Transforming Potential of a myc-Containing Variant of Feline Leukemia Virus In Vitro in Early-Passage Feline Cells

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We studied a naturally occurring variant of feline leukemia virus (FeLV) in which the oncogene myc has substituted for a portion of the viral structural genes (myc-FeLV). myc-FeLV was rescued by replication in the presence of FeLV as helper, and its biological activity was examined in early-passage feline cells in vitro. Infection of leukocytes from peripheral blood, spleen, or thymus, or of kitten fibroblasts did not immortalize these cells or alter them morphologically. Northern blot (RNA blot) analysis of virion RNA prepared from the supernatant of infected cells demonstrated the 8.2-kilobase genome of FeLV, but did not demonstrate the 5.0-kilobase genome of myc-FeLV. Apparently, the myc-FeLV genome was lost in the absence of the selective pressure of transformation. In contrast, infection of embryonic fibroblasts with myc-FeLV(FeLV) rendered these cells capable of greatly increased, if not infinite, proliferative potential. The cells were morphologically altered compared with controls and were only loosely adherent to the substrate. The cells failed to proliferate in semisolid medium and did not form tumors when inoculated subcutaneously into athymic mice. Blot analyses demonstrated the presence and expression of integrated proviral DNAs of both FeLV and myc-FeLV in these cells. They appear, then, to represent cells partially transformed by infection with myc-FeLV(FeLV). The action of feline v-myc in early-passage cells in vitro was compared to that of avian v-myc.

Feline leukemia virus (FeLV) is a horizontally transmissible C-type retrovirus identified as a causative agent of a spectrum of lymphoid malignancies that affect domestic cats and that are collectively referred to as leukemia-lymphoma-sarcoma complex. FeLV is a representative of the slowly transforming retroviruses, since the development of FeLV-mediated leukemia-lymphosarcoma complex in cats occurs after a relatively long latency period of several months to years (23, 29). FeLV contains no genetic information to which its oncogenic potential can be directly attributed, and the molecular mechanism by which it induces malignancy is unknown. FeLV is unusually promiscuous in its genetic interactions with host proto-oncogenes, however, and natural variants of FeLV have been described which contain the oncogenes fes, fms, fgr, abl, sis, kit, and myc (4–7, 15, 19, 35, 37, 38, 51). These observations suggest that the transformation of feline cells by FeLV may contribute significantly to neoplastic induction by this retrovirus.

Among the variants of FeLV which contain oncogenes, all are derived from feline fibrosarcomas except those which contain myc (4–7, 15, 19, 37, 51). The myc-containing variants have been isolated from naturally occurring T-cell lymphosarcomas in domestic cats (31, 35, 38). These variants are significant, then, in two respects. First, their isolation from lymphoid malignancies provides evidence that oncogene-containing derivatives of classical leukemogenic retroviruses play a role in mammals in the induction of hematopoietic neoplasms. The Abelson murine leukemia virus is associated with the induction of such neoplasms following its parenteral introduction in mice (1). Second, they represent the only naturally occurring mammalian retroviruses which contain myc as an oncogene. The tumorigenic potential of the myc-containing FeLV variants remains uncharacterized. These variants may encode significant oncogenic activity, since the avian retroviruses which contain myc as an oncogene induce a remarkable spectrum of tumors in vivo, including myelocytomas, carcinomas, and sarcomas. The broad tumorigenic potential is reflected in the capacity of these viruses to transform hematopoietic, epithelial, and fibroblastic cells in vitro (8, 16, 20–22, 33). Similarly, recombinant murine retroviruses which contain avian v-myc oncogenes have been shown to transform established murine fibroblast lines to anchorage-independent growth, to transform primary mouse fibroblasts morphologically and to anchorage-independent growth, and to transform primary and early-passage murine macrophages (12, 49).

A myc-containing variant of FeLV, termed myc-FeLV, was originally isolated as proviral DNA, 5.5 kilobase pairs (kbp) in length, from a naturally occurring feline thymic lymphosarcoma (31). The myc-FeLV provirus contains a spliced version of the c-myc gene, 1.34 kbp in length, which has replaced the pol gene and part of the gag and env genes of the FeLV parent from which it was derived (11). Nucleotide sequence analysis demonstrates that the coding sequences shared by feline v-myc and c-myc are identical and share 90% identity with murine and human c-myc genes (11, 45). Feline v-myc lacks most of the unusually long, untranslated exon 1 of c-myc, an observation which may be significant in that the transforming function of avian v-myc genes and of some activated c-myc genes is correlated with the absence of an equivalent c-myc exon 1 (2, 3, 11, 24, 40, 42, 44, 45). The reading frame of feline v-myc is continuous with that of the FeLV gag gene, indicating that the v-myc gene product is expressed as a gag-myc fusion protein (11). Reported here are studies of the transforming potential of myc-FeLV in primary and early-passage feline cells in vitro.

(Materials and Methods)

Cell lines and viruses. The D-17 cell line (41; ATCC CCL183) was maintained in Eagle minimal essential medium supplemented with nonessential amino acids and 10% fetal
calf serum (GIBCO Laboratories). The FEA cell line, a continuous line of feline fibroblasts, both uninfected and infected with biologically cloned FeLV-A/Glasgow-1 (27), were the gift of Jennifer Rojko, The Ohio State University. Molecularly cloned, infectious proviral DNA of FeLV-B/Gardner-Arnstein (36) was the gift of J. W. Casey. The EcoRI restriction fragment of the clone, containing full-length proviral DNA and human DNA flanking sequences, was subcloned into plasmid vector pUC9 by standard procedures (34). Proviral DNA of myc-FeLV, molecularly cloned as an 8-kbp BamHI fragment containing full-length proviral DNA and feline DNA flanking sequences, has been described previously (31).

