MOLECULAR AND CELLULAR BIOLOGY, Jan. 2001, p. 73–80 Vol. 21, No. 1
0270-7306/01/$04.00+0 DOI: 10.1128/MCB.21.1.73–80.2001

p45^{NFE2} Is a Negative Regulator of Erythroid Proliferation Which Contributes to the Progression of Friend Virus-Induced Erythroleukemias

YOU-JUN LI,[1] RACHEL R. HIGGINS,[1] BRIAN J. PAK,[1] RAMESH A. SHIVDASANI,[2] PAUL A. NEY,[3] MICHAEL ARCHER,[4] AND YAACOV BEN-DAVID*[1,4]

Division of Cancer Biology Research, Sunnybrook and Women's College Health Sciences Centre and Toronto-Sunnybrook Regional Cancer Centre, Toronto, Ontario, Canada M4N 3M5[1]; Departments of Adult Oncology and Cancer Biology, Dana-Farber Cancer Institute, Boston, Massachusetts 02115[2]; Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, Tennessee 38105[3]; and Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada M5G 2M9[4]

Received 28 June 2000/Returned for modification 31 July 2000/Accepted 16 October 2000

In previous studies, we identified a common site of retroviral integration designated *Fli-2* in Friend murine leukemia virus (F-MuLV)-induced erythroleukemia cell lines. Insertion of F-MuLV at the *Fli-2* locus, which was associated with the loss of the second allele, resulted in the inactivation of the erythroid cell- and megakaryocyte-specific gene *p45^{NFE2}*. Frequent disruption of *p45^{NFE2}* due to proviral insertion suggests a role for this transcription factor in the progression of Friend virus-induced erythroleukemias. To assess this possibility, erythroleukemia was induced by F-MuLV in *p45^{NFE2}* mutant mice. Since *p45^{NFE2}* homozygous mice mostly die at birth, erythroleukemia was induced in +/− and +/+ mice. We demonstrate that +/− mice succumb to the disease moderately but significantly faster than +/+ mice. In addition, the spleens of +/− mice were significantly larger than those of +/+ mice. Of the 37 tumors generated from the +/− and +/+ mice, 10 gave rise to cell lines, all of which were derived from +/− mice. Establishment in culture was associated with the loss of the remaining wild-type *p45^{NFE2}* allele in 9 of 10 of these cell lines. The loss of a functional *p45^{NFE2}* in these cell lines was associated with a marked reduction in globin gene expression. Expression of wild-type *p45^{NFE2}* in the nonproducer erythroleukemic cells resulted in reduced cell growth and restored the expression of globin genes. Similarly, the expression of *p45^{NFE2}* in these cells also slows tumor growth in vivo. These results indicate that *p45^{NFE2}* functions as an inhibitor of erythroid cell growth and that perturbation of its expression contributes to the progression of Friend erythroleukemia.

The transcription factor NFE2 (nuclear factor erythroid 2) plays a critical role in the regulation of erythroid cell-specific gene expression (25). This nuclear factor binds to AP1-like consensus binding sites located in the enhancers and promoters of several erythroid cell- and megakaryocyte-specific genes, including the β-globin locus control region (15, 17, 23, 31), human porphobilinogen deaminase (19), ferrochelatase (29, 34), and thromboxane synthase (9). NFE2 is a heterodimer complex of two basic leucine zipper proteins, consisting of 45-kDa (p45^{NFE2}) and 18-kDa (p18^{NFE2}) subunits (1). The expression of the large subunit, p45^{NFE2}, has been found to be tissue specific, with expression restricted to erythroid cells, megakaryocytes, and mast cells (1). However, p18^{NFE2}, a member of the Maf oncoprotein family (13), is widely expressed in many tissues (2).

p45^{NFE2}-deficient mice display mild abnormalities in erythropoiesis, including hypochromia, anisocytosis, and reticulocytosis (27). However, these mice are severely thrombocytopenic due to arrest in late megakaryocyte maturation which results in hemorrhage after birth. Although most *p45^{NFE2}*-deficient mice die at birth, a small fraction survives and develops primary or secondary phenotypes such as severe megakaryocytosis, splenomegaly, and bone marrow hypercellularity (16).

Previously, we have reported that the *p45^{NFE2}* gene resides in the *Fli-2* locus, a common site for retroviral integration identified in erythroleukemias induced by both FV-P and F-MuLV strains of Friend virus (17). In one erythroleukemia cell line, CB3, proviral insertion within one allele of the *p45^{NFE2}* gene was associated with loss of the second allele, resulting in complete inactivation of *p45^{NFE2}* expression (17). Loss of *p45^{NFE2}* resulted in significant reduction in the expression of both α- and β-globin genes. When *p45^{NFE2}* was reintroduced into CB3 cells, expression of both α- and β-globin was restored, providing evidence that p45^{NFE2} is a positive regulator of globin gene expression (15, 17). Since proviral integration within the *p45^{NFE2}* gene was also identified in other cell lines (17), these results raised the intriguing possibility that this transcription factor functions as a suppressor of tumor growth in Friend virus-induced erythroleukemias.

