p53-independent Tumor Growth and in Vitro Cell Survival for F-MuLV-induced Erythroleukemias

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Abstract
Retroviral insertional activation of the Flt-1 proto-oncogene is the first genetic event associated with the induction of erythroleukemias by the Friend murine leukemia virus (F-MuLV). Mutations within p53, which are only detected in cell lines established from transplanted tumors, have been previously shown to be associated with the immortalization of erythroleukemic cells in culture. In this study, we have demonstrated that primary erythroleukemic cells grown in liquid culture undergo rapid apoptosis independent of the stabilization of wild-type p53 protein. Further confirmation that the programmed cell death observed for liquid-cultured F-MuLV-induced primary erythroleukemic cells is largely p53 independent was provided by experimentation with a transgenic mouse line containing multiple copies of the dominant negative mutant p53\textsuperscript{pro-193} allele. Erythroleukemic cells taken from tumor-bearing transgenic mice expressing high levels of the mutant p53\textsuperscript{pro-193} undergo programmed cell death in culture in a manner that is largely identical to that observed for tumor cells derived from nontransgenic littermates. Furthermore, the rate of development of F-MuLV-induced erythroleukemias for both p53\textsuperscript{pro-193} transgenic and nontransgenic littermates are similar. Moreover, cytogenetic analysis indicates that primary erythroleukemia cells are diploid, whereas chromosomal aberrations were observed in all established cell lines. These results are consistent with the notion that mutations within the p53 tumor suppressor gene affect genomic stability, subsequently leading to changes in gene expression that are associated with the immortalization of erythroid progenitor cells.

Introduction
Friend virus-induced erythroleukemia has emerged as a powerful animal model system to study the multistage nature of cancer progression (1, 2). Similar to human cancers, disease progression in these infected animals involves a multistage process that is directly linked to the accumulation of genetic changes, including the activation of dominantly acting oncogenes and the inactivation of a tumor suppressor gene (1). Two separate isolates of Friend leukemia virus, termed FV-A and FV-P, have been identified. Both FV-A and FV-P are actual complexes of two distinct viral species, namely a unique replication-defective SFFV\textsuperscript{3} strain (SFFV-A and SFFV-P, respectively) and a common replication-competent F-MuLV. The anemia-inducing (FV-A) and polycythemia-inducing (FV-P) strains of the virus produce similar multistage malignancies when injected into susceptible strains of adult or newborn mice. However, F-MuLV can induce, independently of SFFV, a number of hematopoietic neoplasms, including erythroleukemias, which have been shown to be induced in newborn susceptible mice (3).

Toward understanding the molecular mechanisms associated with the induction and progression of leukemias, we have previously identified a common site for retroviral integration, designated Flt-1, that is rearranged in the majority of F-MuLV-induced erythroleukemias (4). Earlier, Moreau-Gachelin et al. (5, 6) had identified Sfpi-1 as a common site for retroviral integration shared by FV-A and FV-P-induced erythroleukemias. Interestingly, the transcriptional domains activated by proviral insertion at these corresponding sites encode two unique transcription factors, both of which are members of the ets oncogene family (5, 7, 8). Retroviral insertional activation of Flt-1 and Sfpi-1 appears to be mutually exclusive with respect to the particular strain of Friend virus used for infection. Specifically, Flt-1 appears to be activated exclusively in erythroleukemias induced by F-MuLV, whereas Sfpi-1 has only been shown to be activated in FV-A/P-induced erythroleukemias.

Upon comparing F-MuLV- and FV-A/P-induced erythroleukemias, it has become evident that these primary tumors display notable biological differences, particularly with respect to their ability to grow in culture. Unlike FV-A/P-derived primary tumors, cells derived from F-MuLV-induced primary erythroleukemias are unable to grow directly in culture, even in the presence of Epo, an important erythroid survival and mitogenic factor (9). However, ED cell lines can be established following serial in vivo passage of these infected primary tumor cells in syngeneic mice (9). Examination of these

