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McDonough Feline Sarcoma Virus: Characterization of the Molecularly Cloned Provirus and Its Feline Oncogene (v-*fms*)

LUDVIK DONNER,[1] LOUIS A. FEDELE,[1] CLAUDE F. GARON,[2] SONI J. ANDERSON,[1] AND CHARLES J. SHERR[1]*

Viral Pathology Section, National Cancer Institute,[1] and Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases,[2] Bethesda, Maryland 20205

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The genetic structure of the McDonough strain of feline sarcoma virus (SM-FeSV) was deduced by analysis of molecularly cloned, transforming proviral DNA. The 8.2-kilobase pair SM-FeSV provirus is longer than those of other feline sarcoma viruses and contains a transforming gene (v-*fms*) flanked by sequences derived from feline leukemia virus. The order of genes with respect to viral RNA is 5'-*gag-fms-env*-3', in which the entire feline leukemia virus *env* gene and an almost complete *gag* sequence are represented. Transfection of NIH/3T3 cells with cloned SM-FeSV proviral DNA induced foci of morphologically transformed cells which expressed SM-FeSV gene products and contained rescuable sarcoma viral genomes. Cells transformed by viral infection or after transfection with cloned proviral DNA expressed the polyprotein ($P170^{gag\text{-}fms}$) characteristic of the SM-FeSV strain. Two proteolytic cleavage products ($P120^{fms}$ and $pp55^{gag}$) were also found in immunoprecipitates from metabolically labeled, transformed cells. An additional polypeptide, detected at comparatively low levels in SM-FeSV transformants, was indistinguishable in size and antigenicity from the envelope precursor ($gPr85^{env}$) of feline leukemia virus. The complexity of the v-*fms* gene (3.1 ± 0.3 kilobase pairs) is approximately twofold greater than the viral oncogene sequences (v-*fes*) of Snyder-Theilen and Gardner-Arnstein FeSV. By heteroduplex, restriction enzyme, and nucleic acid hybridization analyses, v-*fms* and v-*fes* sequences showed no detectable homology to one another. Radiolabeled DNA fragments representing portions of the two viral oncogenes hybridized to different *Eco*RI and *Hin*dIII fragments of normal cat cellular DNA. Cellular sequences related to v-*fms* (designated c-*fms*) were much more complex than c-*fes* and were distributed segmentally over more than 40 kilobase pairs in cat DNA. Comparative structural studies of the molecularly cloned proviruses of Snyder-Theilen, Gardner-Arnstein, and SM-FeSV showed that a region of the feline leukemia virus genome derived from the *pol-env* junction is represented adjacent to v-*onc* sequences in each FeSV strain and may have provided sequences preferred for recombination with cellular genes.

Feline leukemia virus (FeLV) is horizontally transmitted among domestic cats and is a well-established cause of lymphomas and leukemias in infected animals (12, 20, 21, 23, 27, 28, 33, 41). Isolates from naturally occurring tumors generally contain mixtures of different FeLV strains (25, 26, 34), and in the outbred cat, three subgroups of exogenously infectious FeLV (subgroups A, B, and C) have been characterized according to their interference properties (44, 45). Sequences related to FeLV can be detected in the DNA of specific pathogen-free domestic cats (1, 6, 39) as well as in some related *Felis* species (6). The incomplete homology between exogenous and endogenous FeLV sequences could reflect the existence of a different endogenous subgroup ($FeLV_0$) or, alternatively, could be due to integration and subsequent vertical transmission of known subgroups of exogenously acquired FeLV genes.

FeLV can serve as a natural vector and transduce nonviral genetic elements from cat cellular DNA. This has resulted in the formation of recombinant viruses which exhibit altered oncogenicity. Three different isolates of feline sarcoma virus (FeSV), each obtained from naturally occurring fibrosarcomas of FeLV-infected cats, include the Snyder-Theilen (ST) (51), Gardner-Arnstein (GA) (16), and McDonough (SM) (35) strains. Each of these viruses is replication defective, can nonproductively transform mammalian cells in vitro, and induces fibrosarcomas in vivo. By preparing DNA transcripts complementary to ST- and SM-FeSV(FeLV) and selec-

tively removing transcripts which annealed to FeLV, Frankel and co-workers (15) first showed that each FeSV strain contains sarcoma virus-specific sequences (*onc* genes) which were presumed to confer the properties of morphological transformation. These studies established that the sarcoma virus-specific sequences of ST- and GA-FeSV (now designated v-*fes*) are homologous to one another and differ from those of SM-FeSV (here designated v-*fms*). Unlike FeLV sequences, the FeSV *onc* elements are detected in the cellular DNA of all *Felidae*, are more highly conserved among carnivores than endogenous FeLV sequences, and appear not to be reiterated in cat cellular DNA (15).

The structures of the ST- and GA-FeSV genomes have been deduced from chemical, physical, and biological studies of proviral DNA (13, 48, 49). Both viruses have the gene order 5′-*gag-fes-env*-3′, in which only portions of the FeLV *gag* and *env* genes are represented. The v-*fes* sequences of each genome contain similar, but nonidentical, elements (13) derived from a segmented cat cellular gene (c-*fes*) (14). The *gag* and v-*fes* sequences encode fusion polyproteins (4, 38, 43, 50, 53) which exhibit an associated tyrosine-specific protein kinase activity (2, 55) necessary for transformation (3, 9, 40). By contrast, the structure of the SM-FeSV genome has remained unclear, and v-*fms* sequences have not been characterized either with respect to their organization or function. To address these questions, we have molecularly cloned an integrated, biologically active SM-FeSV provirus and have characterized the cloned DNA by chemical and physical methods. The complexity and location of v-*fms* and FeLV-derived sequences in SM-FeSV have been determined, and the different gene products encoded by these sequences have been defined. Comparative studies of SM-FeSV and the cloned proviruses of GA- and ST-FeSV formally establish that v-*fms* and v-*fes* represent different viral oncogenes.

MATERIALS AND METHODS

Cells and virus. A mink cell clone nonproductively transformed by SM-FeSV (G-2/mink) was originally obtained from E. M. Scolnick (National Cancer Institute) and has been used in previous studies (43, 47). The viruses rescued from G-2/mink cells were used to infect normal rat kidney (NRK) cells, and a nonproductively transformed rat cell clone (G-2/NRK) was derived by microtiter cloning procedures (43). Both G-2/mink and G-2/NRK cells produce the polyprotein characteristic of other independently derived SM-FeSV-transformed cell lines (4, 54). Viruses rescued from G-2/mink cells by using an amphotropic murine leukemia virus (MuLV) were used to derive a series of other SM-FeSV transformants of mink CCL64 cells (American Type Culture Collection). Each of two additional transformed nonproducer cell clones (SM-10 and SM-15) derived in the present studies contains a single copy of the SM-FeSV provirus (see Results).

