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Env-derived gp55 gene of Friend spleen focus-forming virus specifically induces neoplastic proliferation of erythroid progenitor cells

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A group of retroviruses carrying truncated viral genes has recently been suggested as the cause of new patterns of diseases. One such virus is the replication defective component of the Friend murine leukemia virus (F-MuLV) complex, called Friend spleen focus forming virus (F-SFFV). This virus induces erythroblastosis, and a virion envelope-related glycoprotein, gp55, encoded by F-SFFV has been suggested as the pathogenic gene. The role of the gp55 gene is, however, yet unclear in the apparently multistep erythroleukemogenesis. By separately producing transgenic mice harboring the whole F-SFFV DNA, the gp55 gene alone under the control of the retroviral long terminal repeat (LTR) and the gp55 gene under the control of cytoplasmic β actin transcriptional regulatory unit, we show here that the gp55 gene is capable of inducing neoplastic proliferation of erythroid progenitor cells specifically in the absence of helper virus and other F-SFFV sequences. Under the control of the viral LTR the gp55 expression was detected only in leukemic tissues, but under the control of cytoplasmic β-actin regulatory sequences, the gp55 was also expressed in a variety of normal tissues including preleukemic normal spleens. The development of erythroleukemia was suppressed under the genetic background of C57B1/6 mouse (resistant to F-MuLV; *Fv-2*^{rr}), and required additional events even under the background of DDD mouse (susceptible to F-MuLV; *Fv-2*^{ss}). The p53 and *Spi*-1 genes were frequently aberrant in transplanted tumors and cell lines derived from them, but were not in primary leukemic spleens.

Key words: erythroleukemia/Friend spleen focus forming virus/glycoprotein/gp55/retroviruses

Introduction

Retroviruses that cause acute neoplasia generally consist of a replication-competent helper virus and a replication-defective component that contains a cell-derived oncogene. However, in a group of defective retroviruses which has recently been identified as causing immunodeficiency or neuronal diseases (Szurek *et al.*, 1988; Aziz *et al.*, 1989; Chattopdhyay *et al.*, 1989; Hartley *et al.*, 1989; Overbaugh *et al.*, 1989; Paquette *et al.*, 1989), truncated viral genes, *env* or *gag* genes, rather than cellular genes, are likely to be responsible for the diseases. Spleen focus forming virus (F-SFFV) component in the acutely polycythemia inducing strain of Friend murine leukemia virus (F-MuLV) complex is representative of such viruses (Amanuma *et al.*, 1983; Clark and Mak, 1983; Wolff *et al.*, 1983).

The F-SFFV component possesses a retroviral *env*-derived gp55 gene and its expression coincides with erythroleukemogenesis (Ikawa *et al.*, 1978; Ruscetti and Wolff, 1984). The virus carrying mutations in this gene did not induce erythroleukemia (Ruta *et al.*, 1981; Linemeyer *et al.*, 1982; Ruscetti and Wolff, 1984). Thus, the gp55 gene products have been considered responsible for the erythroid transformation. The phenomena of defectiveness and interference, however, contribute to a complex series of interactions between the two viral components, and helper virus by itself can induce erythroleukemia (Troxler and Scolnick, 1978); the role of the defective component in the erythroleukemogenesis has therefore been in dispute (Teich *et al.*, 1982; Berger *et al.*, 1985; Bestwick *et al.*, 1985; Wolff and Ruscetti, 1985; Wolff *et al.*, 1986). Erythroleukemia induction by the viral complex is influenced by many mouse gene loci (Teich *et al.*, 1982), and leukemia development is generally considered to be composed of at least two processes characterized by polyclonal expansion of preleukemic cells, followed later by the emergence of clonal leukemic cells (Webdling *et al.*, 1981; Teich *et al.*, 1982; Ruscetti and Wolff, 1984; Sitbon *et al.*, 1986; Wolff *et al.*, 1986). Although the inactivation of the p53 gene (Mowat *et al.*, 1985; Chow *et al.*, 1987; Rovinski *et al.*, 1987; Ben-David *et al.*, 1988; Munroe *et al.*, 1988) or the insertional activation of the putative *Spi*-1 oncogene (Moreau-Gachelin *et al.*, 1988) in the leukemic phase and the role of gp55 products in the preleukemic phase have recently been suggested, the role of the gp55 gene products in these processes is yet unclear. No *in vitro* cell lines are known to be transformed with the F-SFFV DNA.

The gp55 gene in the F-SFFV genome is closely related to the envelope (*env*) gene present in mink-cell focus inducing viruses (Amanuma *et al.*, 1983; Wolff *et al.*, 1983; Clark and Mak, 1983), which have arisen by recombination of ecotropic murine leukemia viruses with xenotropic-like *env* genes endogenously present in the mouse DNA. Although most of gp55 localizes in endoplasmic reticulum as a glycoprotein having a high level of mannose type N-linked oligosaccharides, its processing to plasma membrane or its release from the cells is likely to be required for pathogenesis (Li,J.-P. *et al.*, 1987; Amanuma *et al.*, 1989). The glycoprotein has multidomains and has been considered to interact with specific 'dual-tropic' receptors on erythroid precursor cells which may naturally function in the process of proliferation and differentiation of these cells. Indeed, very recently gp55 was suggested to interact directly with erythropoietin receptor, thereby by-passing the normal

