MOLECULAR AND CELLULAR BIOLOGY, Dec. 1983, p. 2191–2202
0270-7306/83/122191-12$02.00/0

Vol. 3, No. 12

Retrovirus Transduction: Segregation of the Viral Transforming Function and the Herpes Simplex Virus *tk* Gene in Infectious Friend Spleen Focus-Forming Virus Thymidine Kinase Vectors

ALEXANDRA L. JOYNER AND ALAN BERNSTEIN*

Ontario Cancer Institute and Department of Medical Biophysics, University of Toronto, Toronto, Ontario, Canada M4X 1K9

Received 13 April 1983/Accepted 15 September 1983

A series of deletions and insertions utilizing the herpesvirus thymidine kinase gene (*tk*) were constructed in the murine retrovirus Friend spleen focus-forming virus (SFFV). In all cases, the coding region for the SFFV-specific glycoprotein (gp55), which is implicated in erythroleukemic transformation, was left intact. These SFFV-TK and SFFV deletion vectors were analyzed for expression of *tk* and gp55 after DNA-mediated gene transfer. In addition, virus rescued by cotransfection of these vectors with Moloney murine leukemia virus was analyzed for infectious TK-transducing virus, gp55 expression, and erythroleukemia-inducing ability. The experiments demonstrated that deletions or insertions within the intron for the gp55 *env* gene can interfere with expression of gp55 after both DNA-mediated gene transfer and virus infection. In contrast, the gene transfer efficiency of the *tk* gene was unaffected in the SFFV-TK vectors, and high-titer infectious TK virus could be recovered. Revertant viruses capable of inducing erythroleukemia and expressing gp55 were generated after cotransfection of the SFFV-TK vectors with murine leukemia virus. The revertant viruses lost both *tk* sequences and the ability to transduce TK^- fibroblasts to a TK^+ phenotype. These experiments demonstrate that segregation of the TK and erythroleukemia functions can occur in retrovirus vectors which initially carry both markers.

In the accompanying paper (16), it was demonstrated that infectious retroviruses expressing the herpes simplex virus thymidine kinase gene (*tk*) or the bacterial gene for neomycin resistance (*neo*) could be generated by in vivo recombination and deletion events. The resultant TK and NEO vectors contain a single selectable gene regulated by its homologous promoter, by the simian virus 40 (SV40) early promoter region, or by the functional promoter within the retrovirus long terminal repeat (LTR).

In the present paper, we present experiments designed to test whether retrovirus vectors expressing both selectable and nonselectable markers can be constructed. For this purpose, we chose to insert the herpes simplex virus *tk* gene into the genome of the polycythemia-inducing strain of Friend spleen focus-forming virus (SFFV), a murine retrovirus which causes rapid splenomegaly and erythroleukemia in susceptible adult mice. Friend disease is at least a two-stage process, characterized in the early stages by the proliferation of erythroid progenitor cells with the appearance of macroscopic spleen foci (1) and in the later stages by the emergence of tumorigenic cell clones (24, 25, 35). Heteroduplex (4) and nucleotide sequence (8) analyses have shown that the defective SFFV genome shares extensive homology with Moloney murine leukemia virus (MLV) and, in addition, contains deletions and substitutions in the *gag-pol* and *env* regions. The only common SFFV-specific protein expressed by a number of SFFV isolates is an MLV *env*-related glycoprotein, gp55, with a molecular weight of 55,000 (23, 27, 30). This protein consists of *env* sequences derived from both an ecotropic MLV and a xenotropic retrovirus (4) and is translated from a spliced viral mRNA (3). Linemeyer et al. (22) have demonstrated with subgenomic fragments of SFFV that the 3′ *env* region of SFFV encodes the *env*-related gp55. In addition, evidence that the expression of gp55 is required for the induction of erythroleukemia comes from the observation that deletions or insertions within the gp55 coding region abolishes the ability of SFFV to induce the early stages of Friend disease (21).

In this paper, we describe the properties of a series of SFFV vectors that contain deletions of SFFV noncoding sequences or insertions of

sequences encoding the herpesvirus *tk* gene or both. The expression of gp55 and *tk* in fibroblast cells containing these vectors was analyzed, as were the production and characterization of infectious virus conferring either Friend disease in susceptible mice or a TK^+ phenotype to TK^- tissue culture cells. The results demonstrate that (i) deletion of sequences within the SFFV *gag-pol* noncoding region can interfere with expression of gp55, (ii) insertion of the *tk* gene into the SFFV noncoding region inhibits the expression of gp55, whereas *tk* is expressed normally, (iii) infectious TK-transducing virus can be generated from SFFV-TK vectors, but these TK viruses do not induce erythroleukemia, and (iv) infectious erythroleukemia-inducing virus can be isolated from SFFV-TK vectors, but these viruses no longer express the *tk* gene.

MATERIALS AND METHODS

Cells and viruses. The cell lines, viruses, and procedures for virus infection were as described in the accompanying paper (16). Friend MLV was obtained from an NIH 3T3 fibroblast cell clone productively infected with this virus (2). Virus infection of 8- to 10-week-old DBA/2J mice, detection of spleen foci, and the derivation of erythroleukemia cell clones (CFU of Friend virus [CFU-FV]) from the spleens of SFFV-infected mice have been described previously (25).

Construction of recombinant plasmids. Recombinant plasmids were constructed essentially as described previously (18). For cloning, the *Bam*HI, *Bgl*II-*Bam*HI, and *Pvu*II *tk* fragments were isolated by preparative gel electrophoresis and electroelution (11) from the pBR322-derived plasmid pXI (12) carrying the herpes simplex virus *tk* gene. The *Eco*RI pSFFV, *Eco*RI-*Bam*HI 5′ LTR, and *Eco*RI-*Bam*HI 3′ LTR fragments were similarly isolated from phage λ SFFVp502 (37). To construct the plasmid pSFFV, equal amounts (500 ng) of phosphatase-treated, *Eco*RI-digested pBR322 and the *Eco*RI pSFFV fragment were ligated. pAJ032 was constructed by digesting 1 μg of pSFFV with the restriction enzyme *Bgl*II and ligating the mixture at a DNA concentration of 5 μg/ml. Plasmid pAJ011 was constructed by ligating equal amounts (2 μg) of the *Eco*RI-*Bam*HI 5′ and 3′ LTR fragments at a concentration of 100 μg/ml. The ligated DNA was then digested with the restriction enzyme *Eco*RI and ligated to phosphatase-treated, *Eco*RI-digested pBR322. Plasmid pAJ363 was constructed by ligating equal amounts (1 μg) of *Hpa*I-digested pSFFV and the *Pvu*II *tk* fragment. Plasmids pAJ132 and pAJ142 or pAJ232 were constructed by ligating equal amounts (500 ng) of phosphatase-treated, *Bgl*II-digested pAJ032 and the *Bam*HI *tk* or the *Bgl*II-*Bam*HI *tk* fragments, respectively. All plasmids were transfected into *Escherichia coli* HB101 (5) as described previously (9). The construction of the recombinant phages λAJ111 and λAJ121 has been described elsewhere (17).

DNA transfection and virus rescue. DNA transfections of LTA cells and cotransfections of NIH 3T3 cells to recover infectious virus were carried out as described in the accompanying paper (16).