Friend virus-induced erythroleukemia has been an excellent animal model to identify genes involved in multistep malignancies. The induction and progression of erythroleukemias by Friend virus are mainly due to the ability of proviruses to activate cellular oncogenes or inactivate tumor suppressor genes (3). In Friend virus-induced erythroleukemias, the expression of *p53* was first shown to be lost by mechanisms such as proviral integration, mutation, and rearrangement (7, 10, 12,

* Corresponding author. Mailing address: Division of Cancer Biology Research, Sunnybrook and Women's College Health Sciences Centre & Toronto-Sunnybrook Regional Cancer Centre, 2075 Bayview Ave., S-Wing, Room S216, Toronto, Ontario M4N 3M5, Canada. Phone: (416) 480-6100, ext. 3359. Fax: (416) 480-5703. E-mail: bendavid@srcl.sunnybrook.utoronto.ca.

21, 22). Subsequently, the Ets-related transcription factors Spi-1 and Fli-1 were identified, and their expression was shown to be induced as a result of integration of spleen focus-forming virus or F-MuLV adjacent to these genes, respectively (5, 20). While insertional activation of either *Fli-1* or *Spi-1* is an early and critical event during the induction of these two types of erythroleukemia, *p53* mutation is associated with late stages in the progression of the disease (35)

In this study, we utilized $p45^{NFE2}$ mutant mice to study the role of this gene in the progression of Friend virus induced-erythroleukemias. Our results support a role for $p45^{NFE2}$ as a negative regulator of cell growth in Friend virus-induced erythroleukemia.

MATERIALS AND METHODS

Breeding and F-MuLV inoculation of newborn mice. $p45^{NFE2}$ heterozygous (+/−) mice of the 129/Sv strain (28) were mated with BALB/c mice (Jackson Laboratories) for six generations in order to confer upon them sensitivity to Friend virus-induced erythroleukemias (35). The offspring were genotyped by Southern blot analysis of tail DNA as described previously (28). +/− newborn mice from the BALB/c cross were then tested for sensitivity to F-MuLV by a single intraperitoneal injection at birth (26). It was found that these mice were susceptible to F-MuLV-induced erythroleukemia. Accordingly, two breeding pairs of the +/− mice were used to generate offspring for viral inoculation.

Tumor induction and establishment of cell lines. Newborn pups were injected intraperitoneally with F-MuLV clone 57 as described elsewhere (26). They were monitored for (i) enlarged abdomens causing hunched postures, a symptom of splenomegaly, and (ii) a lack of movement, reflecting low energy due to severe anemia, and were sacrificed when moribund. Mice displaying these marked symptoms rarely survive for over a day. For genotyping, tail tissues and tumor cells were processed for Southern analysis. The spleen cells from these erythroleukemias were cultured in α-minimum essential medium supplemented with 15% heat-inactivated fetal bovine serum (Gibco BRL). Cells were cultured in this medium alone or supplemented with either 1 U of erythropoietin (Epo)/ml, 10% stem cell factor (SCF) conditioned medium, or both growth factors as described elsewhere (35). SCF was obtained from SCF-producing BHK-MKL cells (provided by S. Tsai) (33). These conditions were maintained until cells were established in culture, at which time fetal bovine serum was reduced to 10% and growth factors were removed in order to determine the growth factor dependency of the cell lines. To examine cellular growth rates, erythroleukemic cells were cultured in triplicate in the presence of Epo, SCF, or Epo plus SCF and viable cells were determined by trypan blue dye exclusion at various times.

DNA isolation and Southern analysis. High-molecular-weight DNA was isolated from homogenized spleen tissue, tail samples, or cell lines as previously described (12). Fifteen micrograms of genomic DNA from tumors or cell lines was digested overnight with appropriate restriction enzymes and separated on 1% agarose gels. DNA was acid depurinated in 0.1 M HCl for 15 min before denaturation and capillary transferred onto nylon membranes using 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The membranes were hybridized with 100 ng of random-primed DNA probe in a mixture of 50% formamide, 5× SSPE (20× SSPE is 3 M NaCl, 200 mM $NaH_2PO_4 \cdot H_2O$, and 20 mM EDTA), 1× Denhardt's solution (0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrolidone), and 5% dextran sulfate at 42°C. The filters were washed for 15 min twice at room temperature in 2× SSC–0.5% sodium dodecyl sulfate (SDS) and then twice at 65°C in 0.2× SSC–0.1% SDS.

RNA isolation and Northern analysis. Two micrograms of poly(A)$^+$ mRNA isolated from cell lines was dissolved in 2.2 M formamide, incubated at 55°C for 15 min, and separated in a 1% agarose gel containing 0.66 M formaldehyde. Gels were washed twice in transfer buffer (10× SSC) for 20 min and transferred overnight onto nylon filters. The filters were hybridized with 2×10^6 cpm of ^{32}P-labeled random-primed probe per ml of hybridization mixture that contained 50% formamide, 10% dextran sulfate, 1.5× SSC, and 5× Denhardt's solution at 42°C. The filters were washed twice with 2× SSC–0.2% SDS at room temperature for 20 min and then twice with 0.2× SSC–0.1% SDS at 65°C for 15 min. Hybridized probe was removed from the filters by two 30-min washes with a mixture of 0.1% SDS, 10 mM Tris (pH 7.5), and 1 mM EDTA at 70°C.

DNA probes. The F-MuLV *env* probe is a 0.8-kb *Bam*HI segment of plasmid pHC6 (8). The *Fli-1* cDNA probe is a 1.7-kb *Eco*RI fragment of the BB4 plasmid (5). The α-globin probe is a 0.5-kb *Eco*RI fragment from plasmid PB1. The $p45^{NFE2}$ probe used in Northern blot analysis is a 1.5-kb *Eco*RI cDNA fragment derived from the CB7 erythroleukemia cell line (17). For Southern analysis a genomic fragment corresponding to the *Hin*dIII-*Eco*RV fragment of the $p45^{NFE2}$ gene was used (28). The p53 probe is a 0.9-kb *Bgl*II-*Pst*I cDNA fragment from mouse clone 27.1a (14). The 750-bp *Pst*I-*Xba*I fragment of mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA was used to normalize the RNA loaded.

