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Sequence Arrangement and Biological Activity of Cloned Feline Leukemia Virus Proviruses from a Virus-Productive Human Cell Line

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We examined 14 different feline leukemia virus proviruses from the productively infected human cell line RD(FeLV)-2 after cloning in the modified lambda vector Charon 4A. Each isolate was characterized by restriction digestion and Southern blot analysis. The DNA of each isolate was tested for competence to express virus after uptake by sensitive animal cells (transfection). All but one isolate contained an apparently complete provirus, but only four were infectious. Seven isolates (four noninfectious, three infectious) were studied by heteroduplexing followed by electron microscopy or by S1 nuclease treatment and gel electrophoresis. No regions of nonhomology between proviruses were detected by either criterion, and in no case did we observe homology between flanking sequences. Random shearing or removal of flanking sequences by S1 nuclease had no effect on the status of infectivity of the clones. Thus, we were unable to find molecular differences between infectious and noninfectious proviruses. Our data are consistent with either of the following hypotheses: (i) that there is a short host sequence which is essential as a promoter for virus expression; or (ii) that lack of infectivity is due to small mutations within the proviral genome.

Upon infection, the single-stranded RNA genome of a retrovirus is copied by reverse transcription into an unintegrated linear double-stranded DNA copy (47). The latter contains all of the sequences of the RNA in a nonpermuted order. Furthermore, long terminal repeats (LTRs), which are made up of sequences derived from both the 5′ end and the 3′ end of the viral RNA, are found at both ends. Subsequently, one or several copies of the viral genome become integrated into the chromosomal DNA of the host cell (provirus formation). In several cases where a number of independently infected cell lines have been studied, no evidence for preferred integration sites has been found (47).

In virus-productive cells, at least one provirus is actively transcribed, resulting ultimately in the release of virus by the cells. For cases where multiple integration has occurred, it is not known how many of the integrated viral genomes are actually transcribed.

When sensitive cells are exposed under the appropriate conditions to chromosomal DNA from cells expressing viral information, some proviral DNA is taken up; if expressed, this proviral DNA is referred to as infectious (6, 17, 19, 30). This process is called transfection. In some cases, virus expression results in horizontal spread of the virus through the culture by the normal route of infection, so that many of the cells acquire integrated viral genomes and become virus productive. In general, it has been observed that the DNA from cells which produce a high titer of virus is infectious, whereas the DNA from cells which produce a low titer of virus or no detectable virus is not infectious (7, 28, 31).

In some cases, cells in the latter category contain complete viral information, as shown by the low level of virus expression which occurs or can be induced by treating the cells with the halogenated pyrimidines bromodeoxyuridine and iododeoxyuridine (7, 38). These observations support the hypothesis that an integrated viral genome in a configuration in which it is being expressed at a relatively high rate is infectious, whereas integrated viral genomes which are not being expressed at a high rate are not. At present however, the molecular nature of the difference or differences between integrated viral genomes that are infectious and those that are not is not known.

Feline leukemia virus (FeLV) is a particularly interesting retrovirus because it is an infectious

agent which causes leukemia in cats in nature. About one-third of the naturally occurring lymphoid tumors in domestic cats do not contain infectious FeLV or viral structural proteins (13, 22, 43), yet epidemiological evidence suggests that virus-negative tumors are associated with exposure to FeLV (16a; D. P. Francis, M. Essex, S. M. Cotter, N. Gutensohn, R. Jakowski, and W. D. Hardy, Jr., Cancer Lett., in press). It is not known whether the DNAs of virus-negative tumors possess complete viral information. Furthermore, the genomes of healthy domestic cats that are believed to be uninfected contain FeLV cross-hybridizing sequences. There is evidence that some portion of the infectious genome is not present in these endogenous sequences (32).

Several natural FeLV isolates have been described, and these fall into three subgroups (subgroups A, B, and C), based on viral interference (35, 36). Restriction maps have been deduced for two subgroup B isolates, namely, Snyder-Theilen FeLV (ST-FeLV) (mapped by Sherr and co-workers [42]) and Gardner-Arnstein FeLV (GA-FeLV) (mapped in our laboratory [29]). There is significant homology between these two isolates, as determined by a Southern blot analysis (Mullins, unpublished data) and by a comparison of restriction maps. For the 10 enzymes common to both studies, 12 sites map to comparable regions of the two genomes, whereas 10 sites are unique to the ST-FeLV-B genome and 1 site is unique to the GA-FeLV-B genome. Also notable is the fact that although the overall length of the ST-FeLV-B genome is about 0.2 kilobase (kb) shorter than the GA-FeLV-B genome, the ST-FeLV-B LTRs are about 0.2 kb longer and contain *Eco*RI sites which are absent in GA-FeLV-B DNA.

In a previous study, we presented a restriction endonuclease map of the unintegrated linear viral DNA isolated from human cells (RD) freshly infected with GA-FeLV (29). RD(FeLV)-2 is a cloned, virus-productive cell line derived from such an infection. It contains approximately 8 to 10 copies of integrated FeLV per haploid genome, as estimated by blot hybridization intensity (25; Mullins, unpublished data). This corresponds to 16 to 20 different viral integrations per cell, since Southern blotting data has demonstrated that proviruses are not detected in each homologous chromosomal site in these cells (Mullins, unpublished data). The restriction digestion patterns of these integrated viral genomes were also characterized in a previous study (29).

Some of our long-term goals are to determine whether there are acquired FeLV sequences in virus-negative leukemic cats and to characterize and compare such sequences with the acquired sequences in virus-productive leukemic cats and with the endogenous FeLV sequences in all cats. Another goal is to determine the molecular nature of the differences between integrated viral genomes that are infectious and those that are not. As a first step, we have cloned and carried out an initial characterization of some of the integrated viral genomes of the RD(FeLV)-2 cell line.

MATERIALS AND METHODS

RD(FeLV)-2 cells. The RD(FeLV)-2 cell line was derived by soft agar cloning of RD cells 40 passages after mass infection with GA-FeLV-B. DNA from passage 14 after cell cloning was used in subsequent recombinant DNA experiments.

