JOURNAL OF VIROLOGY, Mar. 1983, p. 1004–1016
0022-538X/83/031004-13$02.00/0

Vol. 45, No. 3

Mutant Feline Sarcoma Proviruses Containing the Viral Oncogene (v-*fes*) and Either Feline or Murine Control Elements

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Received 23 September 1982/Accepted 8 December 1982

The sequences required for transformation by the Gardner-Arnstein (GA) strain of feline sarcoma virus (GA-FeSV) were defined by site-directed, in vitro mutagenesis of molecularly cloned proviral DNA. Portions of the Ga-FeSV provirus, subcloned in the plasmid pBR322, were mutagenized by deletion or frameshift at *Xho*I restriction sites flanking the nucleotide sequences presumed to encode the GA-FeSV transforming polyprotein (P108$^{gag\text{-}fes}$). The biological activity of subgenomic and reconstructed full-genome-length molecules was assayed by transfection and focus induction in NIH 3T3 cells. Both mutant and wild-type molecules containing the intact P108$^{gag\text{-}fes}$ coding region induced foci of transformed cells at efficiencies between 10^4 and 10^5 focus-forming units per pmol of DNA; a deletion mutant lacking 3′-terminal v-*fes* sequences was completely nontransforming in parallel assays. Representative subcloned foci of transformed NIH 3T3 cells synthesized P108$^{gag\text{-}fes}$ with associated in vitro protein kinase activity. Focus-forming viruses could be rescued from transformed subclones induced by full-length proviral DNA, but not from cells transformed by subgenomic DNA lacking a 3′ long terminal repeat (LTR). It was concluded that: (i) nucleotide sequences encoding P108$^{gag\text{-}fes}$ and its associated kinase activity are responsible for transformation, (ii) the GA-FeSV 3′ *env* and LTR sequences are not required for focus induction, and (iii) the 3′ LTR is necessary for rescue of infectious FeSV RNA. A chimeric DNA containing the 5′ LTR and P108$^{gag\text{-}fes}$ coding region of GA-FeSV joined to the 3′ LTR of Moloney murine sarcoma virus was both transforming and rescuable at high efficiency. Restriction analysis showed that passaged stocks of rescued transforming virus contained Moloney murine sarcoma virus U3 sequences at both proviral DNA termini, consistent with generally accepted models for LTR formation during reverse transcription. Wild-type GA-FeSV and the chimeric virus (here designated as GAHT), each rescued from NIH 3T3 cells with the same amphotropic murine leukemia virus, yielded approximately equal numbers of foci when titrated on CCL 64 mink cells. By contrast, on mouse NIH 3T3 cells, the focus-forming titer of GAHT was 1 to 2 log higher than that of FeSV. The foci induced on NIH 3T3 cells by GAHT appeared earlier and were reproducibly larger than those induced by GA-FeSV. Differences in transforming activity on NIH 3T3 cells were also found using colony formation in agar, showing that the more rapid appearance and larger size of foci formed in liquid media were not due to virus spread. These data suggest that transcriptional control signals within the viral LTR regulate the levels of the transforming gene product in a species-specific manner.

The Gardner-Arnstein (GA) (20) and Snyder-Theilen (ST) (51) strains of feline sarcoma virus (FeSV) are acutely transforming retroviruses that were generated by recombination of a feline leukemia virus (FeLV) with portions of a cat cellular oncogene (c-*fes*) (18, 19). Both sarcoma viruses lack portions of the FeLV genome necessary for virion formation (16, 44) and must therefore be propagated in a complex with replication-competent helper viruses, which confer

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the viral host range (15, 26). The ST- and GA-FeSV *gag* and v-*fes* sequences code for fusion polyproteins ($p85^{gag\text{-}fes}$ and $P108^{gag\text{-}fes}$, respectively [4, 40, 58]) with associated tyrosine kinase activities (2, 59). Transfection of NIH 3T3 cells with restriction endonuclease-digested cellular DNAs containing integrated ST-FeSV proviruses has suggested that proviral DNA sequences 3′ to the putative polyprotein-coding region are not required for transformation (1, 39). Isolation of transformation-defective FeSV mutants encoding polyproteins lacking this enzyme activity also showed that the fused FeSV *gag* and v-*fes* genes are necessary, but not necessarily sufficient, for transformaton (3, 14, 37).

The control elements regulating transcription of retroviral transforming genes are derived by recombination from the transducing helper virus and are located at the extreme 3′ end of sarcoma viral RNA (the U3 region). These sequences are permuted to the 5′ end of viral DNA during the process of reverse transcription (21), and appear within the long terminal repeats (LTRs) at each end of integrated proviral DNA (29, 41). The order of sequences within each LTR is U3-R-U5, in which U3 and U5 represent unique sequences derived from the 3′ and 5′ ends of viral RNA, and R represents a short redundant sequence present at both ends of the RNA genome. The U3 region of each LTR contains sequences positioning the origin of RNA transcription (the Goldberg-Hogness or TATA box) (11, 12, 23, 30, 34, 36, 53–55, 57), as well as enhancer or activator elements which regulate the efficiency of transcription by cellular RNA polymerase (7, 9, 31, 32). Transcription of proviral DNA begins downstream from U3 at the cap site in the 5′ LTR and proceeds past the polyadenylation signal in the 3′ LTR, thus permitting recovery of the U3 sequence at the 3′ end of viral RNA. Variations within the U3 region of different avian retroviruses have been shown to affect both viral replication and transformation (56).

In the present studies, we wished to determine whether signals within the U3 region of different transforming retroviruses might be recognized in a species-specific manner. A precedent derives from recent experiments involving the exchange of enhancer sequences between papovaviruses and retroviruses which provided evidence for host-specific recognition of these control elements (31). Using a biologically active molecular clone of GA-FeSV proviral DNA (gene order, 5′–LTR–leader–Δ*gag-fes*–Δ*env*–*LTR*–3′) (16), we formally demonstrated that the 5′ LTR and *gag-fes* polyprotein coding region are sufficient for focus-inducing activity, whereas the 3′ LTR is necessary for formation of transmissible FeSV genomes. Hybrid molecules containing the transforming FeSV DNA fragment were joined to the 3′ LTR of the Moloney murine sarcoma virus (MSV) and transfected into NIH 3T3 cells. Rescue of virus from transformed mouse cell clones gave rise to a hybrid sarcoma virus whose genome contained the murine U3 region and encoded FeSV $P108^{gag\text{-}fes}$ polyprotein. When compared with wild-type GA-FeSV, the hybrid virus showed enhanced transforming activity in cultured mouse cells.

