Viral Protein Expression in Producer and Nonproducer Clones of Friend Erythroleukemia Cell Lines

RITA ANAND,¹ FRANK LILLY,^{1*} AND SANDRA RUSCETTI²

Department of Genetics, Albert Einstein College of Medicine, Bronx, New York 10461,¹ and Laboratory of Tumor Virus Genetics, National Cancer Institute, Bethesda, Maryland 20205²

Erythroleukemia cell lines HFL/d and HFL/b, derived from tumors induced in vivo in BALB/c (*H-2^d*) and congenic BALB.B (*H-2^b*) mice, respectively, by a polycythemia-inducing strain of Friend virus, produced both spleen focus-forming virus (SFFV) and its native NB-tropic helper virus (Friend murine leukemia virus [FMuLV]) during early-passage generations in culture. Eventually each line ceased production of both infectious viruses but retained its tumorigenic potential in syngeneic hosts. Virus-producer and -nonproducer clones of these cell lines were examined for expression of proteins encoded by the SFFV or FMuLV genomes. Lysates of labeled cells were treated with various antiviral sera, and the precipitates were examined by gel electrophoresis. Expression of the FMuLV *env* gene-encoded precursor protein, gPr85^{env}, was observed in all producer and most nonproducer clones, but the FMuLV *gag* and *pol* gene products, Pr65^{gag} and Pr200^{gag-pol}, were uniformly undetectable in nonproducer clones. All HFL/d and HFL/b clones expressed appreciable amounts of the SFFV-encoded envelope protein, gp52, including one exceptional clone which had ceased to express any FMuLV-encoded proteins. The molecular weight of this SFFV-encoded envelope protein was consistently smaller in all HFL/b clones than in HFL/d clones, regardless of their producer or nonproducer status. The virus-nonproducer phenotype thus appears to be due to shutdown of expression of the 5' portion of the FMuLV genome in two independent cell lines.

The Friend erythroleukemia-inducing virus complex (FV) consists of the spleen focus-forming virus (SFFV) and a helper retrovirus component, Friend murine leukemia virus (FMuLV). SFFV has been shown to be a replication-defective virus associated with spleen focus induction and the rapid development of splenomegaly and erythroleukemia in susceptible mice (29). Although co-infection with a helper virus is essential for production of infectious SFFV particles, helper virus is not needed for infection and expression of the SFFV genome in fibroblast cultures (3, 31) or possibly even for transformation of erythroid precursor cells (6).

Erythroleukemia cells from tumors induced by FV can be adapted to growth in culture (15). Mice of the BALB/c (*H-2^d*) and BALB.B (*H-2^b*) strains, congenic with respect to the *H-2* locus, have been used in our laboratory to establish a series of *H-2*-congenic erythroleukemia cell lines, designated HFL/d and HFL/b, respectively (12). In these cell lines, the expression of infectious virus and of some viral antigens eventually ceased as each cell line was continuously passed in culture (13). Although virus-nonproducer cell lines were phenotypically virus negative, they still maintained the entire viral genome, since renewed production of infectious FV

could be induced chemically. Recent studies from our laboratory (2) involving molecular hybridization of cellular RNA with viral complementary DNA probes suggested that shutdown of Friend virus expression in these erythroleukemia cell lines was due to a mechanism involving regulation of transcription of proviral DNA. However, due to the unavailability of complementary DNA probes to individual viral genes, *gag*, *pol*, or *env*, the precise and sequential role of these genes in the shutdown of virus production could not be ascertained.

The products of the FMuLV genome are relatively well known (Fig. 1). The *env* gene, located in the 3' portion of the viral genome, codes for a glycosylated precursor protein, gPr85^{env}, which is processed to gp70, the major viral surface molecule, and p15(E) (9, 10, 20). At least three precursor proteins appear to be generated from the 5' portion of the viral genome comprising the *gag* and *pol* genes: Pr200^{gag-pol} is probably a precursor to reverse transcriptase, whereas gPr80^{gag} and Pr65^{gag} have been reported to be two independent primary translation products of the MuLV *gag* gene (7, 9, 18, 21, 28). gPr80^{gag} appears to be further glycosylated and transported to the cell membrane, whereas Pr65^{gag} is processed to form the internal structural pro-

FIG. 1. Diagrammatic summary of the relationship between the genetic maps of ecotropic MuLV and SFFV and the major protein precursors and products translated from these genomes. The dashed lines in SFFV represent the portion of this genome which is not shared with FMuLV, at least part of which originates from the genome of an endogenous xenotropic virus.

teins p30, p15, p12, and p10.