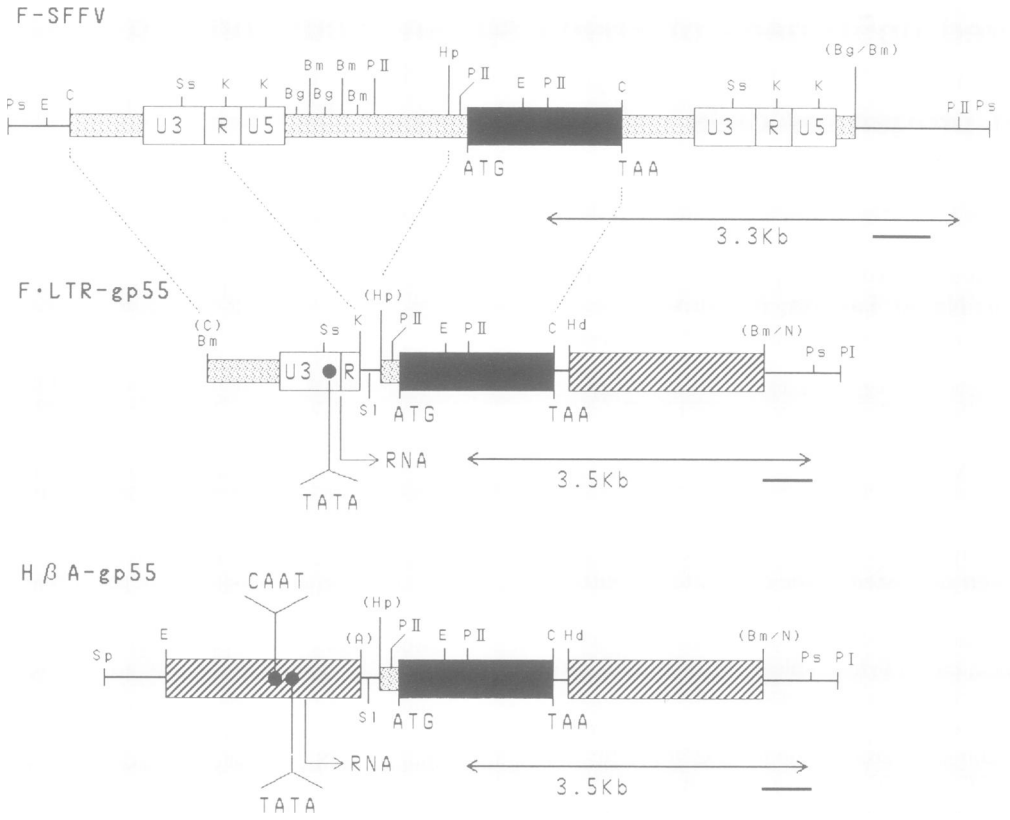

Fig. 1. Schematic representation of transgenes introduced. Filled boxes indicate the coding region of the p53 gene, open boxes LTR, dotted boxes the other F-SFFV sequences, stippled boxes 5′ and 3′ sequences of human β-actin gene, and bars pBR322-derived sequences. Relevant restriction endonuclease sites are indicated, when modified with the original site in parenthesis. Ps, *Pst*I; E, *Eco*RI; C, *Cla*I; Ss, *Sst*I; K, *Kpn*I; Bg, *Bgl*II; Bm, *Bam*HI; Hp, *Hpa*I; PI, *Pvu*I; PII, *Pvu*II; Hd, *Hin*dIII; Sa, *Sal*I; N, *Nde*I; Sp, *Ssp*I; A, *Alu*I. The sizes of the expected fragments in the Southern blot analysis of the transgenes are also shown underneath with the probes used.

requirement of erythropoietin for erythroid proliferation (Li *et al.*, 1990).

In this paper, we examined the role of the F-SFFV genome and the gp55 gene in erythroleukemia induction directly by producing transgenic mice. First, by introducing the whole F-SFFV DNA, we confirmed that the F-SFFV genome can induce not only the early stage but also the malignant stage of erythroleukemia in the absence of helper virus. Second, by introducing the gp55 gene alone under the control of the viral long terminal repeat (LTR), we confirmed that the gp55 gene is responsible for the oncogenicity. Third, we observed that the gp55 gene specifically penetrates erythroid cells, by introducing the gene under the ubiquitous transcriptional regulatory unit of cytoplasmic β-actin gene. The erythroleukemia development in these mice was probably influenced by *Fv-2* locus and required additional events. The aberrations of the p53 and *Spi*-1 genes were associated with the erythroleukemia, but apparently in later stages.

Results

Production of transgenic mice with F-SFFV specific gp55 gene

Figure 1 gives a schematic representation of the transgenes introduced. The F-SFFV genome used throughout the present study was derived from a polycythemic strain of Friend leukemia virus (Amanuma *et al.*, 1983). Among 65 pups born after injection of the whole F-SFFV DNA into zygotes, 25 mice integrated the injected DNA. Four of these F0 mice (SF1 to SF4) had died by 2 months after birth and all showed massive swelling of the spleen (1−4 g) with concomitant liver enlargement. Because of the early onset of the disease, no offspring could be obtained from them. Among the remaining 21 founder mice which had been healthy beyond 1 year, eight were mated and established as lines. Some offspring of the four founders (SF5−SF8) developed the same symptoms by 5 months as shown for SF6 lineage in Figure 2. No increase in the frequency of leukemia development was apparent in homozygous mice or by the transgene dosage.

In the second series of transgenic mice production, the *Hpa*I−*Cla*I fragment containing the gp55 gene was fused to the *Cla*I−*Kpn*I fragment containing the viral LTR, yielding the hybrid gene F·LTR−gp55 (Figure 1). Thirty-five pups were obtained from the zygotes injected with F·LTR−gp55, and seven were identified as harboring the transgene. Three of them (FLGP1−FLGP3) developed the disease by 1.5 months after birth.

In the third series of transgenic mice production, the *Hpa*I−*Cla*I fragment containing the gp55 gene was fused to the 5′ upstream sequences of human cytoplasmic β-actin gene as shown in Figure 1, yielding the hybrid gene HβA−gp55. Among 44 pups born from the zygotes injected with HβA−gp55, six harbored the transgene and three (HAGP1−HAGP3) developed the disease by 2 months after

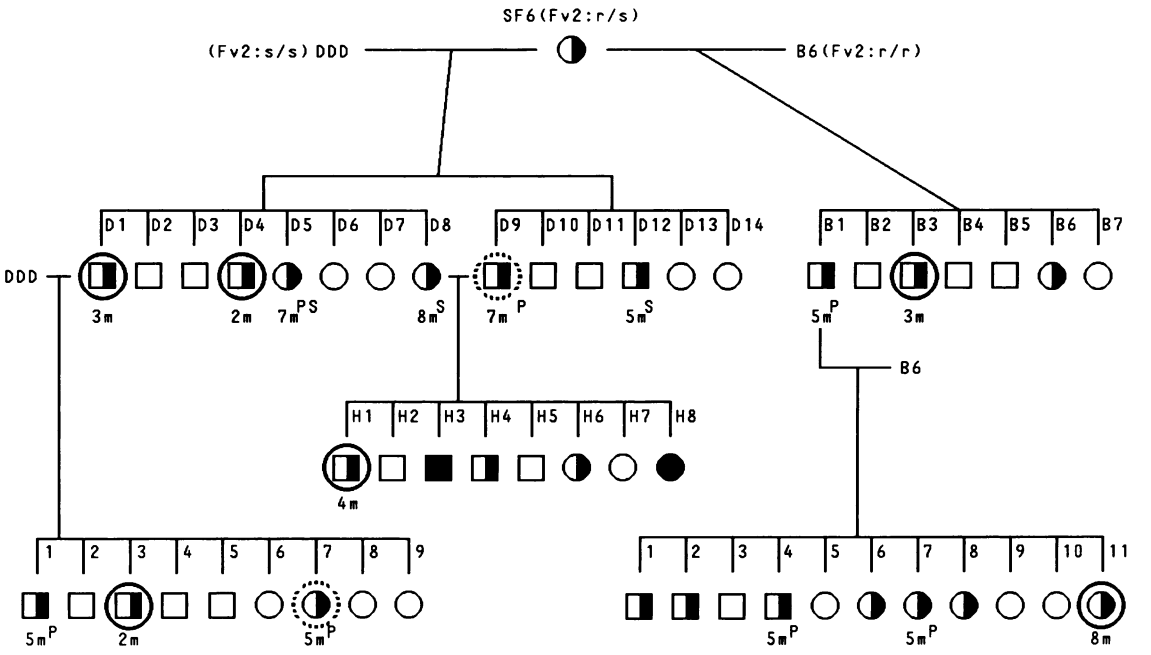

Fig. 2. A partial pedigree of SF6 lineage. An example of pedigree analysis is presented to examine the heritability of erythroleukemia. Females are indicated by open circles, males by open squares, hemizygous transgenic mice by half-filled circles and squares, and homozygous ones by solid circles and squares. Outer circles denote erythroleukemia development, with the age at death shown in months (m). The mice indicated by a superscript S were sacrificed and those by superscript P were treated with phenylhydrazine at the age shown. Outer dotted circles denote tumor development after this drug treatment. All other transgenic mice were autopsied at 10–12 months of age and were normal. The DNA was injected into a zygote obtained by the cross between a C57Bl/6 female and a DDD male, and the founder mouse was mated with DDD and C57Bl/6 mice to generate F1 offspring. The progenies were successively mated with either DDD or C57Bl/6 mice to examine the effects of the genetic background on erythroleukemia development. A part of the progenies were mated with each other to generate homozygous transgenic mice, which were maintained by brother–sister mating.

birth without giving rise to offspring. One founder (HAGP4) developed the leukemia at 3.5 months after birth, and the leukemia developed among its offspring in a stochastic fashion that was observed among the SF6 lines of mice (Figure 2).

