the D6 *p53* gene. RT-PCR was performed on RNA extracted from D6 cells and normal C3H spleen by using the primer *p53* RT for the RT step and primers *p53* U1 and *p53* D1 for the PCR (see Materials and Methods). The products were sequenced by using the *p53* U3 primer. The nucleotide and amino acid sequence of the two products is shown. The ACAG repeats adjacent to the breakpoint are boxed, and the *Dra*III restriction endonuclease site (CACNNGTG), present only in the spleen sequence, is shown. (B) *Dra*III digestion of PCR products amplified from genomic DNA with primers *p53* U2 and *p53* D2. Products from the PCR were gel purified, incubated in the presence (+) or absence (-) of *Dra*III, electrophoresed, blotted onto a nylon membrane, and then hybridized with a 32 P-labelled *p53* U2/*p53* D2 PCR fragment. The map shows the positions of the two primers and the *Dra*III site with relation to the exons (coding, filled boxes; noncoding, empty boxes) of the *p53* gene.

Scanning densitometry of these blots, with the *p53* pseudogene as an internal control, reveals that the ELM cells are hemizygous for the mutant allele (data not shown). Thus, the mutation of *p53* found in these cells is not sufficient to prevent D6 cells from undergoing induced cell death when separated from the feeders or to block differentiation induction.

Expression of Ets family members. We next examined the ELM system for expression of Ets-related proteins. Initially this was done by using known Ets binding sites as oligonucleotide probes in EMSAs with nuclear extract from the I-1 and I-2 cell lines. I-1 was selected because of its phenotypic similarity to Friend erythroleukemia cell lines, all of which express PU-1 or Fli-1; I-2 was used as a comparison cell line to look for correlations between differences in Ets protein expression and differentiation inducibility. No binding was detected in these extracts to the PEA3 oligonucleotide, known to bind various members of the Ets family in *in vitro* assays, such as c-Ets-1, c-Ets-2, Elk-1, and the PEA3 protein (46, 58, 60), or with the PU-1-specific sequence (25) (data not shown). However, nuclear proteins from I-1 and I-2 did give a similar pattern of one major and two minor specific bands in EMSA experiments with the E74 oligonucleotide (data not shown), originally identified as a consensus binding site for the *Drosophila* E74 protein (57), but which is also able to bind to a subset of the known murine Ets proteins, such as Fli-1 (our own observations, data not shown) and the highly homologous Erg protein (47). The E74 binding activity in the I-1 and I-2 extracts was dependent on the integrity of the GGAA motif in the oligonucleotide and could be inhibited inefficiently by other known Ets binding sites. This presented Erg and Fli-1 as candidates to be examined in the ELM system. However, the E74 oligonucleotide must also bind to other (unknown) murine Ets proteins, since it gives specific complexes in EMSAs with nuclear extracts from cells that do not express *Fli-1* or *erg* mRNAs (unpublished data).

Next, we attempted to determine which of the known *ets* genes were being expressed in I-1 and I-2 cells by Northern transfer experiments. Consistent with the EMSAs, no *PU-1*, *ets-1*, or *ets-2* mRNAs were detectable in any of the ELM cells by Northern blotting, although PCR analysis suggests that *PU-1* mRNA is present at the very low levels characteristic of normal erythroid cells (51, 56) (data not shown). In parallel experiments, the F4-12 cell line was found to have a very high level of *PU-1* mRNA. However, *Fli-1* mRNA was found to be highly abundant in the differentiation-resistant I-1 cell line (Fig. 4), in contrast to all other ELM-I clones (the data for three of which are shown in Fig. 4). The low level of *Fli-1* mRNA observed in both the early and late *in vivo*-passaged tumor cells is almost certainly due to a small number of contaminating normal spleen cells. D6 cells cleanly separated from stroma cells by elutriation do not contain detectable *Fli-1* mRNA (Fig. 4). The *Fli-1* mRNA is more abundant in a normal spleen than in an erythropoietically active spleen taken from a phenylhydrazine-treated anemic mouse, suggesting that *Fli-1* is only expressed at low levels, if at all, in normal differentiating erythroid cells (Fig. 4). These results therefore suggest that ectopic *Fli-1* expression is responsible for the block in erythroid differentiation in I-1.