Preparations of RD(FeLV)-2 and Charon 4A DNAs for cloning. RD(FeLV)-2 DNA was purified as previously described (29). *Eco*RI digestion and sucrose gradient fractionation were performed by the procedures described by Maniatis et al. (26). Fractions containing 8- to 25-kb DNA were identified by gel electrophoresis, pooled, and concentrated by ethanol precipitation.

Charon 4A was propagated on KH802 cells (2) by subconfluent plate lysis in Pyrex dishes (18 by 30 cm); 10^7 PFU of Charon 4A and 10^{10} KH802 cells were incubated in 1 ml of SM (50 mM Tris-hydrochloride, pH 7.5, 0.1 M sodium chloride, 5 mM magnesium sulfate, 0.01% gelatin) at 37°C for 15 min and then plated along with a 25-ml top layer of 3X medium (3% NZ amine [Humko-Sheffield], 0.3% Casamino Acids, 0.5% sodium chloride, 1.5% yeast extract, 0.004% thymidine, 0.01% diaminopimelic acid, 0.12% magnesium sulfate) containing 0.6% agar. The bottom layer consisted of 250 ml of 3X medium containing 1.5% agar. After 14 to 18 h at 37°C, top layers were scraped off and extracted with 25 ml of SM containing 1% chloroform. Agar was removed by centrifugation at 7,000 × *g* for 10 min. Phage and phage DNA were purified by the methods of Tiemeier et al. (45). The yields of phage DNA grown by this method ranged between 150 and 600 μg/dish.

*Eco*RI-digested phage arms were prepared and ligation of phage arms and size-selected RD(FeLV)-2 DNA was carried out by the procedures described by Maniatis et al. (26).

Preparation of in vitro packaging extracts. We used the lysogenic bacterial strains NS428 and NS433 (44) and a modification of the procedures of Hohn and Murray (19) and Collins and Hohn (4) to prepare in vitro packaging extracts, as described below.

The presence of heat-inducible lysogens was verified by plating onto M9 medium plates (44) at 42°C (no growth) and 32°C. Overnight cultures were begun from colonies grown at 32°C; subsequently, 500-ml cultures were inoculated with 5 ml of a stationary-phase culture and grown with shaking at 32°C until an absorbance at 600 nm of 0.3 to 0.4 was reached. Lysogens were then induced by placing cultures in a 45°C water bath for 15 min and swirling occasionally. Induced cultures were grown at 37°C for an additional

2 h with vigorous aeration. Cultures were then mixed at a ratio of NS433 to NS428 of 2:1 and chilled in an ice water bath, and the cells were pelleted by centrifugation at 7,000 × *g* for 10 min at 4°C. The cells were then suspended in 0.2 volume of M9 medium without Casamino Acids and irradiated as described by Maniatis et al. (26). The cells were then pelleted as described above, the supernatant was removed by decantation, and the remaining liquid was removed with a pipette. Subsequent steps were carried out quickly at 4°C. 0.004 volume of packaging buffer (40 mM Tris-hydrochloride, pH 8.0, 10 mM spermidine hydrochloride, 10 mM putrescine hydrochloride, 0.1% β-mercaptoethanol, 7.0% dimethyl sulfoxide, 1.5 mM ATP) was stirred into the cell pellet thoroughly, and 50-μl portions were placed into 1.5-ml snap cap polypropylene tubes and immediately frozen in liquid nitrogen. Extracts can be stored at −70 to −80°C for at least 1 year without loss of activity but cannot be allowed to thaw before use.

In experiments conducted since the 1980 change in the National Institutes of Health guidelines, the UV irradiation step was omitted. However, background phage due to packaging of lysogens was effectively zero if the in vitro-packaged phage were grown initially on a Sup III° host, such as KH802.

Packaging reaction. Extracts were placed in an ice water bath for about 2 min. While thawing, 6 μl of a solution containing 20 mM ATP and DNA (in ligation buffer) was added and stirred in well, and the mixture was incubated at 37°C for 60 min. To a second extract 5 μl of DNase I (100 μg/ml; Worthington Diagnostics) in 0.25 M magnesium chloride was added and stirred in well, and the mixture was left on ice for 5 min; 20 μl of the DNase-treated extract was added to the reaction and mixed in well, and this mixture was incubated at 37°C for 30 min. At the end of the reaction, 0.5 ml of SM was added, followed by the addition of 2 drops of chloroform. The reaction mixture was stirred, centrifuged for 10 s, and finally stored at 4°C. When preparations were stored in this way, phage titers decreased roughly about 1 order of magnitude during the first 1 year of storage.

It has been found (D. Goldberg and E. Fritsch, personal communication) that higher packaging efficiencies could be obtained for some preparations in more dilute reactions. We now test each preparation of extracts for optimal efficiency with between 0 and 30 μl of additional packaging buffer. In our hands, packaging efficiencies range between 0.7×10^6 and 1×10^7 PFU/μg of inserted DNA. Efficiencies for uncut vector DNAs are usually about 10-fold higher.

Library amplification and screening. All recombinant phage were grown on DP50 SupF cells. Amplification was performed as described by Maniatis et al. (26), using 3X medium plates.

Screening was conducted by in situ plaque hybridization on plates containing NZCY/DTM medium (1% NZ amine, 0.5% sodium chloride, 0.5% yeast extract, 0.1% Casamino Acids, 0.01% diaminopimelic acid, 0.004% thymidine, 1.2% magnesium sulfate) (1). Absorption of phage and phage DNA to nitrocellulose filters was conducted as described by Maniatis et al. (26). Subsequently, filters were dipped into 0.5 M sodium hydroxide–0.8 M sodium chloride for 30 to 60 s and neutralized by dipping into two changes of 0.5 M Tris-hydrochloride (pH 7.6)–1.5 M sodium chloride (60 s each). After air drying, filters were baked in vacuo at 70°C for 2 to 4 h. Filters were washed with mild rubbing in three or four 500-ml changes of 4× SET (1× SET is 0.15 M sodium chloride, 0.03 M Tris-hydrochloride [pH 8], 2 mM EDTA) at room temperature and then equilibrated in 4× SET containing 50% formamide. The procedures used for pre-hybridization, hybridization (with 10% dextran sulfate; ^{32}P-labeled FeLV complementary DNA probe concentration, 10^6 cpm/ml or 1.2 ng/ml), washing, and exposure to X-ray film have been described previously (29). FeLV-containing phage were purified by rescreening (26) once or twice and subsequent analysis of individual plaques (see below).