Because of the presence of endogenous retrovirus-related sequences (Moreau-Gachelin *et al.*, 1986), some uncertainty remains as to the intactness of the transgenes in the above mice; the intactness was deduced by the presence of the expected 3.3 or 3.5 kb fragment containing half of the gp55 gene and 3′ sequences of the F-SFFV, F-LTR–gp55 or HβA–gp55 transgenes in Southern blot analysis (data not shown, cf. Figure 1).

Erythroid and malignant nature of leukemia

Regardless of the trans-gp55 genes introduced, the macroscopic symptoms observed in the above mice were the same as that induced by the polycythemic strain of the F-MuLV complex: massive swelling of the spleen with concomitant enlargement of the liver. All these transgenic mice showed an increase in the hematocrit value and thus were polycythemic (Figure 3). The increase in cells capable of forming erythroid colonies (CFU-E) was confirmed in three erythroleukemic spleens of the SF6 mice; 135 ± 14, 366 ± 42 and 420 ± 57 CFU-E/10^5 cells of the leukemic spleens in comparison with 0 CFU-E/10^5 cells of normal spleens without apparent effects of erythropoietin (128 ± 27, 392 ± 41 and 510 ± 72). All of the enlarged spleens had histologic findings typical of Friend erythroleukemia as shown in Figure 4. The blastic cells were positively stained

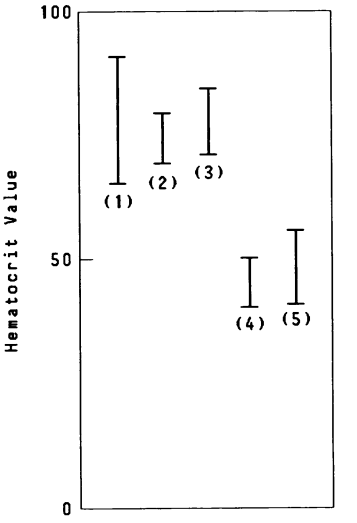

Fig. 3. Increase in hematocrit value in leukemic mice. The values shown are the range of determinations on 12 leukemia-bearing SF series (1), three FLGP series (2) and three HAGP series (3) of transgenic mice, 16 leukemia-free transgenic (4) and 22 nontransgenic control (5) mice.

with the anti-RBC antibody, consistent with the erythroid nature of the leukemias, and frequently infiltrated the liver sinusoid, kidney and lung. Among 14 independent leukemic cells examined, eight were transplantable in nude mice. Six of these primary transplants could be successively passaged

and four give rise to cell lines *in vitro* (Table I). The cultured cells were induced to differentiate into benzidine positive cells by dimethyl sulfoxide, confirming the erythroid nature of the leukemia (Figure 4).

At 5 months after birth, apparently healthy transgenic mice in the F3 generation of SF5 and SF6 lineages under the influence of DDD background (see below) were randomly examined for splenomegaly and polycythemia. No apparent