Several translational products of the SFFV genome have also been identified (Fig. 1). A relatively well characterized one is gp52 (also designated gp55) (5, 24, 26, 33). The gene encoding this protein is apparently the result of recombination between the *env* genes of FMuLV and a xenotropic MuLV (26, 30). The gp52 molecule shows marked immunological cross-reactivity with the gp70 product of a number of mink cell focus-inducing strains of MuLV, which also appear to derive from *env* region recombinational events between ecotropic and xenotropic strains of MuLV (8, 11, 16). Certain strains of SFFV have also been shown to code for another product, p45, which is encoded in the 5' region of the virus and is antigenically related to certain products of the FMuLV *gag* gene (27).

The present study explores the expression of the proteins encoded by the FMuLV and SFFV genomes in relation to the shutdown of infectious virus production in the various erythroleukemia cell clones. Metabolically labeled translational products of FMuLV were identified by immunoprecipitation and gel electrophoresis, using antisera to *gag* and *env* gene proteins. SFFV gene products were identified with antisera raised by immunizing rats with syngeneic nonproducer cells infected only with SFFV.

MATERIALS AND METHODS

Cell lines. Friend erythroleukemia cell lines used in these studies have been previously described (12-14). Their characteristics are outlined in Table 1.

Control cell lines included normal rat kidney cells (NRK) or Fisher rat embryo cells (FRE) infected with SFFV alone, FMuLV alone, both FMuLV and SFFV, or neither. These lines were provided by the generosity of D. H. Troxler and E. M. Scolnick of the National Cancer Institute.

Antisera. Rat anti-SFFV serum was prepared by immunizing Fisher rats with syngeneic SFFV-FRE

cells. The rats were injected subcutaneously at multiple sites with a total of 10^7 cells each and developed solid tumors in 2 to 3 weeks after injection. Antisera from two rats were pooled and inactivated at 56°C for 30 min. Goat antisera to Rauscher MuLV (RMuLV) gp70 and Pr65^{gag} were provided by the Division of Cancer Cause and Prevention, National Cancer Institute. Goat anti-Pr65^{gag} serum was prepared against the purified RMuLV *gag* precursor and reacts with all *gag* proteins.

Metabolic labeling of cells. Subconfluent monolayers of fibroblast lines in 100-mm petri dishes or 6×10^6 cells/ml from each mouse cell suspension culture were labeled with 100 μCi of [^3H]leucine per ml in leucine-free medium containing 1% dialyzed fetal calf serum. Pulsing was carried out for 30 min at 37°C .

For tunicamycin inhibition experiments, cells were treated with 0.5 μg of tunicamycin per ml (E. Lilly & Co., Indianapolis, Ind.) for 1 h and then labeled with 100 μCi of [^3H]leucine per ml for 30 min.

Immunoprecipitation of pulse-labeled extracts. Labeled cells were washed in cold phosphate-buffered saline and then lysed in extraction buffer (0.01 M sodium phosphate buffer, pH 7.6, 0.1 M NaCl, 1% Triton X-100, 0.5% deoxycholate, and 0.1% sodium dodecyl sulfate [SDS]). Extracts were then spun at $2,000 \times g$ for 20 min and pretreated overnight by incubation with normal rat serum (5 $\mu\text{l}/\text{ml}$) and 50 μl of *Staphylococcus aureus* per ml (a 10% Formalin-fixed suspension of Cowan strain I [17]). Extracts were clarified by centrifugation at $2,000 \times g$ for 20 min. Immunoprecipitations were set up at 4°C by incubating 1 ml of each extract with 10 μl of rat anti-SFFV serum per ml or with 5 μl of various goat anti-RMuLV sera per ml. After 16 h of incubation at 4°C , *S. aureus* was added (50 $\mu\text{l}/\text{ml}$) and incubation was continued for 2 h more. Reaction tubes were then centrifuged at $2,000 \times g$ for 15 min, and the pellets were washed three times with extraction buffer and suspended in sample buffer (50 mM Tris-hydrochloride, pH 6.8, 1% SDS, 1% β -mercaptoethanol, 10% glycerol, and 0.001% bromophenol blue) in preparation for polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis. Seven percent polyacrylamide gels with 3.5% stacking gels were prepared, and electrophoresis was carried out as previously described (19). ^{125}I -labeled bovine serum albumin (68,000) and RMuLV p30 (30,000) were used as molecular weight markers. Gels were then processed for fluorography (4) and exposed to Kodak Royal Blue X-Omat film at -70°C .

