

A New Acute Transforming Feline Retrovirus with *fms* Homology Specifies a C-Terminally Truncated Version of the *c-fms* Protein That Is Different from SM-Feline Sarcoma Virus *v-fms* Protein

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The HZ5-feline sarcoma virus (FeSV) is a new acute transforming feline retrovirus which was isolated from a multicentric fibrosarcoma of a domestic cat. The HZ5-FeSV transforms fibroblasts *in vitro* and its replication is defective. A biologically active integrated HZ5-FeSV provirus was molecularly cloned from cellular DNA of HZ5-FeSV-infected FRE-3A rat cells. The HZ5-FeSV has oncogene homology with the *fms* sequences of the SM-FeSV. The genome organization of the 8.6-kilobase HZ5-FeSV provirus is 5' Δ *gag-fms- Δ pol- Δ env* 3'. The HZ5- and SM-FeSVs display indistinguishable *in vitro* transformation characteristics, and the structures of the *gag-fms* transforming genes in the two viruses are very similar. In the HZ5-FeSV and the SM-FeSV, identical *c-fms* and feline leukemia virus p10 sequences form the 5' *gag-fms* junction. With regard to *v-fms* the two viruses are homologous up to 11 amino acids before the C terminus of the SM-FeSV *v-fms* protein. In HZ5-FeSV a segment of 362 nucleotides then follows before the 3' recombination site with feline leukemia virus *pol*. The new 3' *v-fms* sequence encodes 27 amino acids before reaching a TGA termination signal. The relationship of this sequence with the recently characterized human *c-fms* sequence has been examined. The 3' HZ5-FeSV *v-fms* sequence is homologous with 3' *c-fms* sequences. A frameshift mutation (11-base-pair deletion) was found in the C-terminal *fms* coding sequence of the HZ5-FeSV. As a result, the HZ5-FeSV *v-fms* protein is predicted to be a C-terminally truncated version of *c-fms*. This frameshift mutation may determine the oncogenic properties of *v-fms* in the HZ5-FeSV.

Feline leukemia virus (FeLV) is horizontally transmitted in the domestic cat population and is known to be the etiological agent of feline lymphosarcoma, as well as of a large variety of other feline diseases. Several replication-defective *v-myc*-containing feline retroviruses have been characterized in feline lymphosarcoma. From FeLV-associated feline fibrosarcomas, in contrast, acute transforming retroviruses (ATVs) containing a variety of oncogenes have been isolated and characterized (5, 76). Six different *v-onc* genes, *v-fes(fps)*, *v-fms*, *v-sis*, *v-abl*, *v-fgr*, and *v-kit* have been found in the nine known virus strains, ST-, GA-, SM-, GR-, PI-, HZ1-, HZ2-, TP1- and HZ4-feline sarcoma virus (FeSV) (7, 7a, 8, 18, 19, 25, 30, 41, 49, 54, 66, 67, 79). Our past characterization of the PI-, HZ2-, and HZ4-FeSVs indicated that there is no apparent specificity for the transduction of *c-onc* genes by FeLV in viruses recovered from fibrosarcomas. We therefore have continued to isolate and characterize new oncogene-containing feline retroviruses in order to explore further the repertoire of oncogenes that may be found in such viruses. We describe here the isolation and characterization of the HZ5-FeSV, a new feline ATV with *v-fms* homology.

The SM-FeSV originated by the transduction of feline *c-fms* sequences by FeLV (16, 19, 41). The genetic structure of the SM-FeSV is 5' Δ *gag-fms-env* 3'. The *v-fms* amino acid sequence displays features characteristic of a transmembrane receptor, and the cytoplasmic domain of *v-fms* shows homology with tyrosine-specific protein kinases (24). In the SM-FeSV, *v-fms* is expressed as a *gag*-fusion protein which is processed to become a transmembrane

glycoprotein (gp140 *fms*) with tyrosine-specific protein kinase activity (2, 3, 38, 57). The normal cellular homolog *c-fms* is differently expressed in cells of the macrophage cell lineage (60, 65). *c-fms* expression, however, has also been detected in choriocarcinoma cell lines and in placental tissue (46, 47). Sherr and co-workers have shown *c-fms* to be homologous to the macrophage colony-stimulating factor (M-CSF) receptor (65). M-CSF is a lineage-specific growth and differentiation factor which is essential for *in vitro* cell survival, and its receptor (M-CSFR) is a transmembrane 165-kilodalton glycoprotein (42, 45, 56, 75). Like other growth factor receptors, M-CSFR displays tyrosine-specific protein kinase activity upon ligand binding (45, 56). The HZ5-FeSV and SM-FeSV we show here are very similar in structure, although the C termini of HZ5-FeSV and SM-FeSV *v-fms* differ.

MATERIALS AND METHODS

Cells and viruses. FRE-3a rat (8), CCL64 mink (26), NIH 3T3 mouse (31), and FG10 S⁺L⁻ cells (4) were grown in Dulbecco modified Eagle medium supplemented with 10% calf serum, and Fea feline embryo fibroblasts were grown in Dulbecco medium supplemented with 10% fetal calf serum.

Tumor cell extract was prepared according to the method of Moloney (44). Transformation assays with various indicator cells were performed according to standard procedures. Transformed cells were cloned in 0.3% agar by the endpoint dilution method. Amphotropic murine leukemia virus (amph-MuLV) titers were determined by using FG10 S⁺L⁻ indicator cells as described previously (6, 55).