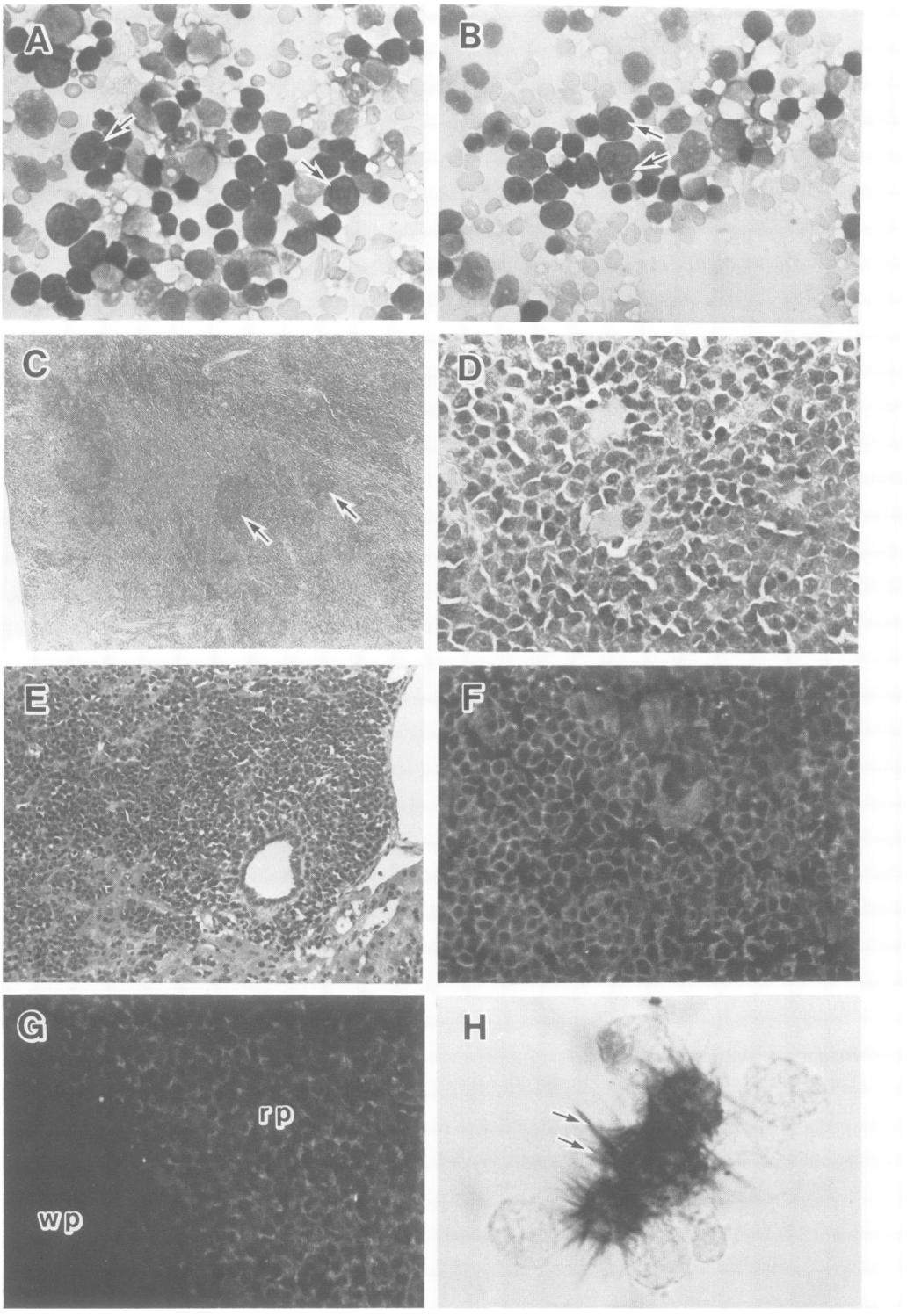

Fig. 4. Histologic appearances of leukemias. Stamp preparations from enlarged spleens stained with a Wright–Giemsa stain showed histologic features of typical Friend erythroleukemia not only in SF series transgenic mice (**A**), but also in FLGP and in HAGP series (**B**); large blastic cells with highly basophilic cytoplasm (arrows) and cells in various stages of erythroid differentiation were abundant, while lymphoid cells decreased significantly. In formalin-fixed preparations (**C,D**), regression of white pulps (arrows) was apparent, and erythroid lineages of cells were growing diffusely. The blastic cells frequently infiltrated into liver (**E**), kidney and lung, and were positively stained with anti-RBC antibody (**F**). Panel (**G**) gives the control staining of normal spleen positively in red pulp (rp) and negatively in white pulp (wp). The leukemic cells cultured *in vitro* were induced to differentiate into benzidine positive cells (arrows) by dimethylsulfoxide (**H**).

increase in the hematocrit value was observed in these mice, but some had mildly enlarged spleens (0.1−0.4 g), and the frequency was six out of 28 examined. Three of these mice remained alive and one developed erythroleukemia, but in two mice the enlarged spleens had regressed by 8 months after birth. The cells of these mildly enlarged spleens expressed gp55 (discussed below), but were not transplantable (Table I).

Gp55 expression and erythroleukemia development

The correlation of gp55 gene expression with neoplasia induction was examined by Western blot analysis; no Northern blot analysis was made because of the presence of the endogenous retrovirus-related products (Moreau-Gachelin *et al.*, 1986). As shown in Figures 5 and 6, under the control of F-SFFV LTR with the F-SFFV or F·LTR−gp55 construct, the trans-gp55 gene was generally expressed in leukemic spleens at high levels (Figures 5A, lane 6 and Figure 6, lanes 2−5), but occasionally at low levels (Figure 6, lane 6). Tissues such as lung, kidney and liver also frequently expressed gp55 (Figure 5, lanes 4, 5 and 7), but the level was well correlated with the extent of infiltration of the leukemic cells. No detectable gp55 expression was observed in any other normal tissues of the leukemic mice (Figure 5, lanes 3 and 8−10). The gene was also not expressed in any normal tissues including spleen of apparently health preleukemic transgenic mice (lanes 12−20). Under the control of the cytoplasmic β-actin regulatory unit, the gp55 gene was expressed at high levels in leukemic spleens of HAGP2 and HAGP3 mice (Figure 6, lanes 19 and 20) and at a moderate level in a HAGP4 line of mice (Figure 7). In these animals, the gene was also expressed at high levels in other tissues such as pancreas, muscle, heart and lung without any apparent histologic lesions in these tissues (Figure 7). Preleukemic normal transgenic mice of the HAGP4 line showed almost exactly the same pattern and level of gp55 expression in each tissue including spleen as the leukemic mice of this line. Thus, the gp55 expression is not adequate to induce erythroleukemia.

To examine the possibility that a low pool size of the target erythroid precursor cells explains the lack of gp55 expression in normal spleens under the control of the viral LTR, apparently healthy mice of SF5 and SF6 lineages were treated with phenylhydrazine, but no gp55 gene products were detected in any enlarged spleens of eight transgenic mice examined (Figure 6, lanes 10 and 11). Erythroleukemia developed in three out of 12 transgenic mice treated twice with the drug 2−3 months later.

Under the control of F-SFFV LTR, gp55 products were detected in the moderately enlarged spleens of up to 0.3 g (Figure 6, lanes 7 and 8), the cells of which were not transplantable (Table I). The gp55 gene was also expressed in erythroid cells cultured *in vitro* from the transplanted leukemic tissues (Figure 6, lanes 12 and 13). Interestingly, gp55 was not expressed in normal cells of the normal kidney, but was expressed in permanent fibroblast-like cells derived from them (Figure 6, lanes 14−17).

Table I. Tumorigenicity in various gp55-induced leukemic cells

Transgenic mouse	Spleen weight	No. transplanted	No. producing tumors	Cell line[a]
SF3	2.5 g	6	0	
SF5-D12-9	2.8 g	6	1	E
SF5-D12-19	1.4 g	4	0	
SF6-D4	2.0 g	6	0	
SF6-D1-3	4.3 g	5	2	E
SF6-D8-H1	2.1 g	5	0	
SF6-D1-1-18	3.1 g	6	3	E
SF7-D5-6	2.3 g	6	3	E
SF8-D1-9	1.2 g	6	1	NT
FLGP1	1.4 g	5	0	
FLGP2	2.1 g	6	1	NT
HAGP2	1.7 g	6	1	NT
HAGP4	1.8 g	7	2	NE
HAGP4-D5	1.3 g	6	0	
SF5-D12-7-1	0.25 g	8	0	
SF6-D1-1-5	0.18 g	9	0	
SF6-D8-H3-7	0.21 g	12	0	

[a]E, established as cell line; NE, not established; NT, not tested.

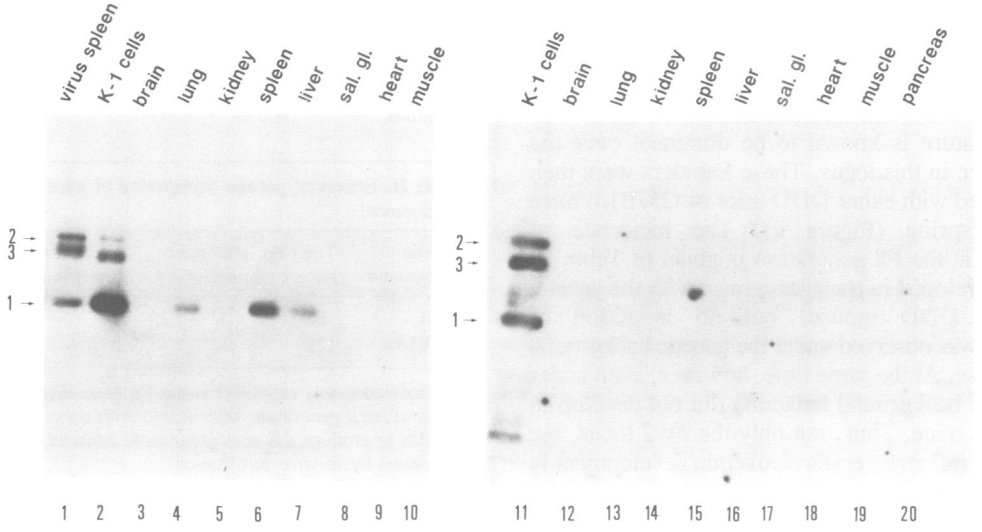

Fig. 5. Western blot analysis of gp55 expression in erythroleukemic and preleukemic SF6 series mice. **Lane 1** represents leukemic spleens of DBA/2 mice infected with the F-MuLV complex; **lanes 2** and **11**, Friend cells (T-3-Cl-2K-1) (Ikawa *et al.*, 1976); **lanes 3−10** and **12−20** represent indicated tissues of leukemic SF6-D1 and normal SF6-D12 mice, respectively. The positions of gp55 (arrow 1), Pr*env*85 (arrow 2) and gp70 (arrow 3) bands are indicated.