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3 The abbreviations used are: SFFV, spleen focus-forming virus; F-MuLV, Friend murine leukemia virus; Flt-1, Friend leukemia integration-1; Epo, erythropoietin; ED, Epo-dependent; EI, Epo-independent; PCD, programmed cell death; wt, wild-type; HCV, hematocrit value.
established ED cell lines revealed that they all contained functional inactivating mutations within the p53 tumor suppressor gene, which is strongly associated with the immortalization of cells in culture. In addition, the large majority of EI cell lines established following the in vivo passage of ED cell lines constitutively express Epo, resulting in the establishment of an autocrine loop involving the Epo receptor, which is also highly expressed in these cells (10). The selection process operating in vivo enabling these tumor cells to acquire growth factor independence is associated with increased genomic instability, as shown by the frequent rearrangement of the Epo gene in these EI cell lines. This increased genetic instability appears to be driven in part by the lack of a functional p53\textsuperscript{tm}.

In the past decade, the study of Friend virus-induced erythroleukemias have shown that p53 mutations appear to be a prerequisite event for the emergence of clonal tumorogenic erythroleukemia cells. In fact, some of the earliest evidence of the tumor suppressor function of p53 emerged from these studies (11). Characterization of the function of this gene in a number of laboratories have shown that wt p53 can trigger G\textsubscript{1} arrest and PCD or apoptosis and, in addition, positively modulate globin gene expression, which is a distinctive marker of erythroid differentiation (12). This raises the intriguing possibility that the cellular death that we observed for primary F-MuLV erythroleukemias grown in liquid culture may be attributed to the expression of wt p53 in these clonal tumor cells. However, this hypothesis is not consistent with the recent findings of Kelly et al. (13), who have demonstrated that splenic polyclonal proerythroblasts derived from FV-A postinfected (2 weeks) mice undergo PCD that is independent of the stabilization of wt p53 protein. In this study, we have examined the status of p53 in the context of primary erythroleukemias induced by F-MuLV in both normal and p53\textsuperscript{pro-193}-transgenic mice. The extensive cell death exhibited by liquid-cultured clonal proerythroblasts derived from both normal and p53\textsuperscript{pro-193}-transgenic animals is characteristic of apoptosis. The PCD induced in erythroleukemia cells derived from normal mice was not associated with the stabilization of wt p53. The presence of the p53\textsuperscript{pro-193} transgene and its subsequent high levels of expression neither accelerated the onset of the primary disease nor blocked the rapid onset of apoptosis induced in these clonal erythroleukemia cells when placed in culture. The role of p53 with respect to F-MuLV-induced erythroleukemia will be discussed.

Results

Apoptosis in Primary Erythroleukemias Induced by F-MuLV Is p53 Independent. We have previously demonstrated that the clonal primary erythroleukemias induced by F-MuLV have acquired an activated Flt-1 and appear to express p53\textsuperscript{mt} protein (9). Although these primary erythroleukemias are capable of generating transplantable tumors in mice, they die rapidly when introduced to cell culture. Moreover, other laboratories have shown that exogenous expression of ts-p53 in a p53-negative FV-P-induced erythroleukemia cell line is associated with the induction of apoptosis (12, 14). These observations raise the possibility that the rapid cell death observed in F-MuLV-induced primary erythroleukemias may be mediated by the activation of wt p53 during in vitro cell culture. To test this hypothesis, primary erythroleukemic cells derived from a F-MuLV-infected mouse were removed and cultured in the absence or presence of 1 unit/ml Epo. Rapid loss of cell viability was observed when these primary tumor cells were cultured in the absence of Epo (Fig. 1A). The addition of Epo to the cell culture medium significantly increased cell survival within the first 3 days of culture but only delayed the onset of cell death. A similar pattern of cell survival can be seen when other independently derived primary erythroleukemias are grown in culture with or without the addition of Epo (data not shown). The rapid cell death observed for these cultured primary erythroleukemic cells is due to apoptosis. This is supported by the ladder pattern of genomic DNA degradation detected within these cells, which is a hallmark of PCD (Fig. 1B). The addition of Epo to the cultures markedly increased cell viability and reduced the extent of the DNA fragmentation compared to cells grown without Epo but could not abrogate the PCD (Fig. 1B).