Bacterial cells, viruses, and plasmids. *Escherichia coli* HB101 was used for the production of plasmid DNA. Trans-

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TABLE 1. Molecular clones of *v-onc* genes

<i>v-onc</i> designation	Viral origin	Molecular clone	Restriction fragments bearing <i>v-onc</i> (size, kb)	Reference
Avian				
<i>src</i>	SR-RSV	psrc	<i>EcoRI</i> (2.95)	15
<i>yes</i>	Y73SV	Y73-11A	<i>SacI</i> (4.0)	32
<i>ros</i>	UR2	pros	<i>PvuII-EcoRI</i> (0.8)	50
<i>rel</i>	REV-T	prel	<i>EcoRI</i> (0.8)	12
<i>erbAB</i>	AEV-ES4	pAE-PvuII	<i>PvuII</i> (2.5)	73
<i>myc</i>	MC29	pVMC3.PstI	<i>PstI</i> (1.5)	74
<i>myb</i>	AMV	pVM2	<i>SacI-EcoRI</i> (0.8)	33
<i>ski</i>	SKV	pski	<i>XhoI</i> (2.8)	E. Stavnezer ^a
Mammalian				
<i>Ha-ras</i>	Ha-MSV	pHEE6	<i>BAMHI-EcoRI</i> (2.2)	17
<i>Ki-ras</i>	Ki-MSV	pHiHi3	<i>EcoRI</i> (1)	17
<i>N-ras</i>	SK-N-SH ^b	pNP-1(R)	<i>HindIII</i> (1)	70
<i>mos</i>	Mo-MSV	pmos	<i>PstI</i> (0.45)	10
<i>abl</i>	Abelson-MuLV	pABsub3	<i>SmaI-HindIII</i> (1.9)	20
		p130abl	<i>HincII</i> (2.5)	
<i>fos</i>	FBJ-MSV	pfos	<i>PstI</i> (1.25)	14
<i>raf</i>	3611-Msv	p3611-MSV	<i>SacII-XhoI</i> (0.7)	53
<i>fes</i>	ST-FeSV	pBfes	<i>PstI</i> (0.5, 0.55)	64
<i>fms</i>	SM-FeSV	pSM-FeSV	<i>KpnI-BglII</i> (1.6)	16
<i>sis</i>	SSV	psis	<i>PstI-XbaI</i> (0.9)	58

^a Personal communication.

^b Human tumor cell line.

formation of *E. coli* HB101 was done according to the CaCl₂ method of Mandel and Higa (37) with appropriate antibiotic selection. Large-scale preparations of DNA were prepared according to the sodium dodecyl sulfate lysis procedure followed by centrifugation in ethidium bromide-CsCl equilibrium gradients. The lambda vector L47.1 was grown in *E. coli* K802, and DNA was prepared according to standard procedures (36).

Molecular cloning of the integrated HZ5-FeSV provirus. High-molecular-weight DNA (120 µg) from HZ5-FeSV FRE rat cells was digested to completion with the restriction enzyme *EcoRI* (New England BioLabs) and fractionated in a 10 to 40% sucrose gradient in 1 M NaCl-1 mM EDTA-10 mM Tris (pH 8.0) (40). The fractions containing the 14.5-kilobase (kb) fragment with the HZ2-FeSV provirus were identified by blot hybridization upon fractionation in a 1% agarose gel, transfer of the DNA to a nitrocellulose membrane, and hybridization with a ³²P-labeled FeLV *rep* probe as described previously (8). The 14.5-kb *EcoRI* DNA (200 ng) was then ligated to 600-ng lambda L47.1 arms (obtained by digestion with *EcoRI*) with T4 ligase (New England BioLabs) and packaged in vitro to form viable phage particles with extracts made from the *E. coli* strains BHB 2688 and BHB 2690 according to the method of V. Pirrotta (J. Ravetch, personal communication [68]). Phage (1.3 × 10⁵) were screened for FeLV hybridization according to the plaque lift procedure of Benton and Davis (39). Prehybridization and hybridization of the filters was done in 50% formamide-6× SPE (0.9 M NaCl, 0.05 M NaH₂PO₄, 0.005 M EDTA; pH 7.0)-0.2% sodium dodecyl sulfate-5× Denhardt solution-200 µg of salmon sperm DNA per ml at 42°C, and an FeLV *rep* probe (specific activity, 1 × 10⁸ to 5 × 10⁸ cpm/µg; 2 × 10⁶ cpm/ml) was used. The filters were washed at 66°C in 0.3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.0). One positive phage was obtained and then plaque purified. For further analysis, the 13.6-kb insert of this phage was subcloned into pBR322.

Analysis of phage and plasmid DNAs. For restriction anal-

ysis, DNAs were digested with one or several restriction enzymes and the resulting restriction fragments were fractionated in agarose gels. To identify specific fragments, the DNA was transferred from agarose gels to nitrocellulose membranes according to the method of Southern and then hybridized with ³²P-labeled probes as described previously (8). Probes with a specific activity of 1 × 10⁷ to 5 × 10⁷ cpm/µg were used to analyze phage and plasmid DNAs. Low-stringency hybridizations were done in 40% formamide-6× SPE at 40°C, and the filters were washed in 2× SSC at 60°C.

Analysis of cellular DNAs. High-molecular-weight DNA from uninfected and HZ5-FeSV-infected cell lines was prepared as described previously (8). For gel analysis 10 µg of DNA was digested with restriction enzyme, fractionated in 1% agarose, transferred to nitrocellulose, and then hybridized with ³²P-labeled probes.

Hybridization reagents. FeLV hybridization probes included (i) the *EcoRI* insert of pFeLVB, which includes the entire FeLV genome, as an FeLV *rep* probe; (ii) a subgenomic 5' *gag* probe derived from 1.13 to 2.1 kb of the FeLV genome; and (iii) a U3 long-terminal-repeat probe derived from the GA-FeLV genome (11, 48). A summary of the *onc* plasmids used in this study is shown in Table 1.

DNA transfections. DNA transfections were done essentially as described previously (27). Various amounts of cloned DNA (1 to 100 ng) were added to 30 µg of sheared NIH 3T3 carrier DNA and then precipitated with ethanol. The precipitated DNA was suspended in 1.25 ml of transfection buffer and then precipitated slowly by the addition of 2 M CaCl₂. The precipitate suspension (0.6 ml) was added to 3.5 ml of medium in a 6-cm dish previously seeded with 4 × 10⁵ NIH 3T3 cells, and after 4 h the cultures were treated for 4 min with 15% glycerol in phosphate-buffered saline. At 12 to 16 h after addition of the DNA the cells were reseeded at 3 × 10⁵ cells per 10-cm dish. Foci of transformed cells appeared after 10 to 14 days and were scored after 16 to 21 days. For further analysis, foci of transformed cells were

picked using glass cloning cylinders and grown to mass culture.

DNA sequence analysis. Nucleic acid sequences were determined by the chain determination method of Sanger et al. after fragments were subcloned into the phage M13 vectors mp18 and mp19 (59, 78).