$p45^{NFE2}$ viral vector and cell infection. The 1.5-kbp *Eco*RI fragment corresponding to full-length $p45^{NFE2}$ cDNA was cloned into the *Eco*RI site of pMX-puro retroviral expression vector (24) and designated pMX-NFE2. To generate the retroviruses, pMX-puro and pMX-NFE2 constructs were transfected into amphotropic GP+envAM12 helper-free packaging cells (18) by the Lipofectin transfection method (Life Technologies). Cells resistant to puromycin (2 μg/ml) were pooled and cocultured with erythroleukemia cell lines for two days. The nonadherent leukemic cells were then removed and selected for 1 week with 0.3 to 0.5 μg of puromycin per ml, and resistant cells were used for expression and cell growth analyses.

In vivo assays. NKH18-C4A cells (10^6) infected with pMX-puro or pMX-NFE2 were injected into nude mice via tail vein injection. Mice were monitored for the development of leukemia and were sacrificed when they exhibited terminal stages of the disease, as described above.

Statistical analysis. Comparisons between two groups were made with Student's *t* test. Differences were considered significant at $P < 0.05$.

RESULTS

In vivo progression of erythroleukemias in $p45^{NFE2}$ mutant and control mice. To render sensitivity to Friend virus, the $p45^{NFE2}$ knockout mice originally generated in the C57BL/6 and 129Sv strains of mice (28) were crossed into the susceptible BALB/c background. After six consecutive crosses, heterozygous breeding pairs of $p45^{NFE2}$ mice were mated and the pups were infected with F-MuLV. Of 39 infected pups, 2 died around 2 weeks after viral infection. The remaining mice were monitored for the development of erythroleukemias and were sacrificed when they became moribund. Genotype analysis indicated that 26 of these erythroleukemias originated from +/− mice, and the remaining 11 were from +/+ mice. −/− mice were not used in this analysis due to their high perinatal mortality (28). The average times between viral injection and sacrifice in +/+ and +/− mice were 44 (standard deviation [SD] = 3.8) and 41 (SD = 4.2) days, respectively. Statistical analysis comparing the spectrum of +/+ and +/− revealed a moderate but significant difference ($P < 0.049$) in the time until these mice succumbed to the disease. In addition, unpaired *t* test comparison of spleen weights between $p45^{NFE2}$ heterozygous (mean, 1.78 g; SD = 0.25) and $p45^{NFE2}$ wild-type mice (mean, 1.552; g; SD = 0.34) indicated a significant difference ($P < 0.03$) between these populations. The shorter time span for tumor development and the significant increase in the volume of tumorigenic spleen suggest that in +/− infected mice the leukemogenic process is accelerated.

Growth of erythroleukemic cells in culture is associated with the loss of the $p45^{NFE2}$ gene. Retroviral insertional activation of the *Fli-1* gene has been detected in the majority of F-MuLV-induced erythroleukemias (4, 12). Thus, we examined the genomic organization of the *Fli-1* locus in tumors induced in $p45^{NFE2}$ mutant mice. Southern analysis revealed rearrangements of the *Fli-1* locus in all tumors derived from +/+ and +/− mice (data not shown). Our previous studies demonstrated that F-MuLV-induced erythroleukemic cells that have acquired activated Fli-1 undergo apoptosis when they are introduced into cell culture (11). However, when F-MuLV was injected into *p53*-deficient mice, tumorigenic cell lines

TABLE 1. Summary of cell lines established from +/− primary F-MuLV-induced erythroleukemias[a]

Tumor	*p45NFE2* genotype in tumors	Cell line	*p45NFE2* genotype in cell lines	Growth factor responsiveness
NKH2-T	+/−	NKH2-C	+/−	Epo or SCF
NKH17-T	+/−	NKH17-C	−/−	Epo
NKH18-T	+/−	NKH18-C2	−/−	Epo or SCF
NKH19-T	+/−	NKH19-C	−/−	Epo
NKH23-T	+/−	NKH23-C	−/−	Epo
NKH24-T	+/−	NKH24-C	−/−	Epo
NKH25-T	+/−	NKH25-C	−/−	Epo
NKH26-T	+/−	NKH26-C	−/−	Epo
NKH31-T	+/−	NKH31-C	−/−	Epo
NKH34-T	+/−	NKH34-C	−/−	Epo

[a] The properties of tumors which gave rise to permanent cell lines are indicated. Growth factors upon which cells are dependent for growth in culture are indicated. Tumors and cell lines were genotyped by Southern analysis.

were established from the majority of the induced erythroleukemias, although their growth was dependent on the presence of Epo and/or SCF (35). To examine whether the absence of *p45NFE2* could also be correlated with the immortalization of primary erythroleukemic cells, tumor cells removed from +/− and +/+ mice were grown in culture medium supplemented with 15% fetal bovine serum alone or further supplemented with both Epo and SCF. After 4 weeks of culturing in the presence of Epo plus SCF, 10 independent cell lines that originated from +/− tumors were established (Table 1). None of the +/+ tumors gave rise to cell lines in the same culture period. Optimal growth of eight of these established cell lines was dependent on the presence of Epo, although two cell lines, NKH18-C and NKH2-C, were capable of growing in the presence of either Epo or SCF (Table 1). Since tumors used to establish these cell lines contained a rearranged *Fli-1* gene, high levels of *Fli-1* mRNA were detected in all of them (see Fig. 3).