Growth and analysis of recombinant DNA clones. Phage stocks of purified recombinant phage (1×10^{10} to 7×10^{10} PFU/ml) were each obtained by inoculating 100 μl of a stationary-phase culture of DP50 Sup F with a phage plug, diluting into 1 ml of fresh medium, and growing overnight at 37°C. Chloroform was added, cell debris was removed by centrifugation, and the stock was stored over chloroform at 4°C.

DNA from each isolate was obtained initially by a slight modification of the miniplate lysis procedure of Davis and co-workers (9). In this procedure, 10^5 PFU of Charon 4A and 10^8 DP50 SupF cells were mixed and incubated at 37°C for 15 min and then plated onto 100-mm NZCY/DTM medium plates, using 0.6% top layer agarose and 1.2% bottom layer agarose to replace the agar. When nearly confluent lysis was achieved (10 to 12 h), the plates were cooled to 4°C for 0.5 hour; 5 ml of chloroform-saturated 10 mM Tris (pH 7.6)–2 mM magnesium chloride was added to cover the top layer, and the plates were stored at 4°C overnight. A 4-ml portion of eluate was added to a solution containing 0.4 ml of 0.5 M EDTA (pH 8.5), 0.2 ml of 2 M Tris (pH 9.7), and 0.2 ml of 10% sodium dodecyl sulfate, and the mixture was blended briefly with a Vortex mixer. A 10-μl portion of diethyl oxydiformate was added, and tubes were incubated at 65°C for 30 min with occasional swirling. The tubes were then chilled on ice, 1 ml of 5 M potassium acetate was added and mixed in, and the mixtures were allowed to stand on ice for 1 h. Precipitates were removed by centrifugation at 20,000 × *g* for 20 min, and the supernatants were decanted carefully into centrifuge tubes containing 10.5 ml of cold 95% ethanol. Tubes were chilled at −20 or −70°C, and precipitates were recovered by centrifugation at 20,000 × *g* for 30 min. Pellets were dried and then suspended and stored frozen in 200 μl of TLE (10 mM Tris, pH 7.8, 0.1 mM EDTA); 5 to 15 μl was used per restriction digest, along with about 2 to 5 U of enzyme.

Large-scale preparations of phage and phage DNA were made by the procedures described above for Charon 4A. Some phage were also grown by the liquid culture procedure described by Blattner et al. (2).

Restriction endonuclease analysis. The procedures used for restriction digestion, gel electrophoresis, Southern blotting, FeLV complementary DNA synthesis, and hybridization were as previously described (29). Nick-translated probes were prepared by a mod-

ification of the procedure of Maniatis et al. (27). Each reaction mixture contained 10 μg of DNA per ml, 5 μM [^{32}P]dCTP (410 Ci/mmol; Amersham), 10 μm dATP, 10 μM dGTP, 10 μM TTP, 1 ng of DNase I (Boehringer Mannheim Corp.) per ml, and 20 U of DNA polymerase I (Boehringer Mannheim) per ml in a solution containing 50 mM Tris-hydrochloride (pH 7.8), 5 mM magnesium chloride, 5 mM dithiothreitol, 50 μg of bovine serum albumin (Pentex, Miles Biochemical) per ml, and 5% glycerol. The reaction mixture was cooled on ice before the addition of enzymes and then incubated at 15°C for 1 to 2 h; 1 volume of column elution buffer (50 mM Tris-hydrochloride, pH 7.5, 250 mM sodium chloride, 0.2% sodium dodecyl sulfate 2 mM EDTA) was added, and unincorporated nucleotides were removed by chromatography on Sephadex G-50 (fine). All hybridizations were done for 17 to 20 h; we used 5×10^4 dpm (0.06 ng) of ^{32}P-labeled FeLV complementary cDNA per ml for restriction mapping, 1×10^6 dpm (1.2 ng) of ^{32}P-labeled FeLV complementary DNA (10% dextran sulfate) per ml DNA for analysis of S1-treated heteroduplexes, 6×10^5 dpm (20 ng) of ^{32}P-labeled Alu family DNA per ml, and 2.5×10^6 dpm (50 ng) of ^{32}P-labeled RD DNA per ml as probes.

Electron microscopy. Heteroduplexes were constructed and spread for electron microscopy by using purified phage, as described by Davis et al. (8). Molecules were examined with a Philips EM 300 electron microscope and photographed with 35-mm film. Negatives were projected onto a table, and images were measured with a Hewlett-Packard digitizer.

Biological analysis. The procedures used for transfection and reverse transcriptase assay have been described previously (30). Complement fixation assays (14) were conducted in the laboratory of R. V. Gilden, Frederick Cancer Research Center. Virus interference assays (21) were conducted in the laboratory of O. Jarrett, University of Glasgow.

S1 nuclease digestion of heteroduplexes. Two λHF clone DNAs (2 μg of each) were mixed and denatured in 60% formamide in TLE at 50°C for 5 min in a volume of 45 μl. The reaction mixture was adjusted to 0.4 M sodium chloride, 50 mM Tris-hydrochloride (pH 8.1), and 2 mM EDTA in a total volume of 50 μl and then incubated at 37°C for 10 min; 1 ml of NTE (0.1 M sodium chloride, 10 mM Tris-hydrochloride pH 7.8, 1 mM EDTA) and 2.8 ml of 95% ethanol were added, and the mixture was chilled at −20°C. Precipitates were collected by centrifugation at 10,000 × *g* for 5 min.

S1 nuclease digestion was performed by a procedure similar to procedures described previously (40, 41). Ethanol precipitates were dissolved in 35 μl of 30 mM Tris-acetate (pH 4.5)–200 mM sodium chloride–3 mM zinc sulfate and digested with 50 U of S1 nuclease (Boehringer Mannheim) at 25°C for 15 min. The amount of S1 nuclease and the conditions of digestion used were such that a similar quantity of closed circular φX174 DNA was converted into a 1:1 mixture of nicked circular and linear molecules. EDTA was then added (final concentration, 10 mM), and samples were loaded directly onto gels; 8.7 kb of S1 nuclease-treated heteroduplex DNA was purified from the gels, as described previously (3).