RESULTS

Origin of HZ5-FeSV. A 3.5-year-old male domestic cat (SKI 3674) with multiple fibrosarcomas was obtained from a pet household in West Palm Beach, Fla. Nodules had been observed for 5 months in subcutaneous tissues; upon autopsy nodules were present in the ventral aspect of the tail (one), the lateral aspect of the ventral chest (four), and the ventral abdominal muscles (one). No metastatic lesions were found in the thoracic or abdominal viscera. The cat was infected with FeLV, as indicated by the presence of FeLV antigens in peripheral blood leukocytes (61).

Extract obtained from one tumor node in the abdominal muscles was used to infect Fea feline embryo fibroblasts. These cells assumed a predominantly transformed phenotype upon passage, indicating the presence of a transforming virus. To obtain HZ5-FeSV pseudotype virus with an extended host range, the Fea/3674-3 cells were infected with amph-MuLV. A filtered virus stock obtained from the amph-MuLV-infected Fea/3674-3 cells was then used to infect rat FRE-3A cells in a standard focus assay. After 7 to 10 days, foci of transformed cells were discernible, and after 11 days individual foci were picked with cloning cylinders, grown up to mass culture, and subsequently cloned twice in semisolid medium. Two of these transformants, the FRE 3674-3/C15B1 cells, were virus nonproducers, i.e., they did not release infectious virus particles. Superinfection of these cells with amph-MuLV produced 10^2 to 10^3 focus-forming units (FFU)/ml of HZ5-FeSV, as determined by focus assay, and 10^5 FFU/ml of amph-MuLV helper virus. Foci of transformed cells were observed on Fea cat, CCL64 mink, FRE-3A rat, and NIH 3T3 mouse cells (see Fig. 3). These experiments provide evidence for the replication-defective nature of the HZ5-FeSV. The FRE HZ5-FeSV nonproducer cells display features characteristic of transformation, i.e., they grow in semisolid medium and produce fibrosarcomas in nude mice.

Structure of the HZ5-FeSV genome. We first investigated the structure of the integrated HZ5-FeSV genome in HZ5-FeSV-infected mink and rat FRE-3A cells by Southern blot analysis. These experiments indicated that the HZ5-FeSV genome did not contain any *EcoRI* restriction sites. The DNA from rat FRE and mink HZ5-FeSV nonproducer cells had *EcoRI* restriction fragments of 14 and >25 kb, respectively, which contained the HZ5-FeSV provirus as determined by hybridization with an FeLV *rep* probe. We have cloned the 14-kb *EcoRI* fragment from the FRE rat HZ5-FeSV nonproducer cell DNA using the lambda vector L47.1 to determine the structure of the HZ5-FeSV provirus. A restriction map of the integrated HZ5-FeSV provirus is shown in Fig. 1. Restriction fragments containing FeLV-related sequences were identified by hybridization of Southern blots with an FeLV *rep* probe (Fig. 2, panel C) and probes specific for 5' *gag* (Fig. 2, panel D), as well as for the U3 sequences of the FeLV long terminal repeat (data not shown). Restriction sites specific for viruses of the FeLV group are seen (48, 63). A pair of *KpnI* and *SmaI* sites in the two long terminal repeats, a pair of *BglII* and *SmaI* sites in the *gag* p15 coding region, and a characteristic combination

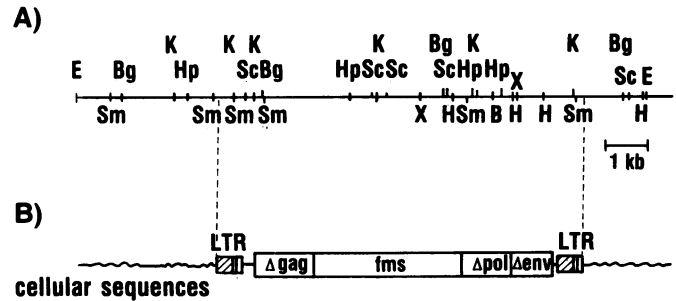


FIG. 1. Restriction map and genetic structure of the molecularly cloned HZ5-FeSV provirus. The restriction enzymes used to construct this map were *EcoRI* (R), *HindIII* (H), *SacI* (Sc), *SmaI* (Sm), *KpnI* (K), *BglII* (Bg), *BamHI* (B), *XhoI* (X), and *HpaI* (Hp).

of sites at the 3' end of the *pol* sequences—*SmaI*, *KpnI*, *BamHI*, and *XhoI* at 9.2, 9.5, 9.8, and 10.4 kb, respectively—are found.

A 1.4-kb *SacI* fragment (7.5 to 8.9 kb) was found to lack homology with FeLV sequences (Fig. 2C, lane 2). A 2.7-kb *HpaI-SmaI* fragment (6.5 to 9.2 kb), on the other hand, showed hybridization with FeLV sequences. These results indicate that the 1.4-kb *SacI* fragment is part of the HZ5-FeSV-specific sequences and, assuming that the *HpaI* site at 6.5 kb is within the HZ5-FeSV-specific sequences (see below), they also indicate that the 3' breakpoint of these sequences is between the *SacI* and *SmaI* sites at 8.9 and 9.2 kb.

The biological activity of the cloned HZ5-FeSV provirus was investigated by DNA transfection experiments. NIH 3T3 mouse cells were transfected with pHZ5 DNA by the calcium phosphate precipitation procedure (22). The appearance of foci of transformed cells was dose dependent, and the efficiency was approximately 10^4 FFU/pmol (Table 2). Foci of transformed cells were picked and grown for analysis. Focus-forming virus could be obtained readily from the transformed cell lines upon superinfection with amph-MuLV (3×10^3 FFU/ml), demonstrating that the transformants contained the whole HZ5-FeSV genome. The morphology of the foci of transformed cells obtained with the rescued virus shown in Fig. 3 is identical with that obtained with pHZ5 DNA and with HZ5-FeSV (amph-MuLV) obtained from FRE HZ5-FeSV nonproducer cells (data not shown).

