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The Spleen Focus-Forming Virus (SFFV) Envelope Gene, When Introduced into Mice in the Absence of Other SFFV Genes, Induces Acute Erythroleukemia

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Previous studies in our laboratory and others have been consistent with the hypothesis that the envelope (*env*) gene of the spleen focus-forming virus (SFFV) is the only gene essential for the induction of acute erythroleukemia. However, no studies have been carried out with the SFFV *env* gene in the complete absence of other SFFV sequences. To accomplish this goal, we isolated the sequences that encode the envelope glycoprotein, gp52, of $SFFV_A$ and expressed them in a Moloney murine leukemia virus-based double-expression vector containing the neomycin resistance gene. The method used to produce retrovirus stocks in tissue culture cells affected the expression of the gp52 gene in the vector and the subsequent pathogenicity of the vector in mice. Highly pathogenic virus stocks were obtained by cotransfection of vector and helper virus DNAs into fibroblasts, followed by virus replication and spread through the cell population. Mice infected with this stock developed a rapid erythroid disease that was indistinguishable from that induced by the entire SFFV genome, and the virus stock transformed erythroid cells in vitro. Spleen cells from the diseased mice expressed the SFFV *env* gene product but not the SFFV *gag* gene product. As expected, mice given the virus containing the SFFV *env* gene in the reverse orientation did not express the SFFV *env* gene product or develop erythroleukemia. This study, therefore, demonstrated (i) that double-expression retroviral vectors can be used under specific conditions to produce viruses expressing high levels of a particular gene and (ii) that incorporation of the SFFV *env* gene into such a vector in the absence of other SFFV sequences results in a retrovirus which is as pathogenic as the original SFFV.

The spleen focus-forming virus (SFFV) is an acutely pathogenic retrovirus which causes an erythroleukemia in adult mice in less than 1 month. The pathogenic effect of the virus results from the direct action of the virus on multiple erythroblast precursor cells, causing them to proliferate out of control (for reviews, see references 16 and 17). In its initial stages, the disease is polyclonal, resulting in rapid hyperplasia of the spleen and liver of infected mice. This alteration in the proliferative capacity of erythroid cells can be mimicked in vitro by inducing, in bone marrow or fetal liver cells, burst-forming activity in the absence of erythropoietin (Epo) (6).

Several studies have focused on the localization of genetic sequences responsible for this pathogenic effect. SFFV is composed entirely of retrovirus sequences, making it impossible to relate its transforming properties to a transduced cellular oncogene. The first molecular dissection of the SFFV genome was carried out by Linemeyer et al. (11, 12), who demonstrated, by using subgenomic fragments and deletion mutants of SFFV, that the pathogenic region of the virus could be localized to a 1.5-kilobase-pair region encompassing both the envelope gene and a portion of the long terminal repeat. This finding lent support to the idea that the envelope gene, which is highly homologous to the envelope gene of pathogenic mink cell focus-inducing (MCF) viruses (16, 17) and which encodes a 52,000-dalton glycoprotein (gp52), is the gene responsible for the initiation of erythroid neoplasia by the virus. Further data which implicated the envelope gene as the leukemogenic component came from Ruta and colleagues (20), who showed that envelope gene mutants of Friend SFFV which were defective in processing the envelope protein were nonpathogenic in mice. That the long terminal repeat sequences of SFFV were not specifically required for its pathogenic effect was determined by recent experiments carried out in our laboratory, in which substitution of the SFFV long terminal repeat sequences by long terminal repeat sequences from other retroviruses (including those from lymphoma-inducing viruses) had virtually no effect on the nature or the latency of the disease (24).

For the present study, we isolated the envelope gene sequences encoding Friend SFFV gp52 and inserted them into another retrovirus vector that has sequences derived primarily from Moloney murine leukemia virus (M-MuLV) and which expresses the neomycin resistance gene. Because the pathogenic effects of this construct were identical to those of SFFV, we have demonstrated in the most conclusive way that SFFV sequences outside the envelope region are dispensible. These studies have also provided us with information on approaches for obtaining, from transfected retrovirus vector DNA, stocks of virus which produce optimal gene expression and biological activity.