DNA-mediated transfection and assay for productive infection. Supercoiled recombinant plasmid vector containing proviral DNA (10 μg) was introduced with D-17 DNA (20 μg) as a calcium phosphate precipitate (48) into 10^7 D-17 cells. For cotransfections, the plasmid containing myc-FeLV proviral DNA was used in 2-fold mass excess over the plasmid containing the FeLV provirus (10 μg to 0.5 μg). After 4 h, the cells were washed and exposed briefly to 15% glycerol as described previously (48). To assay for productive viral infection in D-17 cells, reverse transcriptase activity in the culture supernatants was measured essentially as described previously (30).

Preparation and infection of primary feline cell cultures. Leukocytes isolated from peripheral blood by centrifugation through a Ficoll gradient were maintained in liquid medium in complete RPMI (cRPMI) (RPMI 1640, 10% fetal calf serum, 2 mM glutamine, 5 x 10^{-3} M 2-mercaptoethanol, 0.05 mg of gentamicin per ml). Leukocytes prepared from spleen and thymus by mechanical disruption of the tissue were also maintained in cRPMI. Nonadherent cells were removed from the cultures at 24 h after isolation and periodically thereafter to rid the cultures of contaminating fibroblasts. In some cases, cells were cultured in the presence of concanavalin A (ConA) and chromatographically purified human T-cell growth factor (TCGF; Cellular Products Inc.) as a source of interleukin-2 (IL-2). Primary feline fibroblasts were prepared from muscle tissue of a 3-day-old kitten or from thymus and spleen of adult kittens (crown-rump length, 4 cm) dissected from a single pregnant female. The tissues were digested with trypsin, minced, washed in Hanks balanced saline solution, and suspended in cRPMI. Cells adherent to the surface of the culture dish within 24 h were maintained in cRPMI.

For infection, peripheral blood leukocytes, splenocytes, thymocytes, and fibroblasts were incubated with a 24-h supernatant of D-17 cells productively infected with myc-FeLV(FeLV), an inoculum demonstrated to contain 2 x 10^5 focus-forming units as measured by using the clone 81 feline cell assay (18) [see Results for a definition of myc-FeLV(FeLV)]. As controls, the same number of cells were incubated with a 24-h supernatant of D-17 cells productively infected with FeLV alone, an inoculum also determined to contain 2 x 10^5 focus-forming units, or were mock infected. Polybrene (1 μg/ml) was included in all inocula and in the mock infection (47). An equal number of focus-forming units of FeLV-A/Glasgow-1 (27) was also included in some inocula.

Unless otherwise indicated, viral inocula remained on the cells for 3 days, after which the cells were washed in Hanks balanced saline solution and resuspended in cRPMI with or without exogenously added growth factors. Productive infection of the cultures was monitored each week by using an assay for reverse transcriptase activity in the culture supernatant. A second method of infection was used in which thymocytes or splenocytes (2 x 10^6 cells) were cocultivated in flasks containing 10^6 feline fibroblasts productively infected with either myc-FeLV(FeLV) or FeLV. The leukocytes were cocultivated with the infected fibroblasts for 1 week and then were removed and cleared repeatedly of adherent cells.

Lymphoblast proliferation assay. Leukocytes were prepared by centrifugation of peripheral blood of an adult cat through a Ficoll gradient. The cells were washed in Hanks balanced saline solution and suspended in cRPMI at a density of 10^7/ml. To measure the proliferative response of the cells to exogenously added growth factors (10), an inoculum of 10^6 cells (0.1 ml) was placed in each well of duplicate 96-well plates. Duplicate plates containing various concentrations of human TCGF (chromatographically purified; Cellular Products Inc.) and ConA (Vector Laboratories) were incubated at 37°C in a humidified atmosphere containing 5.0% CO_2. ConA stimulation was continued in the wells of one plate for 24 h, after which the medium was replaced with cRPMI containing the same concentration of TCGF. ConA stimulation was continued for 3 days in the duplicate plate. Both plates were incubated for a total of 3 days. At 18 h before harvest, [3H]thymidine (0.5 μCi) was added to each well of both plates. Cells were disrupted by hypotonic lysis, collected on filter paper on which DNA, with incorporated radiolabel, is adherent, and washed with distilled water by using a mechanical cell harvester (Skatron, Inc.). The amount of radiolabel bound to each filter was then determined by scintillation counting.

Tumorigenicity in nude mice. Cells were tested for tumorigenicity in vivo by inoculating seven athymic mice (BALB/c-C57BL nu/nu; Life Sciences, Inc.) with 5 x 10^6 cells subcutaneously. The mice were observed for 8 to 12 weeks. As a positive control for tumorigenicity in this strain of athymic mice, the same number of BHK cells nonproductively transformed with Rous sarcoma virus (a generous gift of Robert Garry) were inoculated subcutaneously into four mice. These mice developed tumors at the site of inoculation which were readily detectable after 1 week.