To examine whether changes in the stability of p53 were associated with the Epo-sensitive apoptotic process observed in liquid culture, tumor cells derived from two primary erythroleukemias (HB46 and HB47) were cultured for various times in the presence or absence of Epo and collected for Western blot analysis. p53 immunoblot analysis can readily detect changes in the stability of p53\textsuperscript{mt} protein in response to apoptotic stimuli (13). p53\textsuperscript{pro-193}, a stabilized dominant-negative missense mutant of p53 expressed by CB7 cells, has a dramatically extended half-life, which translates into its prominent accumulation and easy detection (Fig. 2). For the primary tumor cells collected at various time points in the presence or absence of Epo, there was no detectable stabilization of wt p53 protein (Fig. 2). Northern analysis of these primary tumor cells demonstrated no change in the RNA expression of p53 for this same extended time course in culture (data not shown). Although wt p53 is actively transcribed in all of these primary tumors (data not shown) the apoptosis observed for these liquid-cultured tumor cells appears to be independent of p53 RNA regulation. Together, these results suggest that the F-MuLV-induced primary erythroleukemic cells undergo apoptosis in culture via a mechanism that is independent of the active stabilization of wt p53 protein or changes in the RNA expression of p53. These observations are consistent with the results reported by Kelly et al. (13), which show that the rapid onset of apoptosis induced in preleukemic p53\textsuperscript{mt} expressing FV-A proerythroblasts is not associated with the stabilization of wt p53 protein.

Apoptosis and Tumor Growth in p53\textsuperscript{pro-193}-transgenic Mice Infected with F-MuLV. The development of both p53 knockout and p53\textsuperscript{pro-193}-transgenic mice have provided researchers with an invaluable model system to directly access the role of p53 in tumorigenesis. In this study, we have used a CD-1/p53\textsuperscript{-3} transgenic mouse line expressing a dominant negative mutant of p53\textsuperscript{pro-193} (Arg-to-Pro-193 mutant) that have previously been shown to develop a spectrum of spontaneous tumors similar to that seen in p53-deficient mice (15). The rationale for this choice is largely based on the fact
Fig. 1. F-MuLV-induced primary erythroleukemia cells undergo apoptosis in vitro that can be temporarily blocked by Epo. A, primary erythroleukemia cells derived from the spleen of a F-MuLV-infected mouse were incubated for different periods of time in the presence or absence of 0.5 unit/ml Epo. B, genomic DNA prepared from primary erythroleukemia cells cultured after 1 or 2 days in vitro in the presence or absence of Epo were electrophoresed on a 1% agarose gel, stained with ethidium bromide, and photographed under UV illumination. HindIII-digested λ DNA was used as a molecular weight.

that the same p53-transgenic line has previously been used by Lavigne and Bernstein (16) to evaluate the role of p53 in FV-P-induced erythroleukemia. This will therefore allow us to directly compare the role of p53 in the in vivo progression of both FV-P and F-MuLV-induced erythroleukemias. When newborn mice derived from five independent breeding pairs

of CD-1/p53-3 transgenic mice were infected with F-MuLV, there was no observed difference in the latency of tumor development between CD-1 and CD-1/p53PRO-193-transgenic littermates (Table 1). We also did not observe any significant differences between infected p53PRO-193 transgenic and nontransgenic mice with respect to the virus-induced anemia (% HCV) or the spleen tumor weight (Fig. 3). The transgenic erythroleukemias express high levels of p53PRO-193 protein (Fig. 4 and Table 1), which is contributed by the mutant transgene (data not shown). Similarly, tumors derived from both nontransgenic and transgenic littermates frequently possessed clonal Fl-1 rearrangements (Table 1). Collectively, these results demonstrate that expression of p53PRO-193 did not accelerate the in vivo progression of F-MuLV-induced erythroleukemias.

To verify whether the expression of the dominant negative p53PRO-193 has any effect on the growth of these cells in culture, tumor cells were placed in liquid culture for different time points in the presence or absence of Epo. As shown in Fig. 5A, no significant differences in cell survival were evident when comparing erythroleukemias that either express (THC-8 and THC-9) or do not express (THC-7 and THC-10) the p53PRO-193 transgene. Interestingly, as shown in Fig. 5B, there appears to be a discernable p53-depend-
in Table 1, Tumor incidence and in vitro cell survival of primary erythroleukemias induced by F-MuLV in p53<sup>mdm2</sup>-transgenic mice.