These observations raised the possibility that *p45NFE2* deficiency contributes to the establishment of erythroleukemic cells in culture. Therefore, we assessed whether the genomic structure of *p45NFE2* was altered in these cell lines. Southern blot analysis demonstrated that eight cell lines derived from +/− tumors had lost the remaining wild-type allele after less than 1 month in culture (Fig. 1; summarized in Table 1). In one cell line, NKH18-C, the intensity of the hybridized band corresponding to the *p45NFE2* wild-type allele was significantly reduced compared to its corresponding tumor (Fig. 1), suggesting an oligoclonal process. Indeed, genomic analysis of the six individual clones isolated from NKH18-C by a limiting dilution experiment resulted in the identification of five cell lines that were homozygous and one (NKH18-C4) that was heterozygous for the *p45NFE2* gene (Fig. 1, right panel). NKH2-C was the only cell line that maintained heterozygosity after 2 months in culture (Fig. 1, left panel).

To assess the clonal relationship between primary tumors and their respective cell lines, we examined the pattern of proviral integration by hybridizing genomic DNA with a F-MuLV-specific *env* probe (12, 35). As shown in Fig. 2 (upper panel), the pattern of proviral integration was similar in cell lines NKH18-C2, NKH19-C, NKH24-C, NKH26-C, NHK31-C and their corresponding tumors. However, five cell lines, NKH2-C, NKH17-C, NKH23-C, NKH25-C and NKH34-C, were clonally unrelated to the dominant cell population present in tumors. Therefore, these cell clones were likely derived from a minor subpopulation of tumor cells that survived in culture following loss of the wild-type *p45NFE2* allele. A clonal relationship was also seen between two representative cell clones isolated from the NKH18-C cell line and its corresponding tumor (Fig. 2, lower panel). A similar clonal relationship between tumors and their corresponding cell lines was previously noted in tumors and cell lines derived from *p53* mutant mice (35).

Loss of the *p45NFE2* gene in erythroleukemias suppresses globin gene expression. Northern blot analysis demonstrated that the nine erythroleukemia cell lines that had lost the wild-type *p45NFE2* allele expressed only the mutated *p45NFE2* mRNA from the targeted allele (Fig. 3). Both normal-size *p45NFE2* mRNA from the wild-type allele and mutant mRNA from the targeted allele were detected in the NKH2-C cell line, which still retained one of the wild-type alleles (Fig. 3). Since *p45NFE2* is an erythroid cell- and megakaryocyte-specific gene, the expression of either mutant or wild-type *p45NFE2* in these cells attests to their erythroid origin.

Our studies have indicated that the *p53* gene is altered in essentially all Friend virus-induced erythroleukemias cell lines (12, 35). Thus, we examined the status of this tumor suppressor gene in the NKH cell lines. Southern analysis using the restric-

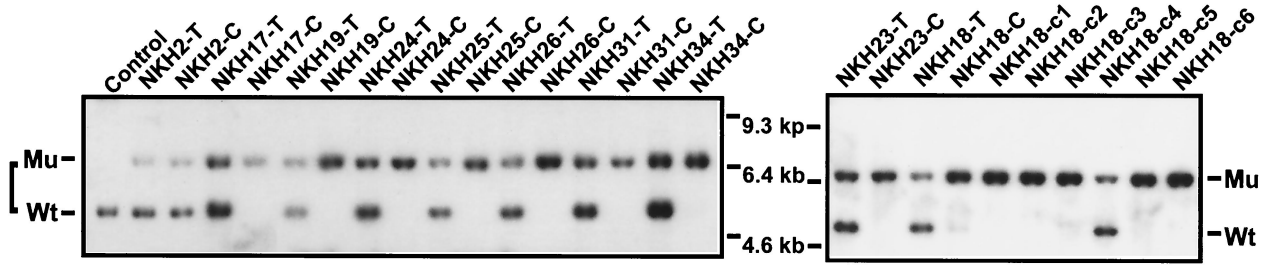

FIG. 1. *p45NFE2* genotype in F-MuLV-induced erythroleukemias and their derivative cell lines. Fifteen micrograms of genomic DNA isolated from the indicated tumors (T) and their derivative cell lines (C) was digested with *Eco*RI, electrophoresed in 1% agarose, transferred onto a nylon filter, and hybridized with *p45NFE2* probe. Bands corresponding to the mutated (Mu) *p45NFE2* from the targeted allele and wild-type (Wt) *p45NFE2* allele are marked. We also included the derivative cell clones isolated from the NKH18-C cell line (designated NKH18-C1 to -C6). Kidney DNA from BALB/c mice was used as a control.