Infectious DNA assay. The biological activity of cloned recombinant phage DNA was assayed by transfection on NIH/3T3 cells by the calcium precipitation method of Graham and van der Eb (19) as described (48). Recipient cultures were trypsinized and replated 1 day after transfection, and foci were scored in confluent monolayer cultures after 14 to 19 days. A recombinant phage containing the transforming GA-FeSV provirus (13) was used as a positive control. Individual foci of transformed cells were subcloned by using microcylinders. This procedure led to the isolation of mixed populations of transformed and uninfected cells which were suitable for viral rescue and metabolic labeling analyses (see below).

Recombinant DNA procedures. DNA extracted from mink SM-10 cells was digested with *Eco*RI, extracted with phenol-chloroform-isoamyl alcohol (25:24:1), and concentrated under ethanol. The DNA fragments (2 mg) were electrophoretically separated in agarose, and 0.3 μg of proviral DNA-containing fragments (mean length, 10 kilobase pairs [kb]) was ligated in a 20-μl reaction (3 h at 15°C) to 1.5 μg of purified vector arms from λgtWES · λB (32). A sample containing 0.5 μg of DNA was packaged in vitro into phage particles (11) and yielded a total of ~5×10^5 PFU. Recombinant phages were screened by plaque hybridization (5), and two phage clones containing SM-FeSV proviral DNA sequences were isolated.

Hybridization reagents and conditions. Recombinant phages containing $FeLV_B$, ST-FeSV (49), and GA-FeSV (13) DNA were used to generate radiolabeled probes specific for v-*fes* and FeLV *gag* sequences. A 1.5-kb *Kpn*I-*Bam*HI fragment of $FeLV_B$ DNA containing U5, *gag* leader, and a portion of the *gag* gene (containing p15, p12, and about half of the FeLV p30 sequences) was used for plaque-blotting analyses. Probes representing the complete ST-FeSV genome or the $FeLV_B$ U5-*gag* leader fragment were used to score SM-FeSV sequences in transformed mink and rat cell clones. Two contiguous *Pst*I fragments (each ~0.5 kb) derived from v-fes^{ST} were separately subcloned into pBR322 and have been previously used to localize homologous sequences in GA-FeSV (13) and in normal cat DNA (14). These have formerly been designated S_L and S_R to indicate their left-to-right (5′-to-3′) orientation with respect to the sarcoma virus-specific sequences of ST-FeSV RNA.

Additional labeled DNA fragments used in these studies included all or part of the cloned SM-FeSV provirus; the positions of these fragments can be located by referring to the restriction map shown in Fig. 3. These included: (i) the entire 10-kb *Eco*RI fragment cloned from SM-10 cells and containing 8.2 kb of SM-FeSV DNA; (ii) a 0.3-kb *Kpn*I-*Sac*I fragment corresponding to U5 and *gag* leader sequences; (iii) a 2.8-kb *Xho*I-*Kpn*I fragment containing *env* and U3 sequences; and (iv) a 0.75-kb *Sac*I-*Xho*I and a 1.05-kb *Xho*I fragment together representing the 3′ 1.8 kb of v-*fms*. Subgenomic proviral DNA fragments and recombinant plasmids were labeled with ^{32}P by nick translation (42) (specific activity, 10^8 dpm/μg) and used for blotting analyses by the method of Southern (52). Restriction enzyme digests of cellular or cloned recombinant DNA were prepared using various lots of enzymes purchased from Bethesda Research Labora-

tories (Rockville, Md.), Boehringer-Mannheim (Indianapolis, Ind.), and New England BioLabs (Beverly, Mass.). The conditions of digestion were those recommended by the vendor. Hybridization under stringent conditions was performed at 42°C in 3× SSC (1× SSC is 0.15 M sodium chloride–0.015 M sodium citrate) containing 50% formamide (46, 48); nitrocellulose papers were washed as described (46) using 0.1× SSC at 50°C to establish the stringency criterion. Annealing under reduced stringency conditions was performed at 37°C in 5× SSC and 40% formamide; the heat step of the low-stringency wash procedure was carried out at 42°C. All hybridization reactions were performed for 18 h; 10% dextran sulfate was used in the annealing buffer to accelerate the rate of reaction.

Heteroduplex analyses. DNA was mounted for microscopy by using the formamide technique essentially as described by Davis and co-workers (8). Grids were examined in a Siemens Elmiskop 101 electron microscope at 40 kV accelerating voltage. Electron micrographs were taken on Kodak Electron Image plates at a magnification of ×6,000. The magnification was calibrated for each set of plates with a grating replica (E. F. Fuller, catalog no. 1000), and contour lengths were measured with a Numonics graphics calculator interphased to a Wang 2200 computer.

Antisera. Goat antisera to the FeLV envelope glycoprotein (gp70), the major structural protein (p30), and disrupted FeLV virions were provided by the Resources Office of the National Cancer Institute. Antisera raised in tumor-bearing rats (rat TB serum) inoculated with G-2/NRK cells have been previously described (43). Antibodies specific for the nonstructural component of the SM-FeSV polyprotein were prepared by absorption of rat TB serum with lyophilized proteins from FeLV (subgroups A, B, and C) (50). FeLV-absorbed rat TB serum did not specifically immunoprecipitate any products from lysates of radiolabeled mink cells infected with FeLV.

Labeling of cytoplasmic polypeptides and polyacrylamide gel electrophoresis. Cells grown to confluence in 75-cm^2 plastic flasks were labeled for 30 min with 2 mCi of [^{3}H]leucine (58 Ci/mmol; Amersham) in 10 ml of Earle balanced salt solution. The cells were lysed in 30 mM Tris-hydrochloride containing 0.5% Nonidet P-40, 0.5% sodium deoxycholate, 3.6 mM $CaCl_2$, 6 mM $MgCl_2$, 0.25 M KCl, and 0.5 mM sodium EDTA, adjusted to pH 7.5 (37). Cytoplasmic proteins were coprecipitated by using antisera and *Staphylococcus aureus* (Cowan I strain) (29). Precipitates were pelleted by centrifugation over 30% sucrose containing 0.2 M Tris-hydrochloride (pH 7.5), 0.05 M NaCl, 0.5% sodium deoxycholate, and 0.5% Nonidet P-40 and washed twice in the same buffer without sucrose. Washed immunoprecipitates were disrupted in sample buffer containing 15% glycerol and applied to continuous 6 to 12% gradient slab gels containing sodium dodecyl sulfate as described (30). After electrophoresis, the gels were permeated with dimethyl sulfoxide–PPO (2,5-diphenyloxazole) (7), dried, and exposed to preflashed Kodak X-Omat film (31) at −70°C.