MATERIALS AND METHODS

Cells and viruses. Virus stocks were grown either in mink CCL64 cells (Mv1Lu; American Type Culture Collection, Rockville, Md.) or in NIH 3T3 cells, both subcloned in our laboratory. PG4 cat cells containing the $S^+ L^-$ MSV genome were a generous gift of Dan Haapala (Frederick Cancer Research Center, Frederick, Md.). F3Cl7 is a nonproductively transformed clone of mink CCL 64 cells containing a single integrated copy of GA-FeSV provirus (27). Molecularly cloned ecotropic Moloney murine leukemia virus (MuLV) (49) was recovered by transfection onto NIH 3T3 cells; nonleukemogenic, amphotropic MuLV (strain 4070A [10]) was obtained similarly. Both MuLV stocks were passaged in NIH 3T3 cells. GA-FeSV(MuLV) pseudotypes were obtained after infection of mink F3Cl7 cells with amphotropic MuLV. Other pseudotype stocks containing focus-forming genomes were prepared by rescue of transfected NIH 3T3 transformants (see below).

Virological assays. Assays for infectious helper virus were performed by using an indirect focus-forming test as described (17, 43), except that cat PG4 cells were used instead of cat CCC clone 81 cells, and the foci were scored 5 days after infection. Assays for focus-forming genomes were performed on NIH 3T3 and mink CCL64 cells as previously described (27, 43). Colony formation in agar was assayed as described elsewhere (14).

DNA transfection. Plasmid DNA containing transforming FeSV fragments was assayed by transfection on NIH 3T3 cells (33). Transformed foci were enumerated 14 days after transfection, and representative foci were subcloned in microcylinders 7 days later. In some cases, mixtures of phenotypically flat and transformed cells were recloned in semisolid medium (14) or in microtiter plates (14, 43). Parallel cultures of cloned, transformed cells were infected with either amphotropic or ecotropic MuLV, and supernatants from infected cultures were titered for focus-forming activity at 1, 2, and 3 weeks after infection on both mouse and mink target cells.

Assays for $P108^{gag\text{-}fes}$. Detailed procedures for detecting $P108^{gag\text{-}fes}$ are described elsewhere (13). In brief, cells grown to confluence in 150-cm^2 plastic flasks were labeled for 2 h with 200 μCi (5 ml) of L-[4,5-^{3}H]leucine (60 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) per ml, and labeled lysates were prepared and precipitated with antisera. Immune precipitates were collected with *Staphylococcus aureus* (Cowan I strain), denatured, and subjected to electrophoresis on polyacrylamide 6 to 12% gradient slab gels containing sodium dodecyl sulfate. In some cases,

unlabeled cell lysates were precipitated as above and incubated with 5 μCi of [γ-^{32}P]ATP (2,000 Ci/mmol; New England Nuclear Corp., Boston, Mass.) (3), and the immune complexes were denatured and electrophoresed as above. After electrophoresis, gels were permeated with dimethylsulfoxide-2,5-diphenyloxazole, dried, and exposed to preflashed Kodak X-Omat film at −70° (13).

Recombinant DNA techniques. All restriction enzymes were obtained from Bethesda Research Laboratories (Bethesda, Md.) and New England BioLabs (Beverly, Mass.) and were used according to instructions supplied by the manufacturer. Endonuclease BAL31 and T4 ligase were purchased from Bethesda Research Laboratories, and AMV polymerase was obtained from the Resources Office of the National Cancer Institute.

A 14-kilobase (kb) *Eco*RI fragment containing the 6.7-kb GA-FeSV provirus was purified by electroelution (50), partially digested with *Hin*dIII, and digested to completion with *Bam*HI (see Fig. 1 for relevant map assignments and structures of different plasmids). Two *Bam*HI-*Hin*dIII fragments were subcloned into pBR322 and transfected into suitable *Escherichia coli* hosts (strain RR-1 or LE-392) (8). Bacteria from ampicillin-resistant colonies were grown in 10-ml cultures, and their plasmid DNAs were extracted (6) and analyzed electrophoretically after restriction endonuclease cleavage. Plasmid DNAs containing the expected viral DNA fragments (Fig. 1, lines b and c) were