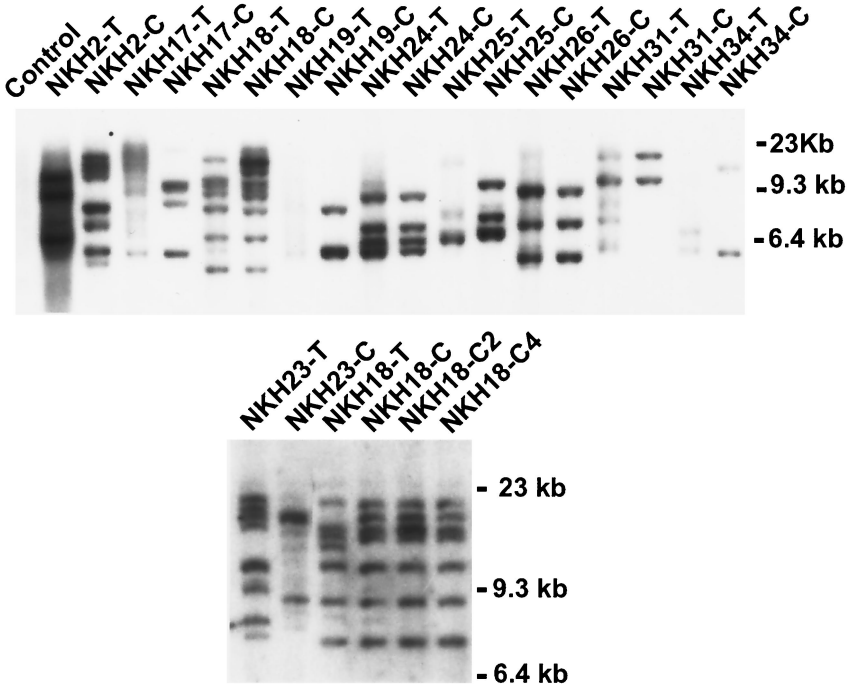

FIG. 2. Clonal analysis of tumors and their derivative cell lines. Fifteen micrograms of genomic DNA isolated from tumors and their derivative cell lines was digested with *Eco*RI, transferred to a nylon filter, and hybridized with the F-MuLV *env*-specific probe. Normal kidney DNA from BALB/c mice was used as a control.

tion enzymes *Bam*HI and *Hin*dIII failed to reveal rearrangement of the *p53* gene in any of the cell lines (data not shown). Moreover, normal levels of *p53* mRNA were detected in nine of the NKH cell lines, and NKH2-C was the only cell line that expressed lower levels of *p53* (Fig. 3). The level of *p53* expression was compared to those in the F-MuLV-induced erythroleukemia cell lines CB3 and CB7, which lost a functional *p53* through deletion and point mutation, respectively (8, 22).

Although globin gene expression was not altered in $p45^{NFE2}$ null mice (28), the expression of globin genes was significantly compromised in the CB3 erythroleukemia cell line, which has a homozygous loss of $p45^{NFE2}$ (17). To determine the generality of this observation, we determined expression of globin in the established NKH cell lines. A negligible level of globin mRNA was detected in all NKH cell lines except NKH2-C and the control $p45^{NFE2}$-expressing erythroleukemia cell line CB7 (Fig. 3). These results further reinforce the notion that $p45^{NFE2}$ is a positive regulator of globin gene expression.

Loss of $p45^{NFE2}$ in erythroleukemic cells accelerates growth in culture. To examine the effect of loss of $p45^{NFE2}$ on erythroleukemia progression, we first compared the growth rates of the NKH18-C derivative cell lines NKH18-C2 and NKH18-C3, which are $p45^{NFE2}$ deficient, and NKH18-C4 cells, which are heterozygous (Fig. 1). In the presence of SCF and Epo, $p45^{NFE2}$-expressing NKH18-C4 cells grew much more slowly than NKH18-C2 and NKH18-C3 cells (Fig. 4A). As expected, NKH18-C4 cells expressed both $p45^{NFE2}$ and α-globin (Fig. 4B). Interestingly, NKH18-C4 cells, which are heterozygous for $p45^{NFE2}$, proliferated rapidly after a month in culture. Southern analysis indicated that this new variant of NKH18-C4 (designated NKH18-C4A) had lost the wild-type $p45^{NFE2}$ allele (data not shown). Loss of $p45^{NFE2}$ in these cells was associated with the downregulation of α-globin expression (Fig. 4D). To further explore the growth suppressor ability of $p45^{NFE2}$ on erythroleukemic cells, we reintroduced this gene into the NKH18-C4A cell line using a retroviral vector. As shown in Fig. 4C, $p45^{NFE2}$-negative cells infected with $p45^{NFE2}$ retrovirus grew at a much lower rate than did cells infected with vector alone. Expression of $p45^{NFE2}$ in these cells also resulted in up-regulation of α-globin (Fig. 4D). Similar results were obtained when an independent $p45^{NFE2}$-negative erythroleukemia cell line NKH23-C was infected with the $p45^{NFE2}$ retrovirus (data not shown). Although globin genes are induced in these cells and are conventionally used as differentiation markers, morphological changes resembling erythroid cell differentiation (32) were not detected in these cells.

Although the difference in the time for development of Friend disease induced in +/− and +/+ mice was moderate but significant, we examined whether reexpression of $p45^{NFE2}$ in nonproducer cells can delay tumor growth in vivo. When NKH18-C4A cells infected with the vector alone or the $p45^{NFE2}$ retrovirus were injected into nude mice, expression of $p45^{NFE2}$ significantly suppressed growth of erythroleukemic cells in vivo (Fig. 5). Overall, these results confirm the role of $p45^{NFE2}$ in the regulation of globin gene expression and suggest that this transcription factor functions as a negative regulator of cell proliferation both in vitro and in vivo.