Preparation and analysis of DNA and RNA. High-molecular-weight DNA was prepared from tissue culture cells as described previously (9). For Southern blot analyses, DNA (10 μg) was digested with restriction enzymes, electrophoresed in a 0.8% agarose gel, and transferred to nitrocellulose (34). Filters were baked at 80°C in vacuo for 3 h and prehybridized at 37°C for 24 h in a solution containing 50% formamide, 1.0 M NaCl, 0.05 M pipervazine-N,N'-bis(2-ethanesulfonic acid) (PIPES) buffer (pH 6.8) (Calbiochem-Behring), 0.1% sodium lauryl sarcosine, 0.01 M EDTA, 0.2 mg of denatured calf thymus DNA per ml, and 5 x Denhardt's solution. Filters were then hybridized for 48 h at 37°C in the same solution containing (per milliliter) 2 x 10^6 cpm of probe DNA radiolabeled to a specific activity of 1 x 10^11 to 2 x 10^12 cpm/μg by nick translation. After being washed twice in 2 x SSC (1 x SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (34)-0.1% sodium lauryl sarcosine at 37°C for 30 min each and twice in 0.1 x SSC-0.1% sodium lauryl sarcosine at 50°C (45°C for heterologous, i.e., nonfeline, probes) for 30 min each, filters were exposed at room temperature or at -70°C for various periods to Kodak XRP-3 film with intensifying screens.

Total cellular RNA was prepared by using guanidine isothiocyanate (13), and poly(A)^+ RNA was isolated by using oligo(dT)-cellulose affinity chromatography (32). Viral RNA was prepared from culture supernatants collected 24 h
after feeding as described elsewhere (L. S. Levy and P. A. Lobelle-Rich, submitted for publication). For Northern blot (RNA blot) analysis, RNA samples were denatured in a formamide-formaldehyde-containing solution, subjected to agarose gel electrophoresis in the presence of formaldehyde as described previously (34), and transferred to nitrocellulose (46). For dot blot analysis, RNA samples were spotted directly onto nitrocellulose as described previously (46). Filters were treated for prehybridization, hybridization, and autoradiography as described above for Southern analyses, except that 10% dextran sulfate was included in the hybridization solution.

For Northern and Southern blot analyses, HindIII-digested lambda DNA and HaelIII-digested φX174 DNA were electrophoresed in parallel as size markers. For Southern analyses, markers were visualized by ethidium bromide staining of the gel. For Northern analyses, markers were denatured exactly as RNA samples were and were visualized after blotting by hybridization to radiolabeled marker DNAs. With respect to blot analyses of myc sequences, a probe representing exon 3 of the feline c-myc gene (31) was used to visualize myc-FeLV transcripts by Northern blot analysis. This probe contains repeated sequences, which, although not transcribed, obscure visualization of the c-myc gene or of integrated myc-FeLV proviral DNA by Southern blot analysis. Therefore a probe representing avian v-myc (50) was used for this purpose.

RESULTS

Rescue of horizontally transmissible myc-FeLV(FeLV). Provirial DNA of myc-FeLV, molecularly cloned from a naturally occurring thymic lymphosarcoma (31), was assumed to be replication defective, since portions of the genes required for its structure and replication have been replaced by feline v-myc (Fig. 1A). Therefore, we attempted to rescue horizontally transmissible myc-FeLV particles in the presence of replication-competent FeLV as helper. Molecularly cloned proviral DNAs of myc-FeLV and of FeLV-B/Gardner-Arnstein were introduced as a calcium phosphate precipitate into D-17 cells, a continuous canine cell line derived from an osteogenic sarcoma (41). Proviral DNAs were introduced in a mass ratio of 20 myc-FeLV to 1 FeLV. As controls, D-17 cells were similarly transfected with FeLV proviral DNA alone or were mock transfected. The recipients of transfected DNA were not isolated from the population, but were allowed to remain in culture with other cells which did not take up DNA. Virions generated by the original recipients of transfected DNA were thus allowed to spread throughout the culture by horizontal infection. These cultures are referred to hereafter as infected D-17 cells.

An assay for the presence of reverse transcriptase activity in the supernatants of cultures into which proviral DNA was introduced was positive initially at 8 weeks after transfection and remained positive thereafter. Such activity has never been detected in the supernatants of mock-transfected cultures. Southern blot analysis of DNA prepared from these cells demonstrated the presence of integrated proviral DNAs of FeLV and myc-FeLV (data not shown). The presence of integrated myc-FeLV proviral DNA in a sufficient number of infected D-17 cells to allow its detection by Southern blot analysis indicates that the myc-FeLV proviral DNA introduced originally by transfection is transcriptionally active and contains the signals required for its encapsidation and horizontal spread.

To document this, viral RNA was prepared as described elsewhere (Levy and Lobelle-Rich, submitted) from the supernatants of productively infected cells collected 24 h after feeding. Northern blot analyses of viral RNA samples hybridized to radiolabeled probes representing the U3 region of the FeLVLTR (31) revealed the 8.2-kilobase (kb) genomic RNA of FeLV in preparations from cells infected with FeLV alone or with FeLV and myc-FeLV (Fig. 1B, lanes A and B). RNA prepared from the culture supernatant of cells infected with FeLV alone contained nothing homologous to a probe representing exon 3 of the feline c-myc gene (3‘5’ cm) (Fig. 1B, lane C); however, the 5.0-kb transcript of myc-FeLV was clearly evident when the 3‘5’ cm probe was used to examine RNA prepared from the supernatants of cultures into which both FeLV and myc-FeLV were introduced (Fig. 1B, lane D). Thus these supernatants contain a complex, referred to

![FIG. 1. (A) Partial restriction enzyme site maps of proviral DNAs of FeLV-B/Gardner-Arnstein (36) and of myc-FeLV (31). Symbols: K, KpnI; P, PstI; Sc, SacI; Sm, Smal. (B) Northern blot analysis of virion RNA prepared from the culture supernatants of D-17 cell lines which produce helper FeLV-B/Gardner-Arnstein only (lanes A and C) or myc-FeLV(FeLV) (lanes B and D). Viral RNA preparations were denatured, electrophoresed in formaldehyde-agarose gels, and transferred to nitrocellulose as described in the text. The amount of viral RNA in lanes A and B is 10% of the amount in lanes C and D. Blots were hybridized with radiolabeled probes representing either the U3 region of the FeLVLTR (31) or the 3‘ exon of myc-FeLV (3‘5’ cm) (31). Molecular lengths, indicated in kilobases, were determined by comparison with the migration of HindIII-digested lambda DNA and HaelIII-digested φX174 DNA, which were denatured, as were RNA samples and electrophoresed in parallel. The RNA species indicated represent the genome length virion RNAs encoded by FeLV (8.2 kb) and myc-FeLV (5.0 kb). The species migrating at <5.0 kb (lane D), although not fully characterized, is thought to represent a degradation product.](http://jvi.asm.org/DownloadedFrom)
by convention as myc-FeLV(FeLV), in which the genomes of both FeLV and myc-FeLV are encapsidated in horizontally infectious particles.