MATERIALS AND METHODS

Cells and viruses. NIH 3T3 cells (9) were recipients for viral DNA transfections and used for viral titrations. Mouse fibroblasts producing M-MuLV were prepared by transfecting M-MuLV DNA (22) into NIH 3T3 cells. $SFFV_A$-L/NIH cells were prepared as previously reported (23). All virus stocks for in vitro transformation assays (burst assays) or for

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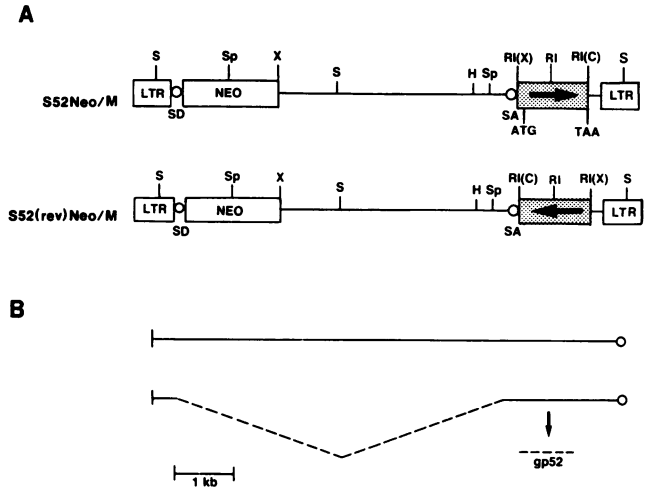

FIG. 1. Retroviral constructs containing SFFV envelope gene. (A) M-MuLV-based vector containing a neomycin resistance gene (NEO) in the 5′ end and the gp52 gene (stippled region) in the 3′ region. Arrows in the stippled region indicate the transcriptional orientations of the gp52 gene. LTR, Long terminal repeat; SD, splice donor; SA, splice acceptor; ATG, translation initiation codon; TAA, translation termination codon; C, *Cla*I; H, *Hin*dIII; RI, *Eco*RI; S, *Sst*I; Sp, *Sph*I; X, *Xma*III. (B) Two transcripts, a full-size genomic transcript and a subgenomic spliced message from which gp52 is translated, expected to be produced from S52Neo/M.

in vivo pathogenicity experiments were 24-h culture fluids from confluent cells.

Retrovirus vectors containing the gp52^{env}-coding sequences of SFFV$_A$-L, either in the correct transcriptional orientation or in the reverse orientation, were prepared as follows. An *Xma*I-*Cla*I *env* fragment from SFFV$_A$-L (23) was isolated, blunt ended with Klenow fragment of *Escherichia coli* polymerase (Bethesda Research Laboratories, Inc., Gaithersburg, Md.), and ligated to *Eco*RI linkers by using standard techniques. Following *Eco*RI digestion and agarose gel purification, this fragment was incorporated into an *Eco*RI site at the 3′ end of a M-MuLV-based double-expression vector as previously reported (2).

Transfection of DNA into cells. DNA transfection into NIH 3T3 cells or M-MuLV-producing NIH 3T3 cells was performed by the calcium phosphate precipitation technique (5). Transfections of vector DNA alone into M-MuLV producer cells was carried out by precipitating 1 μg of DNA in the presence of 5 μg of high-molecular-weight carrier DNA and adding the precipitate to 2×10^5 cells in a 35-mm-diameter dish. After 2 days, cells were seeded at 10^5 per 100-mm-diameter dish in the presence of G418 (GIBCO Laboratories, Grand Island, N.Y.) at a concentration of 400 μg/ml. Selection in the antibiotic was carried out for 10 days. For cotransfections of vector plasmid DNA and pSV2gpt DNA into cells productively infected with helper virus, the procedure was similar, except that a DNA ratio of 10:1 (3 μg of viral vector DNA and 0.3 μg of *gpt* vector DNA) was utilized. Selection of DNA-containing cells, in this case, was carried out by the method of Mulligan and Berg (15), with slight modifications. Cells were seeded at a concentration of 10^5/ml in growth medium supplemented with glycine (10 μg/ml), xanthine (Sigma Chemical Co., St. Louis, Mo.; 250 μg/ml), hypoxanthine (Sigma; 15 μg/ml), thymidine (Sigma; 10 μg/ml), aminopterin (Sigma; 2 μg/ml), and mycophenolic acid (GIBCO; 25 μg/ml) and maintained in this medium for 14 days.

Cotransfections involving the simultaneous introduction of vector DNA and M-MuLV DNA to NIH 3T3 cells were carried out with 3 μg of vector DNA and 0.3 μg of M-MuLV helper virus DNA in the presence of high-molecular-weight carrier DNA (5 μg). To allow for virus spread, cells were passaged two times at a ratio of 1:4 prior to addition of G418 to the medium.