Labeling of cells, immunoprecipitation, and polyacrylamide gel electrophoresis. Tissue culture cells (2×10^6 to 5×10^6) plated 24 h previously or 2×10^7 fresh spleen cells in 100-mm tissue culture dishes were changed to α-minimal essential, methionine-free medium supplemented with 10% dialyzed fetal calf serum and preincubated for 2 h at 37°C. The medium was changed and the cells were labeled for 2 h with 50 μCi (tissue culture cells) or 500 μCi (spleen cells) of [^{35}S]methionine (specific activity, 950 μCi/mmol; New England Nuclear Corp.) per ml. After labeling, cells were washed three times with STE (150 mM NaCl, 10 mM Tris-hydrochloride [pH 7.2], 1 mM EDTA [6]), lysed in 1 ml of RIPA buffer (150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 0.1% sodium deoxycholate, 0.1% Triton X-100, 10 mM Tris-hydrochloride [pH 7.2] [6]), and incubated on ice for 20 min. The lysed cells were then blended hard with a Vortex mixer and centrifuged in a microcentrifuge for 30 min. Portions (400 μl) of the cell extracts were incubated at 4°C for 12 h with 20 μl of monospecific goat antiserum against Rauscher virus gp70 (provided by the Biological Carcinogenesis Branch, National Cancer Institute, Bethesda, Md.) and 200 μl of 10% protein A-Sepharose (Pharmacia Fine Chemicals) in RIPA buffer which had been preabsorbed to normal mouse serum. The suspensions were then centrifuged in a microcentrifuge for 5 min, and the pellets were washed three times in RIPA buffer. The antigen-immunoglobulin complexes were dissociated from the protein A-Sepharose by heating at 100°C for 4 min in 40 μl of protein buffer (0.07 M Tris-hydrochloride [pH 6.8], 11.2% glycerol, 3% SDS, 0.01% bromophenol blue, and 5% 2-mercaptoethanol [6]). After pelleting of the protein A-Sepharose, the supernatant samples were heated to 100°C for an additional 4 min and electrophoresed through an SDS-polyacrylamide horizontal gel containing 5% (stacking gel) and 10% (separating gel) acrylamide as described by Laemmli (20). After staining with Coomassie blue, the dried gels were exposed to Kodak XAR-5 X-ray film with Cronex Lighting-Plus intensifying screens at −70°C.

Blot hybridization and gel electrophoresis. Conditions for restriction enzyme digestion of DNA, agarose gel electrophoresis, and blot gel hybridization are described in the accompanying paper (16).

RESULTS

The protocols used to analyze the properties of the SFFV deletion and SFFV-TK vectors are diagrammed in Fig. 1. In brief, expression of the SFFV *env*-related glycoprotein, gp55, was analyzed in NEO^r fibroblasts by cotransfecting the vector DNA with pSV2*neo*, a plasmid which confers resistance to the antibiotic G418 in animal cells. The levels of gp55 in these cells was determined by metabolic labeling with [^{35}S]methionine followed by immunoprecipitation as described above. The activity of the *tk* gene in the SFFV-TK vectors was quantitated by determining the number of TK^+ colonies obtained after DNA-mediated gene transfer into TK^- Rat-2 cells as described previously (16–18).

To determine whether the SFFV deletion and

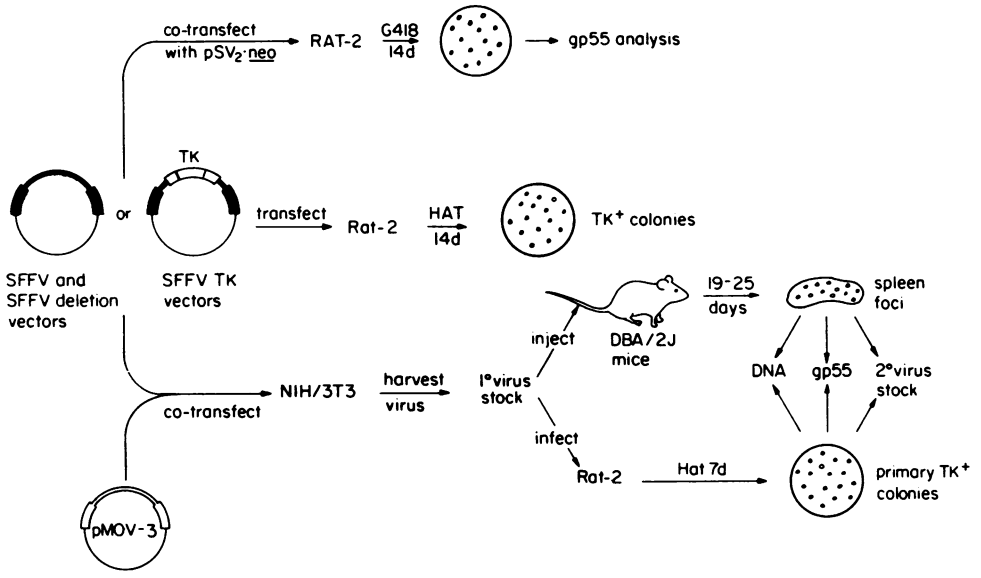


FIG. 1. Protocols used to analyze the properties of the SFFV deletion and SFFV-TK vectors. The *Bam*HI *tk* fragment is shown with the *tk* coding region outlined. Symbols: Thin line, pBR322 sequences; thick line, SFFV sequences; open line, Moloney MLV sequences; large boxes at the ends of each virus, LTRs.

SFFV-TK vectors could generate infectious retroviruses, these DNA clones were cotransfected into NIH 3T3 cells with pMOV-3, a molecular clone of a replication-competent Moloney MLV as described in the accompanying paper (16). The viruses harvested from these cotransfections (the primary virus stocks) were then analyzed for both their TK activity and the presence of SFFV activity by infection of susceptible DBA/2J mice and detection of spleen foci 19 to 25 days later. In addition, the secondary virus stocks harvested from either the TK^+ cell clones or the enlarged spleens of leukemic mice were further analyzed for the presence of *tk* sequences and a functional *tk* gene, gp55 expression, and SFFV activity.

SFFV deletion vectors. As a first step in the construction of SFFV vectors containing the herpesvirus *tk* gene, deletion vectors were constructed in which portions of the SFFV genome outside the gp55 coding region were removed. The *env*-related gp55 of SFFV is translated from a spliced viral mRNA (3), as is the *env* protein of MLV. Comparison of the SFFV and MLV provirus sequences (8) has identified putative splice donor and acceptor sites for the processed SFFV gp55 mRNA (Table 1). The splice donor site is approximately 110 base pairs (bp) upstream from the 5′ *Bgl*II site in SFFV, whereas the splice acceptor site is approximately 20 bp 3′ of the unique *Hpa*I site of SFFV. The *gag-pol* region of SFFV therefore lies within the intron for the gp55 mRNA.