Northern blot analysis of viral RNA indicated that the abundance of particles containing the 8.2-kb genome of helper FeLV was much greater than the abundance of those containing myc-FeLV, particularly since the amount of viral RNA required to visualize the 8.2-kb species was much less than that required to visualize the 5.0-kb species (Fig. 1B). Dot blot analyses of the same viral RNA preparations were performed to quantitate the proportion of myc-FeLV(FeLV) occupied by particles encapsidating the myc-FeLV genome. For this purpose, viral RNA was spotted directly onto nitrocellulose and was hybridized either to the U3 or to the 3'fcm probe, [11]). The data indicated that particles containing the myc-FeLV genome represented only 2% of the total population (data not shown). D-17 cells producing myc-FeLV(FeLV) were then plated at 3 to 5 cells per well in a 96-well dish to isolate a subpopulation enriched for cells producing more abundant myc-FeLV. One subpopulation isolated by this method was shown by quantitative dot blot analysis to contain myc-FeLV as 16% of the total encapsidated viral RNA. This subpopulation was expanded and used for the studies described below.

Infection with myc-FeLV does not transform feline leukocytes. Culture supernatants of D-17 cells producing myc-FeLV(FeLV) were used to infect early-passage feline leukocytes in vitro to assess the biological activity of myc-FeLV in those cells. In initial efforts, leukocytes were separated by centrifugation through a Ficoll gradient from the peripheral blood of adult cats. Leukocytes were maintained in cRPMI for 24 h, and nonadherent cells (2 × 10^6/ml, in duplicate) were then infected with either FeLV-B or myc-FeLV (FeLV), as described in Materials and Methods, or mock infected. Cells were then progressively washed free of the inocula and plated in cRPMI at a range of cell densities between 0.2 × 10^4 and 7 × 10^4/ml. Cells were also plated into semi-solid medium (cRPMI, 0.3% agarose) at the same cell densities. The cells proliferated briefly under both conditions, but began to decline in number by 3 weeks. After 7 weeks, trypan blue exclusion studies of cells in liquid medium demonstrated only 5% living cells. No growth was observed in semisolid medium, and all of the cultures were discarded.

On the basis of the apparent absence of transformation under the conditions used, exogenous growth factors which expand T-lymphocyte populations were included in subsequent studies. Proliferation assays were performed in the presence of various concentrations of ConA and TCGF to optimize the influence of these agents on the growth of feline peripheral blood leukocytes in vitro. Such assays demonstrated that optimal proliferation occurred with a 3-day ConA stimulation of 10 μg of ConA/ml per 10^7 cells plus 10% TCGF, followed by removal of ConA and continued culture in the presence of 10% TCGF (data not shown).

Nonadherent leukocytes prepared from the spleen and thymus of an 8-day-old kitten were cultured in cRPMI in the presence of ConA and TCGF. After 2 weeks, during which considerable blastogenesis was observed in both cultures, cells of both types (2 × 10^6/ml in duplicate) were mock infected or were infected with FeLV-B or with myc-FeLV(FeLV) from the supernatants of producer D-17 cells. It has been observed repeatedly that naturally occurring infections with FeLV are most commonly composed of FeLV subtype A (FeLV-A) alone or of a mixture of FeLV-A and FeLV-B, but never of FeLV-B alone (26, 28). To accommodate what may be a biological dependence of FeLV-B on FeLV-A, an equal number of focus-forming units of FeLV-A/Glasgow-1 (27) was included in the inocula. After being infected for 3 days, cells were washed and suspended in cRPMI–10% TCGF at a density of 10^5/ml. Cells were also plated in semisolid medium (cRPMI, 10% TCGF, 0.3% agarose) at densities of 1 × 10^5, 5 × 10^5, and 2.5 × 10^5/35-mm dish, in duplicate. A second method of infection was used in which splenocytes or thymocytes (2 × 10^6 each) were cocultivated with feline fibroblasts (10^6) productively infected in vitro with FeLV or with myc-FeLV(FeLV) (see below). Leukocytes were cocultivated with fibroblasts for 1 week, separated repeatedly by virtue of their nonadherent properties, and cultured separately. Leukocytes exposed in vitro to the virus-containing supernatants of producer D-17 cells failed to demonstrate evidence of productive infection, as measured by reverse transcriptase activity in the culture supernatant, until 4 weeks after infection. In contrast, the supernatants of cells cocultivated with infected fibroblasts contained demonstrable reverse transcriptase activity immediately after separation from the fibroblasts and thereafter for the life of the cultures. These cells were used for further evaluation of the biological effects of infection.