HZ5-FeSV shows *v-fms* sequence homology. The relationship of the HZ5-FeSV-specific sequences with that of the known oncogenes was then investigated. Recombinant plasmids containing the different retroviral oncogenes were digested with restriction enzymes to obtain fragments which contained *v-onc* sequences free of helper-virus sequences (Table 1). Southern blots containing these DNAs were hybridized with a hybridization probe obtained from the 14-kb insert of the HZ5-FeSV plasmid, and hybridization was carried out at low stringency to detect cross-species homologies due to the different origins of the oncogenes used in this study (Fig. 4). The HZ5-FeSV provirus was found to hybridize to the 1.6-kb *KpnI-BglII v-fms* fragment, as well as with FeLV and other mammalian helper retrovirus sequences (Fig. 4B). No hybridization was seen with restriction fragments containing *h-ras*, *k-ras*, *n-ras*, *src*, *yes*, *ros*, *abl*, and *fes* (Fig. 4) or containing *myc*, *myb*, *ski*, *fos*, *sis*, *raf*, *rel*, *erbAB*, and *mos* (data not shown). These results demonstrate that the HZ5-FeSV provirus contained sequences homologous to the *v-fms* sequences of the SM-FeSV. The *v-fms* sequences in the two viruses appear to be of similar

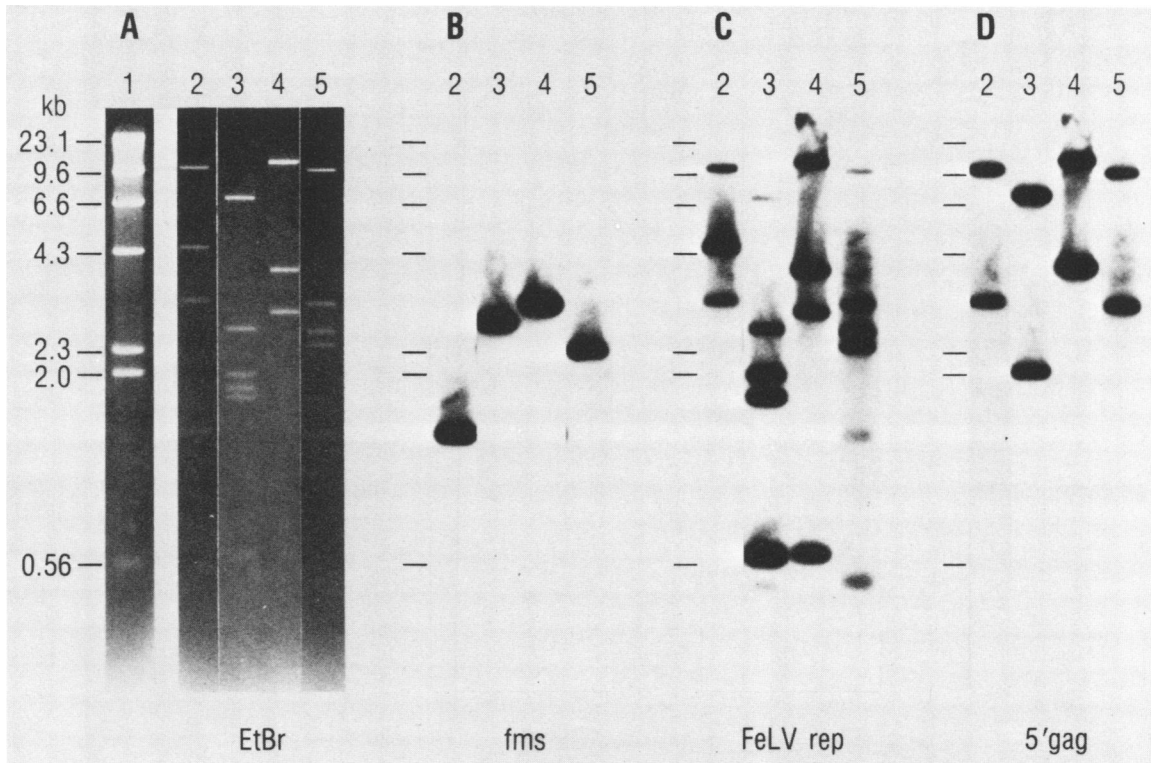


FIG. 2. Identification of FeLV-related and *v-fms*-related sequences in the HZ5-FeSV provirus. Southern blots containing pHZ5 DNA digested with the restriction enzymes *Sac*I (lane 2), *Hpa*I and *Sma*I (lane 3), *Hpa*I (lane 4) and *Kpn*I (lane 5) were hybridized with *v-fms*- and FeLV-specific probes. (A) Ethidium bromide-stained agarose gel before transfer to the nitrocellulose membrane. Lambda DNA digest with *Hind*III is shown in lane 1. (B) Hybridization with 1.6-kb *Kpn*I-*Bgl*II *v-fms* fragment as a probe. (C) Hybridization with an FeLV rep probe. (D) Hybridization with a 5'-*gag*-specific probe.

origin (the domestic cat) since the hybridization signal was retained upon washing in 0.2× SSC at 65°C (Fig. 4, panel C).

Comparative analysis of the *v-fms* sequences in the HZ5- and the SM-FeSVs. To identify the *v-fms* sequences in the HZ5-FeSV genome, DNA blots containing HZ5-FeSV DNA were hybridized with a *v-fms* hybridization probe. These experiments indicated that the *v-fms* sequences in the HZ5-FeSV are located between the *Hpa*I site at 6.5 kb and the *Sac*I site at 8.9 kb of the HZ5-FeSV restriction map (Fig. 2B). A comparison of the restriction maps of the HZ5-FeSV and SM-FeSV *v-fms* sequences revealed that the content of the *v-fms* sequences in the two viruses is very similar. With the exception of the *Sma*I site at 374 base pairs (bp) of the SM-FeSV *v-fms* sequence, which is lost due to sequence polymorphism (an *Ava*I site at 374 bp is retained), all of the following restriction sites known from SM-FeSV *v-fms* are also found in HZ5-FeSV: *Hae*II (37 bp), *Nco*I (104 bp), *Sph*I (150 bp), *Ava*I (374 bp), *Cla*I (666 bp), *Hpa*I (796 bp), *Nco*I (974 bp), *Hae*II (1214 bp), *Sac*I (1,282 bp), *Kpn*I (1,367 bp), *Sac*I (1,633 bp), *Xho*I (2,373 bp), and *Bgl*II (2,813 bp) (24).