Virus titrations. *neo*-containing retroviruses were assayed on the basis of their resistance to G418. Briefly, NIH 3T3 cells, which were seeded at a concentration of 10^5/60-mm-diameter plate, were infected with 10-fold dilutions of virus preparations and treated with G418 at a concentration of 400 μg/ml. After 7 to 10 days, G418-resistant colonies were counted.

Infection of mice with viruses. To test for biological activity, virus stocks containing vector and helper virus were injected intravenously into 5-week-old NIH Swiss mice, and the mice were examined for disease 2 to 4 weeks later. As a means of comparison, mice were also infected with virus containing helper virus and the entire SFFV$_A$-L genome (23).

In vitro erythroid transformation assay. Bone marrow cells from phenylhydrazine-treated mice were infected with viruses and then cultured in methylcellulose in the presence of Epo (0.1 U/ml) as previously described (6, 7). After 5 days in culture, the cells were clotted, stained with benzidine, and examined microscopically for benzidine-positive bursts.

[^{3}H]thymidine incorporation assay. Spleen cells from virus-infected mice were analyzed for their ability to incorporate [^{3}H]thymidine in the absence or in the presence of various concentrations of Epo as previously described (18). Briefly, cells were cultured for 22 h at 4×10^6 cells per ml in 0.1 ml of medium containing 20% fetal calf serum and 0.1 mM 2-mercaptoethanol. The cells were then pulsed with [^{3}H]thymidine (1 μCi per well) for 2 h, harvested onto glass fiber filters with a microharvester, and counted.

Analysis of viral gene products. Cells were analyzed for the expression of viral gene products by labeling with [^{35}S]methionine and immune precipitation of the labeled extracts with specific serum. Proteins precipitated in each case were visualized by autoradiography after electrophoresis on sodium dodecyl sulfate-polyacrylamide gels. Sera used were goat anti-Rauscher MuLV (R-MuLV) gp70 serum, goat anti-R-MuLV p12 serum, and goat anti-M-MCF serum made specific for MCF gp70s by absorption. These procedures have been previously described (19).

RESULTS

Construction of retrovirus vector containing SFFV envelope gene. A double-expression vector for the purpose of expressing both the neomycin resistance gene, *neo*, and the SFFV gp52^{env} gene was constructed (Fig. 1). The placement of sequences encoding SFFV gp52 in the M-MuLV-based vector was just downstream from the splice acceptor site normally utilized for the production of the subgenomic envelope gene transcript. Ligation of vector DNA and gp52 DNA sequences resulted in vector DNAs that had the insert in the correct transcriptional orientation (S52Neo/M), as well as in the reverse orientation (S52Neo[rev]/M). We utilized the vector with the DNA in the reverse orientation for the production of control virus.

Procedures for transfection of retroviral DNA and their effects on expression and pathogenicity of SFFV gp52 gene. In

TABLE 1. Production of pathogenic virus stocks

Transfection technique	Expt no.	*env* gene orientation	Gene selected	gp52 expression[a]	Virus titer (CFU/ml)[b]	% Disease incidence[c]
Vector DNA into M-MuLV producer cells[d]	1A	Correct	*neo*	−	6×10^4	0 (0/10)
	1B	Reverse	*neo*	−	ND[e]	0 (0/10)
	2	Correct	*neo*	−	1×10^4	15 (3/20)
	3A	Correct	*gpt*	−	1×10^4	7 (1/14)
	3B	Reverse	*gpt*	−	ND	0 (0/15)
Cotransfection of vector and M-MuLV DNAs into NIH 3T3 cells[f]	4	Correct	*neo*	+	4×10^5	80 (12/15)
	5A	Correct	None	+	6×10^3	100 (15/15)
	5B	Correct	*neo*	+	6×10^4	93 (14/15)
	6A	Correct	*neo*	+	ND	80 (8/10)
	6B	Reverse	*neo*	−	ND	0 (0/10)

[a] gp52 expression was determined by metabolically labeling cells with [^{35}S]methionine followed by immune precipitation of extracts and their separation on sodium dodecyl sulfate-polyacrylamide gels. Cell lines demonstrating radioactive bands corresponding to gp52 were scored positive (+), others were scored negative (−).

[b] Virus titers were determined on the basis of the neomycin resistance gene activity (see Materials and Methods).

[c] Mice were infected intravenously with 0.5 ml of 24-h tissue culture supernatants. The mice were scored positive for disease if they had enlarged spleens (greater than 0.3 g) by 4 weeks after injection. Values in parentheses are numbers of diseased mice/numbers of mice in test sample.