In neither case were splenic or thymic leukocytes transformed by infection with myc-FeLV(FeLV) as measured by several parameters including rescue from senescence, colony formation in semisolid medium, and release from the requirement for exogenously supplied IL-2 (Table 1). The lifespan of splenic leukocytes infected with myc-FeLV (FeLV) (17 weeks) was somewhat increased, however, compared with that of identical cells which were mock infected or FeLV infected (11 weeks) (Table 1). It is interesting that the myc-FeLV(FeLV)-infected splenic leukocytes were morphologically distinguishable from mock-infected or FeLV-infected cells in that they tended to proliferate in large clusters and individual cells had an elongated and irregular shape (not shown). That the splenocytes and thymocytes infected with myc-FeLV(FeLV) were not transformed by the presence of reverse transcriptase activity in the culture supernatants, but also by Northern blot analysis of viral RNA prepared from those supernatants. Such an analysis with the U3 region of the FeLV LTR (31) as probe clearly demonstrated the presence of the 8.2-kb genomic RNA of FeLV in infected cells, but the 5.0-kb genomic RNA of myc-FeLV was not apparent (Fig. 2A, lanes g to k). An identical analysis with a probe representing the 3' exon of the feline c-myc gene (31) also failed to identify the genomic RNA of myc-FeLV (data not shown). Similarly, Southern blot analysis of DNA from these cells with a probe representing avian v-myc (50) demonstrated only the unaltered feline c-myc gene and did not demonstrate the presence of integrated myc-FeLV proviral DNA (Fig. 2B). Apparently the myc-FeLV genome was lost in the absence of the selective pressure of transformation. It should be noted that the presence and expression of integrated myc-FeLV were readily apparent in cells which were transformed by infection with the same inoculum (see below).

Southern blot analysis of KpnI-digested DNA from infected and mock-infected cells was performed with the U3
region of the FeLV LTR as probe (31). Digestion with KpnI yielded two fragments homologous to the probe from each integrated FeLV provirus: (i) an internal fragment, identical in length among all proviruses, and (ii) the 5′ host-virus junction fragment, whose length depends on the position of the KpnI recognition site in host DNA flanking each provirus (Fig. 1A). Thus the junction fragments in a mass infection are not apparent unless the culture is clonal. This analysis demonstrated a random pattern of proviral integration in splenocytes infected with FeLV alone (Fig. 2C, lane b) and in thymocytes infected with myc-FeLV(FeLV) (Fig. 2C, lane f), since discrete host-virus junction fragments cannot be discerned. In contrast, the appearance of such fragments in the DNA of splenocytes infected with myc-FeLV(FeLV) (Fig. 2C, lane c) indicates that a clonal subpopulation predominated in this culture. The increased lifespan and distinctive morphology observed in this culture may reflect the phenotype of the clonally expanded subpopulation.

A clonal pattern of proviral integration was similarly observed in thymocytes infected with FeLV alone (Fig. 2C, lane e). These cells persisted in culture for 34 weeks, compared with the 12-week lifespan of identical cells which were mock infected or infected with myc-FeLV(FeLV) (Table 1). The cells were morphologically distinguishable from mock-infected and myc-FeLV(FeLV)-infected counterparts, since they proliferated individually or in very large aggregates and individual cells were typically elongated and irregularly shaped (not shown). The predominant population in the culture was apparently T lymphoid, since Southern blot analysis with the β-chain gene of the murine T-cell receptor (a generous gift of E. Palmer, National Jewish Hospital, Denver, Colo.) as probe demonstrated that this gene was rearranged in the DNA of infected cells compared with its organization in the DNA of normal feline kidney (data not shown). This apparent influence of FeLV on the growth of normal thymocytes in vitro has not yet been further characterized.

**Infection with myc-FeLV does not transform kitten fibroblasts but partially transforms embryonic fibroblasts.** Neonal and embryonic fibroblasts were expanded in cRPMI for 1 week after removal from the animals to increase the cell numbers. Fibroblasts of each type (3 × 10^5 each) were then mock infected or infected with the supernatants of D-17 cells producing either FeLV-B or myc-FeLV(FeLV). An equal number of focus-forming units of FeLV-A/Glasgow-1 (27) was added to the inocula. After infection, cells were maintained as monolayer cultures in liquid medium or were plated in semisolid medium (cRPMI, 0.3% agarose) at cell densities of 1 × 10^5 and 5 × 10^4 cells per 35-mm well in duplicate. An assay for reverse transcriptase activity in the supernatant of cultures in liquid medium was positive after 8 days for cells exposed to virus.

Kitten fibroblasts demonstrated no evidence of transformation in response to infection with myc-FeLV (Table 1). Mock-infected, FeLV-infected, and myc-FeLV(FeLV)-infected kitten fibroblasts continued to grow in liquid medium as monolayers of strongly adherent cells with typically fibroblastic morphology for 12 weeks, after which their growth declined (Table 1). The cells failed to form colonies in semisolid medium and failed to proliferate when the serum concentration in liquid medium was decreased from 10 to 0.5% (Table 1). That these cells were indeed infected is demonstrated by the presence of reverse transcriptase activity in the culture supernatant and by Northern blot analysis of virion RNA. Virion RNA samples prepared from the supernatants of kitten fibroblasts exposed to either FeLV or myc-FeLV(FeLV) were shown to contain the 8.2-kb genome of FeLV, but did not contain the 5.0-kb genome of myc-FeLV (Fig. 2A, lanes d to f). Identical analysis with a probe representing the 3′ exon of the feline c-myc gene (31) also failed to demonstrate the myc-FeLV genome (data not shown). As was the case with leukocytes infected with myc-FeLV(FeLV), the myc-FeLV genome is apparently lost in the absence of the selective pressure of transformation.