RESULTS

Integration of SM-FeSV in mink cells. In preliminary experiments, DNA from the nonproductively transformed G-2/mink cell line containing rescuable SM-FeSV was restricted with *Eco*RI and subjected to Southern blotting analysis. Radiolabeled ST-FeSV DNA used as the probe contains portions of the FeLV-derived *gag* and *env* sequences represented in SM-FeSV (see below). Only one viral DNA-containing band (~18 kb) was detected in G-2/mink cellular DNA, consistent with the interpretation that (i) SM-FeSV lacks hexanucleotide recognition sites for *Eco*RI, and (ii) G-2/mink cells contain a single copy of the SM-FeSV provirus.

To study additional SM-FeSV integration events, G-2/mink cells were infected with amphotropic MuLV, and SM-FeSV(MuLV) pseudotype particles released by the cells were used to generate new transformed mink cell clones. Titration of the rescued sarcoma virus showed that infected G-2/mink cells released approximately 3.3×10^3 focus-forming units per ml 7 to 10 days after infection. In two separate experiments, mink cells were infected with these stocks at a multiplicity of 0.1 focus-forming unit per cell and were seeded as single cells in microtiter wells 5 h after infection. Of about 300 colonies examined, 54 transformed colonies were identified, grown, and tested for virus production. Two transformants (SM-10 and SM-15) failed to produce virus, as determined by assays of culture supernatants for focus-forming particles and reverse transcriptase activity.

DNA from 39 clones was extracted, restricted with *Eco*RI, and analyzed for sequences homologous to radiolabeled ST-FeSV DNA. Figure 1 shows a representative result obtained with eight of the DNA samples. A 13.5-kb band was detected in restricted DNA from uninfected mink cells and in all of the DNA samples extracted from transformed clones. This "background" band corresponds to mink cellular sequences (c-*fes*) related to the ST-FeSV transforming gene; as expected, the 13.5-kb band was seen by using radiolabeled recombinant plasmids containing only v-*fes* information. Additional bands containing proviral DNA sequences were detected in each of the transformed clones (see Fig. 1 for representative data). The lengths of these *Eco*RI fragments varied among the different clones, suggesting that the SM-FeSV provirus can integrate at many sites in mink cellular DNA, and that most, if not all, transformants represented clonal progeny of different, single-virus–single-cell interactions. No proviral DNA-containing bands were seen in nontransformed mink cell clones derived from the same experiments, showing that, under stringent hybridization conditions, only the FeLV-derived sequences within the SM-FeSV provirus were detected.

Of 39 clones examined for integrated proviral DNA sequences, 35 clones yielded single provi-

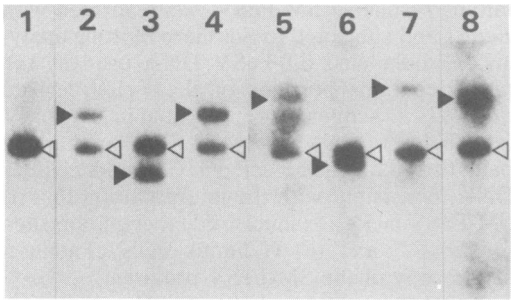

FIG. 1. Sites of integration of SM-FeSV in transformed mink cell clones. Cellular DNA from SM-FeSV–infected mink cells was digested with *Eco*RI and subjected to electrophoresis in 0.8% agarose. The fragments were transferred to nitrocellulose and hybridized to a radiolabeled ST-FeSV probe. A 13.5-kb band (open triangles) detected both in uninfected mink cellular DNA (lane 1) and in SM-FeSV–infected cells (other lanes) represents the host cellular fragment containing sequences homologous to v-*fes*ST (see the text). The positions of *Eco*RI fragments containing SM-FeSV sequences are indicated by the solid triangles in lanes 2 through 8. The DNAs of clones SM-10, SM-12, and SM-15 used in further studies are shown in lanes 3, 4, and 6, respectively.

ral DNA-containing fragments after restriction with *Eco*RI; three clones yielded two proviral fragments, and one clone yielded three. The proviral DNA-containing bands detected among the various clones were all longer than 9.3 kb, suggesting that the genetic complexity of SM-FeSV is probably greater than that of previously characterized FeSV strains (13, 48, 49). The failure to detect multiple proviral insertions in most of the transformed clones was consistent with the presence of only 2 nonproductively transformed clones out of 54 examined; presumably, the apparent excess of helper virus in the stocks used to generate the clones led to rapid viral interference and the subsequent restriction of further proviral integration events.

The DNAs of five transformed mink cell clones were also analyzed for proviral DNA sequences after digestion with *Kpn*I. This enzyme was chosen because it recognizes cleavage sites in the R region of all FeLV and FeSV proviral DNAs so far analyzed (13, 36, 49). An enzyme recognizing cleavage sites within the long terminal repeat (LTR) at each end of proviral DNA (24, 46) would generate internal proviral DNA-containing fragments whose combined lengths would equal that of viral RNA. Because the internal fragments would be derived solely from proviral sequences, any full-length provirus should yield fragments of the same size, regardless of the site of proviral insertion in cellular DNA.

Although each mink cell clone yielded a different proviral DNA-containing fragment after *Eco*RI cleavage (see above), *Kpn*I generated two proviral DNA-containing bands of 4.8 and 2.9 kb from the DNA of each clone. Only the 2.9-kb band was detected with a probe specific for FeLV *gag* sequences (data not shown). From these results we concluded that (i) *Kpn*I recognizes cleavage sites within the SM-FeSV LTR region as well as one additional site within proviral DNA; (ii) the combined length of the internal *Kpn*I fragments (7.7 kb) should approximate the complexity of the SM-FeSV RNA genome; and (iii) the internal *Kpn*I fragments appear in the order 5′–2.9 kb–4.8 kb–3′ with respect to the order of sequences in viral RNA.

Molecular cloning of transforming SM-FeSV sequences. Nonproductively transformed SM-10 and SM-15 cells yielded viral DNA-containing *Eco*RI fragments of 10 and 12.5 kb, respectively (Fig. 1, lanes 3 and 6). Both clones contained rescuable sarcoma viral genomes and expressed products encoded by SM-FeSV (see below). *Eco*RI-restricted DNA from SM-10 cells was fractionated by agarose gel electrophoresis, and a fraction enriched for SM-FeSV proviral DNA sequences was ligated to lambda vector arms and packaged in vitro into phage particles. Of 1.4×10^4 recombinant phages screened by plaque blotting, two contained sequences which hybridized to an FeLV *gag* probe but not to labeled plasmids containing v-*fes* sequences. Both recombinant phages were amplified, and the purified phage DNA was taken for further detailed analyses.