In experiment 1, a p53-3 transgenic mouse that expresses a p53<sup>pro-193</sup> mutant transgene was backcrossed with a normal CD-1 mouse. The newborn offspring were then infected with F-MuLV. Tumors THC-1 through THC-5 represent five erythroleukemias induced in these mice. In experiments 2 and 3, two heterozygous p53<sup>pro-193</sup>-transgenic mice were mated, and the resulting newborn offspring infected with F-MuLV. In experiment 4, newbor

ten difference with respect to the extent of DNA degradation associated with this Epo-sensitive PCD. However, none of the erythroleukemias gave rise to established cell lines in the presence of Epo, despite the high levels of p53<sup>pro-193</sup> transgene expression. The rapid loss of cell viability observed for liquid-cultured p53<sup>pro-193</sup> transgenic erythroleukemic cells could not be attributed to a reduction in the expression of the p53<sup>pro-193</sup> transgene because the level of p53<sup>pro-193</sup> expressed in these cells did not change over the extended time course (Fig. 4B). Therefore, the inability of the dominant negative p53<sup>pro-193</sup> to alter tumor cell survival in vitro, in either the presence or absence of Epo, suggests that the cell death observed for liquid-cultured F-MuLV-induced erythroleukemias may be largely independent of the function of wt p53.

**Karyotypic Analysis of Primary Tumors and Established Erythroleukemia Cell Lines.** Genomic instability directly visualized by cytogenetic techniques often accompanies the transformation process seen in cancer cells. Changes in chromosomal structure or number often have been documented previously in established Friend viral complex-induced erythroleukemia cell lines (17–20). We analyzed the karyotypes of cells derived from several primary erythroleukemias induced in normal or p53<sup>pro-193</sup>-transgenic mice and
In the past decade, the study of Friend virus-induced erythro- leukemia has resulted in the identification of a number of cellular genes that are frequently targeted for mutation, specifically Flt-1, Stp, and p53. Whereas retroviral insertional activation of Flt-1 has been seen in almost all of the primary erythroleukemias induced by F-MuLV, p53 mutations have only been directly identified within cell lines established after the in vivo transplantation of these primary tumors (9). Therefore, the role of p53 in the context of in vivo tumorigenesis for F-MuLV-induced erythroleukemias is not fully understood. In this study, by using primary erythroleukemia cells derived from F-MuLV-infected CD-1 and CD-1/p53-3 transgenic mice and cell lines established from in vivo transplanted primary tumors, we have attempted to analyze the functional importance of p53 mutations during the multistage progression of F-MuLV-induced erythroleukemias.

For primary erythroleukemic cells, low but discernable levels of wt p53 protein expression are detectable. The contrast between high levels of RNA expression and the rather low levels of protein expression for p53 is typical of the normal rapid turnover of this protein. Although the continued growth of these primary tumor cells is permissible in vivo, via transplantation into syngeneic mice, they rapidly undergo PCD in an Epo-sensitive manner when placed in liquid culture. The mechanism of PCD observed for these F-MuLV-induced erythroleukemic cells did not involve p53 protein stabilisation. The same phenomenon has been previously reported by Kelly et al. (13), who examined PCD in polyclonal FV-A-induced proerythroblasts, which also express wt p53. Interestingly, these observations are mirrored by experiments performed with primary erythroleukemic cells obtained from
$p53^{Pro-193}$ transgenic mice. Erythroleukemic cells derived from these tumor bearing transgenic mice express very high levels of the mutant $p53^{Pro-193}$ allele (>50 fold when compared to endogenous $p53^{Wild}$ protein levels), which has been previously shown to be highly oncogenic via its ability to immortalize REFs and to cooperate with ras in vivo transformation assays (21, 22). Tumors obtained from these transgenic mice, like those derived from nontransgenic littermates, have a high frequency of $Flt-1$ rearrangement. However, the high $p53^{Pro-193}$ levels expressed by these tumors did not alter the rate of disease progression when compared to nontransgenic littermates and did not alter the characteristic virus-induced anemia or splenomegaly. In addition, liquid-cultured $p53^{Pro-193}$-expressing erythroleukemic cells undergo PCD in a manner largely characteristic of F-MuLV-induced erythroleukemic cells. The Epo-sensitive survival of in vitro cultured transgenic and nontransgenic tumor cells is similar, if not identical. Although there appears to be a measurable decrease in the degree of DNA degradation profiled by transgene-expressing tumor cells, it does not appear to impart a survival advantage during the Epo culture of these cells in vitro. Together, these results suggest that