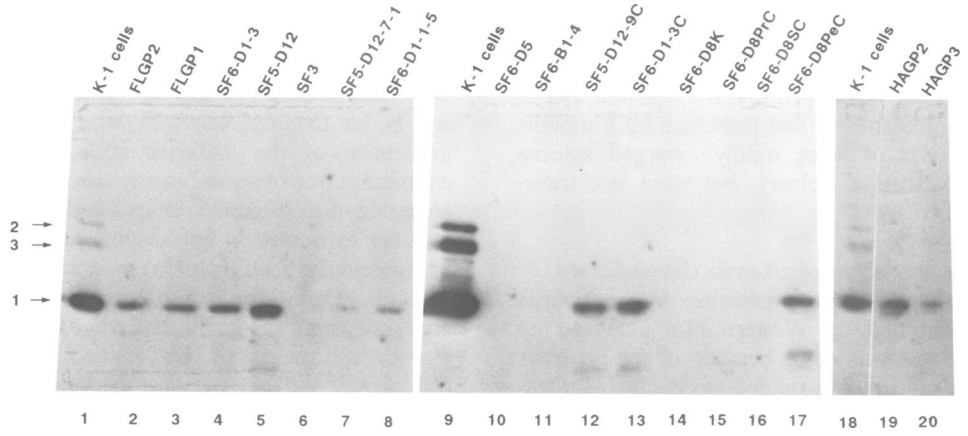

Fig. 6. Western blot analysis of gp55 expression in normal and leukemic spleens and in cells cultured *in vitro*. **Lanes 1, 9** and **18** represent Friend cells (T-3Cl-2K-1); **lanes 2–6, 19** and **20** leukemic spleens of indicated mice; **lanes 7** and **8** moderately enlarged spleens (0.25 and 0.3 g, respectively) of indicated mice; **lanes 10** and **11** spleens of indicated normal mice treated with phenylhydrazine; **lanes 12** and **13** erythroid cells cultured from transplants of leukemic spleens of SF5-D12-9 and SF6-D1-3 mice respectively; **lanes 14–17** normal kidney tissues (K) of the SF6-D8 mouse, their primary culture (PrC), senescent culture (SC) and permanent fibroblast-like cells (PeC) derived from them, respectively.

The Western blot analysis also indicated the absence of the Pr*env* and gp70 bands of helper viruses in all the leukemias examined, demonstrating that helper virus was not present in these leukemic cells. Furthermore, several leukemic spleens were tested for release of virus by inoculating supernatants of leukemic spleen homogenates into DDD mice and were found to be negative (data not shown).

Effects of genetic background of mice

Several mouse gene loci have been known to affect the development of erythroleukemia by the F-MuLV complex (Teich *et al.*, 1982). Most of these have been considered to relate to the infection and replication of the virus, and can be considered irrelevant to erythroleukemia development in transgenic mice. However, *Fv-2* gene might influence the leukemia development in these mice (Odaka and Matsukura, 1969; Suzuki and Axelrad, 1980; Axelrad *et al.*, 1981; Silver and Teich, 1981; Dewey and Eldridge, 1982; Teich *et al.*, 1982; Van Zant *et al.*, 1983; Behringer and Dewey, 1985). We introduced the F-SFFV DNA into F1 zygotes between C57B1/6 (*Fv-2*: r/r) × SJL (s/s) or C57B1/6 × DDD (s/s) (see Materials and methods for the background of each transgenic mouse), and thus the genetic background of all founder mice is r/s at the *Fv-2* locus. The susceptible (s) nature is known to be dominant over the resistant (r) nature in this locus. These founders were then successively mated with either DDD mice or C57B1/6 mice to generate offspring (Figure 2). The incidence of erythroleukemia at the F8 generation is given in Table II. The leukemia developed in transgenic mice with the genetic background of DDD mouse, but no induction of erythroleukemia was observed under the genetic background of C57B1/6 mouse. At the same time, however, even under the DDD genetic background leukemia did not develop in all the transgenic mice. Thus, not only the *Fv-2* locus, but additional factors influence erythroleukemia development in these mice.

Aberration of p53 and Spi-1 genes in leukemic cells

To examine the possible participation of the p53 and *Spi*-1 genes in erythroleukemia development in these transgenic mice (Mowat *et al.*, 1985; Chow *et al.*, 1987; Moreau-Gachelin *et al.*, 1988), Southern blot analysis was carried out on genomic DNAs of leukemic cells with the p53 cDNA covering exons 2–5 as a probe after *Eco*RI, *Hin*dIII or

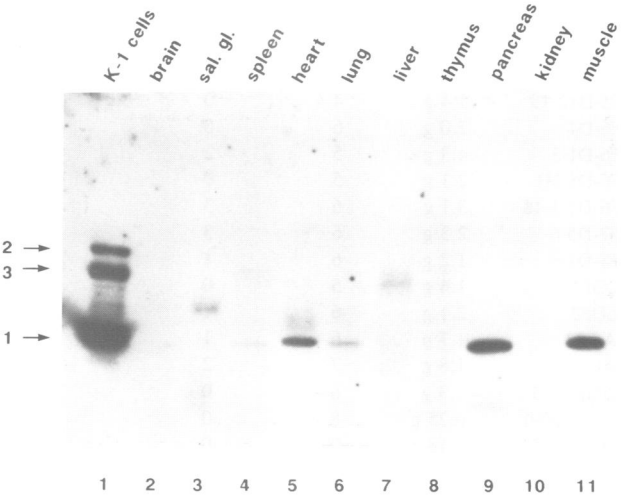

Fig. 7. Western blot analysis of gp55 expression in HAGP4 mouse. **Lane 1** represents Friend cells; **lanes 2–11** represent indicated tissues of leukemic HAGP4 mouse. Tissues other than spleen and liver were histologically normal and had no infiltration of leukemic cells. Preleukemic normal mice of this lineage had almost the same level and pattern of gp55 expression when F1 transgenic offspring were examined.

Table II. Effects of genetic background of mice on leukemia development

Genetic background	Total no. transgenic mice examined	No. developing erythroleukemia	Incidence (%)
DDD	66	12	18
C57B1/6	128	0	0

The incidence was examined at the F8 generation of the SF6 lineage; males of each generation were mated with either DDD or C57B1/6 females to produce the next generation. Most of the matings were performed by *in vitro* fertilization.