RESULTS

Cloning integrated FeLV DNA in Charon 4A. The GA-FeLV genome contains no *Eco*RI endonuclease sites. Electrophoretic fractionation of *Eco*RI digests of RD(FeLV)-2 DNA has shown FeLV hybridizing sequences present as a number of discrete bands in the 8- to 25-kb range (29). As Table 1 shows, RD(FeLV)-2 chromosomal DNA was infectious by transfection, and *Eco*RI digestion did not alter this infectivity. The 8- to 25-kb fraction isolated by sucrose gradient fractionation was infectious at the same level as the undigested DNA.

The cloning capacity of the modified lambda cloning vector Charon 4A is approximately 8 to 22 kb (2). Therefore, we cloned *Eco*RI-digested and size-fractionated RD(FeLV)-2 DNA into the purified end fragments of *Eco*RI-digested Charon 4A DNA by ligation and in vitro packaging, as described above. A total of 3×10^5 recombinant phage were amplified by subconfluent plate lysis, replated, and screened with an FeLV probe. Another 8×10^4 phage were screened without prior amplification to insure that the cloned FeLV isolates were independently ligated and packaged before growth in *Escherichia coli*. A total of 60 FeLV-positive clones were picked from the amplified phage preparation (clones λHF1 through λHF60), and 12 more were picked from the unamplified preparation (clones λHF61 through λHF72). We purified and studied the DNA from 27 clones by restriction mapping, Southern blotting, and transfection.

The restriction map of the proviral genome is shown in Fig. 1. There was a single *Sma*I site within each LTR, and there were three additional *Sma*I sites in the interior of the genome. These sites defined four electrophoretic bands that were within viral sequences and independ-

TABLE 1. *Transfection with RD(FeLV)-2 DNA*

Sample	Treatment	Infectivity[a]
RD(FeLV)-2	None	+[b]
RD(FeLV)-2	*Eco*RI digestion	+
RD(FeLV)-2 (8 to 25 kb)	*Eco*RI digestion and sucrose gradient fractionation	+

[a] Assays for virus infectivity are described in Table 3, footnotes. Dog D-17 cells were used to test all DNA samples. Positive cultures (those producing extracellular reverse transcriptase [30]) were assayed by the complement fixation test and were found to contain FeLV p30. Virus from positive cultures was used to infect cat embryo AH927 cells and was found to contain FeLV subgroup B by virus interference tests (21).

[b] A 1-μg amount of each RD(FeLV)-2 DNA was needed to detect virus production.