Fig. 5. In vitro cell survival of primary erythroleukemias induced in $p53^{Pro-193}$-transgenic and nontransgenic sibling mice. A, primary erythroleukemias induced by F-MuLV infection of newborn mice, derived from breeding two heterozygous CD-1 $p53^{Pro-193}$-transgenic mice, were cultured ($2 \times 10^7$ cells) for various times in the absence or presence of Epo. Cell viability was determined by trypan blue dye exclusion. Tumors designated THC-8 and THC-9 express the $p53$ transgene ($tg$), whereas both THC-7 and THC-10 do not ($tg$), as described in Table 1. B, genomic DNA was prepared from primary erythroleukemia cells, derived from tumors THC-8 ($tg^+$) and THC-10 ($tg^-$), cultured in the presence or absence of Epo (0.5 units/ml) for the indicated times. It was subsequently electrophoresed on a 2% agarose gel, stained with ethidium bromide, and photographed under UV illumination. HindII/EcoRI-digested pBR32 DNA was used as a molecular weight marker.
Fig. 6. Chromosome number range within cells derived from F-MuLV-induced tumors or established cell lines. Each histogram represents the chromosome count for cells derived from the following tumors and cell lines: a F-MuLV-induced primary erythroleukemia (A); ED cell lines HB9.1-ED and HB-39.2-ED (B and C, respectively); El cell lines CB3 and CB7 (D and E, respectively); primary tumors THC-6 and THC-8, which express the p53 transgene (see Table 1; F and H); and primary tumor THC-7, which expresses wt p53 (see Table 1; G).

p53 may have neither a role in the in vivo tumorigenesis of F-MuLV-induced erythroleukemias nor a major role in the process of PCD observed in liquid culture.

The clear lack of synergy between F-MuLV infection and the overexpression of mutant p53 may be explained by the functional redundancy of these two events. This explanation is analogous to that proposed by Marin et al. (50), who observed a similar lack of synergy between p53 and Bcl-2 during in vivo lymphomagenesis. For retroviral mutagenesis, genetically altered mice, particularly transgenic mice, have proven to be a very useful system to explore oncogenic cooperativity during in vivo tumorigenesis (23-26). In this particular study there appears to be no discernable synergy between F-MuLV infection and a mutant p53(550-193) environment in vivo. However, this property of F-MuLV infection is not shared by FV-P (16). This difference raises an important point concerning the in vivo mutational environment defined by these two Friend virus-induced erythroleukemias. It is within this context that we should consider the importance of p53 mutations, and likely any other oncogenic event, because the function of p53 is very much influenced by the cell type and the transforming events that define the system being examined (27-30). Specifically, FV-P activates Sfpi-1 and F-MuLV activates Flt-1, two very important but distinct mutational events that may trigger individual downstream events. This notion is supported by the observation that Flt-1 and Sfpi-1 appear to recognize distinct DNA-binding sequences (31) and thereby transactivate discrete primary target genes. In addition, the Epo mimicry of gp55 in FV-P-induced erythroleukemias, which is absent in F-MuLV-induced erythroleukemias, should also be considered. The mitogenic and survival signaling triggered by the binding of gp55 to the Epo receptor, coupled with the increased self-renewal potential likely conferred by the activation of Sfpi-1 (32), may favor the rapid selection of mutations that immortalize these erythroid progenitor cells. Alternatively, these differences may be linked to the fact that these unique Friend viruses, SFFV-A/P and F-MuLV, may target distinct cell types (CFU-E and BFU-E, respectively) and therefore require a different complement of genetic alterations to successfully transform their respective erythroid progenitor cells. Despite these differences, the ability of F-MuLV-induced primary erythroleukemic cells to proliferate in vivo suggests that the host spleen microenvironment likely supplies the tumor cells with important growth stimuli. This may be communicated by the spleen via growth factors and/or stromal cell interactions (33). The survival of these erythroleukemia cells in culture may therefore require additional genetic events to escape all of the negative growth effects operating in vitro.