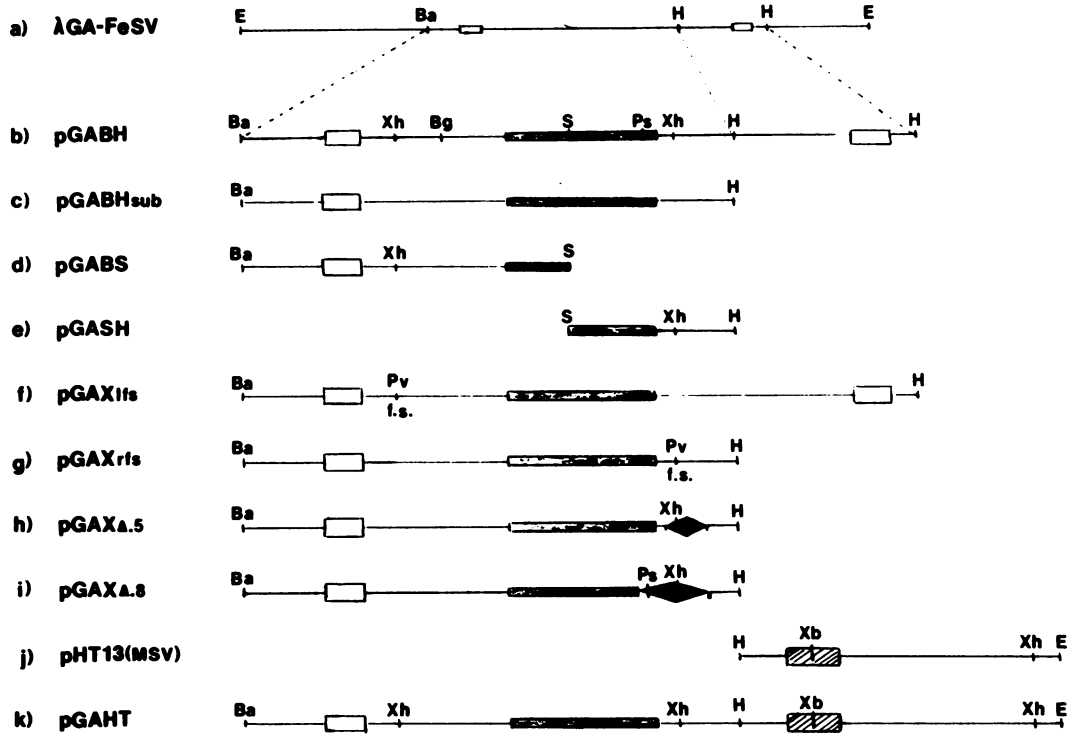

FIG. 1. Schematic maps of cloned DNA fragments inserted into the plasmid pBR322. The top line (a) shows the 14-kb *Eco*RI fragment containing the GA-FeSV provirus, previously cloned into a lambda vector (16). The unshaded rectangles designate the positions of the viral LTRs and define the position of the 6.8-kb provirus within mink cellular DNA. After cleavage with *Bam*HI (Ba) and *Hin*dIII (H), plasmids containing either full-length (line b) or subgenomic (line c) proviral DNA fragments were constructed. Pertinent sites of restriction for several other enzymes are noted and include *Xho*I (Xh), *Bgl*II (Bg), *Sal*I (S), and one of several *Pst*I (Ps) sites. The shaded rectangles define the position of v-*fes* sequences. The plasmid pGABHsub was further subcloned to form pGABS (line d) and pGASH (line e), each of which contained a single site of restriction for *Xho*I. The *Xho*I sites were converted to *Pvu*I (Pv) sites to generate frameshift (f.s.) mutants within full-length (pGAXlfs [line f]) and subgenomic (pGAXrfs [line g]) FeSV DNA. Deletion mutants were generated by BAL31 digestion at the right-hand *Xho*I site contained in pGASH to construct the deleted subgenomic proviruses, pGAXΔ.5 (line h) and pGAXΔ.8 (line i). The respective 0.5- and 0.8-kb deletions are indicated by diamonds; the larger of the two eliminated a *Pst*I site assigned to the 3′ end of v-*fes*. A plasmid containing the 3′ end of the Moloney MSV provirus (strain HT-1) as well as flanking 3′ mink cellular information (60) (line j) was used to construct a chimeric FeSV-MSV provirus (line k). A site of restriction for *Xba*I (Xb) has been assigned to the MSV U3 region within the MSV LTR (crosshatched rectangle). The extreme left-hand and right-hand restriction sites on line drawings b through k were the cloning sites within pBR322.

obtained from 1-liter bacterial cultures and purified by cesium chloride centrifugation (8).

Plasmid DNA containing a subgenomic GA-FeSV DNA fragment (pGABHsub; Fig. 1, line c) was digested with *Sal*I and recircularized with ligase to form pGASH (Fig. 1, line e); the liberated *Sal*I fragment containing both FeSV and pBR322 sequences was digested with *Bam*HI and recloned at the *Sal*I and *Bam*HI sites of pBR322 to generate pGABS (Fig. 1, line d). The latter two plasmids each contained single sites for *Xho*I cleavage which were mutagenized by deletion or frameshift. Frameshift mutations were generated after *Xho*I cleavage by filling in the staggered ends with AMV polymerase in 50-μl reactions containing 2 μg of DNA, 0.14 U of enzyme, 100 mM Tris-hydrochloride, pH 8.0, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, and 0.1 mM dATP, dCTP, dGTP, and dTTP (30 min at 37°C). Deletion mutants were obtained by limited BAL31 digestion, using conditions recommended by the manufacturer and temperatures and times of digestion empirically determined to remove 0.2 to 0.5 kb of DNA from the ends of linear DNA molecules. The blunt ends of linear molecules, generated by either of the two above procedures, were recircularized with T4 ligase and then retreated with *Xho*I to linearize any residual wild-type circular forms. Plasmids containing mutagenized sequences were recovered by transfection into *E. coli* and purified as described above.

The pGABS frameshift variant lacking an *Xho*I site was mixed with a fourfold molar excess of pGABH DNA. The mixture was digested with *Bgl*II, treated with ligase, and then codigested with *Bam*HI and *Eco*RI. A DNA fragment containing full-length proviral DNA was purified by electroelution and recloned in pBR322 (pGAXlfs; Fig. 1, line f). DNAs from pGASH variants containing either deletions or frameshifts at the *Xho*I site were mixed in equimolar amounts with pGABS, digested with *Sal*I, and ligated end-to-end. After codigestion of ligated intermediates with *Bam*HI and *Hin*dIII, 6-kb fragments containing 5′ subgenomic proviral DNA were purified by electroelution and recloned in pBR322 to generate the plasmids, pGAXrfs, pGAXΔ.5, and pGAXΔ.8 (Fig. 1, lines g through i). A recombinant plasmid containing a portion of Moloney MSV was constructed by inserting the purified *Hin*dIII-*Eco*RI fragment of pHT13 (7) (see Fig. 1, line j) into pGABHsub to generate chimeric pGAHT DNA (Fig. 1, line k).

Analyses of integrated proviral DNA. High-molecular-weight DNA was cleaved with appropriate restriction enzymes and analyzed by the technique of Southern (52). A probe containing sequences specific for v-*fes* was prepared by nick translation (38) of the DNA from the previously described plasmids S_L and S_R (18). Hybridizations and washing of blots were performed as previously described (43), except that the monovalent cation concentration was 0.5 M, and annealing was performed at 65°C in the absence of formamide.

RESULTS

Mutagenesis of GA-FeSV plasmid DNAs. Two plasmid subclones of lambda GA-FeSV DNA were generated which contained the complete GA-FeSV provirus flanked by mink cellular sequences (pGABH) and a subgenomic GA-FeSV fragment lacking 3′ *env* sequences and the 3′ LTR (pGABHsub) (Fig. 1, lines b and c, respectively). Restriction endonuclease mapping of cloned FeSV and FeLV genomes previously suggested that the two *Xho*I sites within the GA-FeSV provirus flank the sequences encoding $P108^{gag\text{-}fes}$ (16). Nucleotide sequencing analysis has now confirmed that the 5′ *Xho*I site is 226 bases upstream from the *gag* gene coding sequences, whereas the 3′ *Xho*I site is 298 bases downstream from the 3′ end of the v-*fes* gene and 346 bases from the TGA codon presumed to terminate polyprotein translation (23, 24).

To facilitate mutagenesis at the two different *Xho*I sites, we constructed two additional subgenomic clones containing portions of the FeSV provirus derived from the 5′ and 3′ sides of the single *Sal*I site within v-*fes* (Fig. 1, lines d and e). Mutations were introduced after digestion with *Xho*I by filling in the staggered ends (5′ TCGA 3′) and religating the flush-ended termini to create the sequence 5′ TCGA.TCGA 3′. The four-base addition creates a +1 frameshift. The correctly altered sequence lacks the *Xho*I hexanucleotide recognition site but instead contains a *Pvu*I site (CGAT ↓ CG), thus permitting analysis of the mutation by restriction enzyme digestion. Deletion mutations at the 3′ GA-FeSV *Xho*I site were obtained by limited BAL31 digestion of pGASH followed by recircularization. The mutant plasmids, recovered by bacterial transformation, were extracted and characterized by restriction enzyme analysis. DNAs from selected clones were ligated to one another and recloned to generate the four mutant plasmids shown in Fig. 1. These included two frameshift mutants, pGAXlfs and pGAXrfs (Fig. 1, lines f and g), and two deletion mutants, pGAXΔ.5 and pGAXΔ.8, lacking approximately 0.5 and 0.8 kb, respectively (Fig. 1, lines h and i). The asymmetrical deletion in the latter plasmid eliminated a terminal *Pst*I site within v-*fes*, whereas the smaller deletion did not.