We also tested for the expression of *erg* mRNA in cells from the ELM system. A murine *erg* cDNA fragment was generated by RT-PCR from spleen mRNA, employing primers designed from regions of the human *erg* sequence (48) conserved between species in other *ets* genes. The identity

FIG. 4. Expression of *Fli-1* mRNA in the ELM system. Total cellular RNA (20 μ g) was electrophoresed, blotted, and hybridized to a 32 P-labelled full-length *Fli-1* cDNA probe (Materials and Methods). The tissues and cell lines examined were (right to left) normal C3H spleen, anemic spleen from a phenylhydrazine-treated C3H mouse, late and early passages of *in vivo* ELM tumor cells, MS-5 stroma cells, ELM-D6 cells purified by elutriation, and four ELM-I clones (I-1, I-2, I-5, and I-6). Exposure time was 72 h. The filters were stripped and rehybridized with an actin cDNA probe (32) to assess for equal loading of the samples. The 2.4-, 4.4-, 7.5-, and 9.5-kb markers represent the positions of electrophoresis of RNA markers.

of the cloned fragment was confirmed by comparing its amino acid sequence to human Erg (48): only one difference was found in the Ets domain (data not shown). This cDNA was then used to probe Northern blots of RNAs from the ELM system. These showed the *erg* mRNA to be barely detectable in the ELM tumor *in vivo* or in the D6 cell line growing on MS-5 stroma cells (comprising about 6:1, ELM-D to MS-5 cells) (Fig. 5A). *erg* mRNA is also barely detectable in two other independently derived ELM-D clones, D2 and D3. In contrast, it was relatively abundant in all the stroma-independent cell lines tested (Fig. 5A and B). Once differences in the loading have been normalized by using the actin control, scanning densitometry reveals that the ELM-I cell lines express *erg* at levels on average approximately 20-fold higher (ranging between 4- and 48-fold) than the ELM-D clones growing on stroma cells (Fig. 1). Thus, there appears to be a strong correlation between expression of the *erg* gene and stroma-independent growth. To determine whether this up-regulation of *erg* mRNA is due to epigenetic adaptation to loss of stroma cell contact or is only found in truly stroma cell-independent mutants, pure populations of D6 cells were obtained, either by taking D6 cells that had detached from the stroma cells at high density or by cleanly separating attached D6 cells from MS-5 cells by trypsinization, and following that with size fractionation by centrifugal elutriation and then short-term culture (~2 to 3 h). Subsequent RNA analysis revealed four- to ninefold-higher levels of *erg* mRNA in the pure populations of D6 cells compared with that in the 6:1 D6-MS-5 cocultures, which is much too large to be accounted for by dilution of the *erg* mRNA level by the 15% MS-5 stroma cells in the D6-MS-5 coculture. This compares with the 14- to 40-fold higher levels in the three ELM-I mutants analyzed at the same time (Fig. 5C). Further experiments confirmed that the pure populations of D6 cells were not capable of long-term growth in the absence of MS-5 stroma cells, but died out within 2 to 3 weeks. These results therefore strongly suggest that *erg* mRNA is up-regulated in response to loss of stroma

cell contact but that this is not sufficient per se to allow the long-term stroma cell-independent growth characteristic of the ELM-I mutants. We have also carried out the converse experiment of growing ELM-I lines (I-2 and I-5) on stroma cells at a 6:1 ratio; although these ELM-I lines do not need stroma cell support for permanent growth, they still attach and grow on stroma cells if provided. The results show

FIG. 5. Expression of the *erg* mRNA in the ELM system. Total cellular RNA (20 μ g) was electrophoresed, blotted, and hybridized to a 32 P-labelled *erg* cDNA probe. Exposure time for all *erg*-hybridized filters was 6 days. (A) Right to left: anemic C3H spleen, normal C3H spleen, early and late passages of in vivo ELM tumor cells (ELM), MS-5, a 6:1 coculture of ELM-D6 cells on MS-5 cells, and four stroma-independent cell lines, I-1, I-2, I-5, and I-6. The figures below the lanes give the relative amounts of *erg* mRNA after correction by densitometry for loading differences based on the levels of actin mRNA. (B) Right to left: 10 ELM-I cell lines that were derived in the presence of GM-CSF (Fig. 1), I-2, I-1, and a 6:1 coculture of ELM-D6-MS-5 cells. Filters were stripped and probed with a 32 P-labelled murine actin cDNA (32) to check the relative loading of the samples and the relative amounts of *erg* mRNA calculated as described for panel A. (C) Right to left: MS-5, a 6:1 coculture of D6-MS-5 cells, ELM-D6 cells present in suspension in dense D6-MS-5 cocultures, pure D6 cells fractionated by elutriation, and three ELM-I clones (I-5, I-6, and one of the GM-CSF-derived ELM-I lines (I-3.2.3)). Relative *erg* mRNA levels, after calibration with the actin control, are given below the lanes. (D) MS-5 cells were mixed with a sixfold excess of either I-2 or I-5 cells, and RNA was extracted immediately or after an incubation period of 72 h, as indicated, during which the two cell types adhered. Pure preparations of I-2 and I-5 RNA were also included on the blot. Relative levels of *erg* mRNA, after calibration with the actin control, are given below the lanes.