The proposed functional redundancy for F-MuLV infection and mutant p53 during in vivo tumorigenesis suggests that the p53 mutations commonly observed in established F-MuLV-induced erythroleukemia cell lines are a likely consequence of the in vitro immortalization process. The karyotype analysis of F-MuLV-induced primary tumors and established erythroleukemia cell lines provides strong support for this notion. The aneuploidy associated with F-MuLV-induced cell lines is similar to that which has been previously described for Friend viral complex-induced cell lines and late-stage FV-A-induced primary erythroleukemias (17-20). However, the absence of gross karyotypic abnormalities in F-MuLV-induced primary erythroleukemic cells is striking. This raises an intriguing question concerning the possible role of p53 mutations during the erythroid immortalization process, specifically their proposed involvement in generating genetic
instability. The transcriptional activity of p53 (34) is capable of activating or repressing the transcription of genes that regulate cell cycle progression (35–37), DNA repair (38, 39), and genomic stability (40, 41). The cell cycle control believed to be exerted by p53 at the G1-S transition and the considerable experimental evidence connecting lost control of this specific cell cycle checkpoint and increased genomic instability and malignant progression suggests that p53 plays an important role in maintaining the integrity of the genome (39–43). Therefore, it is possible that the appearance of p53 mutations in F-MuLV-induced erythroleukemic cells may be responsible in part for the emergence of genetic instability. In this respect, a destabilized genome would conceivably predispose these erythroid progenitor cells to further genetic changes that enable them to escape negative growth pressures in culture. Although primary erythroleukemias derived from p53Pro-193-transgenic mice retain their normal ploidy, this may reflect time limitations governed by the morbidity of the disease. However, major chromosomal abnormalities may arise subsequent to additional in vivo passaging of these F-MuLV-induced primary tumor cells. Therefore, it remains to be determined whether the in vivo transplantation of p53Pro-193 primary erythroleukemia cells results in an accelerated rate of erythroid immortalization accompanied by aneuploidy. Although the overexpression of v-raf and c-myc in erythroid progenitor cells derived from the fetal liver of p53-deficient mice (p533-32) has recently been shown to induce immortalization in the absence of gross karyotypic changes, this may largely reflect system differences (44). The specific combination of oncogenic changes enforced on these p533-32 erythroid cells is unique to this system and have not been previously shown to be associated with the in vivo transformation of Friend virus target cells. These particular mutational events may therefore allow for the immortalization of erythroid cells independent of changes in cell ploidy.

In summary, by analyzing the molecular events associated with F-MuLV-induced erythroleukemias we have shown evidence suggesting that p53 is not involved in the in vivo progression of this disease. Primary erythroleukemic cells constitutively expressing Fli-1 by way of retroviral insertional activation undergo PCD in liquid culture independent of the stabilization of p53 protein. The apoptosis observed during the Epo culture of these F-MuLV-transformed erythroid cells appears to be largely mediated by a p53-independent mechanism, as evidenced by the unperturbed survival kinetics of F-MuLV-induced p53Pro193-transgenic erythroleukemic cells. Conceivably, a subpopulation of transplanted primary erythroleukemic cells possessing p53 mutations emerges displaying karyotypic changes and a capacity to be immortalized in culture.

Materials and Methods

Tumors and Cell Lines. The erythroleukemia cell lines CB3, CB7, and HB9.1-ED were previously described elsewhere (9, 45–47). Isolation of established ED cell lines HB 39.2-ED is described elsewhere (10). These cells were maintained in α-MEM supplemented with 10% FCS. The ED cell lines were maintained in medium containing 0.1 unit/ml Epo (Boehringer Mannheim).