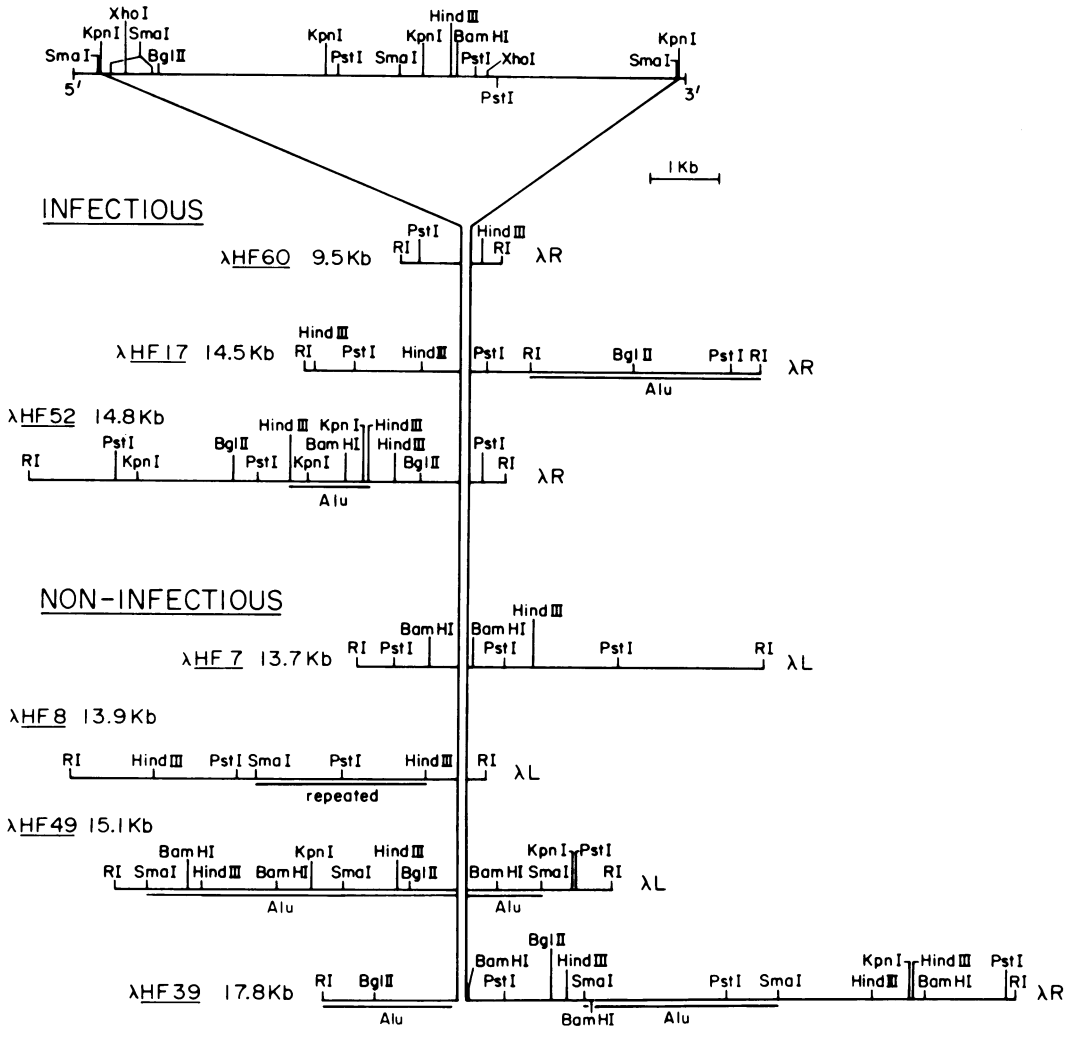

FIG. 1. *Restriction maps of EcoRI insertions in seven provirus clones. Each isolate had the same pattern of sites within the FeLV provirus region. These sites are shown at the top. The positions of the 5′ and 3′ ends of the provirus are as predicted from unintegrated linear viral DNA mapping (29). Additional sequences extending from the SmaI site at the 5′ end of the provirus and from the KpnI site at the 3′ end are indicated in the lower diagrams. λL and λR refer to the positions of the left (19.8-kb) and right (10.9-kb) vector arms, respectively, relative to the insert in each clone. Restriction sites were mapped after digestion with the enzyme indicated alone or with either XhOI or EcoRI. Other double digestions were used to map sites which could not be positioned using Xho I or EcoRI combinations. Additional PstI sites were present in the flanking sequences of some clones but were not mapped. The underlined regions in the maps of isolates λHF17, λHF52, λHF49, and λHF39 correspond to restriction fragments which hybridized to an Alu family repeated sequence probe (see Fig. 3). Alu repeats were found on three or four fragments 5′ to the FeLV provirus in λHF49; however, not all of these repeats could be assigned to a given fragment, and therefore all four fragments are underlined (see legend to Fig. 3). The underlined region in the map of isolate λHF8 corresponds to an additional repeated sequence element identified by hybridization when total RD DNA was used as the probe (see Fig. 3).*

ent of enzyme sites in flanking host sequences. All of these bands were present in all of the cloned isolates. Similarly, there were four *Kpn*I sites in the viral genome, two of which were in the LTRs. All of the cloned isolates except λHF62 contained the four *Kpn*I sites. λHF62 was missing the *Kpn*I site in the left LTR, but a new site was present at a position 200 base pairs 5′ to this position (data not shown).

By using *Eco*RI digestion, all of the cloned

inserts could be correlated with *Eco*RI bands of RD(FeLV)-2 chromosomal DNA (data not shown). By appropriate double digestions, the flanking sequences of the different isolates could be distinguished. We found that the 27 isolates studied could be classified as being derived from 14 different FeLV proviruses, each with different flanking sequences. Of the 14 proviruses, 7 were isolated one time, 4 were isolated twice, 1 was isolated three times, 1 was isolated four times, and 1 was isolated five times. In some cases of multiple isolates (Table 2), the *Eco*RI provirus fragment was present in both orientations relative to the vector arms. This, along with purification from unamplified phage libraries, was decisive proof of independent ligation and packaging.

Transfection experiments. The results of the transfection tests with intact recombinant phage DNA are shown in Tables 2 and 3. Of the 14 different proviruses, 4 gave positive results at a level of 0.1 ng of cloned DNA per dish, one gave a positive result at a level of 10 ng/dish, and 9 were negative even at 2,000 ng/dish. Chromosomal RD(FeLV)-2 DNA gave a comparable positive response at a level of about 1,000 ng/dish.

As a convenient and sensitive test for virus production, we assayed for reverse transcriptase activity in the culture fluid (30). A semiquantitative dose-response comparison of different samples was possible with the assay used (Table 3). Table 3 shows that for an infectious clone, such as λHF60, the reverse transcriptase activity at a given time after the addition of DNA increased with the amount of DNA applied per bottle of cells. The activity was much greater 7 weeks after transfection than 3 weeks after transfection. The minimum infectious dose was about 100 pg/dish for the most infectious clones. In all tests, with several different infectious clones, positive results were never obtained, even 13 weeks after transfection with 10 pg of cloned DNA per dish.

Infectivity was not diminished by *Eco*RI digestion of the cloned DNA (Table 4). The insertion from λHF60 was recloned in pBR322, and it remained infectious (data not shown). In the cases available for study, activity was independent of the orientation of the viral fragment relative to the vector arms. Identical results were obtained after transfection onto cat cells (less sensitive) and dog cells (more sensitive). Therefore, these results were not due to differences in host range. The virus obtained after transfection with each infectious clone contained FeLV p30, as determined by complement fixation, and was identified as FeLV subgroup B by virus interference tests.

TABLE 2. *Transfection with segments cloned in Charon 4A*

Provirus	Insertion length (kb)	Isolate	Orientation relative to vector	Infectivity[a]
1	9.5	λHF21	5′-3′	+[b]
		λHF25	5′-3′	+
		λHF60[c]	5′-3′	+
2[d]	11.2	λHF5	5′-3′	+
	(14.5)	λHF17[c]	5′-3′	+
3[d]	11.3	λHF70	ND[e]	−
	(16.5)			
4	13.5	λHF62	ND	−
5	13.7	λHF7	3′-5′	−
		λHF37	3′-5′	−
6	13.9	λHF8	5′-3′	−
		λHF68	3′-5′	−
7	14.8	λHF52[c]	5′-3′	+
		λHF67	3′-5′	+
8	15.1	λHF32	5′-3′	−
		λHF34	3′-5′	−
		λHF48	3′-5′	−
		λHF49	3′-5′	−
9	15.2	λHF71	ND	−
10	15.6	λHF24	ND	−
11	15.7	λHF40	ND	+
12	17.8	λHF19	5′-3′	−
		λHF26	5′-3′	−
		λHF28	5′-3′	−
		λHF39	5′-3′	−
		λHF64	3′-5′	−
13	18.2	λHF53	ND	+
14	ND	λHF31	ND	−

[a] Assays for virus infectivity are described in Table 3, footnotes. Dog D-17 cells were used to test all DNA samples. In addition, feline embryo fibroblast (AH927) cells were used to test isolates λHF60, λHF52, λHF7, λHF8, λHF49, and λHF39. For other details see Table 1, footnote *a*.

[b] For all isolates except λHF53, 100 pg of recombinant phage DNA was needed to detect virus production; for λHF53, 10 ng was needed.

[c] See Table 3 for an analysis of λHF60, λHF52, and λHF17.

[d] Proviruses 2 and 3 had more than one *Eco*RI fragment cloned into Charon 4A. The first insertion lengths (no parentheses) are the lengths of the fragments containing the FeLV genome, whereas the numbers in parentheses are the total lengths of the inserted fragments.

[e] ND, Not determined.