Transforming activity of plasmid DNAs. The biological activity of the plasmid clones was tested by transfection of their DNAs onto NIH 3T3 cells. Table 1 shows that pGABH containing a full-length provirus was as active as the parental lambda GA-FeSV clone (16). Subgenomic FeSV DNA lacking terminal *env* and 3′ LTR sequences generally gave between 5- to 10-fold fewer foci per pmol of DNA. This agrees with previous observations obtained with Moloney MSV DNA which indicated that, although the 3′ LTR was not required for transformation, its presence increased the efficiency of focus formation (7). For both the full-length and subgenomic DNAs, cleavage with *Bam*HI and *Eco*RI before

TABLE 1. Biological activity of GA-FeSV plasmid clones on NIH 3T3 mouse cells

Plasmid[a]	Restriction enzyme cleavage[b]	Efficiency of transformation (log FFU/pmol)[c]	p108 kinase activity[d]	Rescue of focus-forming virus[e]
pGABH	No	4.4	Yes	Not tested
pGABH	Yes	4.9	Yes	Yes
pGABHsub	No	3.2	Yes	Not tested
pGABHsub	Yes	4.0	Yes	No
pGAXlfs	Yes	5.3	Yes	Yes
pGAXrfs	Yes	4.2	Yes	No
pGAXΔ.5	Yes	4.2	Yes	No
pGAXΔ.8	Yes	<1.3[f]	Not applicable	Not applicable

[a] The different plasmids are summarized schematically in Fig. 1. Plasmid DNAs were transfected as described (16, 33), and foci of transformed cells were enumerated after 14 days. All samples were tested in triplicate by using serial dilutions of plasmid DNA. The 14-kb *Eco*RI fragment of lambda GA-FeSV DNA (16) gave 5-log transforming activity in parallel experiments.

[b] Circular plasmid DNAs were transfected either without treatment or after linearization with *Eco*RI and *Bam*HI.

[c] The efficiency of focus formation was calculated as previously described (33).

[d] Representative transformed foci (at least four from each series) were subcloned and tested for in vitro p108 kinase activity in immune complexes. Results from typical experiments are shown in Fig. 2.

[e] Subcloned foci were infected with either amphotropic or ecotropic MuLV, and the supernatants of cultures were titered on NIH 3T3 mouse cells and CCL64 mink cells.

[f] None detected.

transfection was found to augment the efficiency of focus formation and was therefore used in subsequent transfection experiments.

The two frameshift mutants and the smaller deletion mutant were as efficient as their wild-type counterparts in their ability to induce foci. By contrast, pGAXΔ.8 produced no foci at any DNA concentration tested. Taken together, these results indicated that the GA-FeSV sequences 3′ to v-*fes* were not necessary for transformation, although there appeared to be an absolute requirement for 3′-terminal *fes* sequences themselves.

Expression of GA-FeSV polyproteins. To verify that transformation was due to the expression of GA-FeSV polyprotein in transfected cells, representative foci of transformed cells from experiments with each DNA were subcloned and tested for the presence of P108$^{gag\text{-}fes}$ and its associated kinase activity. In general, at least four different focus-derived clones were assayed from transfection tests performed with each DNA. Cell lysates were subjected to immunoprecipitation with anti-*gag* (p30) sera, followed by incubation of the immunoprecipitates in the presence of [γ-^{32}P]ATP. Figure 2 shows that after denaturation and electrophoresis of the precipitated proteins, P108$^{gag\text{-}fes}$ was itself phosphorylated as were several additional polypeptides (3). Kinase activity was detected in all transformed clones induced either by full-length or subgenomic FeSV DNA. No kinase activity was detected under these conditions with lysates of nontransformed NIH 3T3 cells (Fig. 2, lane 2). Thus, transformed NIH 3T3 clones derived by transfection with either subgenomic or mutant transforming proviral DNAs each expressed an apparently unaltered GA-FeSV transforming protein and its associated enzymatic activity.

Rescue of proviruses from NIH 3T3 transfectants. Cloned foci of transformed cells were infected with amphotropic MuLV. At 2 and 4 weeks after virus infection, supernatants from the cultures were titered for focus-forming virus on both NIH 3T3 and mink CCL 64 cells. At least 3 log transforming virus per ml was obtained by using clones transformed by full-length proviral DNA (pGABH and pGAXlfs). Thus, the frameshift in the 5′ leader sequence did not affect a packaging sequence necessary for rescue (42). By contrast, cells transformed by subgenomic pGABHsub, pGAXrfs, and pGAXΔ.5 did not yield rescuable viral genomes. This was consistently observed in experiments with independently derived foci, amphotropic or ecotropic murine helper viruses, and both NIH 3T3 and mink CCL 64 cells for titration of potential amphotropic pseudotypes. The 3′ end of the GA-FeSV provirus is therefore necessary for the rescue of transmissible focus-forming viral genomes from mouse cells (Table 1).

Chimeric constructions restoring the 3′ LTR sequence. The above data suggested that if the 3′ end of a different retrovirus were substituted for the 3′ FeSV LTR, a rescuable chimeric genome containing a different U3 sequence might be generated. Since the elements controlling transcription of retroviral RNA map within U3 (11,

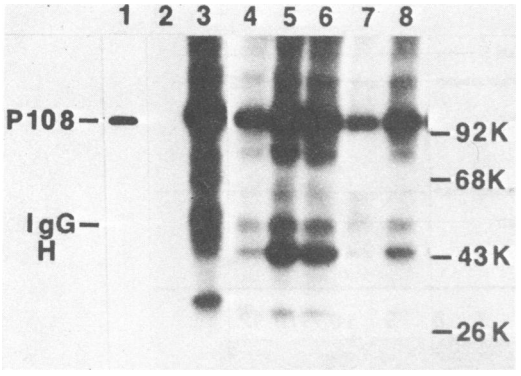

FIG. 2. Assay for P108$^{gag\text{-}fes}$-associated kinase activity in NIH 3T3 transformants. Lysates of transformed cell clones were precipitated with antiserum to p30 protein, and immune precipitates were incubated in the presence of [γ-^{32}P]ATP before denaturation and electrophoresis on acrylamide gels containing sodium dodecyl sulfate (3). Mink cells nonproductively transformed with GA-FeSV (clone F3C17) were labeled with [^{3}H]leucine, and the precipitated P108$^{gag\text{-}fes}$ polyprotein was subjected to electrophoresis as a control (lane 1). Lanes 2 through 8 show representative results of the kinase assay with immune complexes prepared from normal NIH 3T3 cells (lane 2) and F3C17 cells (lane 3) and from cells transformed by transfection with pGABH (lane 4), pGABHsub (lane 5), pGAXlfs (lane 6), PGAXrfs (lane 7), and pGAXΔ.5 (lane 8). The positions of radiolabeled proteins used as molecular weight markers (indicated in kilodaltons [K]) are shown at the right.