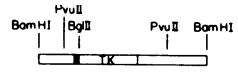
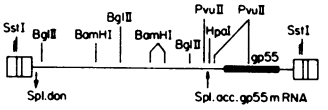
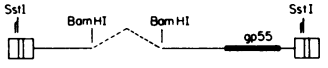
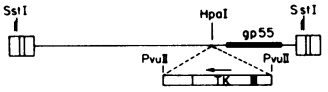
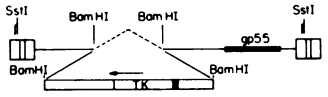
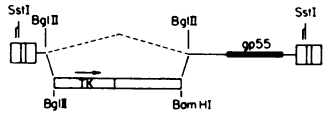
To test whether these intron sequences are required for gp55 expression and rescue of infectious virus, we constructed two SFFV deletion vectors containing specific restriction enzyme-generated deletions in the apparent intron for gp55. Plasmids pAJ011 and pAJ032 were constructed as described above and contain deletions of the sequences between the extreme *Bam*HI and *Bgl*II restriction enzyme sites, respectively, of SFFV (Table 1).

SFFV insertion-substitution vectors. We next constructed a series of vectors in which DNA fragments containing the *tk* gene were inserted into the intact SFFV genome and into the SFFV deletion mutants described above. The insertion vector pAJ363 contains the *tk* gene on a *Pvu*II fragment that includes both *tk* transcription initiation and polyadenylation signals inserted into the unique *Hpa*I site of pSFFV as described above. The transcriptional orientation of *tk* is opposite that of SFFV in pAJ363 (Table 1).

Several SFFV *tk* substitution vectors were constructed in which the *tk* gene was inserted into the unique *Bam*HI or *Bgl*II sites of deletion vectors AJ011 and AJ032, respectively (Table 1). The vector λAJ121 contains the *tk* gene on a *Bam*HI fragment containing the *tk* transcriptional promoter and polyadenylation site inserted into the unique *Bam*HI site of pAJ011 in the opposite transcriptional orientation to the SFFV 5′ LTR (Table 1).

Two SFFV-TK substitution vectors, pAJ132 and pAJ232, were constructed from *Bgl*II-deleted SFFV plasmid pAJ032. pAJ132 contains the *Bam*HI *tk* fragment inserted into the unique

TABLE 1. Characterization of pSFFV and the SFFV deletion and *tk* insertion-substitution vectors derived from pSFFV[a]

Vector	Schematic of vector[a]	Relative TK transfection efficiency[b]	Expression of gp55[c]	Recovery of infectious virus	
				TK virus per ml	Spleen foci[d]
pXI		1	nt	<0.01	–
pSFFV		<0.05	+++	<0.01	+++
pAJ011		<0.05	–	<0.01	+/–
pAJ032		<0.05	+++	<0.01	–
pAJ363		1.07	–	10^2–10^3	++
λAJ121		1.60	–	1–10	+
pAJ232		0.63	–	<0.01	+/–

[a] The schematic diagrams of the vector on each plasmid are shown approximately to scale. The *tk* gene coding region is shown as an open box and the *tk* transcription promoter as a thick vertical line. Horizontal arrows indicate the direction of transcription. The SFFV LTRs are drawn as large boxes at each end of the clone, with the U3, R, and U5 regions (5′ to 3′) separated by vertical lines. The coding region for gp55 is indicated as a thick line, and putative splice donor (Spl. don.) and acceptor (Spl. acc.) sites for the gp55 mRNA are indicated by vertical arrows. Restriction enzyme sites are as indicated and are taken from previously published data (12, 17, 37).

[b] Each plasmid DNA (100 ng) was mixed with 20 μg of carrier DNA and was used to transfect LTA cells by DNA-mediated gene transfer (13). TK^+ colonies were selected in HAT medium and scored at 14 days after gene transfer. The relative TK^+ transfection efficiency was calculated from at least two separate experiments (two plates per experimental group), and the average number of TK^+ colonies per pmol of plasmid per 5×10^5 plated cells for pXI was set as 1. The pXI transfection efficiency is approximately 1×10^4 TK^+ colonies per pmol of plasmid.

[c] Exponentially growing Rat-2 cells (2×10^6 to 5×10^6) cotransfected with each plasmid DNA and pSV2*neo* were labeled with [^{35}S]methionine. Cellular proteins were immunoprecipitated with a monospecific goat antiserum against Rauscher virus gp70 and electrophoresed through a polyacrylamide gel as described in the text (see Fig. 2). nt, Not tested; +++, presence of detectable gp55; –, absence of detectable gp55.

[d] Each plasmid vector (5 μg) was cotransfected with 5μg of the cloned MLV plasmid, pMOV-3 (14), onto NIH 3T3 cells as described in the accompanying paper (16). The TK virus titer was determined by infection of Rat-2 cells with serial dilutions of the harvested virus and selection in HAT medium 24 h later for the presence of TK^+-transduced cell colonies. Spleen foci were detected in DBA/2J mice either 9 to 12 days (pSFFV) or 19 to 25 days after injection with 0.5 ml of virus. +, ++, or +++, low, medium, or confluent numbers of spleen foci; –, absence of spleen foci; +/–, low numbers of spleen foci detected in some, but not all, experiments.

*Bgl*II site of pAJ032 in the same transcriptional orientation as the SFFV 5′ LTR. pAJ232 contains a *Bgl*II-*Bam*HI *tk* fragment with the *tk* coding region and polyadenylation site but not the *tk* promoter inserted into the *Bgl*II site of pAJ032 in the same transcriptional orientation as the SFFV 5′ LTR. We have previously presented data (18) which demonstrate that the SFFV 5′ LTR can activate expression of the *tk* coding region when *tk* is inserted into the 5′ *Bgl*II site of SFFV.

Expression of gp55 by SFFV deletion and SFFV-TK vectors. To determine whether deletions or insertions-substitutions into the SFFV noncoding region would affect gp55 expression, gp55 synthesis was measured in rat fibroblast cells transfected with the SFFV deletion and SFFV-TK vectors. The transfected cells were cotransferred (13) with an excess (1 μg) of each plasmid vector and 100 ng of plasmid pSV2*neo* (32), selecting for cells transformed to NEO^r by growth in the presence of the antibiotic G418. Similar cotransfer experiments by Wigler et al. (36) have demonstrated that cotransfection of an excess of an unselected DNA clone such as pAJ011 with a selectable gene such as SV2*neo* yields cells transformed to NEO^r that have also incorporated the unselected DNA sequences. The NEO^r colonies (20 to 50) from one plate of transfected cells were trypsinized, pooled together, and grown until there were enough cells to test for the expression of gp55 by immunoprecipitation and SDS gel electrophoresis (see above).

Cells transfected with either the intact pSFFV genome or the *Bgl*II deletion vector pAJ032 expressed high levels of gp55 (Fig. 2; Table 1). In contrast, no gp55 was detected in cells transfected with *Bam*HI deletion vector pAJ011, *tk* substitution vectors pAJ232 and λAJ121, or *tk* insertion vector pAJ363 (Fig. 2). These results indicate that deletions in the SFFV noncoding region can interfere with gp55 expression, that substitution of *tk* for SFFV sequences does not restore gp55 expression, and that insertion of *tk* sequences into SFFV genomes expressing gp55 suppresses gp55 synthesis.