In contrast to the results of infection of kitten fibroblasts, the growth patterns of embryonic fibroblasts were dramatically altered by infection in vitro with myc-FeLV(FeLV) compared with growth of identical cells which were mock infected or infected with FeLV (Table 1). Shortly after infection, the capacity of mock-infected and infected embryonic fibroblasts to proliferate when seeded at low density was examined as a parameter of transformation in vitro. Cells were seeded in four 9-cm dishes at a density of 4.5 × 10^4 per dish and were allowed to grow in liquid medium for 4 weeks, with weekly replenishment of the medium. After that time, it was evident by visual examination that mock-infected and FeLV-infected fibroblasts had proliferated only to a limited extent. In fact, direct cell counts demonstrated that these cells had undergone only two or three cell divisions during the experiment (data not shown). In contrast, cells infected with myc-FeLV(FeLV) had formed a nearly

| TABLE 1. Parameters of transformation measured in infected and mock-infected early-passage feline cells |
| --- | --- | --- | --- | --- |
| Cell type | Weeks of survival | Growth in soft agar | Growth without exogenous factors | Tumorigenicity |
| KS(C) | 11 | – | – | ND |
| KS(F) | 11 | – | – | ND |
| KS(mF) | 17 | – | – | ND |
| KT(C) | 12 | – | – | ND |
| KT(F) | 34+ | – | – | ND |
| KT(mF) | 12 | – | – | ND |
| KFi(C) | 12 | – | – | ND |
| KFi(F) | 12 | – | – | ND |
| KFi(mF) | 12 | – | – | ND |
| EF(C) | 15 | – | – | ND |
| EF(F) | 15 | – | – | ND |
| EF(mF) | >52 | + | – | – |

* KS, Kitten splenocytes; KT, kitten thymocytes; KF, kitten fibroblasts; EF, embryonic fibroblasts; (C), mock-infected controls; (F), infected with FeLV alone; (mF), infected with myc-FeLV(FeLV).

* Weeks of survival in vitro after removal from the animal as measured by trypan blue exclusion.

* Leukocytes were seeded in 0.3% low-melting-point agarose in cRPMI with IL-2 at densities of 1 × 10^6, 5 × 10^5, and 2.5 × 10^5 cells per 35-mm well in duplicate. Fibroblasts were seeded in 0.3% low-melting-point agarose in cRPMI at densities of 1 × 10^6 and 5 × 10^5 cells per 35-mm well in duplicate. All plates were observed for 3 weeks for colony formation.

* Leukocytes were tested for the ability to grow independently of exogenous IL-2 by being seeded at 2 × 10^6 cells in 5 ml of cRPMI in duplicate and counting the total number of cells on alternate days for 2 weeks. Fibroblasts were tested for the ability to grow in IL-2 with reduced serum by being plated at a density of 10^6 cells per ml in duplicate in medium containing 0.5% serum. Cells were observed for the ability to proliferate in this medium through at least two passages.

* We tested KT(F) and EF(mF) cells for tumorigenicity by injecting each of seven athymic (nu/nu) mice with 5 × 10^6 cells subcutaneously. Mice were observed for the formation of tumors for 8 weeks or 12 weeks, respectively. ND, Not done.

* This is the result of EF(mF) cells grown in the presence of 1.0% serum. EF(mF) cells were not able to survive beyond one passage in 0.5% serum. Other fibroblasts were not tested in 1.0% serum.
confluent monolayer. The myc-FeLV-infected cells which had grown from low-density seeding were expanded and compared in further analyses with mock-infected and FeLV-infected embryonic fibroblasts.

The proliferative potential of the embryonic fibroblasts infected with myc-FeLV(FeLV) was greatly expanded, if not infinite, since they have been maintained to date for more than 1 year as actively dividing cells in monolayer culture (Table 1). They were morphologically distinguishable from mock-infected or FeLV-infected controls, appearing typically as blunted or broadly scalloped bipolar cells (Fig. 3A to C). Multinucleate cells appeared frequently in the culture (Fig. 3D). The cells were loosely adherent, as is apparent by the presence of numerous highly refractile, rounded cells in the culture (Fig. 3C). Because they were loosely adherent, the monolayer failed to reach a confluent density.

The capacity of mock-infected and infected embryonic fibroblasts to proliferate in medium containing reduced serum was examined as a measure of their release from the requirement for exogenously supplied growth factors. Mock-infected cells failed to grow when plated at a density of 10⁶ cells per ml in medium with the serum concentration reduced from 10 to 0.5%, as did cells infected with either FeLV or myc-FeLV(FeLV) (Table 1). Embryonic fibroblasts infected with myc-FeLV(FeLV) declined rapidly in this medium and failed to survive one passage. They were capable of continued proliferation in the presence of 1.0% serum, however, and demonstrated typical morphology in this medium.

Embryonic fibroblasts appear to have been only partially transformed by infection with myc-FeLV(FeLV), as determined by several parameters. Although the cells were only loosely adherent to the substrate, proliferation remained anchoragedependent. The cells which were released into the medium from the adherent monolayer failed to proliferate as nonadherent cells (data not shown). Further, the cells did not form colonies when plated in semisolid medium (Table 1). These cells did not form tumors when inoculated subcutaneously into athymic mice (Table 1).

Integrated myc-FeLV provirus is readily detectable and is expressed in partially transformed embryonic fibroblasts. It is essential in these studies to document the presence and expression of myc-FeLV in transformed cells to whose establishment its activity is attributed. Southern blot analysis of DNA from partially transformed embryonic fibroblasts was performed and compared with that of mock-infected or FeLV-infected cells (Fig. 4A and B). Hybridization to a probe representing the U3 region of the FeLV long-terminal repeat (LTR) (31) to KpnI-digested DNA demonstrated the presence of the internal 3.7-kb KpnI fragment of integrated FeLV proviral DNA in infected cells. The 5.5-kb KpnI fragment derived from the integrated provirus of myc-FeLV was observed only in the cells into which it was introduced.