DISCUSSION

Analysis of the sites of proviral integration in Friend virus-induced erythroleukemias led to the isolation of the *Fli-2* locus,

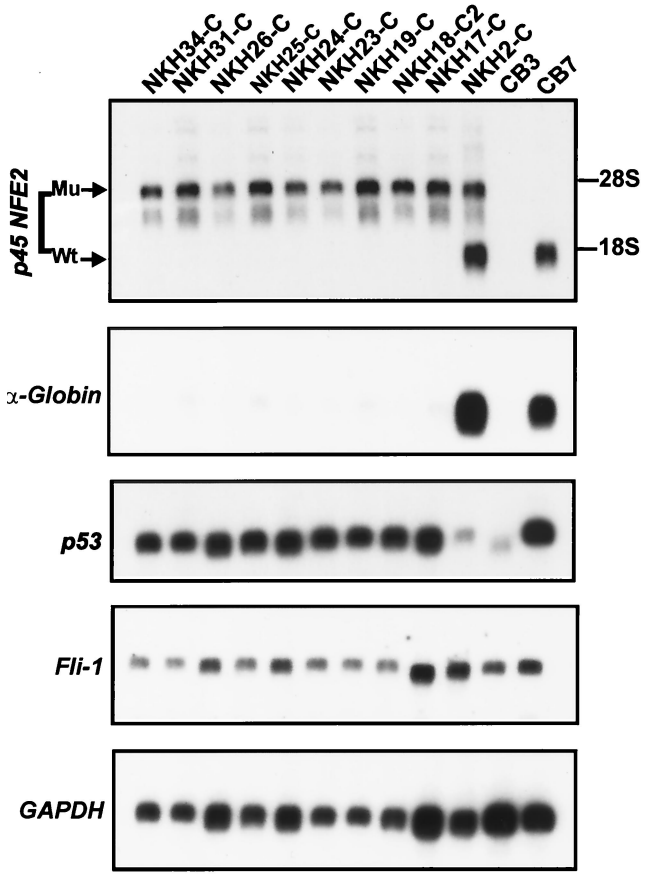

FIG. 3. Analysis of gene expression in erythroleukemia cell lines derived from $p45^{NFE2}$ mutant mice. Two micrograms of poly(A)$^+$ mRNA isolated from the indicated erythroleukemia cell lines was denatured in formamide, electrophoresed on 1% agarose gels, blotted onto nylon filters, and hybridized with the $p45^{NFE2}$ probe. The same blot was subsequently stripped and hybridized with α-globin, *p53*, or *Fli-1* probe and with GAPDH probe to normalize for RNA loading per lane.

a common site for retroviral integration localized within the $p45^{NFE2}$ gene (17). Frequent proviral insertion within the *Fli-2* locus, which was associated with the loss of the second allele in a single cell line, suggested a role for $p45^{NFE2}$ in the progression of erythroleukemias induced by Friend virus. We demonstrate that loss of $p45^{NFE2}$ in tumor cells enhanced proliferation and was associated with the growth of erythroleukemic cells in culture. Moreover, these results confirm a direct role for p45^{NFE2} in the regulation of globin gene expression in erythroid cells.

Since both the targeted and wild-type alleles of $p45^{NFE2}$ are expressed in erythroleukemias, lower expression levels of the functional protein may be responsible for the apparent, albeit modest growth advantage observed in vivo. Alternatively, the presence of a faster-growing subpopulation of erythroleukemic cells that lost the wild-type allele may have increased total cell numbers, resulting in accelerated tumor progression. This hypothesis is consistent with our demonstration that the proliferating erythroleukemic cells, maintained for less than 30 days in culture, are mostly negative for expression of the wild-type $p45^{NFE2}$ allele and that cells expressing the wild-type $p45^{NFE2}$ gene grow slower in vivo and in vitro. Moreover, cell lines established from the NKH18 tumors were shown to be a mixed population of both +/− and −/− erythroleukemic cells in which only the $p45^{NFE2}$ negative cells with higher proliferating rate eventually survived in culture.

We have previously reported a direct association between *p53* mutation, in vitro immortalization, and survival in culture of F-MuLV-induced erythroleukemias (11, 12). These observations were further supported when erythroleukemias were induced in the *p53* mutant mouse background (35). In these studies, *p53*-deficient mice infected with F-MuLV died at a higher rate than control mice and growth of erythroleukemias in culture was mainly seen in erythroleukemias induced in $p53^{-/-}$ and $p53^{+/-}$ mice. Data for the leukemogenic potential of $p45^{NFE2\ -/-}$ mice are not available because they died at birth or shortly after viral inoculation (28). We identified a strong similarity between in vivo and in vitro progression of erythroleukemias induced in $p53^{+/-}$ and $p45^{NFE2+/-}$ mice as follows. (i) Both $p53^{+/-}$ and $p45^{NFE2+/-}$ mice succumb to the disease more rapidly than $p53^{+/+}$ and $p45^{NFE2+/+}$ mice, respectively. (ii) Similar to cell lines derived from $p53^{+/-}$ tumors (35), in vitro establishment of erythroleukemias induced in $p45^{NFE2+/-}$ mice was associated with a loss of heterozygosity in 9 out of 10 established NKH cell lines. (iii) Both the *p53* and $p45^{NFE2}$ genes were frequently targeted for retroviral insertional inactivation, and loss of heterozygosity was commonly seen in erythroleukemias carrying viral integration in the other allele (7). These similarities raise the intriguing possibility that *p53* and $p45^{NFE2}$ have similar or overlapping functions during leukemogenesis and that like *p53*, $p45^{NFE2}$ functions as a tumor suppressor gene in Friend virus-induced erythroleukemias.