12, 23, 30–32, 34, 36, 47, 53–55, 57), this approach could potentially be used to place the feline v-*fes* transforming gene under the control of transcriptional elements derived from retroviruses of another species. A plasmid, pHT13 (7), containing 3′ Moloney MSV sequences (Fig. 1, line j) was ligated at the *Hin*dIII site to the 3′ end of pGABHsub to form the chimeric provirus, pGAHT (Fig. 1, line k). This new proviral DNA contained the left-hand LTR and polyprotein coding region of FeSV and the right-hand LTR of MSV. Transcription of viral RNA in cells transformed by pGAHT was expected to initiate at the cap site (5′ R region) within the FeSV LTR and terminate after the polyadenylation signal (3′ R region) within the MSV LTR. Since the R regions of FeSV and MSV LTRs show ~80% homology (23), it was expected that after packaging and reinfection, chimeric viral RNA molecules would serve as suitable templates for transcription of full-genome-length proviral DNA. Generally accepted models for reverse transcription of retroviral RNA predict that the newly formed proviruses would have U3 regions derived from MSV at both termini, and would give rise to transforming RNA genomes containing only MSV U3 sequences (21, 48).

As expected, the plasmid-containing hybrid viral DNA induced foci of transformed NIH 3T3 cells at the same efficiency as the wild type, full-genome-length GA-FeSV DNA. Transmissible virus was recovered from the cultures of transformed cells after infection with either ecotropic or amphotropic MuLV. The kinetics of appearance of rescuable focus-forming virus after infection were indistinguishable from those obtained with wild-type FeSV-derived transformants rescued in a parallel experiment performed with the same helper virus stocks. Using amphotropic MuLV helper virus and CCL 64 mink cells to score foci, approximately 3 log transforming virus per ml of culture supernatant was obtained from both classes of transfected cultures by 3 weeks after infection.

Restriction analyses of transmitted, chimeric proviral DNA. Foci of NIH 3T3 cells transformed by rescued virus [designated GAHT-(MuLV)] were cloned, and their high-molecular-weight cellular DNA was extracted and studied by Southern blot analyses. Wild-type GA-FeSV DNA is 6.7 kb in length and lacks sites of restriction for *Xba*I (16). By contrast, the GAHT provirus was predicted to be approximately 0.5 kb shorter and to contain *Xba*I sites within both U3 regions (7, 60). Both the wild-type GA-FeSV and GAHT proviruses would contain *Xho*I sites flanking the polyprotein coding region (see schematic, top of Fig. 3). The DNA of three independently derived viral GAHT transformants was analyzed by using a radiolabeled v-*fes* probe. At the stringent conditions chosen for these analyses, interrupted mouse or mink cellular sequences related to v-*fes* (the c-*fes* proto-oncogene) are detected less efficiently than the identical, unsegmented v-*fes* sequences in the provirus.

Figure 3 shows that after digestion with *Xho*I, the characteristic 3.3-kb proviral DNA fragment was detected in mouse cell clones transformed by GAHT (lanes 1 through 3) but not in uninfected NIH 3T3 cells (lane 4). A band of similar length was found in control mink cells nonproductively transformed by a single copy of GA-FeSV proviral DNA (lane 5) but not in uninfected mink cells (lane 6). Digestion with *Xba*I yielded a 5.7-kb DNA fragment from each GAHT transformant (lanes 7 through 9) but not from control mouse cells, showing that, unlike the pGAHT DNA construction (Fig. 1, line k), the GAHT virus contained *Xba*I sites at both proviral DNA termini. The GA-FeSV provirus of a nonproductively transformed mink cell clone lacked *Xba*I sites and was detected within a high-molecular-weight fragment containing flanking mink cellular sequences (Fig. 3, lane

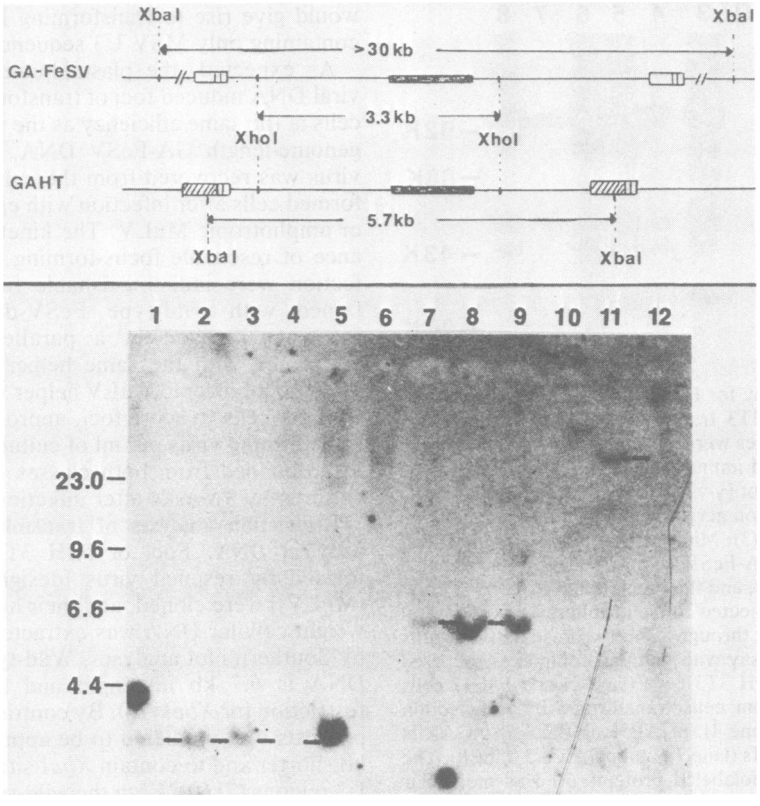

FIG. 3. Restriction endonuclease analysis of integrated proviral DNA detected after Southern transfer with a v-*fes*-specific probe. Lanes 1 through 6 show results after *Xho*I digestion, and lanes 7 through 12 show results after *Xba*I digestion. Approximately 20 μg of DNA were run per lane. The position of marker DNA fragments (in kilobases) is indicated at the left. The different samples included high-molecular-weight DNA from three independently derived NIH 3T3 clones transformed by GAHT(MuLV) (lanes 1 and 7, 2 and 8, 3 and 9, respectively), normal NIH 3T3 (lanes 4 and 10), mink F3C17 cells nonproductively transformed by GA-FeSV (lanes 5 and 11), and uninfected CCL 64 mink cells (lanes 6 and 12). The schematic drawings at the top of the figure indicate the predicted sizes of v-*fes*-containing fragments after digestion with the two enzymes. Note that GA-FeSV lacks sites of restriction for *Xba*I.

11). The presence of *Xba*I sites at each end of GAHT proviral DNA and the size of the internal v-*fes*-containing DNA fragment confirm that sequences from the extreme 3′ end of the MSV genome (U3) are present in the LTR of the GAHT provirus.