FIG. 2. (A) Northern blot analysis of virion RNA prepared from culture supernatants of uninfected cells (lanes a, d, and g), or of cells infected in vitro with FeLV (lanes b, e, and j) or with myc-FeLV(FeLV) (lanes c, f, i, and k). Early-passage feline cells examined in this study included embryonic fibroblasts (lanes a to c), neonatal fibroblasts (lanes d to f), neonatal thymocytes (lanes g to i), and neonatal splenocytes (lanes j and k). The blot was hybridized to a radiolabeled probe representing the U3 region of the FeLV LTR (31). The RNA species indicated measure 8.2 kb in length, compared to the parallel migration of standards, and represent the genome-length viral RNA of FeLV. (B) Southern blot analysis of DNA from mock-infected neonatal splenocytes (lane a), those cells after infection in vitro with myc-FeLV(FeLV) (lane b), mock-infected neonatal thymocytes (lane c), or those cells after infection in vitro with FeLV alone (lane d) or with myc-FeLV(FeLV) (lane e). Indicated at 10 kbp is a band representing the feline c-myc gene. (C) Southern blot analysis of DNA from mock-infected neonatal splenocytes (lane a), those cells infected in vitro with FeLV alone (lane b) or with myc-FeLV(FeLV) (lane c), or from mock-infected thymocytes (lane d), those cells infected in vitro with FeLV alone (lane e), or with myc-FeLV(FeLV) (lane f). Indicated (*) is an internal fragment of FeLV proviral DNA homologous to the probe. For Southern blots, DNA samples (10 µg) were digested with KpnI, electrophoresed in an agarose gel, and transferred to nitrocellulose. Blots were hybridized to radiolabeled probes representing avian v-myc (50) (panel B) or the U3 region of the FeLV LTR (31) (panel C). For Southern and Northern blot analyses, molecular sizes were determined by comparison with the migration of HindIII-digested lambda DNA and HaeIII-digested φX174 DNA electrophoresed in parallel. For Northern blot analysis, molecular size markers were denatured, as were RNA samples before electrophoresis.
FIG. 3. Photomicrographs of embryonic feline fibroblasts which were mock infected (A) or infected in vitro with FeLV (B) or myc-FeLV (FeLV) (C and D). Magnification (panels A, C, and D), ×200; magnification (panel B), ×100.

(Fig. 4A). The presence of discrete host-virus junction fragments in the DNA of embryonic fibroblasts (Fig. 4A, lane c) indicated that a clonal subpopulation predominates in this line. Identical analysis with a probe representing avian v-myc (50) clearly demonstrated the integrated myc-FeLV provirus in the DNA of partially transformed embryonic fibroblasts (Fig. 4B).

Northern blot analysis of the viral RNA species extracted from virions in the culture supernatant of partially transformed embryonic fibroblasts demonstrated that the myc-FeLV genome was both expressed and encapsidated (Fig. 4C). Use in this analysis of the U3 region of the LTR (31) as probe demonstrated the presence of two species of RNA, the smaller of which was of the size predicted for the myc-FeLV genome (Fig. 4C, lane b). Hybridization to a probe representing feline c-myc (31) confirmed the identity of this species as myc-FeLV (Fig. 4C, lane a). Densitometric scanning of the data shown in Fig. 4C, lane b, indicated that the genomic RNA of myc-FeLV represents 14% of the total population in the culture supernatant.

**DISCUSSION**

myc-FeLV was originally isolated as an integrated provirus molecularly cloned from the DNA of a naturally occurring thymic lymphosarcoma of a domestic cat (31). We report here the rescue in canine cells, in the presence of FeLV as helper, of horizontally infectious particles containing myc-FeLV. This is significant because it demonstrates that myc-FeLV proviral DNA, originally isolated from a feline tumor, contains all the regulatory sequences necessary for its expression and packaging. Such functions cannot be contributed by the helper virus, and the finding that myc-FeLV is transcriptionally active and can be encapsidated intensifies our interest in its biological activity.
In the culture supernatant of producer D-17 cells, the encapsidated myc-FeLV genome represented only 2% of the total population, and 16% of a selected subpopulation, even though the myc-FeLV proviral DNA was originally transfected into recipient cells in a 20-fold mass excess over FeLV proviral DNA. This may result from less efficient transcription of myc-FeLV proviral DNA in recipient cells or from less efficient encapsidation of the myc-FeLV genomic RNA relative to that of FeLV. These possibilities can be distinguished by examining the expression of myc-FeLV in the cells which represent the original recipients of transfection; however, those cells were not clonally isolated in the present study. The population of D-17 cells described here as productively infected represents cells that were maintained in the same culture dish as the recipients of transfection and that were horizontally infected by the products of those cells. By comparison, the encapsidated myc-FeLV genome represented 14% of the total population in the culture supernatant of feline embryonic fibroblasts partially transformed by infection with an inoculum derived from the supernatant of productively infected D-17 cells. Since the inoculum contained no more than 16% myc-FeLV, the infected feline fibroblasts can be expected to contain myc-FeLV proviral DNA which represents no more than 16% of the total integrated proviral DNA. That viral RNA isolated from the supernatant of these cells contains 14% myc-FeLV by mass indicates that myc-FeLV is efficiently expressed and encapsidated in feline embryonic fibroblasts.