[d] Vector DNA was transfected into NIH 3T3 cells either by itself (experiments 1 and 2) or in combination with pSV2gpt DNA.

[e] ND, Not determined.

[f] Vector DNA was cotransfected with M-MuLV DNA at a ratio of 10:1.

the course of our studies, we utilized two approaches for obtaining infectious virus from fibroblasts. One technique involved transfection of vector DNA into cells that were already productively infected with M-MuLV helper virus and treatment of these cells after 1 or 2 days with G418, the neomycin analog, to select for cells that contained vector DNA. The other technique involved cotransfection of vector DNA with M-MuLV helper virus DNA at a ratio of 10:1, followed by passage of the cells at low dilution for two times and treatment with G418. The expression of SFFV gp52 from the resulting retrovirus vectors varied significantly. By metabolic labeling of cells with [^{35}S]methionine and immune precipitation, it was possible to demonstrate the expression of gp52 in cells derived by cotransfection of S52Neo/M and helper virus DNAs, but not in cells derived by transfection of S52Neo/M DNA into cells already productively infected with M-MuLV. To ascertain that this result was actually a consequence of the procedures utilized, several more transfections were performed and their results were compared (Table 1 and Fig. 2). In each instance when S52Neo/M DNA was transfected into helper virus-producing cells and transfection was followed immediately by selection for resistance to G418, no SFFV envelope protein was detected (Table 1, experiments 1 and 2; Fig. 2A, lane 1). Even when producer cells were cotransfected with the S52Neo/M and pSV2gpt DNAs and selected for *gpt*, there was no detectable expression of gp52 (Table 1, experiment 3; Fig. 2C, lane 1). Furthermore, viruses produced from the cells derived in experiments 1 through 3 caused a low incidence of disease (0 to 15%) in mice.

In sharp contrast to the results obtained in experiments 1 through 3, a high level of gp52 expression was demonstrated when S52Neo/M DNA was cotransfected with helper virus

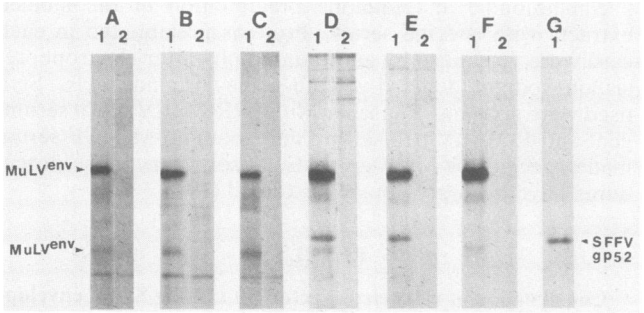

FIG. 2. Expression of SFFV envelope protein in cells transfected with vectors containing the SFFV *env* gene. Cells were labeled with [^{35}S]methionine for 30 min as described in Materials and Methods, and the labeled cell lysates were immune precipitated with goat anti-R-MuLV gp70 serum (lanes 1) or normal goat serum (lanes 2). Cells examined were M-MuLV-infected NIH 3T3 cells (A), M-MuLV-infected NIH 3T3 cells transfected with S52Neo/M DNA (Table 1, experiment 2) (B), M-MuLV-infected NIH 3T3 cells cotransfected with S52Neo/M and pSV2gpt DNAs (Table 1, experiment 3) (C), NIH 3T3 cells cotransfected with M-MuLV and S52Neo/M DNAs (Table 1, experiment 4) (D), NIH 3T3 cells cotransfected with M-MuLV and S52Neo/M DNAs (Table 1, experiment 6A) (E), NIH 3T3 cells cotransfected with M-MuLV and S52Neo(rev)/M DNAs (Table 1, experiment 6B) (F), and NRK cells nonproductively infected with $SFFV_P$ (G).