Biological activity of GAHT virus. Stocks of GA-FeSV(MuLV) and GAHT(MuLV), prepared with a nonleukemogenic amphotropic helper virus, were titered for their ability to transform both mouse and mink cells. Although the host range of both pseudotype virus stocks is determined by the helper MuLV, the transforming efficiency and rate of spread of focus-forming virus in infected cultures would also be a function of the levels of transcription of the transforming viral RNA within the different target cells. Since viral RNA transcription and subsequent expression of transforming polyprotein would be under control of different U3 elements in the two virus stocks, it might be possible to observe different effects in target cells from different species.

Table 2 shows that the titers of MuLV helper virus were approximately 10^7 infectious units per ml for both GA-FeSV and GAHT pseudotypes. Similarly, the focus-forming titers on mink CCL 64 cells were about equal. By contrast, the focus-forming titers of these stocks on mouse NIH 3T3 cells were significantly different. Although GA-FeSV(MuLV) produced more foci on mink cells than on mouse cells, stocks of GAHT(MuLV) were more efficient in inducing foci on mouse cells than on mink cells. Furthermore, on NIH 3T3 cells the foci induced by GAHT appeared earlier after infection than those induced by GA-FeSV. At 4 days after infection, NIH 3T3 foci induced by GAHT were

TABLE 2. Titration of wild-type and chimeric focus-forming viruses on mouse and mink cells

Virus stock[a]	Infectious MuLV titer[b]	Focus-forming titers (FFU/ml) on[c]:		Ratio of focus-forming titers (mink cells/ mouse cells)
		Mink CCL 64 cells	Mouse NIH 3T3 cells	
FeSV(MuLV)	10^7	5.4×10^4	3.2×10^3	17
GAHT(MuLV)	10^7	2.2×10^4	2.4×10^5	0.09

[a] Titrations were performed with pseudotype transforming stocks obtained after rescue of sarcoma virus genomes with a cloned nonleukemogenic, amphotropic MuLV (strain 4070A) (10). Both replicating virus stocks were maintained in CCL 64 mink cells without an appreciable alteration of titer over a 3-month period.

[b] Helper virus titers were determined in an indirect focus-forming assay on cat PG4 S^+ L^- cells as described for CCC 81 cells previously (17).

[c] The average titers from five different experiments performed over a 3-month period are shown.

clearly visible, whereas foci induced by GA-FeSV could not be readily detected. Titers were determined between 7 and 9 days after infection, at which time GA-FeSV foci were visible but smaller and more difficult to score than those induced by GAHT stocks. To illustrate this difference, macroscopic NIH 3T3 foci induced by both virus stocks were stained 14 days after infection. Figure 4A shows a representative experiment in which the chimeric sarcoma virus induced larger and more numerous foci than the wild-type control.

Given the time after infection when foci were scored and the rapid development of interference by excess helper virus, we considered it unlikely that the apparent differences in the sizes and numbers of foci might be due to sarcoma virus spread and secondary focus formation. However, to eliminate this possibility, NIH 3T3 cells were infected, trypsinized 24 h after infection, and replated in semisolid medium before secondary spread of virus could occur. Colonies of transformed cells were counted after 3 weeks. In an experiment performed with the virus stocks used to generate the data shown in Table 2, the titers in agar were 3×10^2 CFU/ml for GA-FeSV(MuLV) and 5×10^3 CFU/ml for GAHT(MuLV). Figure 4B also shows that 24 days after infection the average sizes of colonies induced by the chimeric virus (plate 1) were larger than those induced by GA-FeSV (plate 2). Although the colony-forming efficiencies in agar were 2 to 10% of the values obtained in liquid medium (cf. reference 14), similar relative differences in the numbers and sizes of colonies induced by the two transforming viruses were observed. We conclude that NIH 3T3 cells transformed by GAHT proliferate more rapidly than GA-FeSV transformants, even when cell-to-cell spread of virus is inhibited.

In cloned FeSV transformants, it was previously shown that there is a correlation between the levels of transcription of viral RNA, the amount of polyprotein expressed, and the quantity of FeSV RNA packaged into extracellular virions (14). The titers of focus-forming virus released from cloned mouse cells infected with GAHT(MuLV) or GA-FeSV(MuLV) should therefore reflect the levels of intracellular viral RNA expressed in the different classes of clones. Five agar colonies, selected at random from cells infected with each virus, were grown to confluence in 100-mm dishes, and the titers of transforming virus released into the culture media were determined. Table 3 shows that GAHT-(MuLV), released by independently derived NIH 3T3 clones, transformed mouse cells more efficiently than mink cells, whereas GA-FeSV pseudotypes showed an opposite tropism. The enhanced efficiency of GAHT in transforming mouse cells is therefore not a function of the cells in which the virus is grown (compare Tables 2 and 3) but is an intrinsic property of the hybrid viral genome. Table 3 also shows that GAHT-transformed mouse cells released significantly more virus than their GA-FeSV-transformed counterparts, suggesting that they expressed more viral RNA. Taken together, these results suggest that the MSV U3 region is more efficient than the analogous FeSV sequence in promoting the expression of the feline transforming gene in mouse cells.

Assays for tumor formation in mice. Because of their altered transforming potential in vitro, GAHT pseudotypes prepared with nonleukemogenic, amphotropic MuLV were tested for their ability to induce tumors in NIH/Swiss and BALB/c mice. Newborn animals received intraperitoneal injections of 10^3 focus-forming units (FFU) per animal, and weanling mice received 5×10^3 FFU per animal either subcutaneously or intravenously. Some animals receiving intravenous injections were sacrificed after 2, 4, and 8 weeks and autopsied. Mice inoculated with GA-FeSV or GAHT were followed for 3 to 5 months without any evidence of tumor formation, spleen