In SM-FeSV the *Sph*I site is 150 nucleotides away from the 5' junction between the FeLV *gag* and *v-fms* sequences. To analyze the 5' junction between *gag* and *v-fms* in the HZ5-FeSV, we determined the nucleotide sequence 5' of the same *Sph*I site in HZ5-FeSV. The nucleotide sequence displayed in Fig. 5A revealed that the HZ5- and SM-FeSVs have identical 5' recombination sites with respect to the FeLV, as well as the *c-fms* sequences. Minor differences exist, however, between the HZ5- and SM-FeSV sequences (8 of 200 nucleotides). Five of these changes are contained in

the 66-nucleotide *gag* segment, indicating that the two viruses derive from different and divergent FeLV parents.

HZ5- and SM-FeSV *v-fms* differ at the 3' end. Inspection of the restriction map at the 3' end of HZ5 *v-fms* indicated restriction sites in the *v-fms* segment which were not present in the SM-FeSV. To investigate these sequences and to analyze the 3' junction between *v-fms* and FeLV *pol* in the HZ5-FeSV, we determined the nucleotide sequence between the *Bgl*II and the *Sma*I restriction sites 8.7 and 9.2 kb of the HZ5-FeSV genome, respectively (Fig. 5B). The 3' junction between the *v-fms* and the *pol* sequences in the HZ5-FeSV was determined by comparing the HZ5-FeSV sequence with *pol* sequences known from the HZ2-FeSV and the GA-FeLV (7a). The 3' junction was located at nucleotide 444 of the sequence shown in Fig. 5. The sequences of FeLV *pol* which

TABLE 2. In vitro transforming activity of the molecularly cloned HZ4-FeSV provirus^a

Input DNA	Dose (μg)	Efficiency of transformation (FFU/μg. 10 ³)
pHZ5 FeSV	0.15	0.7
pHZ5 FeSV	0.05	0.7
pHZ5 FeSV	0.015	1.0
Carrier DNA alone	30.0	<0.0001

^a DNA transfections were done as described previously except that the proviral DNA was coprecipitated with 30 μg of NIH 3T3 cell DNA as carrier in 1.25 ml of transfection buffer (19). NIH 3T3 cells were used as recipient indicator cells, and foci of transformed cells were scored 12 to 18 days after transfection.

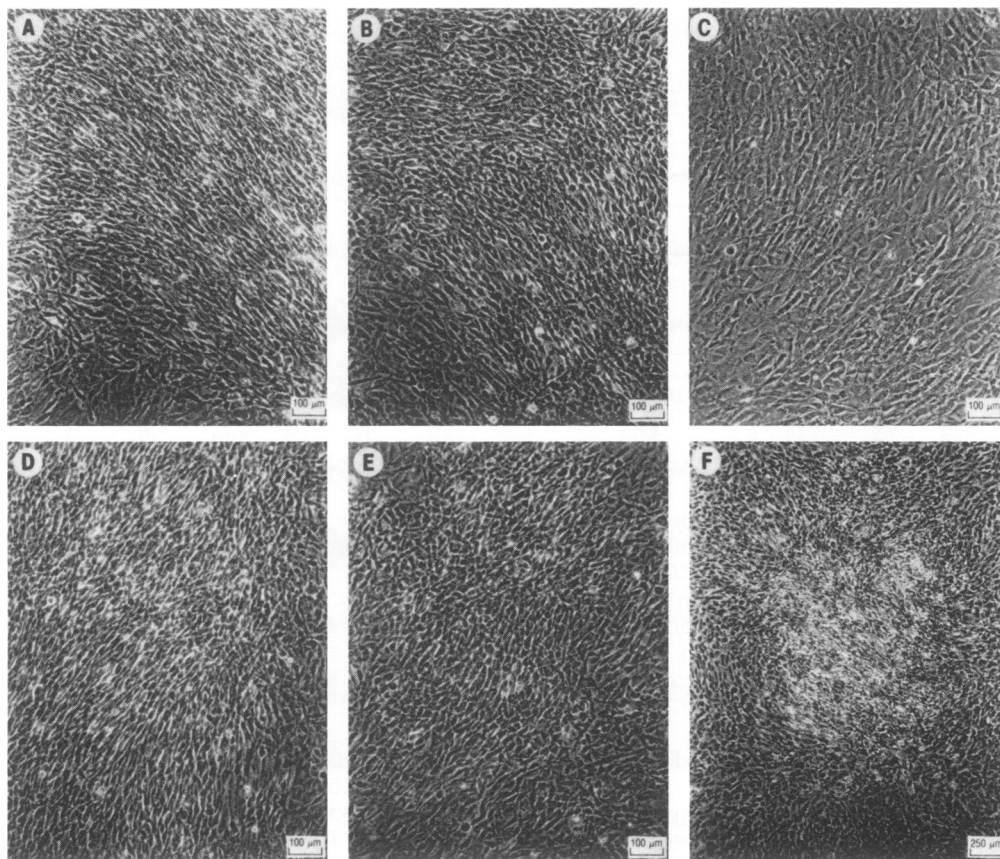


FIG. 3. Morphology of HZ5- and SM-FeSV transformed NIH 3T3 cells. Foci of transformed cells induced by HZ5-FeSV (amph-MuLV) (panels A, B, and F) and by SM-FeSV (amph-MuLV) (panels D and E) 9 days after infection are shown. Uninfected NIH3T3 cells are shown in panel C.

correspond to the 3' junction in the HZ5-FeSV are 1.4 kb 5' of the 3' end of the *pol* gene, located between the 3' recombination sites of the HZ2- and HZ4-FeSVs.

The 3' *v-fms* sequences of the HZ5-FeSV were then compared with those of the SM-FeSV. Perfect homology between the two sequences is seen for the first 82 nucleotides 3' of the *Bgl*II site. The sequences 3' of nucleotide 82, however, are not related at all in the two viruses. The divergence of the 3' HZ5-FeSV *v-fms* sequences, a segment of 362 nucleotides, and the 3' SM-FeSV *v-fms* sequences raises the question of their respective origins. Their relationship to *c-fms* is discussed below in the light of the recent characterization of a human *c-fms* cDNA clone (13a).

In the HZ5-FeSV the known open reading frame of *v-fms* extends from the *Bgl*II site (nucleotide 1) to nucleotide 162 (Fig. 5), where a TGA termination signal is reached. The predicted C terminus of the HZ5-FeSV *v-fms* gene product, on the other hand, contains 11 amino acids which are present in the HZ5-FeSV.