RESULTS

HFL/d and HFL/b cell lines: virus production and tumorigenicity in vivo. The HFL/d and HFL/b cell lines and their clonal derivatives originated from the spleens of BALB/c and BALB.B mice, respectively, infected with FV of NB-tropic host range (14). Each cell line produced both infectious SFFV and FMuLV (titers, $>10^2$ infectious units/ml of culture supernatant) in early passages in culture. The HFL/d line ceased producing both viruses very early, and no frozen stocks of this line

TABLE 1. Tumorigenicity and expression of infectious virus by clones of rat and mouse cell lines

Cell line	Symbol	Tumor ^a	SFFV (FFU/ml) ^b	FMuLV (FFU/ml) ^c
Rat				
SFFV-FRE/FMuLV			8.5×10^2	2.5×10^5
FMuLV/NRK			0	6.25×10^2
SFFV/NRK			0	0
SFFV/FRE			0	0
NRK			NT	NT
Mouse				
HFL/d-NB cl. A	V ⁺ gp70 ⁺	+	15.8×10^4	6.25×10^6
HFL/d cl. 4	V ⁻ gp70 ⁺	+	0	0
HFL/d cl. A	V ⁻ gp70 ⁻	+	0	0
HFL/b cl. B2	V ⁺ gp70 ⁺	+	7.1×10^2	2.5×10^3
HFL/b cl. C4	V ⁻ gp70 ⁺	+	0	0

^a 5×10^6 cells were inoculated subcutaneously into young syngeneic recipients, and mice were observed for tumor growth.

^b Culture supernatants were tested by the spleen focus assay in vivo. FFU, Focus-forming units.

^c Culture supernatants were tested by the XC plaque assay.

before shutdown exist. The HFL/b line ceased virus production much later in its passage history, and this shutdown has occurred repeatedly at approximately the same cumulative passage level when stocks frozen before shutdown were reestablished in culture (22).

Characteristics of the various clones of the cell lines used in these studies, representing different phenotypes with respect to virus expression, are summarized in Table 1. Clones representing the HFL/d cell line include: (i) a typical nonproducer clone, HFL/d clone 4, which expresses the viral envelope antigen (VEA) of FV (12) and its major component, gp70 (14), called V⁻gp70⁺; (ii) a unique nonproducer clone, HFL/d clone A, derived from a V⁻gp70⁺ population but lacking expression of VEA or gp70, called V⁻gp70⁻; and (iii) two virus-producer clones obtained from the V⁻gp70⁻ line by either treatment with 5-bromodeoxyuridine (HFL/d-Bu) or superinfection with SFFV-free NB-tropic FMuLV (HFL/d-NB clone A), both called V⁺gp70⁺. Clones representing the HFL/b cell line include: clone B2, which produces both SFFV and FMuLV and expresses VEA antigens (V⁺gp70⁺); and clone C4, which is a virus nonproducer but expresses VEA antigen (V⁻gp70⁺). No HFL/b clones which have ceased to express VEA antigen have as yet been observed.

Each of these clones was studied for the production of infectious virus detectable in either the XC plaque assay for FMuLV (25) or the spleen focus assay for SFFV (1). All lines, virus producers as well as nonproducers, gave rise to tumors in syngeneic mice 1 to 3 weeks after subcutaneous injection of 4×10^6 cells (Table 1).

Demonstration of specificity of antiviral sera. Various antisera to be used in characteri-

zation of the erythroleukemia cell clones were analyzed for their specificity, using rat fibroblasts infected with FMuLV alone, SFFV alone, or both FMuLV and SFFV. Anti-serum to Pr65^{gag} precipitated not only FMuLV *gag* gene products (Pr200^{gag-pol}, Pr65^{gag}, and p40 [21]; Fig. 2, lanes 11 and 12), but also the homologous SFFV *gag* protein (p45) (lane 13). Anti-gp70 serum precipitated the FMuLV *env* gene precursor, gPr85^{env} (lanes 9 and 10), as well as the analogous protein encoded by SFFV, gp52 (lanes 7, 8, and 10). Rat antiserum prepared against SFFV-nonproducer cells precipitated FMuLV *gag* gene products (lanes 3 and 4) but not FMuLV *env* gene products. The rat serum also precipitated the SFFV-encoded gp52 (lanes 2 and 4).