The recombinant phage DNAs were restricted with *Eco*RI, *Kpn*I, or sequentially with both enzymes. *Eco*RI yielded the expected 10-kb proviral DNA-containing fragment characteristic of SM-10 nonproducer cells. *Kpn*I cleaved the 10-kb fragment at three sites, yielding four smaller bands, two of which measured 4.8 and 2.9 kb in length (see Fig. 3 for restriction map assignments). As predicted, digestion of phage DNA with *Kpn*I alone yielded only the internal 4.8- and 2.9-kb bands characteristic of the SM-FeSV provirus, as well as *Kpn*I junctional fragments containing terminal cloned DNA sequences linked to lambda DNA. The sizes of different classes of junctional fragments obtained with several enzymes were identical for both clones, showing that the two independent phage recombinants contained the same DNA fragment oriented in the same direction with respect to the vector arms. Blotting experiments confirmed that the 4.8- and 2.9-kb *Kpn*I fragments contained sequences homologous to an FeLV probe, although only the 2.9-kb fragment hybridized to FeLV *gag* sequences. By these criteria, then, the cloned DNA fragments were each identical to the SM-FeSV proviral DNA-containing fragment of SM-10 cells.

The above results suggested that the cloned DNA should "back-hybridize" to proviral DNA sequences in other SM-FeSV transformants, regardless of the genetic background of host cellular DNA. The 10-kb *Eco*RI fragment was therefore purified, labeled in vitro, and hybridized to the DNA of mink and rat cell clones nonproductively transformed with SM-FeSV. Figure 2 shows the results obtained after *Kpn*I digestion of cellular DNA from uninfected mink cells (lane 1) and three different mink cell transformants (lanes 2 through 4). The 4.8- and 2.9-kb *Kpn*I bands characteristic of the SM-FeSV provirus were detected only in the DNA of transformed cells. Similar results were obtained with the DNA of G-2/NRK cells (lane 5) as opposed to uninfected rat cells (lane 6). These results provided additional chemical evidence that the cloned DNA fragment contained SM-FeSV sequences.

Biological activity of cloned proviral DNA. The biological activity of the recombinant DNA was tested by transfection on NIH/3T3 cells. A phage containing the GA-FeSV provirus served as the positive control (13). DNA of both phages was digested with *Eco*RI, and the fragments containing proviral DNA sequences were each purified by electroelution and mixed with carrier NIH/3T3 DNA prior to transfection. Table 1 shows that the DNA of both sarcoma viral phage recombinants induced foci of transformed cells at about the same efficiency. At the DNA concentrations tested, the efficiency of focus formation was independent of the DNA dose, suggesting that all information required for transformation resided on a single class of DNA molecules. Neither wild-type lambda phage, transfected under identical conditions, nor carrier DNA alone yielded any foci.

TABLE 1. Biological activity of cloned SM-FeSV proviral DNA[a]

DNA dose[b] (μg per plate)	No. of foci	Efficiency of transformation (log FFU/pmol)
SM-FeSV		
0.01	4	3.3
0.05	16	3.2
0.20	53	3.1
GA-FeSV		
0.01	3	3.1
0.10	26	3.0

[a] NIH/3T3 cells were transfected with purified *Eco*RI fragments containing proviral DNA from SM-FeSV and GA-FeSV, respectively. The 10-kb *Eco*RI fragment cloned from mink SM-10 cells contains an 8.2-kb provirus with an estimated molecular weight of 5.2×10^6. The 14.4-kb *Eco*RI fragment containing the 4.2×10^6-dalton GA-FeSV provirus (6.7 kb long) was used as a positive control (13). Molecular weights were calculated assuming a value of 622 per base pair. FFU, Focus-forming unit.

[b] The DNA doses shown indicate the calculated quantities of proviral DNA in the 10- and 14.4-kb *Eco*RI fragments, containing SM-FeSV and GA-FeSV, respectively.

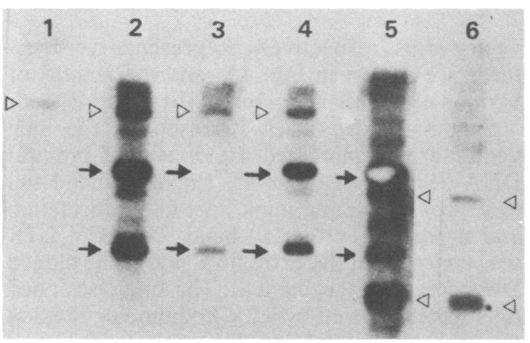

FIG. 2. DNA sequences in SM-FeSV-transformed cells homologous to cloned SM-10 DNA. The DNA of uninfected mink (lane 1), SM-12 (lane 2), SM-15 (lane 3), G-2/mink (lane 4), G-2/NRK (lane 5), and uninfected NRK (lane 6) cells was restricted with *Kpn*I and subjected to electrophoresis in 0.8% agarose. After transfer to nitrocellulose, the fragments were hybridized to the radiolabeled 10-kb *Eco*RI fragment cloned from SM-10 cells. The positions of the internal 4.8 and 2.9-kb bands derived from the SM-FeSV provirus are indicated by arrows. Major background bands detected in uninfected cells are indicated by open triangles.

Foci of transformed cells were recloned and tested for SM-FeSV gene products. A mixed cell population containing approximately 10% transformed cells was metabolically labeled with [^{35}S]methionine, and the radiolabeled lysate was reacted with rat TB serum containing antibodies to the SM-FeSV polyprotein. Polyacrylamide gel analysis of the immunoprecipitates showed the presence of the characteristic SM-FeSV P170 product. Infection of these cultures with amphotropic MuLV led to the rescue of focus-forming genomes which induced secondary foci on mink cells in subsequent rounds of infection. Thus, the phage recombinants were biologically active and contained competent v-*fms* genetic information.

Chemical and physical mapping of SM-FeSV DNA. A restriction map of the 10-kb *Eco*RI fragment cloned from SM-10 cells is shown in Fig. 3. The proviral DNA sequences are shown in 5′-to-3′ orientation with respect to viral RNA; within the recombinant phages, however, the viral sequences appear in the opposite orientation with respect to the long and short vector arms. *Eco*RI and *Sal*I did not cleave SM-FeSV DNA. *Hin*dIII, *Bam*HI, and *Bgl*II cleaved SM-FeSV DNA at single sites, whereas *Xho*I, *Sac*I,