clearly that both ELM-I lines maintain elevated levels of *erg* mRNA even when grown on stroma cells, unlike ELM-D lines (Fig. 5D). This implies that the mutation(s) responsible for creating the ELM-I lines directly or indirectly prevents the down-regulation of *erg* mRNA by stroma cell contact.

Characterization of a rearrangement at the *Fli-1* locus. Although no rearrangement of the restriction map of the *erg*

FIG. 6. Southern blot analysis showing a rearrangement at the *Fli-1* locus in I-1 cells. The Southern blot was prepared as described in Materials and Methods by using DNA from C3H spleen (lanes labelled S) or I-1 and then hybridized with 32 P-labelled SU-9 probe (7) (exposure time, 72 h) (A) or with the 5' noncoding region of the *Fli-1* gene (exposure time, 96 h) (B). (C) Restriction map of the normal *Fli-1* locus. The positions of the SU-9 probe and the ELM breakpoint are marked with open boxes. Viral integration sites observed in F-MuLV and Cas-Br-E-induced leukemias are indicated by black boxes, along with their orientation (5, 7). Sites for restriction endonucleases *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Nsi*I (N), *Pst*I (P), and *Xba*I (X) are shown.

gene region could be detected by using the available partial *erg* cDNA probe, with the SU-9 probe from the *Fli-1* locus (7) (Fig. 6C), an additional band was seen in the I-1 DNA digested with *Hind*III or *Xba*I (Fig. 6A), implying that one *Fli-1* allele has been rearranged with the breakpoint between the *Eco*RI and *Hind*III sites (Fig. 6C). When the same filter was probed with the 5' noncoding region of *Fli-1* (generated by PCR from spleen DNA), an additional band was also observed in the I-1 DNA digested with *Bam*HI or *Pst*I (Fig. 6B), placing this region of the gene very close to the breakpoint.

To elucidate the nature of the mutation at the *Fli-1* locus, we constructed a genomic library from I-1 DNA partially digested with *Sau*3AI and ligated into λ GEM11 (see Materials and Methods). This was screened with SU-9, and two positive clones were isolated and characterized. By restriction mapping, clone A was shown to be derived from the rearranged allele and contained 10.5 kb from the *Fli-1* locus and 2 kb from the other side of the breakpoint (Fig. 7A). (Clone B did not span the breakpoint or hybridize to the *Fli-1* cDNA and was derived from 15 kb of the large first intron of

FIG. 7. (A) Restriction map of lambda phage clone A derived from the rearranged *Fli-1* allele. Restriction endonuclease sites for *Bam*HI (B), *Eco*RI (E), *Hind*III (H), *Nsi*I (N), *Pst*I (P), and *Xba*I (X) are shown. The black boxes represent the arms of the lambda clone. Probe BP1 is marked with an open box. (B) Position of the long terminal repeat and part of the *gag* gene in clone A determined by sequencing. The location of the first exon of the *Fli-1* gene is indicated by the black box; the arrow shows the direction of transcription. The position of the lambda phage clone B insert, which overlaps the clone A insert and does not hybridize to the *Fli-1* cDNA, is also marked.

Fli-1 [Fig. 7B].) Fragments excised from the clone A insert were subcloned and sequenced. The sequence generated from the portion inserted into the *Fli-1* locus was almost identical to the *gag* gene and long terminal repeat of F-MuLV (Fig. 7B). Probing a Southern blot with the *env* gene of F-MuLV confirmed the presence of exogenous viral DNA in the I-1 cell line (data not shown). Sequence data from the *Fli-1* locus showed that the first exon, which contains the 5' noncoding region and the first six amino acids, is about 900 bp from the insertion site of the retrovirus and is in the opposite orientation (Fig. 7B).