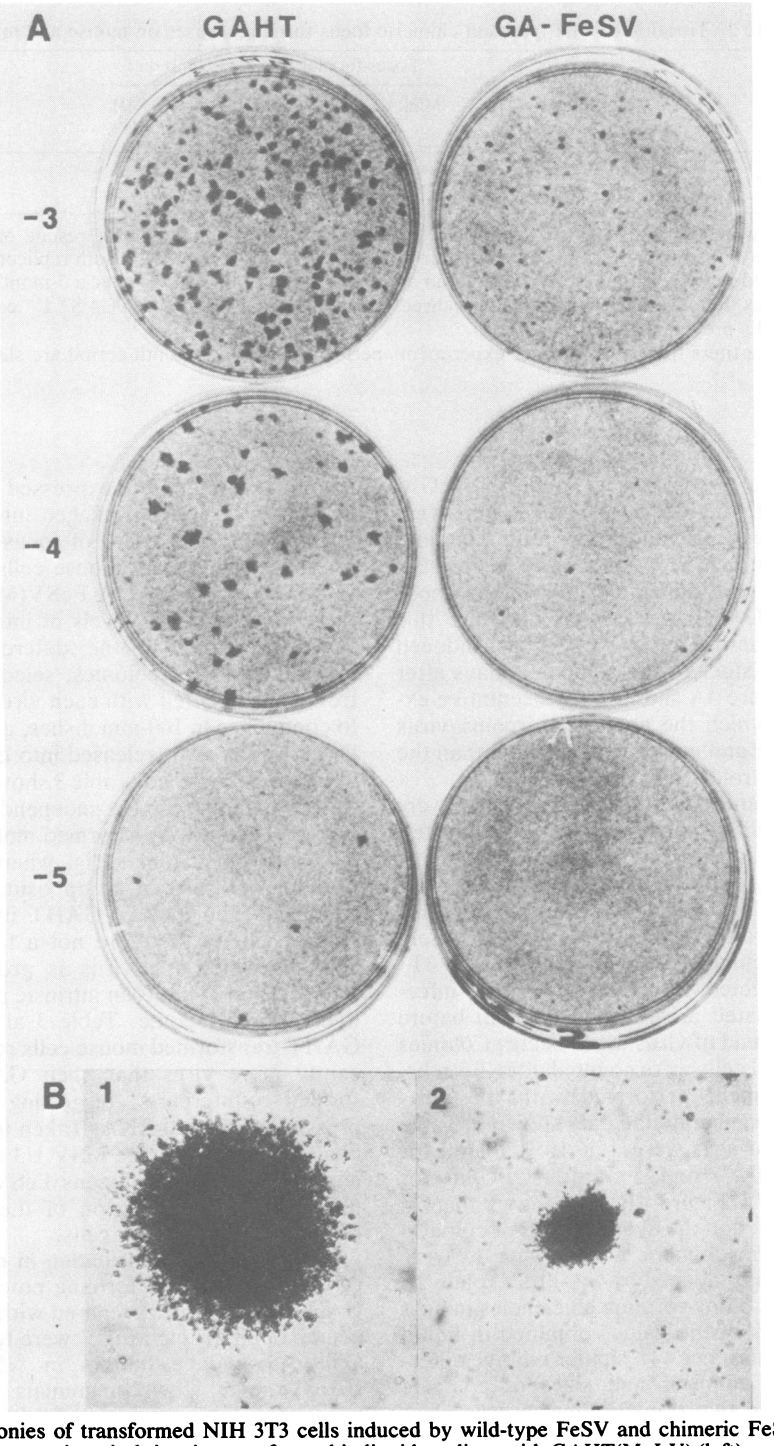

FIG. 4. Colonies of transformed NIH 3T3 cells induced by wild-type FeSV and chimeric FeSV-MSV. (A) Results of representative viral titrations performed in liquid medium with GAHT(MuLV) (left) versus wild-type GA-FeSV(MuLV) (right). The cells were infected with 2.0 ml of diluted culture supernatants from virus-producing cells. The dilutions shown are 10^{-3}, 10^{-4}, and 10^{-5} as indicated at the left. The transformed foci were stained with Giemsa 14 days after infection. Both stocks of virus exhibited equal apparent titers on CCL 64 mink cells (Table 2). Note the relative size and number of foci. (B) Single agar colonies generated 3 weeks after infection in a parallel experiment. The colonies chosen were photographed at the same magnification and represent colonies of average size in their respective plates.

TABLE 3. Titration of focus-forming viruses released from cloned NIH 3T3 transformants[a]

Virus	Focus-forming titers (FFU/ml) on:		Ratio of focus-forming titers (mink cells/ mouse cells)
	Mink CCL 64 cells	Mouse NIH 3T3 cells	
GA-FeSV(MuLV) from:			
Clone 1A	1.2×10^2	<10	>12
Clone 2A	3.4×10^2	18	19
Clone 3A	1.4×10^2	<10	>14
Clone 4A	1.6×10^2	Not tested	
Clone 5A	6.0×10^1	Not tested	
GAHT(MuLV) from:			
Clone 1B	5.0×10^3	8.1×10^4	0.06
Clone 2B	8.2×10^3	1.0×10^5	0.08
Clone 3B	1.1×10^3	8.0×10^3	0.14
Clone 4B	1.2×10^4	Not tested	
Clone 5B	2.3×10^3	Not tested	

[a] NIH 3T3 cells infected with viruses (Table 2) were cloned 24 h after infection in semi- solid medium. Transformed agar colonies selected at random from cells infected with either GA-FeSV(MuLV) or GAHT(MuLV) were grown and tested for virus production as described in Table 2. Foci were scored 5 to 9 days after infection.

focus formation, or splenomegaly. Thus, although FeSV is highly oncogenic in cats, dogs, and several other species (15, 26), our preliminary results suggest that neither the wild-type nor the hybrid-transforming virus efficiently induces tumors in these mouse strains.

DISCUSSION

Determination of the nucleotide sequences of portions of the GA-FeSV provirus has defined an open reading frame able to encode the P108$^{gag\text{-}fes}$ transforming protein (23, 24). The open reading frame begins 344 bases from the 5′ end of viral RNA in the leader sequence preceding the *gag* gene and includes two ATG codons, both in frame with each other, separated by 228 nucleotides. The first ATG codon is just two bases upstream from the 5′ *Xho*I site subjected to mutagenesis in these studies, whereas the second ATG codon corresponds to the 5′ end of the viral *gag* gene. At the 3′ end of the open reading frame, a single TGA termination codon is found 51 nucleotides before the 3′ end of the v-*fes* gene. These biochemical data imply that translation of the polyprotein begins at either the first or second ATG codon and terminates within v-*fes*, suggesting that sequences 3′ to v-*fes* are not required for transformation.

We have formally tested these assumptions by constructing full-genome-length and subgenomic proviral DNAs containing frameshift and deletion mutations near the putative initiation sites and termini of the polyprotein coding sequences. The biological activity of pGAXlfs shows that translation of the transforming polyprotein is initiated at the second ATG codon. Our data do not exclude the possibility that the first ATG codon can be used, but show that if a larger polyprotein is synthesized, it is not required for transformation. Similarly, the ability of pGAXΔ.5 and pGAXrfs to induce foci shows that 3′ *env* and LTR sequences are not required for transformation, in general agreement with previously published data obtained with restriction digests of uncloned proviral DNA (1, 39). By contrast, the inability of pGAXΔ.8 to transform cells indicates that 3′ terminal v-*fes* coding sequences are necessary for focus formation. Restriction mapping analysis showed that the 0.8-kb deletion extended symmetrically from the *Xho*I site and included 400 to 450 base pairs upstream from *Xho*I (data not shown). The deletion eliminated a *Pst*I restriction site located 22 bases 5′ to the putative polyprotein termination codon and 371 bases 5′ to the *Xho*I site (24). This suggests that the terminal 8 to 33 amino acids of P108$^{gag\text{-}fes}$ may be necessary for transformation.