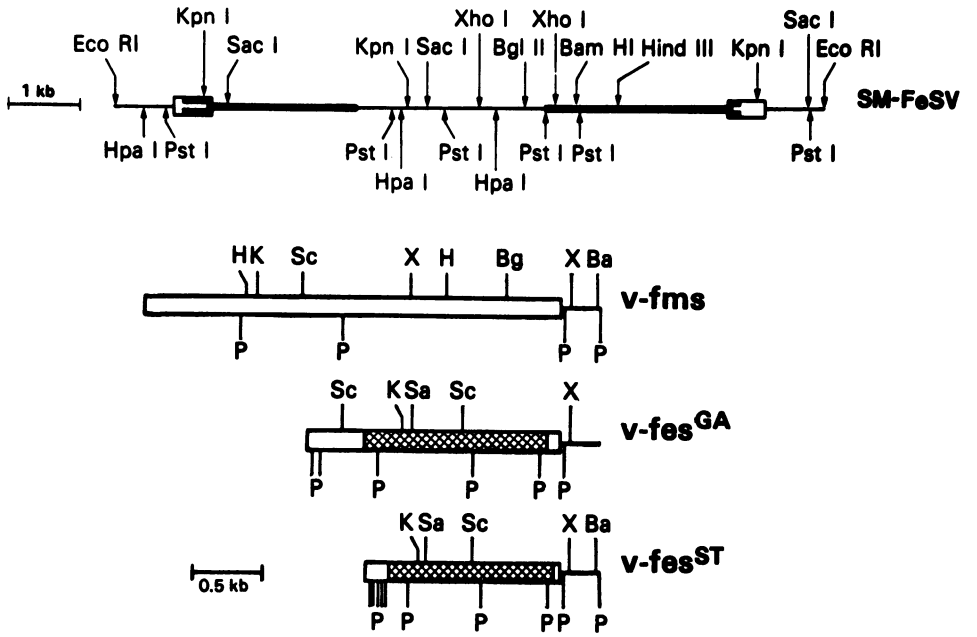

FIG. 3. Physical maps of the SM-FeSV provirus, its transforming gene (v-*fms*), and the *onc* elements (v-*fes*) of ST- and GA-FeSV. Sites of restriction within the cloned 10-kb *Eco*RI fragment containing the 8.2-kb SM-FeSV provirus are indicated in the top line drawing. The upper bar standard (1.0 kb) refers only to the top line. The orientation of the provirus is 5′ to 3′ with respect to viral RNA. The rectangles indicate the positions of LTR sequences, and heavy lines indicate the sequences homologous to FeLV. The v-*fms* region at the center of the cloned DNA fragment is indicated on an expanded scale by the rectangle in the drawing on line 2. The lower bar standard (0.5 kb) refers to the line drawings on lines 2 through 4. The maps of v-fes^{ST} and v-fes^{GA} are indicated by the rectangles on lines 3 and 4, respectively; the shaded areas refer to regions of exact homology between v-*fes* regions of the two independent FeSV isolates (13). The 3′ portion of each *onc* gene terminates at a similar FeLV-derived sequence (horizontal line) originating from near the 5′ end of the FeLV *env* gene (13, 49). Sites of restriction for various enzymes are indicated on lines 2 through 4 as follows: Ba, *Bam*HI; Bg, *Bgl*II; H, *Hpa*I; K, *Kpn*I; P, *Pst*I; Sc, *Sac*I; Sa, *Sal*I; X, *Xho*I.

*Hpa*I, *Kpn*I, and *Pst*I recognized multiple sites in proviral DNA. The position of terminally redundant sequences was verified by using a labeled SM-FeSV U5-*gag* leader probe (the ~300-base pair *Kpn*I-*Sac*I fragment) which hybridized to homologous sequences at both proviral termini. Assuming that the LTR sequence is approximately 0.5 kb long (36, 48), the length of the SM-FeSV provirus is estimated to be 8.2 kb.

The complexity of sarcoma virus-specific sequences in SM-FeSV was determined from measurements of heteroduplexes formed between DNA from a recombinant phage containing $FeLV_B$ and the 10-kb *Eco*RI fragment containing SM-FeSV. Unlike SM-FeSV, the provirus of $FeLV_B$ (and also that of ST-FeSV) contains *Eco*RI sites within the U3 portion of the LTR. Thus the FeLV DNA clone is only 7.8 kb in length and lacks sequences distal to the *Eco*RI sites at each end of the cloned DNA fragment. Annealing reactions were performed using phage DNA containing viral sequences in 5′-to-3′ orientation with respect to the long and short vector arms, respectively. Representative structures are shown in Fig. 4A, and the lengths of various segments are given in the figure legend.

Single-stranded DNA representing the long vector arm demarcated the 5′ end of proviral DNA sequences. A short, single-stranded tail (0.95 kb) containing mink host cell information and a portion of the left-hand SM-FeSV LTR was observed at the extreme 5′ end of the cloned DNA fragment (segment a). The single-stranded tails were followed by a 2.4-kb homology region (segment b) containing U5, *gag* leader, and most, but not all, of the FeLV *gag* gene. Segment b terminated in a symmetrical region of nonhomology. The unpaired region of the FeLV genome was estimated to correspond to residual *gag* sequences and the complete *pol* gene (single-strand segment c). By contrast, the unpaired region of the SM-FeSV clone (3.1 ± 0.3 kb, labeled d in Fig. 4A) defined the maximal complexity of the v-*fms* gene. A second 2.45-kb homology region (segment e) which corresponded to FeLV *env* sequences and a portion