Expression of the *tk* gene by SFFV-TK vectors. The specific activity of the *tk* gene in the SFFV-TK vectors was determined by measuring the number of TK$^+$ colonies after DNA-mediated gene transfer of the SFFV-TK vectors into TK$^-$ LTA cells. The *tk* insertion and substitution vectors expressed the *tk* gene at an efficiency similar to that of pXI, the parental *tk* plasmid (Table 1). This was also true of plasmid pAJ232, in which a DNA fragment, including the *tk* coding sequence but deleted of the *tk* promoter, was inserted 0.2 kilobases (kb) downstream and in the same transcriptional orientation as the SFFV 5′ LTR (Table 1). This observation confirms and extends previous observations from this laboratory showing that the SFFV 5′ LTR can activate the herpesvirus *tk* gene (18).

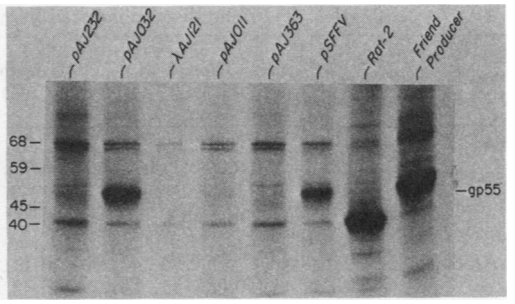


FIG. 2. gp55 analysis of cells cotransfected with pSV2*neo* and each of the vectors indicated in the figure (see text and footnote *c* in Table 1). As controls, the recipient Rat-2 and an NIH 3T3 cell lines producing SFFV were analyzed for gp55 production.

Rescue of infectious virus. The ability of the SFFV deletion and SFFV *tk* insertion-substitution vectors to generate infectious virus was determined by cotransfecting equal quantities (5 μg) of each of the SFFV vectors with a molecular clone (pMOV-3) of Moloney MLV into NIH 3T3 cells (14, 16). Primary virus stocks were harvested after several cell passages and assayed for in vivo spleen focus-forming ability and, where appropriate, infectious TK virus.

Cotransfection of pSFFV DNA with pMOV-3 generated very high titers (>5 × 10^3 spleen focus-forming units per ml) of SFFV (Table 1). In contrast, cotransfection experiments with the *Bam*HI deletion vector, pAJ011, resulted in the generation of only the occasional primary virus stock which contained low titers of virus capable of inducing spleen foci (Table 1). In general, the SFFV titers recovered after transfection with pAJ011 were 10^2- to 10^3-fold lower than those observed with pSFFV. Although cells transfected with pAJ032 express high levels of gp55, no detectable SFFV was generated from this plasmid in two separate cotransfection experiments.

The results obtained with *tk* substitution vectors λAJ121 and pAJ232 and *tk* insertion vector pAJ363 paralleled those described above for parental vectors pAJ011, pAJ032, and pSFFV. Low levels of spleen focus-forming activity were produced after cotransfection of λAJ121 and pMOV-3 into NIH 3T3 cells. In three separate experiments, little or no SFFV activity was detected from vector pAJ232 (in one experiment, one of three mice injected with AJ232 virus had numerous spleen foci). Finally, moderate levels of SFFV could be detected in all cotransfection experiments with pAJ363 and pMOV-3 (Tables 1 and 2).

TABLE 2. Friend disease induced by novel SFFV derived from SFFV-TK vectors[a]

Vector	Days after injection into mouse	Spleen wt (g)	Hematocrit (%)	Spleen foci	gp55	Virus recovered from spleen	
						Spleen foci	TK virus
λAJ121	19	0.11/0.09	53/52	−/−			
	28	0.09/0.11/0.45	nt[b]/nt/48	−/−/+	nt/−/+	nt/−/+	−/−/−
	41	1.85/nt/1.74[c]	86/47/76	+/−/+	+/−/+	+/−/+	−/−/−
	51	nt/2.38	nt/84	−/+	nt/+	nt/+	nt/−
pAJ363	21	1.15/0.50	59/nt	+/+			
	28	0.08/0.45	nt/48	−/+	−/+	−/+	−/−
	41	nt/2.27/1.75	51/78/80	−/+/+	nt/+/+	nt/+/+	nt/−/−
	51	1.63[c]	74	+	+	+	−

[a] Adult DBA/2J mice (6 to 8 weeks of age) were injected intravenously with 0.5 ml of primary virus harvested after cotransfection of λAJ121 or pAJ363 and pMOV-3 as indicated. At the times shown, hematocrits were measured, spleens were weighed, and cells from individual spleens were taken for gp55 analysis (see text), virus extraction, or plating in methylcellulose to detect CFU-FV (25). The virus recovered from individual spleens was analyzed for TK virus and induction of spleen foci as described in footnote *d* of Table 1. + or −, Presence or absence, respectively, of indicated characteristic.

[b] nt, Not tested.

[c] CFU-FV were obtained from these spleen cells (cell clones 2_1-1 from λAJ121 and cell clones 6_1-1, 6_1-5 from pAJ363).

The primary virus stocks, obtained after cotransfection of the SFFV-TK vectors and pMOV-3, were also analyzed for their levels of infectious TK virus by infecting TK^- Rat-2 cells and determining the number of HAT (0.1 mM hypoxanthine, 1.0 μM aminopterin, 40 μM thymidine)-resistant colonies 7 days later. The primary virus stocks generated from *tk* insertion vector pAJ363 contained 10^2 to 10^3 TK virus per ml (Table 1), whereas the virus stocks generated from *tk* substitution vector λAJ121 yielded only low titers (1 to 10) of TK virus. No detectable TK virus was recovered from vector pAJ132 or pAJ232. This latter observation is consistent with the observation that the parental molecule, pAJ032, does not generate detectable levels of infectious SFFV virus.

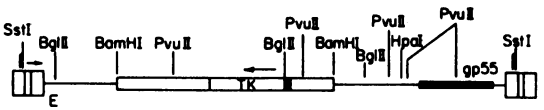
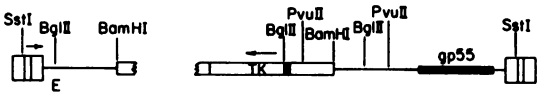
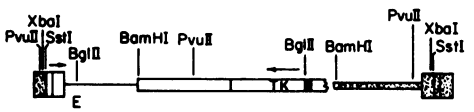
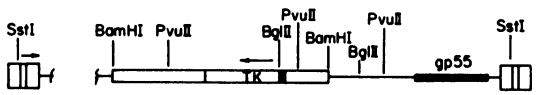
Infectious TK virus derived from SFFV-TK vectors. To characterize the infectious TK virus present in the primary virus stocks generated from the SFFV-TK vectors, three TK^+ Rat-2 cell clones (121-2A, 121-3A, 121-1B) infected with primary TK virus generated from the *Bam*HI substitution vector λAJ121 were isolated and grown in HAT medium. These cells were then analyzed for the structure of the integrated TK provirus present in each cell line, their levels of gp55 expression, and the secondary titers of TK virus and SFFV activity released by these cells.