Surprisingly, when a Southern blot of *Nsi*I-cleaved DNA from other ELM cells was probed with the *Bam*HI-*Pst*I fragment from clone A (BP1 [Fig. 7A]), they all were found to contain the rearranged *Fli-1* locus (Fig. 8). Thus, it appears that insertion at the *Fli-1* locus was an important event in the evolution of this erythroleukemia, but what remains a puzzle is why *Fli-1* expression is restricted to the ELM-I-1 cell line. Possible explanations of this are discussed below.

FIG. 8. Southern blot analysis of *Nsi*I-digested DNA samples showing the presence of the retroviral insertion at *Fli-1* throughout the ELM system. The blot was hybridized with 32 P-labelled BP1 probe (Fig. 7A). Exposure time was 72 h. The cells and tissues tested were normal spleen (S), in vivo-passaged ELM tumor cells (ELM), and ELM cell lines D, D6, I-1, I-2, I-5, and I-6. The increased intensity of the normal band relative to the additional band in lanes 3 and 4 arises as a result of contaminating feeder cell DNA.

DISCUSSION

In this article, we describe studies of a novel erythroleukemia cell system that is distinct from Friend erythroleukemia in a number of respects. Like the Friend virus-induced malignancies, cells from the ELM tumor are transplantable and rapidly develop leukemia in recipient mice (20). However, growth of these cells in vitro requires direct contact with stromal feeder cells (19, 21), whilst exposure to EPO is able to induce differentiation. Separation from the feeders induces cell death, an event that can be delayed by the addition of growth factors such as GM-CSF, IL-3, and SCF. The interaction between TM SCF on MS-5 cells and c-Kit expressed on the leukemic cells is necessary for their optimum growth in vitro (19), but it seems possible that other components of the stroma are required to maintain the long-term viability of the leukemic cells. Thus, certain characteristics of normal erythroid progenitor cells are still retained in these leukemic cells. Fifteen individual clones that have lost their stromal cell dependence have been established in culture by four different selection procedures, and these cells show variation with respect to their ability to differentiate. In particular, I-1 has lost the capability to differentiate both in vivo and in vitro in response to EPO and IL-3 and thus appears to be phenotypically equivalent to most Friend cell lines. This phenotype is associated with specific genetic changes in Friend cells: mutation of the *p53* suppressor gene and deregulated expression of either the *Fli-1* or the *PU-1* gene, both of which are members of the *ets* transcription factor family (4). We have exploited the progressive loss of normal progenitor characteristics in the ELM system in an attempt to define a role for these mutational events in generating erythroleukemias and overcoming stromal cell dependence.

Exogenous overexpression of wild-type *p53* has been implicated in the suppression of cell growth in several cell types (1, 10, 14), in the induction of apoptosis (24, 49, 62), and in the stimulation of erythroid differentiation of K562 and Friend cells (17, 24). However, ELM-D cells have a mutation in the *p53* gene, which is accompanied by loss of the normal allele, but are still able to undergo induced cell death when separated from the stromal cells and will differentiate in response to erythropoietin. The same mutation is present in ELM tumor cells (and, thus, in all cells of the ELM system) and was probably a key event in the development of the original malignancy. Recent work suggests that *p53* blocks the division of cells that have sustained DNA damage (reviewed in reference 26); therefore, mutation of *p53* in an erythroid progenitor cell would be a favorable event in tumor progression which would allow the accumulation of other mutations (28, 61) but would still permit differentiation and induced cell death. The normal development and control of erythropoiesis observed in mice lacking any *p53* protein supports this theory (16), whilst the apoptosis induced in Friend erythroleukemia cells by reconstituting wild-type *p53* expression may be a consequence of DNA damage detection in these cells (24, 49).

The nature of the mutation present in the ELM cells is unusual in that it is an 18-bp deletion in a region of the gene not often targeted by mutational events (11). Interestingly, this deletion results in the removal of part of the epitope for the mutant-specific PAb240 anti-*p53* antibody (54), which becomes exposed by point mutations in other regions of the protein. This implies that conformational changes of this epitope are associated with loss of the *p53* suppressor

function, and it seems likely that the deletion observed in the ELM system would have a similar effect.