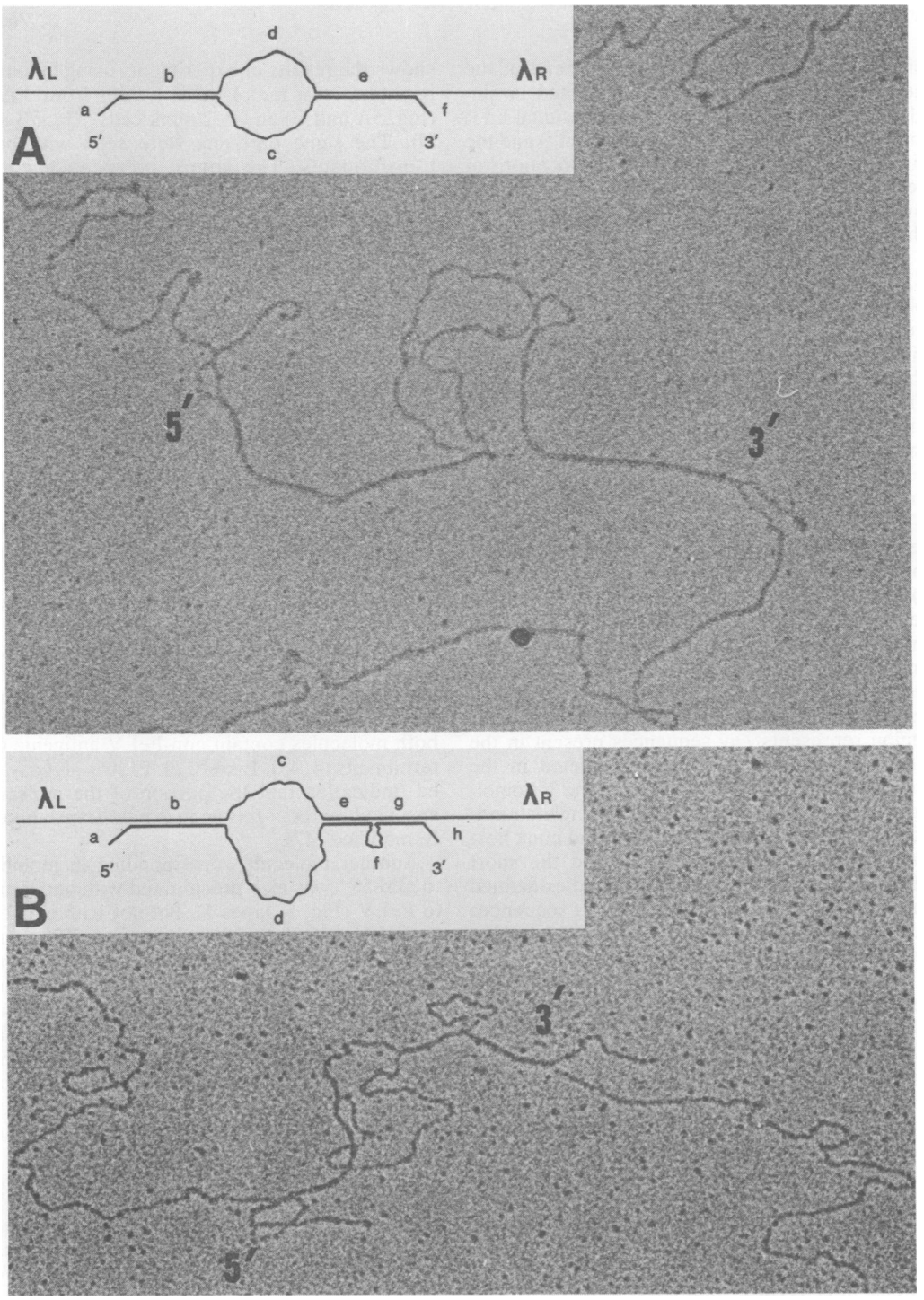

FIG. 4. Heteroduplexes between SM-FeSV DNA and $FeLV_B$ DNA (top) or ST-FeSV DNA (bottom). The 10-kb *Eco*RI fragment containing the SM-FeSV provirus was annealed to λ phages containing the other viral DNAs; both $FeLV_B$ and ST-FeSV DNA are oriented 5′ to 3′ both with respect to viral RNA and in relation to the left and right vector arms, respectively. Measurements in kb for various segments were computed for 15 molecules (standard deviation less than ±10% for all segments). The various segments are indicated in the schematic illustrations at the upper left of each panel. (A) SM-FeSV versus $FeLV_B$: segment a, 0.95 kb; segment b, 2.4 kb; segment c, 3.2 kb; segment d, 3.1 kb; segment e, 2.45 kb; segment f, 1.1 kb. (B) SM-FeSV versus ST-FeLV: segment a, 1.0 kb; segment b, 1.75 kb; segment c, 1.3 kb; segment d, 3.2 kb; segment e, 0.7 kb; segment f, 1.6 kb; segment g, 0.7 kb; segment h, 1.0 kb.

of the U3 region was seen at the 3′ end of the heteroduplex. This segment terminated in single-stranded tails corresponding to residual LTR and mink host cell sequences (segment f) and the right vector arm (segment λR). The locations of sequences homologous to FeLV are indicated by the heavy lines in the physical map shown in the top line of Fig. 3.

Heteroduplexes were also prepared using the SM-FeSV-containing *Eco*RI fragment and a phage recombinant containing ST-FeSV sequences. Figure 4B shows that, following the single-stranded tails demarcating the 5′ ends of the proviral DNA sequences (segments λL and a), a 1.75-kb homology region (segment b) was observed. This segment was ~0.65 kb shorter than the 5′ homology region seen in duplexes between SM-FeSV and $FeLV_B$ and reflects the presence of fewer *gag*-derived sequences in the ST-FeSV genome (4, 38, 43). The 5′ homology segment ended at an asymmetrical loop containing 1.3 kb of ST-FeSV v-*fes* sequences (segment c) and 3.2 ± 0.32 kb of SM-FeSV DNA representing unpaired *gag* plus v-*fms* sequences (segment d). Two base-paired segments (segments e and g) located 3′ to the nonhomology region were interrupted by a deletion loop measuring ~1.6 kb (segment f). The latter single-stranded region represents *env* sequences present in the SM-FeSV genome which were deleted in the formation of ST-FeSV (49). Again, the 3′ homology segment was contiguous with single-stranded tails representing residual LTR and mink host flanking sequences (segment h) and the short vector arm (segment λR). These studies defined the location and complexity of v-*fms* sequences and failed to demonstrate any homology between v-*fms* and v-*fes* elements.

Proteins encoded by SM-FeSV in transformed cells. Several laboratories have reported that SM-FeSV encodes a polyprotein of 170 to 180 kilodaltons ($P170^{gag\text{-}fms}$) that contains part of the FeLV *gag* precursor (Pr65) fused to a novel antigenic portion (designated x) (4, 43, 54). Chemical studies of P170 have shown that the polyprotein contains several tryptic peptides apparently specified by the v-*fms* gene (4, 54). In addition, a protein of 120,000 molecular weight (P120) has been identified by immunoprecipitation with certain sera; P120 lacks *gag* but contains x antigenic determinants (4, 43) and includes the tryptic peptides thought to be encoded by v-*fms* sequences (4). Another protein, immunologically and structurally similar to FeLV $gPr85^{env}$, has also been detected in mink cell transformants but was presumed to be encoded by a defective helper viral genome (4).

We performed similar studies using nonproductively transformed cells, each containing a single copy of the SM-FeSV provirus. Figure 5 shows the results of experiments using immunoprecipitates of radiolabeled lysates from SM-10 (Fig. 5A and C) and G-2/mink cells (Fig. 5B and D). The same products were seen with both transformants. The largest polypeptide corresponded to the full-length gag-x polyprotein (P170), which was precipitated with goat antiserum to FeLV (lanes 1), goat antiserum to FeLV p30 (lanes 3), and rat antiserum to SM-FeSV–transformed cells (rat TB serum) (lanes 4). Particularly in SM-10 cells, P170 appeared as a doublet; labeling with [^{3}H]mannose has shown that this protein can be glycosylated, suggesting that several forms of the molecule may be synthesized (47). A second product corresponding to P120 was only precipitated with TB rat serum (lanes 4). Since no subgenomic mRNA species containing v-*fms* sequences has been detected in transformed cells (our unpublished data), P120 appears to represent a proteolytic cleavage product of P170. A reciprocal *gag* cleavage product ($pp55^{gag}$) was also precipitated with antisera to FeLV and to FeLV p30 (lanes 1 and 3), but not with TB rat serum absorbed with FeLV proteins (Fig. 5C and D, lanes 5). Both P170 and P120 were immunoprecipitated with TB rat serum after absorption with FeLV proteins, in agreement with earlier findings that both molecules contain non-FeLV antigenic determinants (4, 43). Like P170, P120 is glycosylated, indicating that the portion of the molecule specified by the v-*fms* gene is post-translationally modified (47).