Of 10 cell lines established from $p45^{NFE2+/-}$ tumors, NKH2-C was the only cell line that remained heterozygous and showed dramatically reduced expression of *p53*. Similarly, *p53* inactivation was also seen in two previously established erythroleukemia cell lines, CB7 and DP28–9, which were also heterozygous for $p45^{NFE2}$ due to proviral insertion within the *Fli-2* locus (6, 22). Interestingly, the other nine cell lines with the $p45^{NFE2-/-}$ genotype did not appear to have abnormalities in *p53* expression, and sequence analysis of one of these cell lines confirmed wild-type *p53* status (unpublished results). To our knowledge, this is the first demonstration of an erythroleukemia cell line that expresses wild-type *p53* (35). These results suggest that mutations within either *p53* or $p45^{NFE2}$ may be required for immortalization of erythroleukemias.

The strong selective advantage for $p45^{NFE2}$ nullizygosity raises the possibility that expression of this gene in erythroleukemic cells negatively regulates their proliferative capability. Indeed, reintroduction of $p45^{NFE2}$ into the nonproducer erythroleukemia cell lines significantly attenuated cell growth rates in vitro and in vivo. In addition, in the case of the NKH18-T tumor that contained a clonally related population of +/− and −/− cells, erythroleukemic cells lacking this transcription factor proliferated faster in culture. This suggests that $p45^{NFE2}$ expression confers a negative growth advantage to erythroleukemic cells. The higher rate of tumor development and the increase in the size of tumorigenic spleens observed in +/− infected mice suggest that erythroleukemias induced in these mice may contain heterogeneous populations of both +/− and