(i) Structure of the SFFV-TK provirus in TK^+ transductants. The structure of the rescued TK provirus genomes was investigated by Southern gel (31) and blot hybridization (34) analyses of total cellular DNA from the three TK^+-transduced Rat-2 clones with a ^{32}P-labeled *Bam*HI *tk* fragment probe. These results were compared with the restriction maps of parental viruses AJ121 and MOV-3, and tentative restriction maps are shown in Table 3. To determine the size of the rescued TK proviruses, cellular DNA was cleaved with *Sst*I, an enzyme which cleaved within the U3 region of the Friend LTR. Each of the three primary TK viruses analyzed had a different *Sst*I *tk* fragment size (Fig. 3) which was less than the genome size of virus AJ121.

To determine the origin of the LTRs of the TK-transducing viruses, the restriction enzyme *Xba*I was used. This restriction enzyme cleaves within the LTRs of MOV-3 but does not cut the SFFV genome. *Xba*I digestion of DNA from clone 121-2A yields a single *tk* DNA fragment which appears to be identical in size to the fragment generated by *Sst*I cleavage of the same DNA. *Xba*I digestion of DNA from the remaining two clones yields *tk* DNA fragments larger than the *Sst*I *tk* fragments. Thus, as discussed in the accompanying paper (16), the U3 regions of both the 5′ and 3′ LTRs in cell clone 121-2A appear to be derived from the helper Moloney virus, whereas the U3 regions of the two LTRs in clones 121-1B and 121-3A appear to be from SFFV.

The TK provirus genome structures were further analyzed with restriction enzymes which cleave the SFFV-TK genome internally (Fig. 3). Cleavage of clone 121-3A DNA with each of the restriction enzymes *Pvu*II, *Bam*HI, and *Bam*HI plus *Sst*I yielded DNA fragments of 2, 3.4, and 3.4 kb, respectively, the sizes obtained with vector λAJ121. *Pvu*II digestion also gave an expected 1.4-kb weakly hybridizing band equivalent in size to the 5′ *tk*-SFFV junction fragment in AJ121. However, digestion with *Bgl*II yields a

TABLE 3. Characterization of infectious TK virus derived from the TK substitution vector λAJ121

Cell line	Schematic of provirus	Expression of gp55[b]	Virus production[c]		
			TK virus/ml	Spleen foci	XC plaques
121		−	<0.01	−	+
121-1B		−	$>10^3$	−	+
121-2A		−	$>10^4$	−	+
121-3A		−	<0.01	−	+

[a] The structure of three TK proviruses generated by cotransfecting pMOV-3 and λAJ121 are shown schematically for the TK$^+$ infected Rat-2 cell lines 121-1B, 121-2A, and 121-3A. The TK$^+$ Rat-2 cell line 121 was transfected, with DNA-mediated gene transfer, with the vector λAJ121. The schematic diagrams are drawn to scale with one LTR representing 500 bp and the symbols described in footnote *a* of Table 1. The spotted areas in the provirus present in cell line 121-2A represent MOV-3 sequences (see accompanying paper for a complete restriction map of MOV-3 [16]). The restriction maps of the proviruses present in 121-1B, 121-2A, and 121-3A were deduced from the blot hybridization and restriction enzyme analysis shown in Fig. 3 and comparison of these results with the restriction maps of MOV-3 and AJ121. Because the restriction enzyme analysis was carried out with the unique *Bam*HI *tk* fragment as a probe, only those cleavage sites nearest to *tk* can be distinguished, and only these sites are shown. The presence of a new 3′ LTR in cell clone 121-2A was deduced with enzymes which cleave within the Moloney LTR. The position of the new LTR was determined from the distance the diagnostic enzyme sites were from the sites within *tk*.

[b] See footnote *c* of Table 1.

[c] TK virus titers and spleen foci were determined as described in footnote *d* of Table 1. Each cell line was tested for its ability or inability (+ or −, respectively) to form plaques when cocultivated with rat XC cells (29).

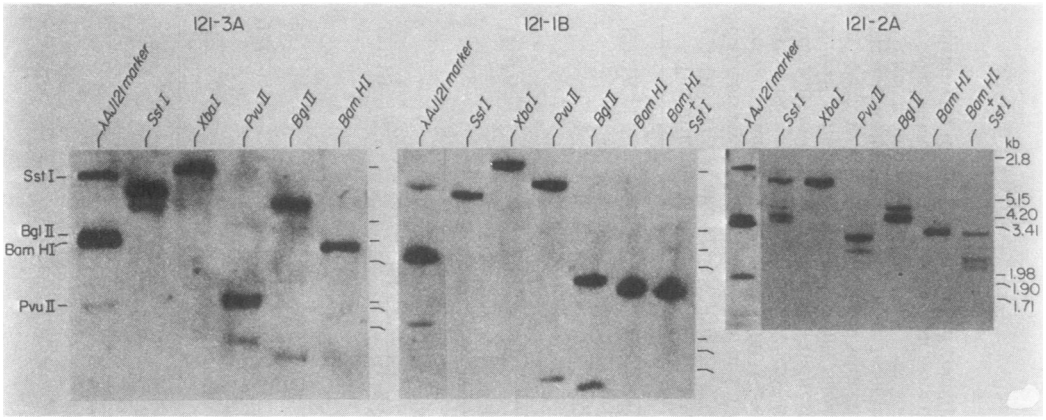


FIG. 3. Blot hybridization and restriction enzyme analysis of TK proviral DNA in cell lines infected with rescued primary TK virus generated by cotransfection of λAJ121 and pMOV-3. Total cellular DNA (20 μg) from the TK$^+$ Rat-2 cell lines was cleaved with the indicated restriction enzymes. After gel electrophoresis and transfer to nitrocellulose, the baked filters were hybridized to a ^{32}P-labeled *Bam*HI *tk* probe as described in the accompanying paper (16). The control lane contains 50 pg of the indicated marker vector digested separately with each of the enzymes shown. The lower-molecular-weight DNA bands observed after digestion of 121-2A and 121-3A DNA with *Sst*I are the result of a contaminating endonuclease in the *Sst*I preparation.

larger-than-genome-sized *tk* fragment, as well as the weakly hybridizing 1.3-kb band corresponding in size with the 5′ *tk*-SFFV junction fragment. These results indicate that the 5′ *Bgl*II site of SFFV was lost in this clone. The results obtained by digestion with *Sst*I indicate that the TK provirus in cell line 121-3A has a deletion of 1 kb, and the *Bgl*II digestion results suggest that the deletion is in the 5′ end of its genome including the *Bgl*II site.

Similar analysis of DNA from clone 121-1B indicates that the TK provirus in this clone is similar to vector AJ121 but has a 1-kb deletion, including the 5′ *Pvu*II site in the *Bam*HI *tk* fragment (Fig. 3). The results of the restriction enzyme and blot gel analysis of the TK provirus in clone 121-2A can be interpreted to indicate that this virus arose as the result of a single recombination event between the SFFV-TK genome of AJ121 and the MOV-3 helper virus genome. The point of recombination in the SFFV-TK vector must be within the *Bam*HI *tk* fragment between the 3′ *Bgl*II and *Pvu*II sites and within the helper virus genome at least 2 kb from the 3′ end of MOV-3, including the *Bam*HI restriction enzyme site.