Restriction endonuclease analyses. Characterization of some of the cloned proviral genomes by various methods is described below; our principal goal was to find a property that correlated with positive or negative infectivity, as listed in Table 2. Detailed restriction endonuclease maps were deduced for the seven proviruses that were obtained as multiple isolates (Fig. 1 and 2). The enzymes used recognized 17 sites in the unintegrated viral DNA genome (29)

TABLE 3. *Transfection assays*[a]

Infective recombinant clone[b]	Reverse transcriptase activity with the following DNA doses:[c]						No. of weeks after DNA treatment
	1,000 ng/culture	100 ng/culture	10 ng/culture	1 ng/culture	0.1 ng/culture	0.01 ng/culture	
λHF60	2.5–3.1 (4/4)[d]	1.8–2.1 (6/6)	0.8–1.5 (6/6)	0.1–0.4 (4/6)	Bkg (0/6)[e]	Bkg (0/6)	3[f]
	NT[g]	2.7–3.6 (6/6)	2.0–3.4 (6/6)	2.2–3.4 (6/6)	2.7–3.2 (6/6)	Bkg (0/6)	7
λHF17	2.4–2.7 (2/2)	1.2–1.4 (6/6)	0.6–0.8 (6/6)	0.07–0.3 (2/6)	Bkg (0/6)	Bkg (0/6)	3
	NT	2.6–2.9 (6/6)	2.4–3.0 (6/6)	2.5–2.8 (6/6)	2.1–2.8 (5/6)	Bkg (0/6)	6
λHF52	2.1–2.0 (2/2)	1.9–2.0 (8/8)	1.1–1.5 (6/6)	0.03–0.1 (3/6)	Bkg (0/6)	Bkg (0/6)	3
	NT	2.3–3.3 (6/6)	2.4–3.1 (6/6)	2.6–2.9 (6/6)	2.4–3.0 (5/6)	Bkg (0/6)	7

[a] Assays were conducted as described previously (30) by using 10^6 dog D17 cells per assay. Virus production was measured at 3- to 4-week intervals by the reverse transcriptase assay.

[b] Noninfective DNA clones were designated noninfective after they were tested at the highest dose (≧1 μg) for the longest time (≧12 weeks).

[c] Reverse transcriptase assay results are reported as counts per minute ($\times 10^5$) of [*methyl*-^{3}H]TMP incorporated per milliliter of culture fluid (5×10^6 to 10×10^6 cells) per 60-min incubation.

[d] Numbers in parentheses are number of cultures positive for reverse transcriptase/number tested. Cultures scored as negative for virus production were less than or equal to background (Bkg) in the reverse transcriptase assay. All positive cultures were assayed by the complement fixation test for identification of viral p30 antigen. In all cases tested, p30 was FeLV. Values for cultures considered positive by the reverse transcriptase assay were at least 20 times the background values.

[e] The background value for the reverse transcriptase assay was ≦500 cpm.

[f] Cultures negative for reverse transcriptase were tested for up to 11 to 13 weeks. No positive cultures were detected later than 8 weeks after DNA treatment.

[g] NT, Not tested.

TABLE 4. *Infectivity of cloned proviral DNAs after various treatments*[a]

Isolate	Infectivity after the following treatments:					
	Treatment of DNA before transfection[b]				S1 nuclease digestion of heteroduplexes[c]	
	None	*Eco*RI digest	Gel purification of *Eco*RI fragment	Random shear[d]	Total mixture	8.7-kb DNA
λHF60	+	+	+	+		−
λHF17	+	+	+	+		
λHF52	+	+		+		
λHF60/λHF52						+
λHF7	−	−		−		
λHF8	−	−		−		
λHF49	−	−	−	−		
λHF39	−	−		−		
λHF7/λHF8					−	
λHF7 + λHF8 + λHF49 + λHF39	−					

[a] Assays for virus infectivity are described in Table 3, footnotes.

[b] All positive cultures had values of $>3 \times 10^5$ cpm/ml in the reverse transcriptase assay at 4 weeks. Negative cultures were assayed up to 16 weeks. The amounts of DNA used per dish were 2 μg (no treatment), 2 μg of each clone (cotransfection), 1 μg (*Eco*RI digest), 0.2 μg (gel-purified *Eco*RI fragment), and 4 μg (randomly sheared DNA).

[c] Heteroduplexes were formed by using 2 μg of each phage DNA preparation. This total amount was tested for infectivity for the heteroduplex between λHF7 and λHF8. The 8.7-kb DNA recovered from the gel was not quantitated. Assuming a typical 40% recovery for this procedure, the 8.7-kb band from the heteroduplex was positive at a 10-ng level at 9 weeks and negative at a 1-ng level. Each dilution comparable to 100, 10, or 1 ng of material migrating with 8.7 kb after S1 nuclease treatment of 4 μg of a self-renatured preparation of λHF60 was negative even after 12 weeks.

[d] Duplex DNA samples were sheared by several passages through a 26-gauge syringe needle so that, as determined by gel analysis, there was a broad distribution of lengths from about 5 to 30 kb (median, about 14 kb).

(one *Pst*I site and one *Sma*I site were not detected in our previous study). With the exception of the heterogeneity that arose during phage amplification (see below), these analyses did not reveal any internal sequence differences among the proviruses studied. As Fig. 2 shows, the sets of bands derived from within each provirus were identical, as determined by mobility in gel electrophoresis.

The pattern of restriction endonuclease sites within the host flanking sequences was different for each of the 14 proviruses. Furthermore, there was no consistent pattern of sites near the points of provirus integration. Thus, each provirus was integrated at a different place in the host genome, and there was no evidence for any common sequence features in these flanking sequences.

Repeated sequences. A prevalent set of interspersed repeated sequences, designated the Alu family, has been identified within the human genome (20, 24). We used an available probe for this family from a human δ-globin gene cluster (kindly provided by E. Fritsch) (15) to search for this sequence in the RD(FeLV) insertions (Fig. 1 and 3). Alu family repeats were found in two of three infectious clones and two of four noninfectious clones. These repeats were found both 5′ and 3′ to the proviral sequences. Furthermore, this sequence had no detectable homology with FeLV proviral DNA.

Highly repeated sequences in the human genome (not necessarily related to the Alu family) were mapped by using total nick-translated human RD DNA as a hybridization probe (15; T. Sargent, personal communication). All of the Alu family repeats were detected by this method, and an additional repeat was mapped to a position upstream to the provirus in λHF8.