The above antisera were then used below to characterize the FMuLV and SFFV proteins expressed in the various mouse erythroleukemia cell clones.

Expression of FMuLV-coded proteins in mouse tumor cell clones. Suspension cultures of clones of the HFL/d and HFL/b cell lines were pulse-labeled with [³H]leucine for 30 min, and lysates of the cells were treated with antiviral sera. Electrophoresis of the proteins precipitated with anti-RMuLV gp70 serum revealed that gPr85^{env} was detectable in all clones, irrespective of their virus-producer or -nonproducer status, with the exception of HFL/d clone A (V⁻gp70⁻) (Fig. 3, lane 4). The gp70 product of gPr85^{env} was detected only at very low levels after a 30-min pulse, but a 2-h chase after the labeling period led to a marked enhancement of gp70 detection (data not shown) and permitted confirmation that its presence or absence correlated with that of gPr85^{env}.

Similarly prepared cell lysates were also

FIG. 2. Identification of viral proteins in clones of rat cells infected with SFFV, FMuLV, or both. Cells were metabolically labeled with [^{3} H]leucine for 30 min, and lysates were treated with various antisera. Antigen-antibody complexes were extracted with a 10% Formalin-fixed suspension of *S. aureus* and analyzed by electrophoresis on a 7% polyacrylamide gel containing SDS. Rat anti-SFFV antiserum (lanes 1–4), goat anti-RMuLV gp70 serum (lanes 6–10), or goat anti-RMuLV Pr65^{gag} serum (lanes 11–14) was used to precipitate uninfected NRK (lanes 1, 6, and 14), SFFV-FRE (lane 7), SFFV-NRK (lanes 2, 8, and 13), FMuLV/NRK (lanes 3, 9, and 12), or SFFV-FRE/FMuLV (lanes 4, 10, and 11) cells. Lane 5 represents ^{125} I-labeled molecular weight markers bovine serum albumin (68,000) and RMuLV p30 (30,000).

FIG. 3. Immunoprecipitation of *env* gene products from mouse erythroleukemia cell lines. Clones of HFL/d and HFL/b cell lines were labeled with [^{3} H]leucine for 30 min. Cell lysates were precipitated by goat anti-RMuLV gp70 serum. Different virus-producer and -nonproducer cell lines used were HFL/d-Bu, V⁺gp70⁺ (lane 1); HFL/d-NB clone A V⁺gp70⁺ (lane 2); HFL/d clone 4, V⁻gp70⁺ (lane 3); HFL/d clone A, V⁻gp70⁻ (lane 4); HFL/b clone B2, V⁺gp70⁺ (lane 5); and HFL/b clone C4, V⁻gp70⁺ (lane 6).

treated in different experiments with goat antiserum prepared against the RMuLV *gag* gene precursor, Pr65^{gag} (Fig. 4). In contrast to the *env* gene product of FMuLV, the expression of Pr65^{gag} and gPr80^{gag} correlated with the virus-producer phenotype in the various clones (lanes

1 and 5). Virus-nonproducer clones showed no detectable levels of this protein (lanes 2–4). This same correlation with virus production was seen with respect to expression of the high-molecular-weight precursor, Pr200^{gag-pol}, a finding confirmed in separate studies using goat anti-RMuLV reverse transcriptase antiserum (data not shown).

These experiments demonstrated that the virus-nonproducer phenotype among the erythroleukemia clones examined correlated with the absence of gene products of the 5' portion but not with those of the 3' portion of the FMuLV genome.