(ii) Expression of gp55 in cells transduced by SFFV-TK virus. Expression of gp55 was measured in primary cell lines 121-2A, 121-3A, and 121-1B by immunoprecipitation and SDS gel electrophoresis. The results are shown in Fig. 4, and it can be seen that the cell lines producing virus capable of forming XC plaques (Table 3) all produce a protein of 70 kilodaltons which cross-reacts with the Rauscher anti-gp70 antibody. This is consistent with the MOV-3 helper viruses present in the producer cell lines expressing a Moloney gp70 *env* protein. None of the cell lines produces detectable amounts of gp55. Cell line 121-2A would not be expected to express gp55 since the restriction enzyme analysis of the provirus described above suggests that the 3′ gp55 SFFV sequences present on parent plasmid AJ121 have been replaced by 3′ MOV-3 sequences. The DNA structure of the TK proviruses present in the two other cell lines would suggest that the gp55 coding sequences are intact. Thus, the lack of expression of gp55 by these TK viruses is consistent with the absence of gp55 expression in cells transfected with the parental vector AJ121 used to generate these viruses.

(iii) TK virus and SFFV production by TK$^+$ transductants. The TK virus and SFFV titers produced by the three cell clones listed in Table 3 were determined by harvesting virus from each cell line and testing for the presence of TK virus and SFFV as described for the primary virus stocks. Cell lines 121-2A and 121-1B produce high titers of TK virus (>10^3/ml), but no SFFV was detected after injection of many mice with these virus stocks (Table 3). Cell line 121-3A, carrying a TK virus with a deletion including the *Bgl*II site 200 bp downstream from the 5′ LTR of AJ121, does not produce any detectable TK virus or SFFV, although high titers of helper virus are produced.

Infectious SFFV virus derived from SFFV-TK vectors. Characterization of the TK virus present in the primary SFFV-TK virus stocks indicates that the stocks contain a heterogeneous population of structurally different TK viruses

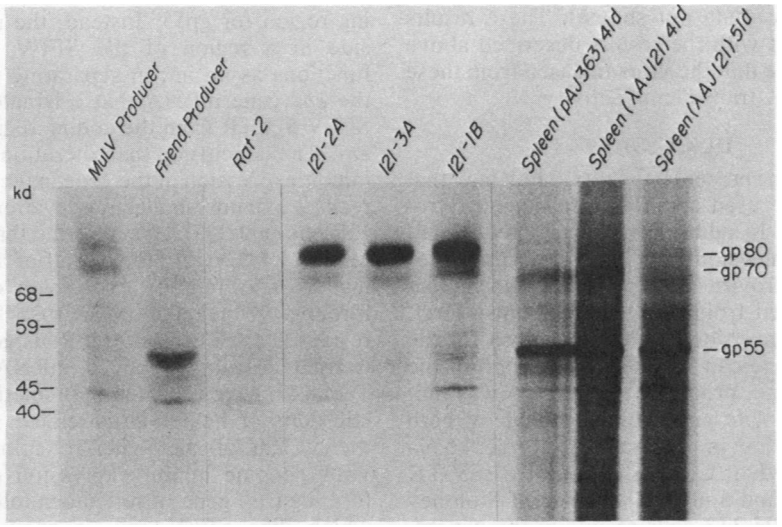


FIG. 4. gp55 analysis of TK$^+$ Rat-2 cells infected with rescued TK virus generated from λAJ121 (cell lines 121-2A, 121-3A, and 121-1B) and spleen cells from leukemic mice infected with virus generated from AJ121 and AJ363. The days after infection at which the spleens were taken are indicated. As controls, the uninfected Rat-2, MLV-infected, and SFFV-infected NIH 3T3 cell lines were analyzed for the production of gp55. See text for details of gp55 analysis. kd, Kilodaltons.

which do not express gp55 or SFFV activity. Also, the SFFV-TK vectors from which these viruses were generated do not express gp55. It was therefore of interest to document the progression of Friend disease induced by the primary virus stocks, to determine whether gp55 is expressed by leukemic spleen cells infected with the SFFV-TK virus, and to analyze the virus recovered from these spleen cells for erythroleukemia induction and TK transduction.

(i) Friend disease induced by novel SFFV derived from SFFV-TK vectors. A summary of the analysis of induction of Friend disease in vivo by these novel SFFVs is shown in Table 2. The early stages of Friend disease were observed in six of eight mice infected with the primary virus generated by SFFV-TK insertion vector pAJ363, as assayed by the presence of spleen foci, by the increase in mouse spleen weight, and by the elevated hematocrit. Also, two transformed cell lines (6_1-1 and 6_1-5) which are characteristic of the later stage of Friend leukemia were derived from the spleen cells of one mouse taken 51 days after infection. Similarly, at least 4 of the 10 mice infected with the primary virus generated from the SFFV-TK substitution vector λAJ121 acquired an erythroleukemia. Again, one transformed cell line (2_1-1) was obtained from the spleen cells of a mouse 41 days after infection. These results demonstrate that the virus present in the primary SFFV-TK virus stocks is capable of producing both the early and the late stages of Friend disease.

(ii) TK virus production by leukemic spleens. The secondary virus stocks harvested from the enlarged spleens of leukemic mice were tested for the presence of TK and SFFV virus. No TK virus was detected in any of the eight secondary virus stocks obtained from leukemic spleens. However, spleen foci were observed in mice infected 9 to 11 days previously with all eight of the secondary spleen virus stocks (Table 2).

(iii) gp55 expression in leukemic spleen cells. The expression of gp55 by leukemic spleen cells 28 days or more after infection with primary virus was determined by immunoprecipitation with an anti-Rauscher gp70 antibody and SDS gel electrophoresis as described above (Fig. 4). Spleen cells from mice showing the early stages of Friend disease expressed gp55, whereas spleen cells of infected mice not showing erythroleukemia did not express gp55.

(iv) Structure of the SFFV-TK proviruses in erythroleukemic cell lines. The DNA from the spleens of two leukemic mice and the two erythroleukemic mouse cell clones, 6_1-1 and 6_1-5, were analyzed by gel electrophoresis and blot hybridization for the presence of *tk* sequences. The spleens and cell lines were obtained from mice infected with the primary virus generated from TK insertion vector pAJ363. The ^{32}P-labeled *Bam*HI *tk* probe and an 800-bp SFFV probe derived from the 3′ half of the SFFV genome were used to analyze *Sst*I- and *Xba*I-digested cellular DNA from the enlarged spleens and the two cell lines. No *tk* sequences were detected in the DNA from these cells, whereas several bands were observed with the SFFV-

derived probe (data not shown). These results are consistent with the results described above which indicate that the virus released from these cells lacks TK transducing activity.

DISCUSSION

This paper presents results showing that SFFV can be used as a TK-transducing retrovirus vector. In addition, two unexpected but important conclusions that have implications for the use of retroviruses as gene transfer vectors can be derived from these experiments. First, deletions or insertions within the intron for the SFFV *env* gene can suppress expression of the gp55 *env* gene product. Second, it was not possible to isolate virus clones capable of both TK transduction in vitro and erythroleukemia induction in vivo. Cotransfection of SFFV-TK vector DNA and a molecular clone of Moloney MLV into NIH 3T3 cells results in the generation of a heterogeneous population of infectious viruses in the primary virus harvest. Virus harvested from TK^+ transductants in vitro lost the potential to induce erythroleukemia in vivo, whereas virus harvested from the erythroleukemic spleens lost the *tk* gene.