On the average, Alu family repeats occur approximately once per 6 kb in the human genome (20). Repeated sequence elements occur approximately once per 2 kb over 50 to 60% of the human genome (10, 39). We found at least one repeated sequence on each of nine restriction fragments in the 32 kb of human DNA studied, a frequency of at least one repeat per 3.6 kb. Thus, the frequency of repeated sequences in the host sequences flanking the proviral genomes was about as expected for random integration. Furthermore, the position or presence of repeated sequence elements did not correlate in any obvious way with the infectivities of the provirus clones studied.

Instability of FeLV sequences in λ clones. We observed that certain phage preparations developed heterogeneity when they were propagated under relatively nonselective growth conditions, such as subconfluent plate lysis, compared with the more selective method of liquid culture propagation. Heterogeneity occurred within the eucaryotic portion of the phage, resulting in minor bands either 8.2 kb larger or 8.2 kb smaller than the major band observed after *Eco*RI digestion (Fig. 2, isolates 60 [λHF60] and 39 [λHF39]). Heterogeneity was observed only in phage in which the maximum and minimum size limits on the eucaryotic insert (22 and 8.8 kb, respectively, for Charon 4A) were not exceeded by the insertion or deletion of 8.2 kb. Specifically, for λHF60 propagated in liquid culture, the insert was 9.5 kb long. When grown by subconfluent plate lysis, a few phage gave an *Eco*RI band 17.7 kb long. This band was weak as determined by ethidium bromide staining but more intense as determined by hybridization with an FeLV probe. Similarly, when λHF39 (containing a DNA insert 17.8 kb long) was grown by plate lysis, it produced a 9.6-kb *Eco*RI band, which had an ethidium bromide intensity about 5% of that of the 17.8-kb band. However, this 9.6-kb band gave a very weak signal when hybridized with an FeLV probe. (The faint band at 10.9 kb in all of the autoradiographs of Fig. 2B was due to a λ arm, as discussed in the legend to Fig. 2.)

The length of the FeLV genome lacking one LTR is 8.2 kb (29). Therefore, these observations suggested that homologous site recombination occurred between the proviral LTRs, resulting in either a deletion (λHF39) or a tandem duplication (λHF60) of the FeLV genome. Further restriction mapping experiments supported this interpretation (data not shown). This interpretation was confirmed by the presence of the expected heteroduplex structures in self-renatured preparations (Fig. 4). Similar recombinants have been observed in λ clones of the spleen necrosis virus (33). There is no evidence that this recombination phenomenon is related to infectivity.

Electron microscopic heteroduplex analysis. We studied homology between proviral sequences and the possible homologies between host flanking sequences of the different proviruses by heteroduplex analysis. Representative structures are shown in Fig. 4, and the data are summarized in Table 5. In all cases, an uninterrupted duplex region 8.8 ± 0.2 kb long was observed, corresponding to the position of the retrovirus genome in the cloned DNA. This was the case irrespective of whether the heteroduplex was between two infectious clones, between two noninfectious clones, or between an infectious clone and a noninfectious clone. We believe that internal nonhomologies (either deletions, insertions, or substitutions) more than about 200 base pairs long would have been observed if