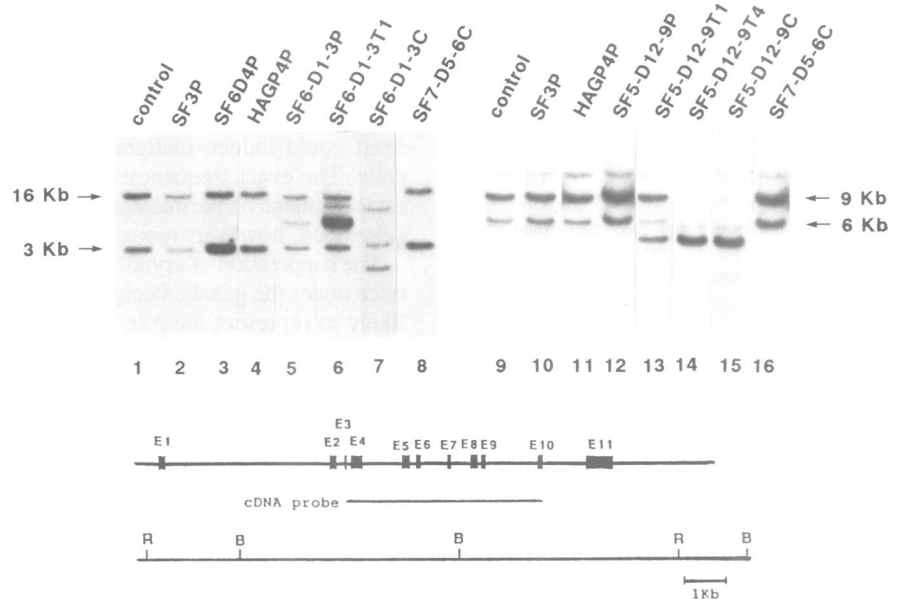

Fig. 8. Southern blot analysis of the aberration of the p53 gene in leukemic cells. An example of Southern blot analysis of the p53 gene is given for normal spleen cells of C57Bl/6 mice (**lanes 1** and **9**), leukemic spleen cells (**lanes 2−5** and **10−12**), transplanted tumors passaged once (**lanes 6** and **13**) and four times (**lane 14**) and cell lines (**lanes 7, 8, 15** and **16**) respectively originating from the indicated mice, with the p53 cDNA probe after the *Eco*RI (lanes 1−8) and *Bam*HI (lanes 9−16) digestions. The positions of the fragments expected from the normal gene and pseudogene are indicated by arrows.; the normal gene gave rise to a 16 kb *Eco*RI fragment, and 9 and 6.0 kb *Bam*HI fragments and the pseudogene to a 3.3 kb *Eco*RI fragment and 9 kb *Bam*HI fragment (Zakut-Houri *et al.*, 1983). Suffix P denotes the DNAs prepared from primary leukemic spleens, T from transplants with the passage number and C from cultured cells.

*Bam*HI digestion (Zakut-Houri *et al.*, 1983) and with the A or B probe (Moreau-Gachelin *et al.*, 1988) after *Eco*RI, *Eco*RV or *Bam*HI digestion, respectively. The details of the analyses will be published elsewhere, but an example of such an analysis is given in Figures 8 and 9. Twelve out of 14 leukemic spleens examined (cf. Table I) had normal configurations of both p53 and *spi*-1 genes (Figures 8, lanes 2−4 and 10−12; Figure 9, lanes 2, 3, 8 and 9). However, in the remaining two leukemic spleens of SF6-D1-3 and SF8-D1-9 mice, minor populations of the spleen cells had aberrant configurations of the p53 and *Spi*-1 genes (Figure 8, lane 5; Figure 9, lane 4). These leukemic spleens were successfully transplanted, and transplanted tumors had the aberrant configurations of the p53 and *Spi*-1 genes (Figure 8, lane 6. Figure 9, lane 5). The transplanted tumors originating from the SF6-DF1-3 mouse could be cultured and the p53 gene was further changed in these cells (Figure 8, lane 7). Out of 12 leukemic spleens which did not have any aberrant p53 or *Spi*-1 genes, six could be transplanted and three of these had aberrant forms of the p53 and *Spi*-1 genes in minor populations of the first passage of the tumors and in the major populations of the later passages (Figure 8, lanes 12−14; Figure 9, lanes 9 and 10). Two of these could be cultured and retained the aberrant forms of the p53 (Figure 8, lane 15) and *Spi*-1 genes. Only one tumor originating from the SF7-D5-6 mouse retained the normal configurations of these genes even after the *in vivo* passage and *in vitro* culture within the limit of the assay (Figure 8, lanes 8 and 16; Figure 9, lanes 6 and 11). Thus, the aberrations of the p53 and *Spi*-1 genes were frequently associated with transplantable tumors and cells derived from them as summarized in Table III. It is worthy of notice that the aberration of the p53 gene and that of the *Spi*-1 gene coincided in these tumors.

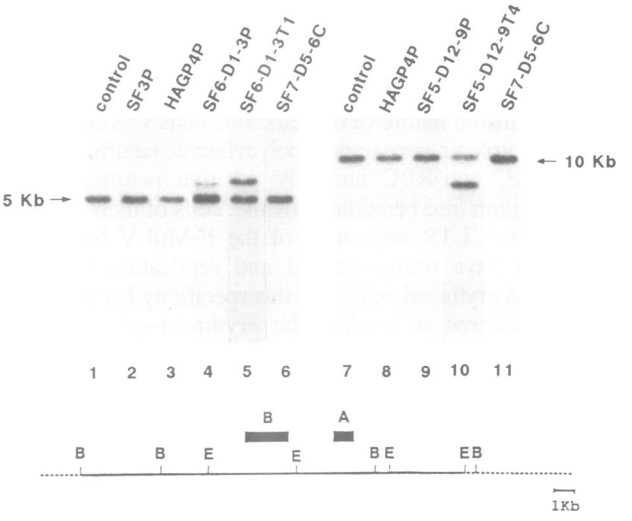

Fig. 9. Southern blot analysis of the aberration of the *Spi*-1 gene in leukemic cells. An example of Southern blot analysis of the *Spi*-1 gene is given for normal spleens of C57Bl/6 mice (**lanes 1** and **7**), leukemic spleen cells (**lanes 2−4** and **8−9**), transplanted tumors passaged once (**lane 5**) and four times (**lanes 10**) and cell lines (**lanes 6** and **11**), respectively originating from the indicated mice, with the A probe (Moreau-Gachelin *et al.*, 1988) after the *Eco*RI digestion (lanes 1 to 6) and with the probe B after the *Bam*HI digestion (lanes 7 to 11). The positions of the fragments expected from the normal gene are indicated by an arrow.

Discussion

Previous studies of F-MuLV-induced erythroleukemia have suggested that the disease evolves from a polyclonal stage to a clonal disease characterized by the emergence of tumorigenic cells. Using helper-free stocks, Berger *et al.*

Table III. Aberrations of p53 and *Spi*-1 genes at various stages of erythroleukemia

Genes	Enlarged spleens		Transplanted tumors		Cell lines
	<0.4 g	>1.0 g	Primary[a]	Late[a]	
p53	0/3	0/14 (2)[b]	2/8 (3)[b]	5/6	3/4
Spi-1	0/3	0/14 (2)[b]	2/8 (3)[b]	5/6	3/4

[a]'Primary' indicates tumors transplanted once and 'late' more than three times.
[b]In the cases indicated in parenthesis, aberrant forms of the genes were detected as minor populations.