Comparison of the transfection efficiencies of the different plasmid DNAs linearized by *Bam*HI and *Eco*RI digestion showed that full-length proviruses were 5 to 10-fold more efficient in focus induction than subgenomic DNAs lacking the 3′ LTR. Similar results have been obtained with DNA of Moloney MSV (7), in which the viral oncogene sequences map closer to the 3′ LTR of proviral DNA (60). We presume that the modest reduction in transforming efficiency of subgenomic DNAs is explained by the absence of a 3′ viral polyadenylation signal,

necessitating termination of transcripts within adjacent host cellular DNA. Circular full-length molecules are also more active than circular subgenomic molecules, but they are less efficient in inducing foci than their respective linearized counterparts. Possibly, the presence of pBR322 DNA is inhibitory, or alternatively, the circular molecules may randomly linearize with interruption of tandem regulatory and coding sequences.

The inability to efficiently rescue a transforming virus from cells transfected with subgenomic FeSV fragments lacking the 3′ LTR was expected (22, 39, 48). Since 5′ and 3′ R sequences are required for reverse transcription of the proviral DNA negative strand, allowing the first jump between templates (21), regeneration of a replicating virus could only occur by recombination with the helper virus used in rescue. Indeed, after focus induction with circularly permuted Harvey MSV DNA, recombination during rescue was occasionally detected and gave rise to transforming viral genomes of various lengths, each containing the v-*ras* gene (22). Thus, the relative inability to obtain infectious virus from cells transformed by fragments lacking the 3′ LTR was not due to a defect in packaging viral RNA in pseudotype particles but rather to a block in the synthesis of infectious proviral DNA in the next round of infection. We assume that in the case of GA-FeSV, subgenomic viral RNA was similarly packaged into virions, but after infection of new cells it could neither serve as a template for the formation of proviral DNA nor recombine with the heterologous MuLV helper viruses used for rescue.

By religating a heterologous 3′ LTR to the transforming 5′ FeSV subgenomic fragment, it was possible to obtain transmissible focus-forming viruses after infection with replicating helper virus. This was predicted from generally accepted models for retroviral reverse transcription (21) and from experimental data obtained with molecularly cloned avian retroviral DNA (48). Although the latter experiments were performed with variant LTRs from a single class of viruses, we used LTR elements derived from viruses of different species, predicting that the level of homology within their R sequences would be sufficient to allow negative strand DNA synthesis. As predicted, the rescued focus-forming viruses were of the expected length of complete chimeric FeSV-MSV genomes and contained restriction sites for *Xba*I characteristic of MSV U3 sequences. These viruses were rapidly generated after rescue with either ecotropic or amphotropic MuLV, and their kinetics of appearance paralleled those of wild-type FeSV rescued in control experiments. Given the inability to rescue viruses from cells transformed by subgenomic FeSV DNA and the reproducible lengths of rescued GAHT proviral DNAs generated from different, independently derived transformants, it seems highly unlikely that the reconstitution of the LTR sequences occurred by recombination with helper virus sequences.

The chimeric transforming viruses contain the polyprotein-coding region of FeSV, transcriptionally regulated by control elements derived from the MSV genome. Pseudotype viruses containing GAHT focus-forming genomes are more efficient than wild-type FeSV in transforming mouse cells and induce more rapidly developing foci in either liquid or semisolid medium. The differences between GAHT and GA-FeSV in transforming mouse cells are not due to the rate of spread of the transforming virus between cells. We favor the possibility that substitution of the MSV U3 sequence can differentially affect the growth rate of cells transformed by the v-*fes* gene, possibly by increasing the relative levels of viral RNA transcripts and, in turn, transforming protein in susceptible cells.

The 73-base-pair repeat within the U3 region of Moloney MSV has been shown to substitute for the 72-base-pair repeat of simian virus 40 in enhancing the transcription of papovaviral early genes (32). Both simian virus 40 and MSV enhancer elements have recently been assayed for their ability to activate gene expression in different cells and were shown to be more efficient in monkey and mouse cells, respectively (31). Thus, the enhancer elements may interact with host-specific molecules and thereby exert differential effects on transcription in different species. The GA-FeSV U3 region contains a single copy of sequences homologous to the MSV enhancer element (23). In FeSV, the comparable sequence is only 63 nucleotides in length and shows interrupted homology (85%) with the MSV sequence. It is therefore possible that even subtle alterations within this region of the genome are able to significantly alter the expression of viral genes in different cells.

Cat, dog, and mink cells transformed by ST- and GA-FeSV express relatively high levels of transforming polyprotein, whereas mouse and rat cells transformed by the same viruses express comparatively reduced levels (L. A. Fedele, L. P. Turek, J. Even, and C. J. Sherr, unpublished observations). When mink and rat cells were infected at low multiplicity with FeSV(FeLV) and cloned immediately after infection, many different nonproductively transformed clones containing single copies of the FeSV provirus were isolated. Liquid hybridization analyses performed with radiolabeled DNA complementary to the ST-FeSV v-*fes* gene showed that the levels of expression of FeSV RNA in different mink cell clones were routinely

quite high (average $C_{rt1/2}$, 150 mol · s/liter) (14), whereas the corresponding levels in several rat cell transformants were about 10-fold lower ($C_{rt1/2}$, 2,000 mol · s/liter, unpublished observations). Similarly, high levels of focus-forming virus could be rescued from mink, but not rat, clones. In general, the FeSV LTR may be regulated differently in carnivore versus rodent cells.

The feline v-*fes* gene shows a high degree of nucleic acid sequence homology to a viral oncogene (v-*fps*) transduced by the Fujinami and PRCII strains of avian sarcoma virus (46). As was predicted from nucleic acid hybridization studies, the polyprotein products of each of these avian isolates are biochemically related to those of ST- and GA-FeSV (5) and encode tyrosine-specific protein kinase activities necessary for transformation (25, 28, 35). Determination of the nucleotide sequences of the FeSV (24) and Fujinami sarcoma virus (45) proviruses shows that v-*fes* and v-*fps* were derived from cognate proto-oncogene loci of mammals and birds. Thus, the same viral oncogene can be naturally transduced by helper viruses derived from different species, and it can be regulated by evolutionarily divergent transcriptional control elements. It seems likely that the capacity of transforming retroviruses to induce tumors in different species depends, at least in part, on species-specific recognition of control elements regulating oncogene expression.

ACKNOWLEDGMENTS

We thank Robert Bassin for his help in cloning NIH 3T3 transformants and titration of helper viruses, Marianne Oskarsson and George Vande Woude for their gift of pHT13(MSV) DNA, David Baltimore for cloned Moloney MuLV DNA, and Thomas Shaffer for his able assistance with tissue culture.

This work was supported by the Carcinogenesis Intramural Program, Division of Cancer Cause and Prevention, National Institutes of Health.

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