Other genetic events in addition to the *p53* mutation were obviously required to generate the ELM erythroleukemia and alter the phenotype of the cell lines in vitro. The other class of mutation(s) identified involves *ets*-related genes, thus extending the findings with various other Friend virus-induced erythroleukemia cell lines selected by growth in suspension (5) or subcutaneous growth (36) in which *Fli-1* or *PU-1* has been found to be activated (5, 33–35). However, a novel feature of our stromal cell-independent ELM cell lines, selected by growth in suspension in various ways, is that they show elevation of *erg* expression, with only one showing activation of *Fli-1*. Moreover, those lines expressing *erg* can still differentiate in response to EPO, whereas the one expressing *Fli-1* cannot. Thus, it seems significant that the ELM suspension line showing the most abnormal characteristics (I-1) expresses *Fli-1* and is phenotypically very similar to previously isolated Friend cell lines induced by F-MuLV (5). Although it is supported by only a single example, this evidence suggests that expression of *Fli-1* might be responsible for the differentiation arrest in I-1, and we are planning to test this hypothesis directly by expressing the *Fli-1* cDNA in cells.

A second novel finding to emerge from the present studies is that activation of *ets* gene expression is a relatively late event in the ELM system associated with the transition to stromal cell-independent growth: *erg* or *Fli-1* expression is barely detectable in stromal cell-dependent ELM-D6 cells or in the ELM tumor maintained in vivo. The molecular mechanisms whereby *erg* and *Fli-1* come to be highly expressed in the stromal cell-independent ELM cell lines are clearly different. *erg* mRNA is up-regulated in response to loss of stroma cell contact, but this is not sufficient by itself to permit long-term survival without stroma cell contact. The fact that *erg* mRNA is not down-regulated in ELM-I mutants grown in contact with stromal cells suggests that ELM-I cells have undergone a second genetic event which directly or indirectly prevents this regulation of *erg* mRNA expression by stromal cells. Such a mutation may be a key event in the development of stromal cell-independent growth. As far as *Fli-1* is concerned, it is intriguing that the ELM tumor cells in vivo and all the ELM cell lines derived from it in vitro contain an inserted retrovirus in one allele of the *Fli-1* locus upstream of the promoter site. The fact that the tumor cells are monoclonal with respect to this insertion implies that it was selected for during tumor development. There are precedents in other systems for such insertions being selected because of activation or inactivation of adjacent genes. However, in the ELM leukemia, inactivation seems unlikely, since we have no evidence for *Fli-1* expression in normal erythroid progenitors. Furthermore, only one allele appears to be affected by mutation in the ELM tumor. There seem to be two plausible interpretations of our data involving gene activation by retroviral insertion. First, *Fli-1* could have been activated in the original tumor but then became inactivated during subsequent passage in vivo, i.e., *Fli-1* expression was required for initiation of the leukemia but not for its subsequent maintenance. However, if this hypothesis is correct, inactivation of *Fli-1* must have occurred very early, since tumor cells obtained from the very first passage of the original leukemic spleen do not express detectable levels of *Fli-1*. An alternative explanation is that the retroviral insertion upstream of the *Fli-1* gene activates another gene nearby. Although *Fli-1* and *erg* are on different chromosomes, *Fli-1* is known to be linked to *c-ets-1* (5), but we

have shown that this is not expressed in the ELM tumor. A further question concerns the mechanism whereby *Fli-1* becomes expressed in I-1. No structural differences have been detected at the *Fli-1* locus between I-1 and other ELM cell lines (data not shown), although we cannot exclude the possibility that small sequence changes may be affecting expression of the *Fli-1* gene. Alternatively, I-1 may express certain transcription factors absent from other ELM cell lines that permit *Fli-1* transcription. We are presently attempting to elucidate these various possibilities by further experiments.