The primary erythroleukemias were induced by a single i.p. injection of F-MuLV into newborn BALB/c or CD-1 p53-transgenic mice as described previously (9). The generation of the p53-3-transgenic mouse line containing multiple copies of a mutant p53 allele, bearing an alanine-to-proline mutation at residue 193, has been described previously (15). The majority of these infected animals usually develop erythroleukemia between 2 and 3 months postinfection. The F-MuLV-induced murine primary erythroleukemias were cultured for various time periods in the presence or absence of 1 unit/ml Epo and used for DNA, RNA, or protein analysis.

Protein Determination. Western blotting was performed by lysing the cells (104 cells/ml) directly in loading buffer containing 50 mM Tris (pH 8.6), 100 mM β-mercaptoethanol, 2% SDS, 0.1% bromophenol blue, and 1% glycerol. DNA contained within each of the samples was sheared by passing the cell lysate twice through 18 and 26 gauge syringes. After removing the cell debris by centrifugation for 15 min, 20 μl of protein extract were loaded on a 10% acrylamide gel. The separated proteins were then blotted onto a nitrocellulose filter and hybridized to 1/1000 dilution of pAb240 antibody (Santa Cruz Biotechnology; Ref. 9) using the ECL (enhanced chemiluminescence) system (Amersham). Equal loading in each sample was determined by blotting the filters with 1/1000 dilution of an anti-MAPK (ERK-1) antibody (Santa Cruz). This antibody recognizes a 44-kDa band on Western blotting.

Cell Viability Determination. Cells (5 x 104) were cultured in medium containing 10% FCS in the presence or absence of 1 unit/ml Epo at 37°C. The number of viable cells were determined by staining with trypan blue.

Isolation of DNA. Genomic DNA was prepared as described previously (48). In brief, cells were lysed in TNE buffer consisting of 10 mM Tris (pH 7.5), 10 mM EDTA, 100 mM NaCl, 1% SDS; digested with proteinase K; and subjected to organic extraction and ethanol precipitation. DNA (10 mg) was loaded onto a 1% agarose gel and stained with ethidium bromide. For Southern blotting, DNA was digested with restriction enzymes and electrophoresed on agarose gels. The DNA was acid depurinated before denaturation and transferred to nitrocellulose. The filters were hybridized with 2 x 106cpm of random primed probe per ml of hybridization mixture that contained 10% dextran sulfate, 5 x SSPE (20 x SSPE = 3 x NaCl, 200 mM NaH2PO4, H2O, 20 mM EDTA), 5 x Denhardt’s solution (1 x Denhardt’s solution = 0.02% BSA/0.02% Ficol/0.02% polyvinylpyrrolidone), 1–2% SDS, and 100 mg/ml denatured salmon sperm DNA at 65°C, overnight. The filters were washed twice at 65°C for 20 min with a 0.1 x SSC and 0.1% SDS solution. Hybridized probe was removed from the filters by two 20-min washes with 0.1% SDS, 10 mM Tris, pH 7.5, and 1 x EDTA at 95°C.

DNA Probes. The Fli-1 probe was an EcoRI genomic fragment subcloned from the Fli-1 locus (4). The mouse p53 probe was a 900-bp BglII-PstI fragment from mouse p53 cDNA 27.1a (49).

Karyotypic Analysis. Erythroleukemia cell lines and cells derived from F-MuLV-induced primary erythroleukemias were grown in α-MEM supplemented with 10% FCS. Epo (1 unit/ml) was added to growth medium of ED cell lines HB9.1-ED and HB 39.2-ED and primary erythroleukemia cells. Ten μl of colcemid solution (1 mg/ml) were added to the cultured cells, which were subsequently incubated at 37°C for 30 min. The cells were then pelleted, resuspended in 1 ml of warmed 0.075 M KCl, and then fixed in methanol-acetic acid (3:1 v/v) for 30 min at room temperature. After three changes of fixative, the cells were dropped onto cold dry slides, dried, and stained for 2 min with a Giemsa solution. Chromosome numbers were then determined.

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