(1985) observed that the F-SFFV genome was able to induce the early stage of Friend leukemia but the leukemia regressed without the emergence of malignant cells. On the other hand, Wolff and Ruscetti (1985), Wolff *et al.* (1986) reported that single infection of helper-free F-SFFV induced neoplastic transformation of erythroid cells. In addition, the gp55 gene in the absence of other F-SFFV sequences, when introduced into a retrovirus vector derived from Moloney murine leukemia virus, could induce the early stage of the disease (Wolff and Ruscetti, 1988). The present results with transgenic mice extend these observations by Wolff *et al.* with helper-free virus and strongly suggest that not only the F-SFFV genome, but the gp55 gene by itself can cause malignant transformation of erythroid cells uniquely. It could be questioned whether leukemia induction by the F-SFFV whole genome was mediated by viruses somehow formed. This possibility, however, was ruled out by the absence of leukemia induction with homogenates of leukemic spleens as well as by the absence of Pr85*env* and gp70 products in leukemic tissues.

The erythroid nature of the leukemic cells was confirmed by their histologic appearance, polycythemic nature, increase in CFU-E, anti-RBC antibody positive nature and the differentiation into benzidine positive cells of their cultured counterparts. LTR sequences of the F-MuLV have been known to have transcriptional and replicational activity uniquely in erythroid cells, and this specificity has generally been considered to explain the erythroid-specific transformation by the viral complex (Chatis *et al.*, 1983, 1984; Evans and Morrey, 1987; Holland *et al.*, 1987; Li,Y. *et al.* 1987; Thiesen *et al.*, 1988). On the other hand, Wolff and Ruscetti (1986) reported that the recombinant virus in which the 3'LTR of F-SFFV was replaced by the corresponding regions of Moloney murine leukemia virus or mink-cell focus forming virus, induced erythroleukemia. It is also well known that even if it is expressed, the gp55 gene cannot transform NIH3T3 cells and cells of other known cell lines. Our present result that erythroleukemia developed uniquely even under the control of the ubiquitous transcriptional regulatory unit of the cytoplasmic β-actin gene strongly suggests that gp55 products themselves specifically penetrate erythroid precursor cells. The target specificity may be explained by the interaction of gp55 with molecules specific to erythroid cells. Indeed, gp55 was recently reported to bind directly to erythropoietin receptor (Li *et al.*, 1990).

Putative preleukemic and leukemic phases in erythroleukemia development by the F-MuLV complex present in the same clinical picture, and there have been no definite markers to distinguish them. Transplantability and availability of culture have been hallmarks of the malignant stage of Friend disease (Webdling *et al.*, 1981). The leukemias developed in the transgenic mice were infiltrative and at least some of them were transplantable. Some of these transplantable leukemias could be cultured *in vitro*. Thus, not only the F-SFFV genome, but also the gp55 gene by itself could induce malignant transformation of erythroid cells. The exact frequency of transplantable tumors and of establishment of permanent cultures among the leukemias developed, however, requires more extensive examination.

The suppression of erythroleukemia induction in transgenic mice under the genetic background of C57B1/6 mice is most likely to represent the role of the *Fv-2* locus. The result in mice supports the assumption that this locus is not a gene involved in viral infection and replication *per se* (Odaka and Matsukura, 1969; Suzuki and Axelrad, 1980; Axelrad *et al.*, 1981; Silver and Teich, 1981; Dewey and Eldridge, 1982; Teich *et al.*, 1982; Van Zant *et al.*, 1983; Behringer and Dewey, 1985). The role of immunologic loci such as H-2 (Teich *et al.*, 1982; Chesebro *et al.*, 1974), however, cannot be ruled out from the present experiments. Congeneic strains have been established in which the $Fv\text{-}2^s$ allele was introduced into the C57B1/6 ($Fv\text{-}2^{rr}$) background and in which $Fv\text{-}2^r$ allele was introduced into the DDD background (Odaka, 1970; Axelrad *et al.*, 1972). Introduction of the gp55 gene into these congeneic mice will more clearly demonstrate the role of the *Fv-2* locus in erythroleukemogenesis.

Leukemia developed only in a fraction of the transgenic members of the SF6 family even under the DDD genetic background. The gp55 gene products were expressed only in leukemic spleens and not in any normal spleens under the control of the viral LTR. There are two possible explanations for this. One is that the gene is expressed in target erythroid cells but it could not be detected because of the limited pool size of these cells; the stochastic occurrence of tumors can then be explained by the necessity of secondary events. The target cells of the F-MuLV complex have been postulated as being the erythroid precursor cells at a proerythroblastic stage, or more specifically, at the differential stage between burst-forming unit erythroid (BFU-E) and colony-forming unit erythroid (CFU-E) (Shibuya and Mak, 1983). The effects of the increase in pool size of these cells were tested by treating the mice with phenylhydrazine. No expression of the p53 gene, however, was detected in any enlarged spleens of these mice. The effects on the erythroleukemia development were also not remarkable, though more extensive experiments are required to draw a definite conclusion. The other explanation is that the transcriptional activity of the viral LTR is suppressed in normal cells during the course of development and differentiation, and relaxation of suppression and expression occur stochastically in the normal target cells which gives rise to stochastic occurrence of the leukemias. Such regulation of transgenes *in vivo* was observed with the SV40 large T gene (Suda *et al.*, 1987, 1988). Primary cells from kidneys of the transgenic mice did not express the gp55 gene, but permanent fibroblast-like cells derived from them did. However, even if this were the case, the gp55 expression in the target erythroid cells is less likely to be adequate for erythroleukemia development; the cells of mildly enlarged spleens expressed gp55, but were not transplantable. Among those, some developed into malignant erythroleukemia, but some appeared to regress, though the exact frequency

remains for further examination. Furthermore, the observation in the HAGP4 line of mice that the gp55 gene was expressed in preleukemic, normal spleens and there was no apparent increase in the expression associated with leukemia development may suggest that some additional events are required to induce erythroleukemia. The effects of the level of gp55 expression on leukemia induction could be another factor influencing tumor incidence. In many cases the gene was expressed at a high level, but in some cases the level was moderate even in transplanted tumors. A modest level of gp55 expression may be sufficient to induce leukemia in conjunction with secondary events as it is in some oncogene-induced tumors (Quaife *et al.*, 1987; Suda *et al.*, 1987).

The changes of the putative suppressor p53 gene and the putative proto-oncogene *Spi*-1 have frequently been observed in leukemic cells as they were in Friend virus-induced erythroleukemias (Mowat *et al.*, 1985; Chow *et al.*, 1987; Rovinski *et al.*, 1987; Ben-David *et al.*, 1988; Munroe *et al.*, 1988; Moreau-Gachelin *et al.*, 1988; Finlay *et al.*, 1989). In addition, the changes of these genes coincided, although they did not in the Friend virus-induced tumors (Moreau-Gachelin *et al.*, 1988). Three out of the four cell lines established and five out of eight transplanted tumors had aberrations in these genes; this frequency is unusually high when one considers the limit of the assay in which mutations cannot be detected (Arai *et al.*, 1986; Eliyahu *et al.*, 1988; Finlay *et al.*, 1988; Hinds *et al.*, 1989; Nigro *et al.*, 1989). However, the aberrations were more apparent in progressed tumors and not in the primary erythroleukemic spleens. Thus, the changes of the p53 and *Spi*-1 genes are more likely to be associated with the later stages of erythroleukemia or with the processes of adaptation to transplantation or culture (Ruscetti and Scolnick, 1983). Other unknown events may be required to induce erythroleukemia primarily in these transgenic mice. One promising approach to target the genes of which changes cooperate with the gp55 gene is the gene-inactivation or activation by retrovirus infection into these mice (Van Lohuizen *et al.*, 1989). Indeed, Moloney leukemia virus could induce erythroleukemia uniquely in all of the transgenic mice of the SF6 line preliminarily tested at 25–55 days after inoculation. More importantly, in a similar time course the virus also induced erythroleukemia in the HAGP4 line of transgenic mice. Efforts are in progress to identify the gene(s) possibly targeted by the virus.