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REFERENCES

- Baker, S. J., S. Markowitz, E. R. Fearon, J. K. V. Wilson, and B. Vogelstein. 1990. Suppression of human colorectal carcinoma cell growth by wild-type *p53*. *Science* 249:912-915.
- Bartek, J., R. Iggo, J. Gannon, and D. P. Lane. 1990. Genetic and immunochemical analysis of mutant *p53* in human breast cancer cell lines. *Oncogene* 5:893-899.
- Bartholomew, C., K. Morishita, D. Askew, A. Buchberg, N. A. Jenkins, N. G. Copeland, and J. L. Ihle. 1989. Retroviral insertions in the *CB-1/Fim-3* common site of integration activate expression of the *Evi-1* gene. *Oncogene* 4:529-534.
- Ben-David, Y., and A. Bernstein. 1991. Friend virus-induced erythroleukemia and the multistage nature of cancer. *Cell* 66:831-834.
- 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.
- Ben-Ishay, Z., and J. M. Yoffey. 1972. Ultrastructural studies of erythroblastic islands of rat bone marrow. II. The resumption of erythropoiesis in erythropoietically depressed rebound marrow. *Lab. Invest.* 26:637-648.
- Bergeron, D., L. Poliquin, C. A. Kozak, and E. Rassart. 1991. Identification of a common viral integration region in Cas-Br-E murine leukemia virus-induced non-T-, non-B-cell lymphomas. *J. Virol.* 65:7-15.
- Bessis, M. 1973. *Living blood cells and their ultrastructure*. Springer-Verlag, New York.
- Broxmeyer, H. E., S. Cooper, L. Lu, G. Hangoc, D. Anderson, D. Cosman, S. D. Lyman, and D. E. Williams. 1991. Effect of murine mast cell growth factor (c-kit proto-oncogene ligand) on colony formation by human marrow hematopoietic progenitor cells. *Blood* 77:2142-2149.
- Chen, P.-L., Y. Chen, R. Bookstein, and W.-H. Lee. 1990. Genetic mechanisms of tumor suppression by the human *p53* gene. *Science* 250:1576-1580.
- de Fromental, C. C., and T. Soussi. 1992. *TP53* tumor suppressor gene: a model for investigating human mutagenesis. *Genes Chrom. Cancer* 4:1-15.
- Delattre, O., J. Zucman, B. Plougastel, C. Desmaza, T. Melot, M. Peter, H. Kovar, I. Joubert, P. de Jong, G. Rouleau, A. Aurias, and G. Thomas. 1992. Gene fusion with an ETS DNA-binding domain caused by chromosome translocation in human tumours. *Nature (London)* 359:162-165.
- Dexter, T. M., L. H. Coutinho, E. Spooncer, C. M. Heyworth, C. P. Daniel, R. Schiro, J. Chang, and T. D. Allen. 1990. Stromal cells in haemopoiesis. *CIBA Found. Symp.* 148:76-95.
- Diller, L., J. Kassel, C. Nelson, M. A. Gryca, G. Litwak, M. Gebhardt, B. Bressac, M. Ozturk, S. J. Baker, B. Vogelstein, and S. H. Friend. 1990. *p53* functions as a cell cycle control protein in osteosarcomas. *Mol. Cell. Biol.* 10:5772-5781.
- Dolci, S., D. E. Williams, M. K. Ernst, J. L. Resnick, C. I. Brannan, L. F. Lock, S. D. Lyman, H. S. Boswell, and P. J. Donovan. 1991. Requirement for mast cell growth factor for primordial germ cell survival in culture. *Nature (London)* 352:809-811.
- Donehower, L. A., M. Harvey, B. L. Slagle, M. J. McArthur, C. A. Montgomery, Jr., J. S. Butel, and A. Bradley. 1992. Mice deficient for *p53* are developmentally normal but susceptible to spontaneous tumours. *Nature (London)* 356:215-221.
- Feinstein, E., R. P. Gale, J. Reed, and E. Canaani. 1992. Expression of the normal *p53* gene induces differentiation of K562 cells. *Oncogene* 7:1853-1857.
- Flanagan, J. G., D. Chan, and P. Leder. 1991. Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the *Sl^d* mutant. *Cell* 64:1025-1035.
- Itoh, K., K. Klingler, C. Laker, U. Just, K. Mannweiler, J. Mori, S.-I. Nishikawa, S. Bittner, L. Fang, C. Wagener, R. J. B. Nibbs, P. R. Harrison, and W. Ostertag. Stromal cells confer stable alterations in growth factor response to murine erythroid precursor cells. Submitted for publication.