Another molecule corresponding in mobility to $gPr85^{env}$ was also precipitated with antiserum to FeLV (Fig. 5, lanes 1), but not with antisera to FeLV p30 (lanes 3) or with rat TB serum (lanes 4). This protein reacted with antiserum directed against FeLV gp70 (lanes 2), suggesting that SM-FeSV encodes an envelope precursor protein in addition to $P170^{gag\text{-}fms}$. Thus, the entire coding capacity of the 7.7-kb SM-FeSV RNA genome can be translated into identifiable protein products.

Sequences related to v-*fms* in cat cellular DNA. Subgenomic fragments representing portions of v-*fms* were used to screen for homologous sequences in cat cellular DNA. As the probe in these studies, we used the 0.75-kb *Sac*I-*Xho*I fragment and the 1.05-kb *Xho*I fragment, together representing the 3′ 1.8 kb of v-*fms*. We estimate from the physical map that the latter fragment includes no more than 0.1 kb of FeLV *env* sequences; the combined length of the two fragments represents about 60% of the v-*fms* gene.

Cat cellular DNA was restricted with either *Eco*RI or *Hin*dIII electrophoresis, subjected to, and transferred to nitrocellulose. Each of these enzymes does not recognize cleavage sites with-

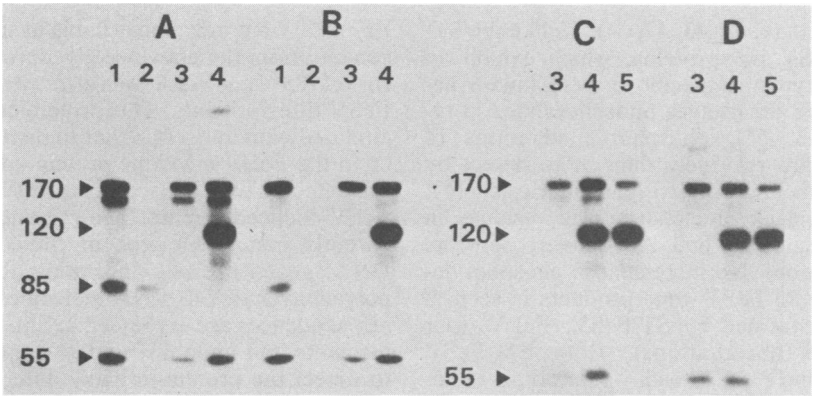

FIG. 5. Viral polypeptides in SM-FeSV–transformed mink cells. Results with SM-10 cells are shown in (A) and (C); results with G-2/mink cells are in (B) and (D). The different antisera included: anti-FeLV (lanes 1); anti-FeLV gp70 (lanes 2); anti-FeLV p30 (lanes 3); rat TB serum raised against SM-FeSV–transformed NRK cells (lanes 4); and rat TB serum absorbed with disrupted FeLV (lanes 5). The molecular weights of the proteins ($\times 10^3$) calibrated from the positions of known protein standards (43) are indicated at the left of the lanes.

in the v-*fms* gene (Fig. 3, line 2). Figure 6 shows that several *Hin*dIII and *Eco*RI bands were detected which hybridized strongly to the v-*fms* probe (lanes 1 and 3). Several other weakly hybridizing bands were also observed. The above results differed substantially from those obtained with a v-*fes* probe which detected unique *Hin*dIII and *Eco*RI fragments in cat cellular DNA (lanes 2 and 4; cf. reference 14). We estimate that the combined lengths of the *Eco*RI or *Hin*dIII fragments which hybridized to the v-*fms* probe were greater than 40 kb. Thus, c-*fms* sequences are relatively more complex than c-*fes* and are distributed separately in cat cellular DNA.

Lack of detectable homology between v-*fms* and v-*fes*. Although we were unable to demonstrate homology between v-*fms* and v-*fes* by heteroduplex analysis, this technique may have failed to detect short or highly mismatched regions of base pairing between the different DNA molecules. Purified fragments corresponding to portions of the SM-FeSV provirus were electrophoretically separated, transferred to nitrocellulose, and hybridized, using low-stringency conditions, to labeled plasmids (S_L and S_R [13, 14]) containing cloned *Pst*I fragments representing ~1.0 kb of v-*fes*. Reciprocal hybridization experiments were also performed using GA-FeSV proviral DNA and the radiolabeled v-*fms* probe representing 60% of the SM-FeSV sarcoma virus-specific sequences. In no case was specific hybridization detected, showing that v-*fes* and v-*fms* represent two different viral oncogenes transduced by FeLV from cat cellular DNA.

DISCUSSION

Several lines of evidence have previously suggested that two different viral transforming genes are represented among the three known strains of FeSV. Molecular hybridization experiments first showed that the sarcoma virus-specific sequences (v-*fes*) of ST- and GA-FeSV were homologous to each other but were unrelated to *onc* sequences (v-*fms*) of SM-FeSV (15). The polyprotein encoded by SM-FeSV ($P170^{gag\text{-}fms}$) was subsequently shown to differ antigenically and chemically from the ST- and GA-FeSV

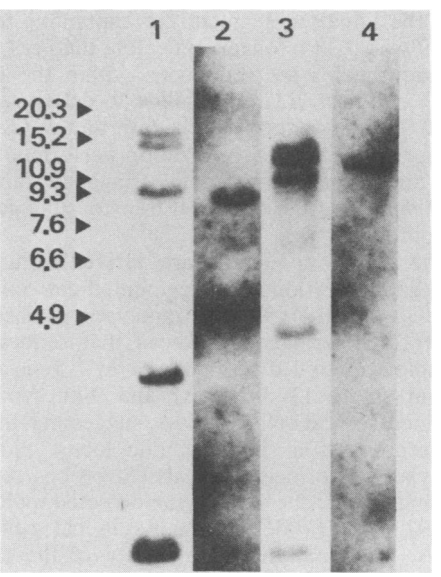

FIG. 6. Cat cellular sequences homologous to v-fes^{ST} and v-*fms*. Uninfected cat cellular DNA was digested with *Hin*dIII (lanes 1 and 2) or *Eco*RI (lanes 3 and 4), separated electrophoretically, transferred to nitrocellulose, and hybridized with v-*fms* (lanes 1 and 3) or v-*fes* (lanes 2 and 4) probes. The lengths in kb of restriction fragments from adenovirus type 2, used to calibrate the gel, are indicated at the left.

gene products (4, 38, 43, 47, 54). Unlike the ST- and GA-FeSV polyproteins, which exhibit an associated tyrosine-specific protein kinase activity and are themselves phosphorylated in tyrosine (2, 3, 55), phosphorylated forms of P170$^{gag\text{-}fms}$ are relatively difficult to detect by metabolic labeling with [^{32}P]phosphoric acid (43, 47, 54) and lack phosphotyrosine residues in vivo (M. Barbacid and A. Lauver, personal communication). No increment in phosphorylation of the SM-FeSV gene products is seen in cells cotransformed by ST-FeSV (FeLV) (our unpublished observations). Both SM-FeSV P170$^{gag\text{-}fms}$ and P120^{fms} readily incorporate radiolabeled sugars (47) and are highly membrane-associated glycoproteins.