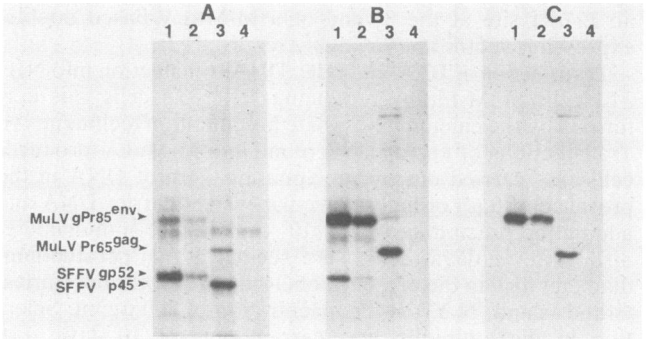

FIG. 3. Expression of SFFV proteins in spleen cells from mice infected with vectors containing the SFFV *env* gene. Cells were labeled with [^{35}S]methionine for 60 min as described in Materials and Methods, and the labeled cell lysates were immune precipitated with goat anti-R-MuLV gp70 serum (lanes 1), goat anti-Moloney-MCF gp70-specific serum (lanes 2), goat anti-R-MuLV p12 serum (lanes 3), and normal goat serum (lanes 4). Cells examined were spleen cells from mice infected with the entire $SFFV_A$ genome pseudotyped with Friend-MuLV (A), spleen cells from mice infected with S52Neo/M pseudotyped with M-MuLV (B), and spleen cells from mice infected with S52Neo(rev)/M pseudotyped with M-MuLV (C).

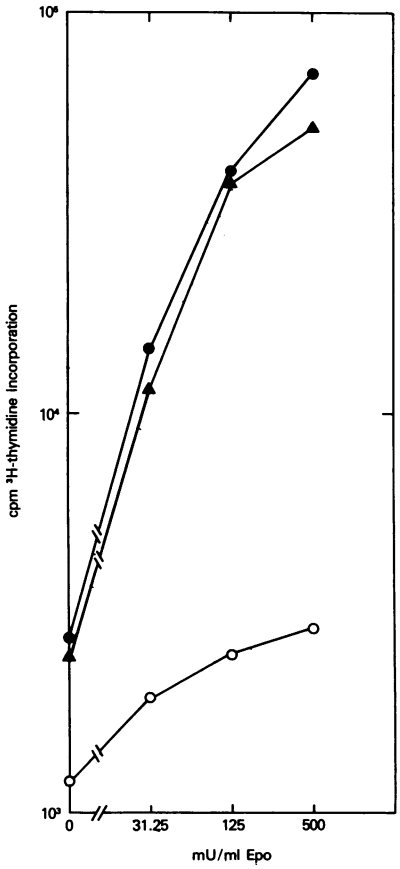

FIG. 4. [^{3}H]thymidine incorporation into spleen cells from mice infected with vectors containing the SFFV *env* gene. Spleen cells from mice were cultured for 22 h in the presence of various amounts of Epo and then pulsed for 2 h with [^{3}H]thymidine. Cells examined for the amount of [^{3}H]thymidine incorporated into DNA were spleen cells from a leukemic mouse that had been infected with the entire $SFFV_A$ genome pseudotyped with Friend-MuLV (●), spleen cells from mice infected with S52Neo/M pseudotyped with M-MuLV (▲), and spleen cells from mice infected with S52Neo(rev)/M pseudotyped with M-MuLV (○). Proliferation is expressed as counts per minute per 4×10^5 cells.

DNA and the resulting virus was allowed to spread by infection through the culture prior to selection (Table 1, experiments 4 to 6; Fig. 2D and E, lanes 1). Even in the absence of antibiotic selection, the NIH 3T3 cells which had been transfected with both helper virus and S52Neo/M DNAs and passaged long enough for the virus to spread expressed gp52 (Table 1, experiment 5A). Cells cotransfected with S52Neo(rev)/M, the vector having the gp52 sequences in the reverse orientation, did not express gp52 (Table 1, experiment 6B; Fig. 2F, lane 1).

Virus stocks from cells expressing high levels of gp52 (Table 1, experiments 4, 5, and 6A) were very potent in vivo, inducing leukemia with incidences ranging from 80 to 100% in less than 1 month. The increased pathogenicity of these stocks compared with those prepared by transfection of helper virus-producing fibroblasts did not appear to be due to differences in virus titers (Table 1). Enlarged spleens taken from mice infected with viruses obtained by cotransfection with helper virus DNA expressed gp52 (Fig. 3B, lanes 1 and 2) but not p45, the *gag-pol* protein expressed by the entire SFFV genome (compare Fig. 3A and B, lanes 3). Confirmation of the erythroid nature of the disease induced by S52Neo/M was obtained by taking spleen cells from mice infected with the vector and demonstrating the responsiveness of these cells to Epo in a [^{3}H]thymidine incorporation assay. Spleen cells from mice infected with S52Neo/M responded to Epo with incorporation values that were similar to those of cells infected with the standard SFFV (Fig. 4). Almost no incorporation was obtained in spleen cells from mice infected with S52Neo(rev)/M, the vector having the gp52 gene in the reverse orientation.