- Itoh, K., K. Ono, H. Sawada, H. Tezuka, H. Sakoda, H. Nakane, T. Uchiyama, H. Uchino, and K. J. Mori. 1988. Establishment and characterization of a transplantable erythroblastic leukemia in C3H mice. *Leukemia Res.* 12:471-478.
- Itoh, K., R. Sasaki, K. Ono, H. Tezuka, H. Sakoda, K. Hitomi, K. Nakane, T. Uchiyama, H. Uchino, and K. J. Mori. 1988. Stromal cell dependent growth of the leukemic cells from murine erythroblastic leukemia. *Jpn. J. Cancer Res. (Gann)* 79:931-935.
- Itoh, K., H. Tezuka, H. Sakoda, M. Konno, K. Nagata, T. Uchiyama, H. Uchino, and K. J. Mori. 1989. Reproducible establishment of hemopoietic supportive stromal cell lines from murine bone marrow. *Exp. Hematol.* 17:145-153.
- Jego, N., G. Thomas, and R. Hamelin. 1993. Short direct repeats flanking deletions, and duplicating insertions in *p53* gene in human cancers. *Oncogene* 8:209-213.
- Johnson, P., S. Chung, and S. Benchimol. 1993. Growth suppression of Friend virus-transformed erythroleukemia cells by *p53* protein is accompanied by hemoglobin production and is sensitive to erythropoietin. *Mol. Cell. Biol.* 13:1456-1463.
- 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.
- Lane, D. P. 1992. *p53*, guardian of the genome. *Nature (London)* 358:15-16.
- 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 (London)* 343:762-764.
- Livingstone, L. R., A. White, J. Sprouse, E. Livanos, T. Jacks, and T. D. Tlsty. 1992. Altered cell cycle arrest and gene amplification potential accompany loss of wild-type *p53*. *Cell* 70:923-935.
- Macleod, K., D. Leprince, and D. Stehelin. 1992. The *ets* gene family. *Trends Biochem. Sci.* 17:251-256.
- Mager, D., T. W. Mak, and A. Bernstein. 1980. Friend leukemia virus-transformed cells, unlike normal stem cells, form spleen colonies in *Sl/Sl^d* mice. *Nature (London)* 288:592-594.
- Maisel, J., V. Klement, M. M.-C. Lai, W. Ostertag, and P. Duesberg. 1973. Ribonucleic acid components of murine sarcoma and leukemia viruses. *Proc. Natl. Acad. Sci. USA* 70:3536-3540.
- Minty, A. J., S. Alonso, M. Caravatti, and M. Buckingham. 1982. A fetal skeletal muscle actin mRNA in the mouse and its identity with cardiac actin mRNA. *Cell* 30:185-192.
- Moreau-Gachelin, F., D. Ray, N. J. de Both, M. J. M. van der Feltz, P. Tambourin, and A. Tavitan. 1990. *Spi-1* oncogene activation in Rauscher and Friend murine virus-induced acute erythroleukemias. *Leukemia* 4:20-23.
- Moreau-Gachelin, F., D. Ray, P. Tambourin, A. Tavitan, 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* 61:1166.
35. Moreau-Gachelin, F., A. Tavitan, and P. Tambourin. 1988. *Spi-1* is a putative oncogene in virally induced murine erythroleukaemias. *Nature (London)* 331:277-280.
 36. Moreau-Gachelin, F., F. Wendling, P. Bucau-Varlet, M. Charon, and P. Tambourin. 1981. Detection of tumorigenic cells in Friend virus-infected mice: an *in vivo* methodological investigation. *JNCI* 66:1121-1126.
 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. Nalbantoglu, J., D. Hartley, G. Phear, G. Tear, and M. Meuth. 1986. Spontaneous deletion formation at the *aprt* locus of hamster cells: the presence of short sequence homologies and dyad symmetries at deletion termini. *EMBO J.* 5:1199-1204.
 39. Nishioka, Y., and P. Leder. 1979. The complete sequence of a chromosomal mouse α -globin gene reveals elements conserved throughout vertebrate evolution. *Cell* 18:875-882.
 40. Nocka, K., J. Buck, E. Levi, and P. Besmer. 1990. Candidate ligand for the c-kit transmembrane kinase receptor: KL, a fibroblast derived growth factor stimulates mast cells and erythroid progenitors. *EMBO J.* 9:3287-3294.
 41. Ohneda, O., N. Yanai, and M. Obinata. 1992. Combined action of c-kit and erythropoietin on erythroid progenitor cells. *Development* 114:245-252.
 42. Ostertag, W., and K. Itoh. Unpublished data.