DISCUSSION

Similar origins and structures of HZ5- and SM-FeSV. HZ5-FeSV and SM-FeSV were both isolated from multicentric fibrosarcomas of domestic cats; the morphology of cells transformed by them is indistinguishable (Fig. 3), and both viruses were similarly generated by the transduction of feline *c-fms* sequences with FeLV. Our studies show that the genomes of the two viruses are very closely related in

structure. Compared with the SM-FeSV, the HZ5-FeSV *gag-fms* transforming gene consists of an identical portion of the FeLV *gag* gene; the 5' recombination sites in the two viruses are identical with regard to both the FeLV *gag* and the *c-fms* sequences, and the *v-fms* coding region in the HZ5-FeSV appears to be indistinguishable from that in the SM-FeSV, within the limits of the analysis presented here, with the exception of the C terminus.

Identical 5' recombination sites in HZ5- and SM-FeSV. The finding that HZ5- and SM-FeSVs have identical 5' recombination sites (*gag-v-fms* junctions) is of interest. The pathway by which ATVs are formed is thought to involve five steps: (i) upstream integration of an FeLV provirus, (ii) a deletion at the DNA level in which FeLV *gag* sequences become fused with *c-onc* sequences, (iii) transcription of a hybrid RNA consisting of retrovirus and *onc* sequences, (iv) packaging of the hybrid RNA into a retrovirus particle, and (v) recombination of 3' retroviral sequences with this RNA chimera upon infection of cells with virus particles containing this RNA (21, 28, 69). The formation of 5' recombination sites may be determined by at least three factors: (i) short homologies at the recombination site, (ii) recombinant sequences, and (iii) functional constraints for the expression of the transforming protein. Four of the known characterized feline ATVs, the GA-, HZ4-, and HZ2-FeSVs and the M-FeLV *myc* virus, have 5' recombination sites which involve a preferred region in FeLV p30 *gag* (7a). A possible consensus sequence, AAAGAGG/CCTCTTT, which is repeated in the p30 region, is also found in the HZ5- and

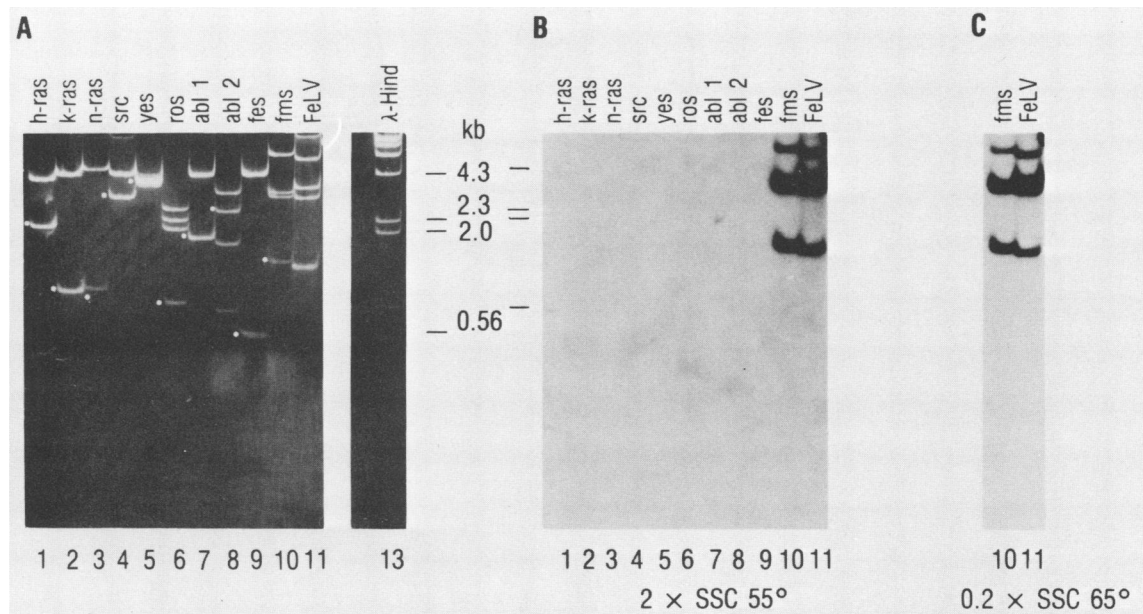


FIG. 4. Determination of homology between the known retroviral oncogenes and the HZ5-FeSV provirus. Oncogene-containing plasmids were digested with appropriate restriction enzymes, separated in a 1% agarose gel, transferred to nitrocellulose paper, and hybridized with ^{32}P -labeled nick-translated HZ5-FeSV insert (14 kb) using nonstringent hybridization conditions in $6\times$ SSCPE (0.72 M NaCl–0.09 M sodium citrate–0.075 M sodium phosphate–0.006 M EDTA, pH 7.2)–40% formamide– $2\times$ Denhardt–0.25 mg of yeast RNA per ml–0.125 mg of salmon sperm DNA per ml–0.2% sodium dodecyl sulfate at 37°C . Blots were washed under nonstringent washing conditions ($2\times$ SSC, 55°C) (Fig. 4B) and at 65°C (Fig. 4C). Ethidium bromide-stained gels before the transfer are shown in Fig. 4A. Restriction fragments containing *v-onc* sequences are marked by a white asterisk. The *v-onc* DNAs pHEE6 (*v-h-ras*), pHiHi3 (*v-k-ras*), pnras (*n-ras*), psrc (*v-src*), lambda Y73-IIA (*v-yes*), pros (*v-ros*), pAB3sub3 (*v-abl*), p130abl (*v-abl*), pvfes (*v-fes*), and pSMFeSV (*v-fms*) were digested with the restriction enzymes indicated in Table 1. The FeLV plasmid pGAFeLV was digested with *Kpn*I. Lambda DNA digested with *Hind*III is shown in lane 13.

SM-FeSV recombination sites in FeLV p10. The known site in p10 was used twice although a similar recombination, in which the *gag* reading frame is sustained, is possible in the p30 region (amino acid 445 of FeLV *gag*).

The nucleotide sequence of the recently characterized human *c-fms* cDNA shows that there is an 8-base homology between the *c-fms* and the FeLV *gag* sequences at the 5' recombination site in the HZ5- and SM-FeSVs (13a). Unless functional constraints for the expression of the transforming protein are determining the recombination event, this finding suggests that short sequence homologies at retroviral recombination sites are a determining factor in the generation of retrovirus *v-onc* junctions. These observations are in good agreement with those known from prokaryotes where short sequence homologies determine hot spots for deletion formation (1).