Characterization of molecularly cloned SM-FeSV proviral DNA showed that v-*fms* sequences are 3.1 ± 0.3 kb in length and are centrally positioned within the 8.2-kb provirus. Like ST- and GA-FeSV, the order of genes in SM-FeSV is 5′-*gag-onc-env*-3′. However, the SM-FeSV genome is substantially more complex since it contains an almost complete *gag* sequence, a twofold larger *onc* element, and an intact *env* gene. Heteroduplexing of cloned SM-FeSV and ST-FeSV DNA generated structures whose unpaired strands corresponded in length and position to v-*fes*ST and v-*fms* sequences. Similar results were obtained by using GA-FeSV instead of ST-FeSV (data not shown), except that the unpaired segment corresponding to v-*fes*^{GA} was 0.3 kb longer, reflecting the presence of additional v-*fes* sequences within the GA-FeSV provirus (13). The failure to detect reciprocal homology between v-*fms* and v-*fes* by using nucleic acid hybridization performed under conditions of reduced stringency further confirmed that the two viral transforming genes are different.

The v-*fes*ST sequences are derived from discontiguous portions of a segmented cat cellular gene (c-*fes*) (14). Blotting analyses performed under high stringency showed that sequences homologous to 1.0 kb of the v-*fes*ST gene map within single 13-kb *Eco*RI and 9-kb *Hin*dIII fragments of cat cellular DNA, suggesting that c-*fes* represents a single genetic locus. Similar analyses performed with labeled probes representing 60% of the v-*fms* gene detected multiple *Eco*RI and *Hin*dIII fragments in cat cellular DNA. The combined complexity of these sequences was greater than 40 kb. Although c-*fms* could represent a single but highly complex locus, these data do not exclude the possibility that several different c-*fms* genes are present in the cat genome, analogous to the family of different genes that encode the transforming proteins of rat sarcoma viruses (10).

A polypeptide antigenically unrelated to P170$^{gag\text{-}fms}$ but indistinguishable in size and antigenicity from the envelope glycoprotein precursor (gPr85^{env}) of FeLV was also detected in SM-FeSV transformants. This protein could be precipitated with antisera either to disrupted FeLV or to the FeLV envelope protein gp70, but was not detected with antisera from rats bearing SM-FeSV–induced tumors. The presence of an apparently intact *env* gene in cloned SM-FeSV DNA, recent results indicating that spliced, polyadenylated 24S RNA molecules containing *env* sequences are expressed in SM-FeSV transformants (our unpublished data), and our ability to detect the protein in many different nonproductively transformed clones all suggest that these molecules are encoded by SM-FeSV. This result is surprising since, with the single exception of the Rous sarcoma virus, none of the other acutely transforming avian and mammalian viruses isolated to date encodes complete envelope glycoproteins. The presence of such molecules in SM-FeSV transformants might confer resistance to infection by certain FeLV subgroups and could also lead to the formation of phenotypically mixed particles during rescue. Indeed, if these products were to elicit an immune response in vivo, SM-FeSV variants defective in *env* gene functions might be isolated from virus-induced tumors.

The three FeSV strains contain two different *onc* genes which have recombined at their 5′ ends with different portions of the FeLV *gag* gene. This is in agreement with studies showing that SM-FeSV specifies p30 antigens (4, 38, 43, 54) whereas ST- and GA-FeSV lack some of the p30 coding region (13, 48, 49). The major differences between the v-*fes* sequences of ST- and GA-FeSV, detected by heteroduplexing and restriction enzyme analyses, are also located at the 5′ ends of the *onc* elements (see Fig. 3 and reference 13). By contrast, the 3′ ends of both v-*fes* and v-*fms* are contiguous with similar FeLV-derived sequences originating from a region near the 5′ end of the FeLV *env* gene (Fig. 3 and reference 49). For ST-FeSV, the *Pst*I and *Xho*I sites mapped to this region fall within 0.1 kb of the 3′ end of v-*fes* (unpublished sequencing data in collaboration with A. Hampe, I. Laprevotte, and F. Galibert). Similar sites of cleavage are found adjacent to the 3′ ends of v-*fes*^{GA} and v-*fms*. These FeLV-derived regions fall outside the polyprotein coding sequences (Hampe et al., unpublished data) and do not appear necessary for transformation (J. Even and C. J. Sherr, unpublished data).

The observations that helper proviruses can integrate upstream and promote transcription of an avian *onc* gene (22) suggest one mechanism for the formation of recombinant FeSV's. Insertion of FeLV proviral DNA upstream from a cat

onc gene and subsequent deletion of the right-hand portion of the provirus could result in the synthesis of spliced mRNA molecules containing fused *gag* and cellular sequences. Rescue of the hybrid RNA species by helper virus and reverse transcription in a subsequent round of infection (18) could lead to the synthesis of a DNA negative strand, initiated on an FeLV RNA template and completed by copying the transduced hybrid RNA by a copy choice mechanism. If a "jump" between templates were to occur after reverse transcription of the FeLV *env* gene, negative-strand DNA could have the structure:

$$5'\text{-}(tRNA_{pro})\text{-leader-}gag\text{-}onc\text{-}env\text{-U3}$$

where only a portion of the *gag* gene but all of the *env* gene were represented. Such a minus strand would be suitable as a template for plus-strand synthesis and, by conventional mechanisms (17), would permit the formation of a linear DNA intermediate containing two LTRs. Subsequent deletion of *env* sequences in the formation of ST- and GA-FeSV (but not SM-FeSV) would not affect the ability of the recombinant viruses to transform cells or induce fibrosarcomas. Recombination with the different *onc* elements could therefore involve preferred sites of limited homology between FeLV sequences near the *pol-env* junction and cellular *onc* genes. The location of the putative crossover point with respect to viral RNA raises the possibility that sequences at the gene junctions (for example, splice recognition points) might provide the necessary homology between cellular and viral information. Sequencing of the relevent portions of v-*fes* and v-*fms* and their cellular homologs should help in resolving certain of these questions.

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