Expression of SFFV-coded proteins in mouse tumor cell clones. The SFFV gene product, gp52, was precipitated by antibodies present in rat anti-SFFV and goat anti-RMuLV gp70 antisera. When these sera were used to precipitate radiolabeled lysates of the panel of erythroleukemia cell lines (Fig. 3 and 5), gp52 could be detected in each of the HFL/d clones whether or not they produced virus or expressed FMuLV *env* gene products (Fig. 3, lanes 1–4; Fig. 5, lanes 1, 2, 5, and 6). This was also true of the virus-producer and -nonproducer HFL/b clones, but the size of the major protein precipitated with anti-gp70 and anti-SFFV sera was always slightly smaller, approximately 48,000 daltons (Fig. 3, lanes 5 and 6; Fig. 5, lanes 3, 4, 7, and 8). The smaller protein was also a glycoprotein, since it, like gp52, incorporated [^{3} H]-mannose during a 1-h pulse (data not shown). Smaller amounts of a 52,000-dalton protein could also be detected in these cells.

FIG. 4. Immunoprecipitation of *gag* gene-coded proteins from mouse cell lines. Virus-producer and -nonproducer clones of the HFL/d and HFL/b cell lines were pulse-labeled with [^{3} H]leucine for 30 min. Cell lysates were precipitated with goat anti-RMuLV Pr65^{gag} serum and separated on a 7% polyacrylamide gel containing SDS. Clones analyzed were HFL/d-NB clone A, V⁺gp70⁺ (lane 1); HFL/d clone 4, V⁻gp70⁺ (lane 2); HFL/d clone A, V⁻gp70⁻ (lane 3); HFL/b clone C4, V⁻gp70⁺ (lane 4); and HFL/b clone B2, V⁺gp70⁺ (lane 5).

To ascertain whether the difference in molecular weight of HFL/d gp52 and HFL/b gp48 resided in the protein or carbohydrate portion of the molecule, the virus-producer clones of both lines were treated with tunicamycin, an inhibitor of glycosylation, before labeling with [^{3} H]leucine. Smaller gp52-related proteins were expressed in both clones after tunicamycin treatment of the cells (Fig. 6, lanes 2 and 3), and the difference in molecular weight between these unglycosylated proteins appeared to be about the same as that between gp52 and gp48. Conversion of FMuLV gPr85^{env} to Pr66^{env} in the presence of tunicamycin (lanes 2 and 3) confirmed that glycosylation was indeed inhibited in this experiment. It thus appears that the difference between the HFL/d gp52 and the HFL/b gp48 resides in their protein moieties.

In experiments not shown, spleen cells from both BALB/c and BALB.B mice infected 9 days

earlier with FV expressed a gp52 but not a gp48. Thus, the expression of gp48 in clones of the HFL/b cell line is most likely an idiosyncrasy of this line which is not related to its H-2^b type.

Although the SFFV-encoded *gag*-related protein, p45, could be consistently immunoprecipitated with goat anti-Pr65^{gag} and rat anti-SFFV sera from SFFV-infected rat cell lines (Fig. 2), its presence in any of the mouse cell lines was not readily detected, suggesting that it was expressed only at very low levels, if at all. It has previously been shown that certain strains of SFFV do not express p45 (27), and the strain of SFFV in the lines studied here may be such a p45-negative strain. Rat anti-SFFV sera did, however, precipitate Pr65^{gag} in all producer clones (Fig. 5, lanes 5 and 8), but not in any nonproducer clones (Fig. 5, lanes 6 and 7), confirming the data obtained with goat anti-Pr65^{gag} serum.

DISCUSSION

Two FV-induced erythroleukemia cell lines of independent origin, HFL/d and HFL/b, have given rise to clones representing both the virus-producer and virus-nonproducer phenotypes. Present studies demonstrate that virus nonproduction is correlated with the absence of gene products encoded near the 5' end of the helper FMuLV component of the FV complex. Gene products encoded in the 3' *env* region of FMuLV continued to be detectable in nonproducer cells,

FIG. 5. Comparative analysis of viral proteins of mouse cell lines by immunoprecipitation with goat anti-RMuLV gp70 serum and rat anti-SFFV serum. HFL/d and HFL/b virus-producer and -nonproducer clones were labeled with [^{3} H]leucine for 30 min. Cell lysates were divided into two portions and immunoprecipitated separately with goat anti-RMuLV gp70 serum (lanes 1-4) and rat anti-SFFV serum (lanes 5-8). Various cell lines used were HFL/d-NB clone A, V⁺gp70⁺ (lanes 1 and 5); HFL/d clone A, V⁻gp70⁻ (lanes 2 and 6); HFL/b clone C4, V⁻gp70⁺ (lanes 3 and 7); and HFL/b clone B2, V⁺gp70⁺ (lanes 4 and 8).