Both HZ5-FeSV and SM-FeSV *v-fms* are C-terminally truncated versions of *c-fms*. The protein product of the proto-oncogene *c-fms*, M-CSFR, is an integral transmembrane receptor with an outer cellular domain (ligand-binding domain), a transmembrane domain, and an intracellular domain (24, 56, 57, 65). The intracellular domain contains the protein kinase which is activated upon ligand binding. In analogy with other receptor systems, the activation of the M-CSFR kinase is thought to be an essential step in M-CSFR-mediated signal transduction (56, 65). The human *c-fms* cDNA nucleotide sequence predicts that the *c-fms* sequences at the 5' junction of both HZ5- and SM-FeSV derive from the 5'-untranslated region of *c-fms*, such that the *gag* open reading frame is sustained through the untranslated region into the correct reading frame of *c-fms* (13a). As a result, the *v-fms*

gene product of the HZ5- and SM-FeSVs is predicted to contain the entire outer cellular domain of the feline M-CSF receptor.

At the C terminus the HZ5-FeSV and SM-FeSV differ. We have compared the HZ5-FeSV 3' *v-fms* sequence with the 3' human *c-fms* cDNA sequence in order to determine their relationship (Fig. 6) (13a). The two sequences are clearly related, although differences do exist. The most striking feature is an 11-bp deletion of *c-fms* sequences in *v-fms* at nucleotide 155 (Fig. 5). As a result of this frameshift mutation, a TGA termination signal is reached two amino acids downstream. We therefore predict that the HZ5-FeSV *v-fms* protein is missing the 24 C-terminal amino acids of feline *c-fms*. In contrast, comparison of the 3' SM-FeSV *v-fms* sequences with *c-fms* and HZ5-FeSV *v-fms* revealed no relationship. The origin of these sequences is not known; they could derive either from downstream sequences of the *c-fms* locus or possibly from sequences of another locus. In the SM-FeSV *v-fms* protein, as a consequence of the sequence insertion in SM-FeSV *v-fms*, a 48-amino-acid segment at the C terminus of the *c-fms* protein is replaced by a sequence of 11 amino acids of unknown origin.

At the point where HZ5-FeSV and SM-FeSV *v-fms* sequences diverge a unique repeat sequence $(\text{AGC})_x$ is found. In the HZ5-FeSV the $(\text{AGC})_{11}$ segment is furthermore followed by a 33-nucleotide sequence which very likely derives from the same AGC repeat unit. Compared with the human *c-fms* sequence, HZ5-FeSV *v-fms* contains nine additional AGC repeats and SM-FeSV *v-fms* contains four. This suggests that the additional AGC repeats seen in the viral genomes derive from feline *c-fms*. Direct repeat sequences

A

5' Recombination site in the HZ5- and the SM-FeSV :

p30 — | — p10 (T) (N)

H K E M T K V L A T V V A Q N R D K D R
 CATAAGGAGATGACTAAAGTTCTGGCCACAGTAGTTGCTCAGAATAGAGATAAGGATAGA HZ5
 -----A-----C-----A-C-----A-----SM

p10 --||←start v-fms

E E R M P S G P G H Y G A S A E T P G P
 GAGGAACGTATGCCAGTGGTCCCAGGCTACTACGGAGCTAGCGCTGAGACTCCAGGGCC T HZ5
 -----SM

(R) (L)

C P P L C P A S S C C P P T E A M G P R HZ5
 TGCCACCCCTCTGCCCTGCATCATCTGCTGCCCCCACTGAGGCCATGGGCCAAGG HZ5
 C-----T-----SM

(M)

A L L V L L V A HZ5
 GCTCTGCTGGTCTGCTGGTGCCA SM
 -----A-----

B

3' End of v-fms in the HZ5-FeSV and the SM-FeSV :

BglII

I C S L L Q K Q A Q E D R R V P N Y T N 60 HZ5
 ATCTGCTCCTCCTCCAGAACGAGCCCAAGAGGACAGGAGAGTGCCGAACCTACCAAC SM

L P S S S S S S S S S S S S C R T G S G G 120 HZ5
 TTGCCAAGCAGCAGCAGCAGCAGCAGCAGCAGCAGCTGCCACTGGCAGTGGCGGT HZ5
 R L L R P W R G P P L * SM
 -----GCTCCTTAGGCCCTGGCGAGGACCCCTGTAGCCA SM

G S S S E P E E E S S S L L * 180 HZ5
 GGCAGCAGTAGTGAGCCCGAGGAGGAGAGCTAGCTGTGAGCAGGGGATATCGC HZ5
 GGTGAAACGGCAAAGCAGACAGTGCTCAGAGACTG 3'end v-fms SM

CCAGCCCTGCTGCAGCCCAACACTACCAGTTCTGCTGAGGGGACAACAGGGAAGCC 240 HZ5
 GCTTCCCCTTCCCAAGCTCAACTCCTCCGTGGATGGGGACATGGGGAGAACCAC HZ5
 AGACTGCCCTTGGTCATTCTCACTCAACAGCCAGTTCAGACAACATAAATGGCCATGC HZ5
 ACACAGACATCCAGGCCCTAGAAAGTCAATAGTGCCCTTAGAGAAAGTCCTGACCTCC HZ5

end v-fms --||←start FeLV pol

TTTCTGAAGTAGTCTTACAAAACAGCCGGGCTGCTCACTCAGAAGGAAAAGAAATTA HZ5

3' Recombination Site in FeLV pol :

S R Y A F A T A H V H G E I Y R R R G L ←start FeLV homology
 CAGCCGATGCTCTTGTCTACAGCTATGTACACGGGAAATCTACAGCCGGGGCT HZ2-FeSV
 S R Y A F A T A H I H G E I Y R R R G L Mo-MuLV