Mechanisms of segregation of SFFV and TK-transducing activity. Segregation of the TK- and erythroleukemia-inducing functions in the recovered viruses appears to be mediated by different mechanisms. The generation of infectious SFFV capable of inducing erythroleukemia and expressing gp55 is associated with loss of *tk* expression and, in two proviruses analyzed, loss of the *tk* sequences. Although it cannot be concluded directly that loss of the *tk* gene was required for the expression of the gp55 *env* gene, the observation that insertion of the *tk* gene into the SFFV genome results in suppression of gp55 expression suggests that the recovery of SFFV activity and loss of the *tk* insert are related phenomena. In contrast, the generation of infectious TK virus from the SFFV-TK insertion-substitution vectors does not require loss of the gp55 coding region. Two of the three TK proviruses analyzed appear to contain the 3′ end of the SFFV genome, likely including the entire SFFV gp55 region. In these viruses, loss of the coding region for the transforming function of SFFV is not required for the loss of erythroleukemia induction in vivo. Instead, the data indicate that insertion of the *tk* gene within the 5′ half of the SFFV genome is sufficient to inhibit expression of the gp55 glycoprotein and the erythroleukemic potential of SFFV.

Suppression of gp55 expression and erythroleukemogenic activity. The deletion mutant pAJ011 and the *tk* insertion-substitution mutants described here do not express the SFFV *env* gene, even though the alterations lie outside the coding region for gp55. Instead, the mutations reside in a region of the SFFV genome that functions as an intron separating the 5′ end of the *env* gene mRNA that originates within the SFFV 5′ LTR from the coding region for SFFV *env*. The conclusion that alterations in an intron inhibit expression of the gene in which the intron resides extends similar observations by others. Varmus et al. (33) have reported that insertion of Moloney MLV into the 5′ half of a Rous sarcoma provirus inhibits expression of the transforming *src* gene of this virus. The *src* gene resides at the 3′ end of the Rous genome and is translated from a spliced mRNA. Chen and Temin (7) have reported that deletions and substitutions of helper virus-related sequences in the 5′ half of the avian reticuloendotheliosis virus genome inhibit expression of the transforming (*rel*) gene of reticuloendotheliosis virus which, like SFFV *env*, is expressed from a spliced mRNA. In addition, Hawley et al. (15) have observed that spontaneous insertions of intracisternal A-type particle genomes into the intron of an immunoglobulin gene in mouse plasmacytoma cells markedly inhibits expression of that gene.

It is interesting to note that deletion of 1.4 kb of the SFFV noncoding region in the *Bam*HI deletion variant drastically lowers gp55 expression, whereas the larger 2.9-kb deletion in the *Bgl*II deletion variant expresses normal levels of gp55. The *Bgl*II deletion includes all but 0.6 kb of the putative gp55 mRNA intron. Chen and Temin (7) have also observed that there does not appear to be a direct relationship between the size of the intron deletion and the levels of gene expression.

Design of retrovirus vectors. Structural analysis of the proviruses present in the TK^+ transductants and in the erythroleukemia cell lines indicates that deletion and recombination events are associated with the generation of these infectious viruses. These genetic changes result in the generation of a heterogeneous population of viral genomes in the primary virus stocks and the functional segregation of the *tk* and erythroleukemia-inducing functions after selection for either marker.

These results have several implications for the design and utilization of retrovirus vectors. First, vectors containing more than one genetic marker may segregate upon passage of the virus or, more likely, during the initial DNA transfection. This segregation may occur randomly and nonselectively or there may be positive selection for the loss of one gene if its presence inhibits either viral infectivity or expression of a second selected gene. Second, the expression of coding sequences located downstream from the splice acceptor site normally utilized in *env* mRNA

biosynthesis may be very sensitive to deletions or insertions-substitutions within the intron for this spliced mRNA. Thus, strategies for the design of retrovirus vectors in which, for example, cDNAs are inserted downstream from the *env* splice acceptor site may have to be modified to ensure expression of these coding sequences.

Transformation and transduction by SFFV. Studies by Linemeyer et al. have demonstrated that the SFFV *env* gene is necessary to induce the early preleukemic stages of Friend erythroleukemia (21, 22). Our results confirm that expression of the *env* gene-encoded gp55 is necessary for at least the early stages of Friend leukemia. In addition, the results presented here, paralleling similar studies with the avian retrovirus reticuloendotheliosis virus (7), indicate that the presence of an intact SFFV *env* gene is not sufficient to induce disease. Deletions or insertions within the SFFV noncoding region can suppress transformation by this virus. The SFFV genome contains multiple deletions and substitutions within its *gag-pol* and *env* regions when compared with the Moloney MLV genome (4, 8). Although many of these genetic alterations may be neutral mutations that do not affect the pathogenicity of the virus, some of these alterations in the SFFV noncoding region may favor, or be essential for, the expression of gp55 and induction of Friend disease. Thus, SFFV may have arisen by a multistage process that included both the acquisition of the gp55 *env* gene and deletions-substitutions within the *gag-pol* region. Other retroviruses have been described that have acquired a cellular oncogene whose expression may also utilize the *env* splice acceptor, including reticuloendotheliosis virus (7), avian myeloblastosis virus (19, 26), Moloney murine sarcoma virus (28), and simian sarcoma virus (10). Like SFFV, the evolution of these transducing viruses may have involved a multistep process that included the stable acquisition of a dominant transforming gene, as well as alterations in the *gag-pol* region.

ACKNOWLEDGMENTS

We thank G. Cheong and V. Prideaux for their excellent technical assistance, Steve Clark for plasmid pSFFV, and J. Low for help in the preparation of this manuscript.

This work was supported by grants from the Medical Research Council and National Cancer Institute of Canada. A. Joyner is a recipient of a studentship from the Medical Research Council of Canada.