FIG. 6. Viral gene products in tunicamycin-treated cells. HFL/d-NB clone A, $V^{-}gp70^{+}$ (lanes 2 and 4) and HFL/b clone B2, $V^{+}gp70^{+}$ (lanes 3 and 5) were incubated either in the presence (lanes 2 and 3) or absence (lanes 4 and 5) of tunicamycin for 1 h, labeled with [^3H]leucine for 30 min, and then immunoprecipitated with goat anti-RMuLV gp70 serum. Lane 1 represents ^{125}I -labeled molecular weight markers.

although one of the nonproducer lines (HFL/d, $V^{-}gp70^{+}$) eventually gave rise to a different variant (HFL/d, $V^{-}gp70^{-}$) which expressed neither 5' nor 3' genes of FMuLV. In contrast, all clones, producer and nonproducer alike, clearly expressed the gp52 product of the 3' end of the SFFV genome.

Our present results are consistent with other recent studies in this laboratory which involved analysis by nucleic acid hybridization techniques of the expression of FMuLV-specific and SFFV-specific RNA sequences in the cytoplasm and nucleus of these same producer and nonproducer clones (2). These studies indicated that both types of clones expressed essentially all SFFV-specific RNA sequences and all RNA sequences shared by both SFFV and FMuLV. However, nonproducer clones were partially or totally deficient in expression of FMuLV-specific RNA sequences. The present studies have permitted us to identify the portion of the FMuLV genome whose expression is deficient in these cell lines. Clones found earlier to express only 75% of FMuLV-specific RNA sequences are those which express gene products of the 3' but not the 5' end of FMuLV, and the unique clone found earlier to express no detectable FMuLV-specific RNA sequences also fails to express products of either end of the viral genome.

The nonproducer erythroleukemia cell clones analyzed in this study are apparently different from the single nonproducer clone analyzed by Racevskis and Koch (23), who concluded that the nonproducer status of cells that they were studying was due to a defect in the processing of Pr65^{gag} rather than to its failure to be transcribed. Thus, more than one mechanism may be responsible for the nonproducer phenotype of a given erythroleukemia clone.

The SFFV-encoded gp52 molecule was detected in all clones of both the HFL/d and HFL/b cell lines. However, in HFL/b clones, it was routinely fainter in the autoradiographs, and a new band, absent in HFL/d clones, was always present at a position corresponding to about 48,000 daltons. This new protein showed the same pattern of reactivity with the various antisera as gp52. Treatment of the cell lines with tunicamycin to inhibit glycosylation produced parallel effects on the electrophoresis patterns of gp52 in HFL/d cells and gp48 in HFL/b cells, the apoprotein of gp52 being about 46,000 daltons and that of gp48 being about 42,000 daltons. Preliminary observations of electrophoretic patterns of products of partial enzymatic digestion of gp52 and gp48 also show them to be very similar (data not shown). The presence of gp48 in HFL/b cells appears to be an idiosyncrasy of this particular line, since only gp52 and not gp48 could be detected in cells from the enlarged spleens of BALB.B mice infected *in vivo* with FV. A possible explanation for the phenomenon is that gp48 originates from a variant SFFV *env* gene; since gp52 is also detected in these cells, it seems possible that the HFL/b line originated from a progenitor cell containing more than one SFFV proviral genome, one of which bears a mutation in the gp52 region. Consistent with this idea is the observation that some strains of SFFV code for slightly smaller gp52 molecules (27, 32).

Each of the clones examined in these studies has retained its capacity to form tumors upon reintroduction into syngeneic mice. The finding that one of these clones, HFL/d clone A, fails to express any FMuLV gene products while continuing to express the SFFV gp52 lends support to the idea that SFFV and not FMuLV is responsible for the oncogenic potential of the FV complex. Also, the observation that the SFFV-encoded gp52 is expressed in each of the erythroleukemia cell clones, whereas the SFFV-encoded p45 is not, is consistent with the hypothesis that the oncogenic potential of SFFV resides in its *env* gene. However, additional $V^{-}gp70^{-}$ clones of FV-induced erythroleukemia cell lines, tumorigenic as well as nontumorigenic, must be analyzed in order to establish a firm correlation

between SFFV gp52 expression and leukemic transformation.

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