In vitro properties of envelope gene expressed by vector. Viral stocks from experiments 4 and 6 (Table 1) were utilized in in vitro experiments to demonstrate the ability of the vector expressing gp52 to transform erythroid cells. Bone marrow-derived erythroid precursor cells were transformed by S52Neo/M (Table 2), resulting in demonstrable Epo-dependent erythroid bursts after 5 days in methylcellulose. The number of bursts obtained in this assay was comparable with the number obtained with a standard stock of $SFFV_A$. No erythroid bursts were seen in the assay when bone marrow cells were infected with S52Neo(rev)/M, the vector containing gp52 sequences in the reverse orientation.

DISCUSSION

Previous experiments in our laboratory and others have been consistent with the hypothesis that the SFFV envelope gene, which encodes gp52, is the only part of SFFV essential for the induction of erythroleukemia. We have confirmed this hypothesis in this study by completely isolating the SFFV gp52-coding sequence and expressing gp52 in another vector. This vector, when introduced into mice along with helper virus, produced a disease indistinguishable from that caused by wild-type virus. Furthermore, the vector transformed erythroid cells in vitro.

The mechanism by which gp52 transforms erythroid cells is still unknown, but the unusual structural features of gp52 may provide hints for elucidating its pathogenic mechanism. The glycoprotein appears to have evolved from a retroviral envelope glycoprotein precursor molecule (e.g., $gPr85^{env}$) such that portions of MCF-like gp70 and p15E have been fused by an internal deletion. In addition, the molecule is truncated at its carboxy terminus (1, 3, 25). Because this aberrant retroviral envelope protein is transported poorly to the cell surface, only a small percentage of the total viral envelope protein in the cell can be detected on the cell membrane at a given time (19, 21). Nevertheless, there is evidence to suggest that the cell surface form of the molecule is the form responsible for the transforming effects that are observed (10).

TABLE 2. Hemoglobinized erythroid bursts in the presence of Epo

Virus	No of erythroid bursts per 10^6 cells[a]
$SFFV_A$-L	62
S52Neo/M[b]	41
S52Neo(rev)M[c]	0
M-MuLV alone	0

[a] Each value represents the average of triplicate determinations in four separate assays of bone marrow cells from phenylhydrazine-treated mice 5 days after in vitro infection and after incubation in the presence of 0.1 U of Epo per ml.
[b] Virus stock was from experiment 6A (Table 1).
[c] Virus stock was from experiment 6B (Table 1).

The gp52-encoding construct reported here proved useful as a vector for comparing transfection procedures that were aimed at good infectious-virus production. The reason for this usefulness is that gp52 expression can be easily monitored by internal labeling of cells and immune precipitation, and disease induction in this system is very rapid. We found that virus stocks prepared by a procedure in which vector and helper virus DNAs were transfected together into NIH 3T3 cells and passaged prior to G418 treatment were significantly more-potent inducers of erythroleukemia than stocks prepared by transfection of vector into cells that were already productively infected. The majority of cells harboring stably integrated vector DNA following cotransfection of helper virus DNA and vector DNA most likely acquired virus by the natural infection and integration process rather than by transfection, because virus had time to spread during the subculturing of cells that preceeded antibiotic selection. The decreased pathogenic effect of virus stocks from cells receiving DNA by transfection rather than by infection was apparently due to the decreased overall ability of the vector to express gp52 in cells and not due to lower virus production (Table 1). This may in part be explained by the fact that transfected DNA can undergo deletions or other alterations. Dougherty and Temin (4) have demonstrated in studies using spleen necrosis virus-based vectors that mutations in transfected DNA can occur at rates which are 20-fold greater than that in DNA introduced by infection. Others (8, 13, 14) have reported that vectors introduced into cells by infection are transcribed more efficiently and/or can be produced at higher titers, presumably because retroviruses integrate into transcriptionally active genes and/or are not significantly deleted. Deletions resulting in the lack of some biological function are more likely obtained after transfection of double-expression vectors, such as the vectors presented here, and selection for only one gene.

Our study has provided an approach to the study of the leukemogenic effect of a specific gene and demonstrates the importance of utilizing vectors that efficiently express the gene of interest, as well as the importance of utilizing a virus-producing technique which does not inhibit that expression. Inadequate procedures for virus production could result in nonpathogenic vectors that otherwise would be pathogenic. Optimally prepared virus stocks in our study were as pathogenic as the original SFFV.

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