Materials and methods

Transgene construction

The source of the F-SFFV DNA used in the present study was pLSF4 (Amanuma *et al.*, 1983). To construct pHβA-gp55, the gp55 gene encompassing the *Hpa*I and *Sst*I sites of pLSF4 was inserted into pUC19 at *Sma*I and *Sst*I sites and released as a *Pst*I–*Cla*I fragment. The fragment was reintroduced into pBR322 at the *Pst*I and *Cla*I sites and released as a *Sal*I–*Hin*dIII fragment. This fragment was inserted into *Sal*I and *Hin*dIII sites of pHβAPr-3-neo, an expression vector with human β-actin 5′ and 3′ sequences kindly provided by Dr L.Kedes (Stanford University School of Medicine). The F-LTR–gp55 was constructed as follows: pLSF4 was cleaved with *Eco*RI and *Kpn*I, and the fragment containing the U3 region was cloned into *Eco*RI and *Kpn*I sites of pUC19, which was released by *Eco*RI and *Hin*dIII and reinserted into the *Eco*RI–*Hin*dIII sites of pBR322. The recombinant was digested with *Eco*RI and *Cla*I, and the sites were ligated with *Bam*HI linker. The fragment containing the U3 region was then released as a *Pvu*I–*Sal*I fragment and substituted with the fragment containing the *neo* gene and 5′ sequences of human β-actin gene in pHβA-gp55 at *Pvu*I and *Sal*I sites.

Production of transgenic mice

In injecting DNAs into mouse zygotes, the plasmid pLSF4 was digested with *Pst*I, pF-LTR-gp55 with *Bam*HI and *Pvu*I, and pHβA-gp55 with *Pvu*I and *Ssp*I. The digested DNAs were separated by agarose electrophoresis, purified and dissolved in an injection buffer (10 mM Tris–HCl pH 7.4, 0.25 mM EDTA) to a final concentration of 1 μg/ml. Fertilized eggs were obtained from superovulated C57B1/6 females by mating with SJL or DDD males; the founders SF1–SF2 were obtained with SJL males, and SF3–SF8, FLGP1–FLGP3 and HAGP1–HAGP4 with DDD males. DNA injection into the pronucleus of mouse zygotes was performed as previously described (Suda *et al.*, 1987).

DNA analysis

High mol. wt DNAs extracted from ~1 cm tail sections of newborn mice were digested with *Pvu*II for the SF series of mice or with *Pst*I and *Pvu*II for the FLGP and HAGP series, and the digests were electrophoresed on 1% agarose gels. The fractioned DNAs were then transferred to nitrocellulose paper (Southern, 1975). The presence of the trans-gp55 genes was probed by hybridizing the filters with [^{32}P]dCTP-labeled pBR322 sequences (a bp 548–1205 *Nar*I fragment or a bp 2297–3609 *Nde*I–*Pst*I fragment), as previously described (Suda *et al.*, 1987).

For analysis of the p53 gene, high mol. wt DNAs extracted from spleens, transplants or cells cultured *in vitro* were digested with *Hin*dIII, *Eco*RI or *Bam*HI. After electrophoresis, the DNAs were probed with a [^{32}P]dCTP-labeled p53 cDNA (a *Bgl*II–*Pst*I fragment) as described (Mowat *et al.*, 1985; Chow *et al.*, 1987). For analysis of the *Spi*-1 gene, the DNAs were probed with a [^{32}P]dCTP-labeled A or B fragment (Moreau-Gachelin *et al.*, 1988) kindly given by Dr F.Moreau-Gachelin.

Western blot analysis

For analysis of the gp55 protein, each minced tissue was homogenized in RIPA buffer (20 mM Tris–HCl pH 7.2, 1% NP-40, 0.1% SDS, 150 mM NaCl, 2 mM PMSF) and the homogenates were centrifuged at 12 000 r.p.m. for 10 min. Supernatants containing 30 μg protein were mixed with 5-fold sample buffer (10 mM Tris–HCl pH 7.2, 1% SDS, 0.1 M mercaptoethanol, 10% glycerol, 0.001% BPB) and the proteins were solubilized by boiling. The solubilized protein was subjected to SDS–PAGE on 10% resolving gels and the fractioned proteins were electrophoretically transferred to Durapoa membrane (Nihon Millipore). The membranes were blocked with BSA and incubated with goat antiserum to Rauscher-MuLV gp70 (NCI 80-S-18) at a dilution of 1/333. After washing with rinse buffer (10 mM Tris–HCl pH 7.5, 0.15 M NaCl, 1 mM EDTA, 0.1% Triton X-100), the membranes were incubated with [^{125}I]protein A (Amersham 0.2 Ci/ml) and autoradiographed.

Histologic analysis

Complete autopsies were performed whenever possible and organs were observed both grossly and histologically. For the latter, tissues were fixed with 4% formaldehyde in PBS and embedded in paraffin. The paraffin blocks were sectioned at 4 μm thickness and the sections were routinely stained with hematoxylin and eosin. The spleen stamps were stained with Giemsa. Immunohistology was performed in frozen sections with anti-mouse RBC antibody (Cappel) as previously described (Suda *et al.*, 1987).

Transplantation

Enlarged spleens were chopped into small pieces and dispersed by rubbing with two pieces of ground slide glass in PBS containing 10% serum. Singly dispersed cells were collected by passing through 100 μm mesh and 1×10^7 cells were transplanted into the abdominal cavity or under the skin of nu/nu mice (Charles River, Japan) as described (Webdling *et al.*, 1981).

Phenylhydrazine treatment of mice

Phenylhydrazine solution (0.2 ml of 6 mg/ml) was injected intraperitoneally for two consecutive days, and the gp55 expression was analyzed on the third day. To examine the effects on erythroleukemia induction, the mice were again treated with the drug 2 weeks later; mice surviving 5 months after the drug treatment were killed and autopsied to examine for signs of disease.

Cell culture

Cells from erythroleukemic spleens and their transplanted tumors were cultured in RPMI-1640 supplemented with 10% fetal calf serum without any additives such as erythropoietin as previously described (Ikawa *et al.*, 1976). To determine the CFU-E, spleen cells from normal and

erythroleukemic mice were cultured for 2 days in a plasma clot system (Stephenson *et al.*, 1971) in the presence (0.5 U/ml) or absence of erythropoietin, and the clots were then stained with benzidine and hematoxylin and examined microscopically for benzidine positive colonies.

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