LITERATURE CITED

1. **Axelrad, A. A., and R. A. Steeves.** 1964. Assay for Friend leukemia virus: rapid quantitative method based on enumeration of macroscopic spleen foci in mice. Virology **24**:513–518.
2. **Bernstein, A., T. W. Mak, and J. R. Stephenson.** 1977. The Friend virus genome: evidence for the stable association of MuLV sequences and sequences involved in erythroleukemia transformation. Cell **12**:287–294.
3. **Bilello, J. A., G. Coletta, G. Warnecke, G. Koch, D. Frisby, I. B. Pragnell, and W. Ostertag.** 1980. Analysis of the expression of spleen focus-forming virus (SFFV)-related RNA and gp55, a Friend and Rauscher virus-specific protein. Virology **107**:331–344.
4. **Bosselman, R. A., L. J. Van Griensven, M. Vogt, and I. M. Verma.** 1980. Genome organization of retroviruses. IX. Analysis of the genomes of Friend spleen focus-forming (F-SFFV) and helper murine leukemia viruses by heteroduplex-formation. Virology **102**:234–239.
5. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41**:459–472.
6. **Brugge, J. S., and R. L. Erikson.** 1977. Identification of a transformation-specific antigen induced by an avian sarcoma virus. Nature (London) **269**:346–348.
7. **Chen, I. S. Y., and H. M. Temin.** 1982. Substitution of 5' helper virus sequences into non-*rel* portion of reticuloendotheliosis virus strain T suppresses transformation of chicken spleen cells. Cell **31**:111–120.
8. **Clark, S. P., and T. W. Mak.** 1983. Complete nucleotide sequence of an infectious clone of Friend spleen focus-forming provirus. Proc. Natl. Acad. Sci. U.S.A. **80**:5037–5041.
9. **Dagert, M., and S. D. Ehrlich.** 1979. Prolonged incubation in calcium chloride improves the competence of *Escherichia coli* cells. Gene **6**:23–28.
10. **Devare, S. G., E. P. Reddy, K. C. Robbins, P. R. Andersen, S. R. Tronick, and S. A. Aaronson.** 1982. Nucleotide sequence of the transforming gene of simian sarcoma virus. Proc. Natl. Acad. Sci. U.S.A. **79**:3179–3182.
11. **Edgell, M. H., and F. I. Polsky.** 1980. Use of preparative gel electrophoresis for DNA fragment isolation. Methods Enzymol. **65**:319–326.
12. **Enquist, L., G. Vande Woude, M. Wagner, J. Smiley, and W. Summers.** 1979. Construction and characterization of a recombinant plasmid encoding the gene for the thymidine kinase of herpes simplex type I virus. Gene **7**:335–342.
13. **Graham, F., and A. Van der Eb.** 1973. A new technique for the assay of infectivity with human adenovirus 5 DNA. Virology **52**:456–467.
14. **Harbers, K., A. Schnieke, H. Stuhlman, D. Jahner, and R. Jaenisch.** 1981. DNA methylation and gene expression: endogenous retroviral genome becomes infectious after molecular cloning. Proc. Natl. Acad. Sci. U.S.A. **78**:7609–7613.
15. **Hawley, R. G., M. J. Shulman, H. Murialdo, D. M. Gibson, and N. Hozumi.** Mutant immunoglobulin genes have repetitive DNA elements inserted into their intervening sequences. Proc. Natl. Acad. Sci. U.S.A. **79**:7425–7429.
16. **Joyner, A., and A. Bernstein.** 1983. Retrovirus transduction: generation of infectious retroviruses expressing dominant and selectable genes is associated with in vivo recombination and deletion events. Mol. Cell. Biol. **3**:2180–2190.
17. **Joyner, A., Y. Yamamoto, and A. Bernstein.** 1981. Construction and transfer of recombinant retrovirus clones carrying the HSV-1 thymidine kinase gene, p. 535–546. *In* D. D. Brown (ed.), Development biology using purified genes, Academic Press, Inc., New York.
18. **Joyner, A. L., Y. Yamamoto, and A. Bernstein.** 1982. Retrovirus long terminal repeats activate expression of coding sequences for the Herpes simplex virus thymidine kinase gene. Proc. Natl. Acad. Sci. U.S.A. **79**:1573–1577.
19. **Klempnauer, K.-H., T. J. Gonda, and J. M. Bishop.** 1982. Nucleotide sequence of the retroviral leukemia gene v-myb and its cellular progenitor c-myb: the architecture of a transduced oncogene. Cell **31**:453–463.
20. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
21. **Linemeyer, D. L., J. G. Menke, S. K. Ruscetti, L. H. Evans, and E. M. Scolnick.** 1982. Envelope gene sequences which encode the gp52 protein of spleen focus-

forming virus are required for the induction of erythroid cell proliferation. J. Virol. 43:223–233.
22. **Linemeyer, D. L., S. R. Ruscetti, E. M. Scolnick, L. H. Evans, and P. H. Duesberg.** 1981. Biological activity of the spleen focus-forming virus is encoded by a molecularly cloned subgenomic fragment of spleen focus-forming virus. Proc. Natl. Acad. Sci. U.S.A. 78:1401–1405.
23. **MacDonald, M. E., F. H. Reynolds, W. J. M. Van De Ven, J. R. Stephenson, T. W. Mak, and A. Bernstein.** 1980. Anemia- and polycythemia-inducing isolates of Friend spleen focus-forming virus. Biological and molecular evidence for two distinct viral genomes. J. Exp. Med. 151:1477–1492.
24. **Mager, D., M. E. MacDonald, I. B. Robson, T. W. Mak, and A. Bernstein.** 1981. Clonal analysis of the late stages of erythroleukemia induced by two distinct strains of Friend leukemia virus. Mol. Cell. Biol. 1:721–730.
25. **Mager, D., T. W. Mak, and A. Bernstein.** 1981. Quantitative colony method for tumorigenic cells transformed by two distinct strains of Friend leukemia virus. Proc. Natl. Acad. Sci. U.S.A. 78:1703–1707.
26. **Papas, T. S., K. E. Rushlow, D. K. Watson, J. A. Lautenberger, B. Perbal, M. E. Baluda, and E. P. Reddy.** 1982. Nucleotide sequence and organization of the transforming region and large terminal redundancies (LTR) of Avian Meloblastosis virus (AMV). J. Cell Biochem. 20:95–103.
27. **Racevskis, J., and G. Koch.** 1977. Viral protein synthesis in Friend erythroleukemia cell lines. J. Virol. 21:328–337.
28. **Reddy, E. P., M. J. Smith, and S. A. Aaronson.** 1981. Complete nucleotide sequence and organization of the Moloney murine sarcoma virus genome. Science 214:445–450.
29. **Rowe, W. P., W. E. Pugh, and J. W. Hartley.** 1970. Plaque assay techniques for murine leukemia viruses. Virology 42:1136–1139.
30. **Ruscetti, S., D. Troxler, D. Linemeyer, and E. Scolnick.** 1980. Three laboratory strains of spleen focus-forming virus: comparison of their genomes and translational products. J. Virol. 33:140–151.
31. **Southern, E. M.** 1975. Detection of specific sequences among the DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98:503–517.
32. **Southern, P. J., and P. Berg.** 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. J. Mol. Appl. Gen. 1:327–341.
33. **Varmus, H. E., N. Quintrell, and S. Ortiz.** 1981. Retroviruses as mutagens: insertion and excision of a nontransforming provirus alter expression of a resident transforming provirus. Cell 25:23–36.
34. **Wahl, G., M. Stern, and G. Stark.** 1979. Efficient transfer of large DNA fragments from agarose gels to diazobenzyloxymethyl paper and rapid hybridization by using dextran sulphate. Proc. Natl. Acad. Sci. U.S.A. 76:3683–3687.
35. **Wendling, F., F. Moreau-Gachelen, and P. Tambourin.** 1981. Emergence of tumorigenic cells during the course of Friend virus leukemia. Proc. Natl. Acad. Sci. U.S.A. 78:3614–3618.
36. **Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel.** 1979. Transformation of mammalian cells with genes from procaryotes and eukaryotes. Cell 16:777–785.
37. **Yamamoto, Y., C. L. Gamble, S. P. Clark, A. Joyner, T. Shibuya, M. E. MacDonald, D. Mager, A. Bernstein, and T. W. Mak.** 1981. Clonal analysis of the early and late stages of erythroleukemia induced by molecular clones of integrated spleen focus-forming virus. Proc. Natl. Acad. Sci. U.S.A. 78:6893–6897.