L T S E G K E I K N K N E HZ2-FeSV
 GCTAACTTCAGAAGGAAAAGAAATTAATAAATAAATGAA HZ2-FeSV
 L T S E G K E I K N K D E Mo-MuLV

FIG. 5. Nucleotide sequence of the HZ5-FeSV 5' and 3' v-fms sequences. The nucleotide sequence of the 3' HZ5-FeSV v-fms sequences was determined on both strands after subcloning of restriction fragments into the M13 vectors mp18 and mp19 by the chain-termination method of Sanger et al. (59). The 5' v-fms sequences, on the other hand, were determined on one strand only. The predicted amino acid sequence is shown above using single-letter amino acid notations. Where different from the HZ5-FeSV nucleotide sequence, the SM-FeSV sequence is shown below and the amino acid sequence differences are shown above in brackets. The junction between the gag proteins p30 and p10 is indicated, as well as the 5' and 3' boundaries of the v-fms sequences. To determine the 3' recombination site between the fms sequences and FeLV pol, the fms-pol sequence was compared with pol sequences of the HZ2-FeSV and Moloney-MuLV (amino acid sequence is shown). (A) Recombination site between FeLV gag and c-fms sequences in the HZ5- and SM-FeSVs. (B) 3' End of v-fms in the HZ5- and the SM-FeSVs.

A

Comparison of the 3' fms sequences in HZ5-FeSV and human c-fms :

R R V P N Y T N L P S S S S S S S S S S 96 HZ5
 AGGAGAGTCCGAACCTACCAACTTGCCAAGCAGCAGCAGCAGCAGCAGCAGCAGCAGC
 -----A-G-G---T---TC---G--- hMCSFR
 R R E R D Y T N L P S S S

S C R T G S G G G S S S S E P E E E S S S 155 HZ5
 AGCTGCCGACTGGCAGTGGCGGTGGCAGCAGTAGTGAGCCCGAGGAGAGACTCTAG hMCSFR
 A-A-GC---TG-CA---CA---C-----TG-----T
 R S G G S G S S S S S E L E E E S S S

L L * 205 HZ5
 CCTGCTGTGAGCAGGGGATATCGCCACGCCCTGTCTGCAGCCAACAAC hMCSFR
 GAGCACCTGA-----C---A-----T-----T-----
 E H L T C C E Q G D I A Q P L L Q P N N

TACCAGTTCTGCTGAGGGG ACAACAGGGAAGGCCCTTTCCCTTCTCCAAGCT 261 HZ5
 --T-----A-GTTG--G-----TA--A--C-----A-- hMCSFR
 Y Q F C *

TCAACTCTCCG TGGGGC ACATGGGGAGAACCACAGACTC GCCCTGGTTCATT 315 HZ5
 -----ATGGA-----G---C-----AT---A---T---T-C----- hMCSFR

TCACTCAACAGCCGAGTTCAGACAACAATGGCCATGCACAGACATCCAGGCCCT 375 HZ5
 -----T-G-CC---CTCTGAAACTTGGGAAGGTGAGGATTCAGGGGAGGTCA hMCSFR

AGAAGAGTCAATTAGTGCCTTAGAGAAGTCCTGACCTCCCTTTCTGAAGTAGCTTACA 435 HZ5
 GAGGATCCCACTCCTGAGCATGGGCCATCACTGCCAGTCAGGGGCTGGGGCTGAGGCC hMCSFR

AAACA HZ5
 TCACC hMCSFR

B

Comparison of the predicted C-terminal protein sequences of human M-CSFR, HZ5-FeSV and SM-FeSV v-fms:

R R V P N Y T N L P S S S S S S S S S C - T - S G - G - - - - hMCSFR
 -----S-----S-----S-----S-----S-----S-----S----- hMCSFR
 -----S R L L R P W R G P P L * SM

L E E E S S S E H L T C C E Q G D I A Q P L L Q P N N Y Q F C * hMCSFR
 P-----L L * HZ5

FIG. 6. (A) Comparison of the 3' HZ5-FeSV v-fms sequence with the 3' human c-fms cDNA sequences. The HZ5-FeSV (HZ5) sequence is shown on top, and the human M-CSF receptor (hMCSFR = c-fms) is shown below. The corresponding amino acid sequences are shown above and below, respectively. The numbering of the HZ5 sequence is as in Fig. 5. Nucleotide identities are indicated by a dash, and gaps are indicated by an empty space. The hMCSFR sequence was kindly provided by I. M. Verma and C. Van Bevern before publication. (B) Comparison of the predicted C-terminal protein sequences of human c-fms, HZ5-FeSV, and SM-FeSV v-fms. Amino acid identities are indicated by a dash and gaps by an empty space.

are known to facilitate the generation of deletions and amplifications (1). It is therefore likely that the polymorphism seen between the feline and the human fms sequences is based on the repeated nature of these sequences. Protein kinase oncogenes like src and erbB (EGFR) are thought to derive their oncogenic properties from structural alterations of their protein products rather than from a disruption of the mode of expression as is observed with "nuclear oncogenes" such as myc, myb, and fos (9, 29, 35, 43, 52, 62, 71). The v-erbB protein of AEV-H is a truncated version of EGFR in which N-terminal and C-terminal sequences are missing (72, 77). In avian leukosis virus-induced erythroblastosis, EGFR is truncated at the N-terminus only (34, 51). Both src and EGFR contain in their C-terminal domains important tyrosine phosphorylation sites, which are

known to contribute to the modulation of their respective associated protein kinase activities (13, 23). Both HZ5- and SM-FeSVs have truncated C termini, and it seems possible that this alteration is a determinant of the oncogenic properties of the two *v-fms* proteins. An alternative mechanism of the transformation mediated by *v-fms* has recently been proposed by Sherr et al. (65). Fibroblasts, as well as a number of other tissues, are known to produce M-CSF. Since *v-fms* contains the entire outer cellular domain of the M-CSF receptor, it is feasible that M-CSF could bind to the *v-fms* protein in HZ5-FeSV- or SM-FeSV-infected cells and thus establish an autocrine loop. HZ5-FeSV and SM-FeSV readily transform nonfeline mammalian cells; if the proposed autocrine mechanism is true then M-CSF should display cross-species reactivity that parallels the transformation specificity of the *fms* viruses. Single-amino-acid differences between the SM-FeSV *v-fms* protein and the human *c-fms* proteins exist, particularly in the extracellular domain. These could be species differences; however, they may also be activating mutations. A critical evaluation of amino acid differences between the *v-fms* proteins and the human and cat *c-fms* proteins may therefore be necessary in order for us to understand the oncogenic activation of *v-fms*.

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