We initially investigated the transforming potential of myc-FeLV in leukocytes derived from peripheral blood, spleen, and thymus whose proliferation in vitro was influenced by the addition of ConA and IL-2 to the culture medium, i.e., T lymphocytes. The choice of these cells was based on (i) the observation that myc-FeLV was isolated originally from a thymic lymphosarcoma (31), (ii) our preliminary evidence of a causative association between infection in vivo with myc-FeLV(FeLV) and the relatively rapid appearance of thymic lymphosarcoma (L. S. Levy, R. Fish, and G. Baskin, unpublished observations), and (iii) the observation that FeLV induces lymphosarcomas in vivo which are primarily of T-lymphoid origin (25). We wished to determine initially the transforming potential of myc-FeLV in leukocytes cultured under the described conditions in vitro.

The results indicate that following infection with myc-FeLV(FeLV), the growth parameters of leukocytes from peripheral blood, spleen, or thymus are not altered in a significant way from those of identical cells which were mock infected. This is an intriguing finding, since preliminary data indicate that myc-FeLV is capable of transforming T lymphocytes in vivo. In studies to date, three of seven kittens inoculated with myc-FeLV(FeLV) developed thymic lymphosarcoma at 14 to 20 weeks after inoculation. Littermates inoculated with helper FeLV alone developed no disease after as long as 10 months. DNA isolated from each tumor contains integrated myc-FeLV proviral DNA (Levy et al., unpublished observations). It is possible that the circumstances under which myc-FeLV is capable of transforming T lymphocytes in vitro have not yet been identified.

The transforming potential of myc-FeLV was also examined in early-passage feline fibroblasts prepared from the tissues of a newborn kitten and of mid-gestational embryos. Infection with myc-FeLV(FeLV) failed to alter the growth patterns of kitten fibroblasts in vitro compared with those of identical cells which were mock infected or infected with FeLV alone (Table 1). In contrast, embryonic fibroblasts infected in vitro with myc-FeLV(FeLV) demonstrated dramatic alterations in growth patterns compared with identical cells which were mock infected or infected with FeLV alone. The reason for the difference in embryonic fibroblasts and neonatal fibroblasts with respect to the interaction with the feline v-myc gene is as yet unknown. Embryonic fibroblasts infected with myc-FeLV(FeLV), selected on the basis of their potential to proliferate when seeded at low density, are morphologically distinctive, rescued from senescence, and loosely adherent and have a reduced requirement for serum in the culture medium (Table 1). Embryonic fibroblasts are not fully transformed by infection with myc-FeLV(FeLV), however, since the cells remain anchorage dependent for proliferation and do not form tumors when inoculated subcutaneously into athymic mice (Table 1). The evidence that
these cells do not represent spontaneous transformants includes the following observations: (i) mock-infected embryonic fibroblasts and identical cells infected with FeLV alone exhibited no such alteration in phenotype (Table 1), and (ii) in cells which were infected with myc-FeLV(FeLV) but not transformed, the myc-FeLV component was apparently lost from the population (Fig. 2). In contrast, the immortalized embryonic fibroblast lines contain and express retrovirally transduced mammalian v-myc genes, all of which have been identified in derivatives of FeLV (31, 35, 38). It is useful to compare the biological activity of this v-myc gene with that of the avian v-myc genes and of the other mammalian v-myc genes identified to date. Like myc-FeLV, the avian myc-containing viruses exhibit a transforming potential in vitro in fibroblasts which appears to be insufficient for expression of the fully transformed phenotype. Chicken fibroblasts transformed by MC29 exhibit a dramatically increased growth rate, morphological alteration, and the capacity for anchorage-independent growth in semisolid medium; however, the cells are not tumorigenic, as determined by inoculation in syngeneic chickens (22, 43). MC29 and its relatives, CMII, OK10, and MH2, transform quail fibroblasts morphologically and render them loosely adherent, capable of anchorage-independent growth, and capable of increased, if not infinite, proliferation (16, 21, 33, 39). The cells fail to exhibit a fully transformed phenotype, however, in that they are not tumorigenic when inoculated into athymic mice (39). Recombinant murine retroviruses constructed to contain the v-myc gene of MC29 (12) or of OK10 (49) retain the capacity to alter the morphology of early-passage murine fibroblasts and to render them capable of unlimited proliferation and anchorage-independent growth. These activities parallel those of myc-FeLV in embryonic feline fibroblasts, except that the partially transformed feline cells fail to form colonies in semisolid medium. They are distinctly less adherent to the substrate, however, and fail to reach confluence as a monolayer for this reason.

Although little information is available about the biological activity of other naturally occurring mammalian v-myc genes, myc-FeLV appears to be distinct from other isolates examined which fail to transform fibroblastic cells in vitro (38, 45). Assays with other isolates were performed in established fibroblastic lines, however, and it is not clear whether other myc-containing variants of FeLV are capable of transforming early-passage feline fibroblasts in vitro. In fact, myc-FeLV has no effect on established murine fibroblasts when introduced by DNA-mediated transfection (31). myc-FeLV can be compared in coding content to CT4, another myc-containing variant of FeLV also isolated from a naturally occurring feline thymic lymphosarcoma (38, 45). The v-myc genes of both of these viruses contain the entire coding sequence from exons 2 and 3 of c-myc and only a short remnant of exon 1 (11, 45). The v-myc protein encoded by myc-FeLV is identical to that encoded by feline c-myc, except that v-myc sequences are expressed as a gag-myc fusion product (11, 45). The v-myc coding sequence of CT4 differs from c-myc at three coding positions and is probably expressed independently of FeLV gag (45). Continuing investigation of the structure and biological activity of the naturally occurring mammalian v-myc genes is important to evaluate both the function of the myc oncogene and the role it may play in virally induced oncogenesis in mammals.

ACKNOWLEDGMENTS

This work was supported by grant MV-241 from the American Cancer Society to L.S.L.

We acknowledge the excellent technical assistance of Sue Ann Sullivan.

LITERATURE CITED