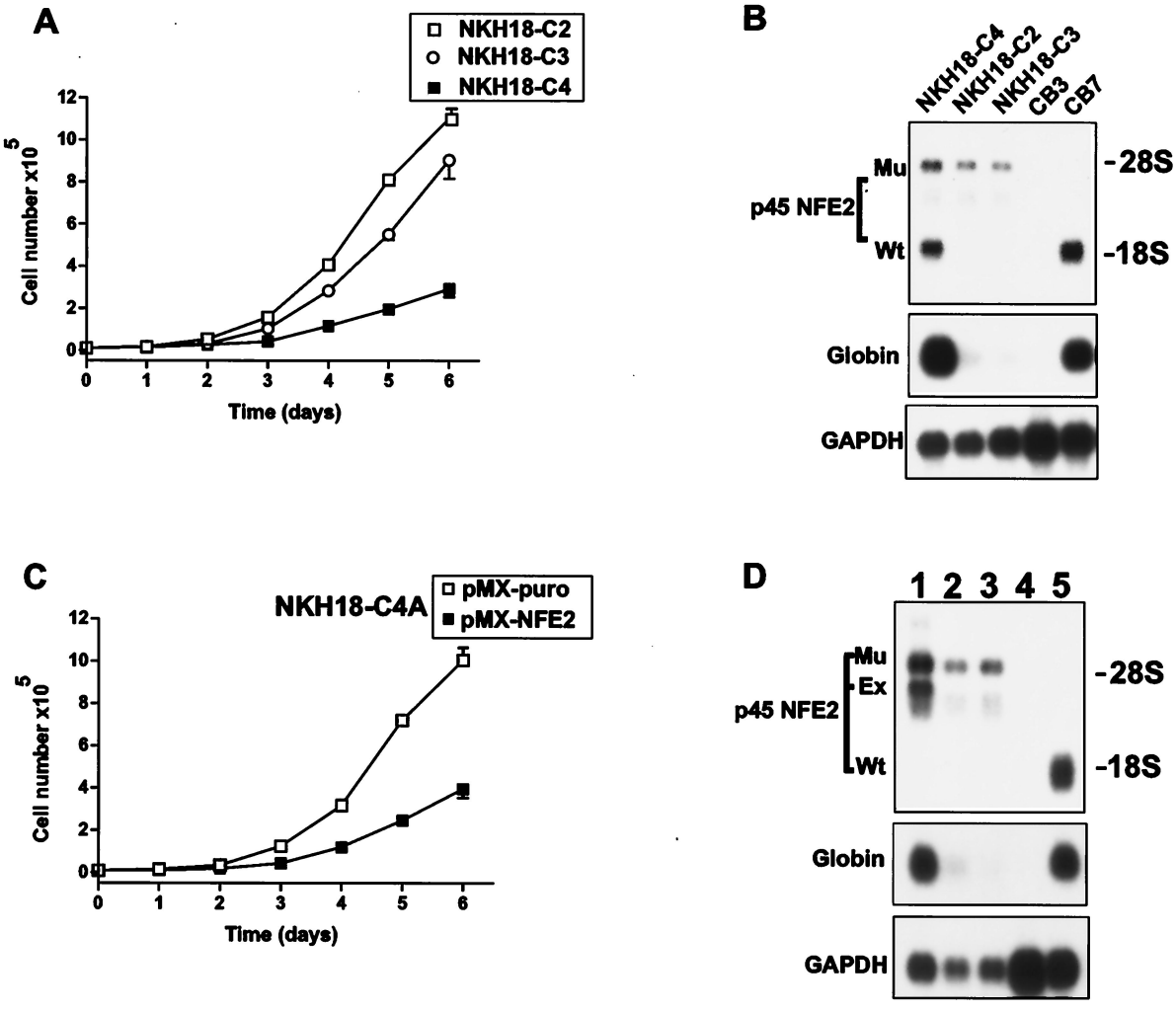

FIG. 4. Expression of $p45^{NFE2}$ in nonproducer erythroleukemia cell lines suppresses cell proliferation. (A) Triplicate cultures of the indicated cells (10^4) were cultured in the presence of Epo plus SCF, and the number of viable cells was determined for days 1 to 6 by trypan blue dye exclusion. (B) RNAs extracted from the indicated erythroleukemic cells were Northern blotted and sequentially hybridized with $p45^{NFE2}$, α-globin, and GAPDH probes. The $p45^{NFE2}$ producer cell line CB7 and the nonproducer cell line CB3 were used as controls. (C) Triplicate cultures (10^4) of NKH18-C4A cells that were infected with either PMX-puro or PMX-NFE2 retroviruses were grown in the presence of SCF plus Epo. The growth rate of these cells was determined for days 1 to 6 by trypan blue dye exclusion. (D) Northern blot analysis of NKH18-C4A cells (lane 3) infected with the PMX-puro (lane 2) and PMX-NFE2 (lane 1) retroviruses was performed as described for panel B. Cell lines CB3 (lane 4) and CB7 (lane 5) were used as controls for $p45^{NFE2}$-negative and -positive cell lines, respectively.

−/− cells. Since retroviral insertional activation of *Fli-1* is required for the initial transformation of erythroblasts by F-MuLV, the loss of $p45^{NFE2}$ in a subpopulation of these leukemic cells could result in the appearance of a cell population with a higher proliferative capability. Although clonal dominance of the −/− cells was not obvious by Southern analysis of the primary tumors, this could be explained by the early (~40 days) death of mice after viral inoculation, due to the development of severe anemia (35). This is supported by the observation that only −/− cells survive after 3 weeks of growth in culture. Furthermore, while the number of erythroid burst-forming unit and erythroid CFU progenitor cells were identical in +/+ and −/− mice (27), splenomegaly with active erythropoiesis throughout life has been observed in the surviving adult −/− mice (16). Together, these results support a negative role for $p45^{NFE2}$ in the proliferation of erythroblasts.

Previous studies using transgenic mice and cell lines indicated that $p45^{NFE2}$ plays a major role in globin gene expression (15, 17, 30). However, in mice lacking $p45^{NFE2}$, erythroid lineages were mildly affected and a small decrease in the hemoglobin content per cell was detected. Interestingly, −/− erythroleukemia cell lines independently derived from +/− mice were severely defective in globin gene expression, and reintroduction of $p45^{NFE2}$ into these cells resulted in restored globin expression. These observations support the notion that $p45^{NFE2}$ expression is critical for globin gene expression and further suggest that in −/− mice, the function of this protein may be compensated for by another factor capable of restoring globin gene expression. In contrast to adults, severe erythrocyte abnormalities, including extensive reticulocytosis, hypochromia, target cells, and dysmorphic cell forms, were detected in −/− neonates (27). Since F-MuLV induces erythroleuke-

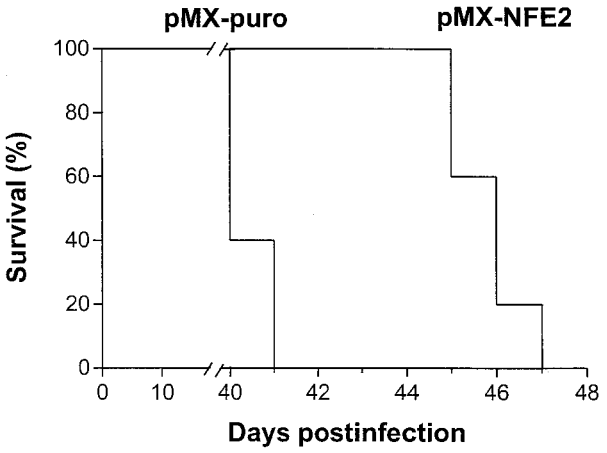

FIG. 5. Expression of *p45*^{NFE2} in nonproducer erythroleukemic cells suppresses growth in vivo. NKH18-C4A cells (10^6) that were infected with either pMX-puro or pMX-NFE2 retroviruses were injected into nude mice ($n = 5$ for each group). Days indicate the time postinfection at which the mice succumbed to the disease.

mias when injected into newborn mice, it is possible that this virus targets a subpopulation of erythroid progenitors in which globin expression is independent of p45^{NFE2}. It is also possible that these neonate-derived erythroid cells do not express the compensatory factor that overlaps the p45^{NFE2} function. Therefore, identification of such a factor could significantly enhance our understanding of globin gene regulation.

In summary, the results of this study demonstrate that loss of *p45*^{NFE2} expression is required for the establishment of permanent erythroleukemic cells in culture. The absence of *p45*^{NFE2} in erythroleukemic cells promotes tumor growth by accelerating the rate of cellular proliferation. Moreover, we provided comprehensive and direct evidence to support the requirement of *p45*^{NFE2} in globin gene expression.

ACKNOWLEDGMENTS

We thank Jorge Filmus for his comments on the manuscript and Lynda Woodcock for help in preparation of the manuscript.

This work was supported by a grant from the Medical Research Council of Canada to Y.B.-D. and the National Cancer Institute of Canada to M.A. R.A.S. was supported in part by a grant from the National Institutes of Health. P.A.N. was supported in part by National Institutes of Health grant ROI DK53469, NIH Cancer Center Support grant p30 (CA21762), and the American Lebanese-Syrian Associated Charities. B.J.P and Y.-J.L. were supported by fellowships from the Sunnybrook Trust Fund for Medical Research. R.R.H. was supported by fellowships from the Natural Science and Engineering Research Council (Canada) and the Leukemia Research Fund of Canada.

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