 43. Ostertag, W., H. Melderis, G. Steinhelder, N. Kluge, and S. Dube. 1972. Synthesis of mouse haemoglobin and globin mRNA in leukaemic cell cultures. *Nature (London) New Biol.* 239:231-234.
 44. Plumb, M., J. Frampton, H. Wainwright, M. Walker, G. Goodwin, and P. Harrison. 1989. GATAAG: a *cis*-control region binding an erythroid-specific nuclear factor with a role in globin and non-globin gene expression. *Nucleic Acids Res.* 17:73-92.
 45. Quesenberry, P. J. 1992. Stroma-dependent hematolymphopoietic stem cells, p. 151-166. In C. Muller-Sieburg, B. Torok-Storb, J. Visser, and R. Storb (ed.), *Hematopoietic stem cells: animal models and human transplantation*. Springer-Verlag, Berlin.
 46. Rao, V. N., and E. S. P. Reddy. 1992. A divergent *ets*-related protein, Elk-1, recognizes similar c-ets-1 proto-oncogene target sequences and acts as a transcriptional activator. *Oncogene* 7:65-70.
 47. Reddy, E. S. P., and V. N. Rao. 1991. *erg*, an *ets*-related gene, codes for sequence-specific transcriptional activators. *Oncogene* 6:2285-2289.
 48. Reddy, E. S. P., V. N. Rao, and T. S. Papas. 1987. The *erg* gene: a human gene related to the *ets* oncogene. *Proc. Natl. Acad. Sci. USA* 84:6131-6135.
 49. Ryan, J. J., R. Danish, C. A. Gottlieb, and M. F. Clarke. 1993. Cell cycle analysis of p53-induced cell death in murine erythroleukemia cells. *Mol. Cell. Biol.* 13:711-719.
 50. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
 51. 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.
 52. Sels, F. T., S. Langer, A. S. Schulz, J. Silver, M. Sitbon, and R. W. Friedrich. 1992. Friend murine leukemia virus is integrated at a common site in most primary spleen tumors of erythroleukemia animals. *Oncogene* 7:643-652.
 53. Steel, K. P., D. R. Davidson, and I. J. Jackson. 1992. TRP-2/DT, a new early melanoblast marker, shows that steel growth factor (c-kit) is a survival factor. *Development* 115:1111-1119.
 54. Stephen, C. W., and D. P. Lane. 1992. Mutant conformation of p53. Precise epitope mapping using a filamentous phage epitope library. *J. Mol. Biol.* 225:577-583.
 55. Toksoz, D., K. M. Zsebo, K. A. Smith, S. Hu, D. Brankow, S. V. Suggs, F. H. Martin, and D. A. Williams. 1992. Support of human hematopoiesis in long-term bone marrow cultures by murine stromal cells selectively expressing the membrane-bound and secreted forms of the human homolog of the steel gene product, stem cell factor. *Proc. Natl. Acad. Sci. USA* 89:7350-7354.
 56. Tsai, S.-H., 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.
 57. Urness, L. D., and C. S. Thummel. 1990. Molecular interactions within the ecdysone regulatory hierarchy: DNA binding properties of the *Drosophila* ecdysone-inducible E74A protein. *Cell* 63:47-61.
 58. Wasylyk, B., C. Wasylyk, P. Flores, A. Begue, D. Leprince, and D. Stehelin. 1990. The *c-ets* proto-oncogenes encode transcription factors that cooperate with *c-Fos* and *c-Jun* for transcriptional activation. *Nature (London)* 346:191-193.
 59. Witte, O. N. 1990. *Steel* locus defines new multipotent growth factor. *Cell* 63:5-6.
 60. Xin, J.-H., A. Cowie, P. Lachance, and J. A. Hassell. 1992. Molecular cloning and characterization of *PEA3*, a new member of the *Ets* oncogene family that is differentially expressed in mouse embryonic cells. *Genes Dev.* 6:481-496.
 61. Yin, Y., M. A. Tainsky, F. Z. Bischoff, L. C. Strong, and G. M. Wahl. 1992. Wild-type *p53* restores cell cycle control and inhibits gene amplification in cells with mutant *p53* alleles. *Cell* 70:937-948.
 62. Yonisch-Rouach, E., D. Resnitzky, J. Lotem, L. Sachs, A. Kimchi, and M. Oren. 1991. Wild-type *p53* induces apoptosis of myeloid leukaemic cells that is inhibited by interleukin-6. *Nature (London)* 352:345-347.