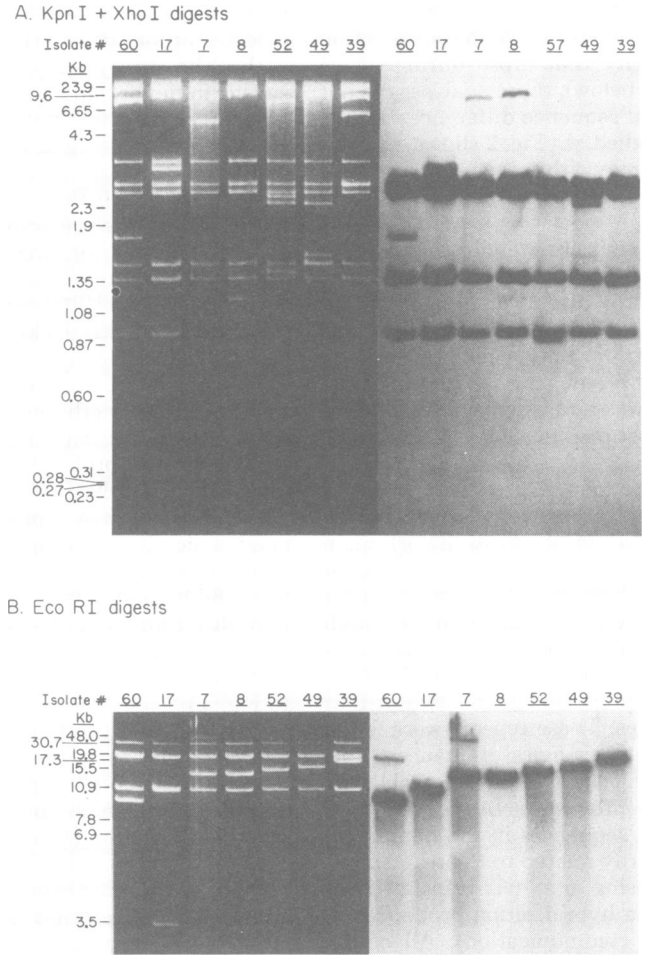

FIG. 2. *Restriction digests of seven provirus clones. Recombinant phage DNA was isolated as described in the text. Restriction digestion, gel electrophoresis, Southern blotting, hybridization, and autoradiography were performed as described in the text and by Mullins et al. (29). The left side of each panel shows the ethidium bromide staining pattern after electrophoresis in a 1.5% (A) or 0.7% (B) agarose gel. The right side of each panel is a Southern blot of the gel on the left probed with [^{32}P]FeLV complementary DNA rep, as described in the text. The molecular weight markers run in adjacent lanes were an HindIII digest of λ and an HaeIII digest of φX174 in (A) and uncut λ DNA, an EcoRI digest of Charon 4A, a Kpn digest of Charon 4A, and a SalI digest of λ in (B). (A) A 0.75-μg amount of each DNA sample was digested sequentially with KpnI and XhoI under the conditions recommended by the supplier (New England BioLabs). All isolates had five FeLV hybridizing bands in common (at 2.8, 2.6, 1.34, 0.89, and 0.33 kb), corresponding to internal FeLV provirus bands. Each isolate had a unique set of hybridizing fragments, corresponding to the junction of FeLV provirus sequences and human flanking sequences. High-molecular-weight fragments (>10 kb) and low-molecular-weight fragments(<1 kb) were underrepresented, as determined by hybridization intensity, due to relatively poor transfer to or retention by nitrocellulose. (B) A 0.3-μg amount of each DNA sample was digested with EcoRI and electrophoresed in a 0.7% agarose gel. Each isolate had a single EcoRI insertion, except λHF17, which had two. The 11.2-kb insertion in λHF17, which was not resolved from the short (10.9-kb) vector fragment in this gel, contained sequences homologous to FeLV, whereas the 3.4-kb insertion did not. A small percentage of the phage in λHF60 preparations contained a duplication of the FeLV provirus; a similar percentage of the λHF39 phage had 8.2 kb of the FeLV provirus deleted. These insertions are present as relatively faint bands on both sides of the panel at 17.7 kb (λHF60) and 9.6 kb (λHF39). The large vector fragment (19.8 kb) and annealed vector fragments (30.7 kb) were revealed by ethidium bromide staining. Our FeLV probe had a small amount of homology to the shorter λ arm (10.9 kb), resulting in its detection by hybridization. The ~48-kb band present in the λHF7 lanes was undigested phage DNA.*

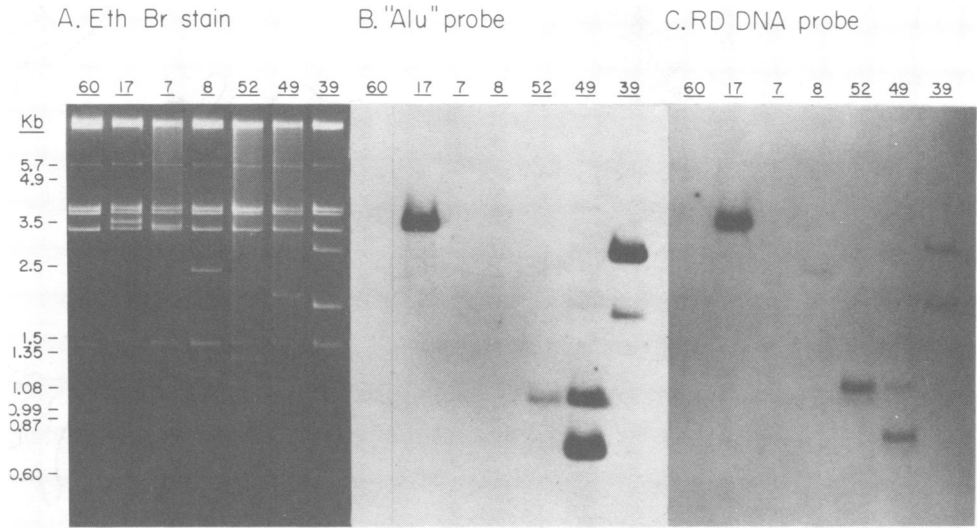

FIG. 3. *Mapping of repeated sequence elements in the DNA flanking FeLV proviruses. A 0.75-μg amount of DNA from each isolate was digested sequentially with HindIII, SmaI, and EcoRI by using the conditions recommended by the supplier (New England BioLabs). The procedures used for gel electrophoresis (1.2% agarose gel), Southern blotting, hybridization, and autoradiography were as described in the text and by Mullins et al. (29). (A) Ethidium bromide (Eth Br) staining pattern. The molecular weight markers used were a partial KpnI-EcoRI digest of Charon 4A DNA and an HaeIII digest of φX174 DNA. (B) Autoradiogram of a blot of the gel in (A) produced by using a nick-translated ^{32}P-labeled Alu family repeated sequence fragment (24) as the hybridization probe. The fragment used was the 0.45-kb TaqI-BglII fragment described by Fritsch et al. (15), which was derived from a region 5' to the human δ-globin gene. This sequence was identified as a member of the Alu family first by its homology with an Alu family repeat found elsewhere in the human β-like gene cluster (12, 24) and more recently by DNA sequence analysis (S. Weismann, personal communication). Three ~0.75-kb SmaI/HindIII fragments were generated by digestion of λHF49 DNA. They occurred in a region adjacent to the 5' end of the provirus. At least two and possibly all three of these fragments contained an Alu family repeated sequence. (C) Autoradiogram of a blot of a gel similar to that in (A) which was hybridized by using ^{32}P-labeled RD cellular DNA as the probe. We believe that under the conditions of hybridization used, the repeated sequences in the probe DNA hybridized to all homologous sequences in the cloned DNA.*

present (8).

We observed there were characteristic intrastrand secondary structure features in several of the host flanking sequences (e.g., the hairpin loop in λHF39) but in no case did we detect a homology between flanking sequences.

S1 nuclease analysis of heteroduplexes. A sensitive method for searching for a mismatch between two closely related genomes is S1 nuclease digestion of heteroduplexes (41). Double-stranded S1 nuclease cuts can be detected by gel electrophoresis.

Shenk and co-workers (41) found that double-stranded cleavages are made at high frequencies when an insertion 35 nucleotides long or longer is present in one strand of a heteroduplex and not in the other. Dodgson and Wells (11) found that a mismatch of at least a 5 base pairs is necessary for detectable cleavage.

We prepared heteroduplexes between various λ clones of the proviral sequences and treated them with S1 nuclease (see above). The digestion products after native gel electrophoresis were detected by ethidium bromide staining or by the more sensitive method of blotting with an FeLV probe (Fig. 5). In all analyses, the following four bands were visible by staining: 40- to 49-kb homoduplex molecules, vector arms 19.8 and 10.9 kb long, and an 8.7-kb provirus band which migrated with a mobility similar to that of unintegrated linear FeLV DNA. The intensity of the 8.7-kb band was lower when the proviral DNAs were in opposite orientations relative to the vector arms (e.g., λHF7 versus λHF60, λHF5, or λHF39) because of the lower frequency of formation of the desired heteroduplexes.

Thus, within the limits of resolution of S1 nuclease digestion and neutral gel analysis, we found no evidence for a mismatch in any of the proviral genomes, infectious or noninfectious.

Cotransfection experiments. In the few cases tested, it has been observed that the small fraction of cells which are competent for the uptake of DNA in DNA-mediated cell transformation experiments incorporate many copies of

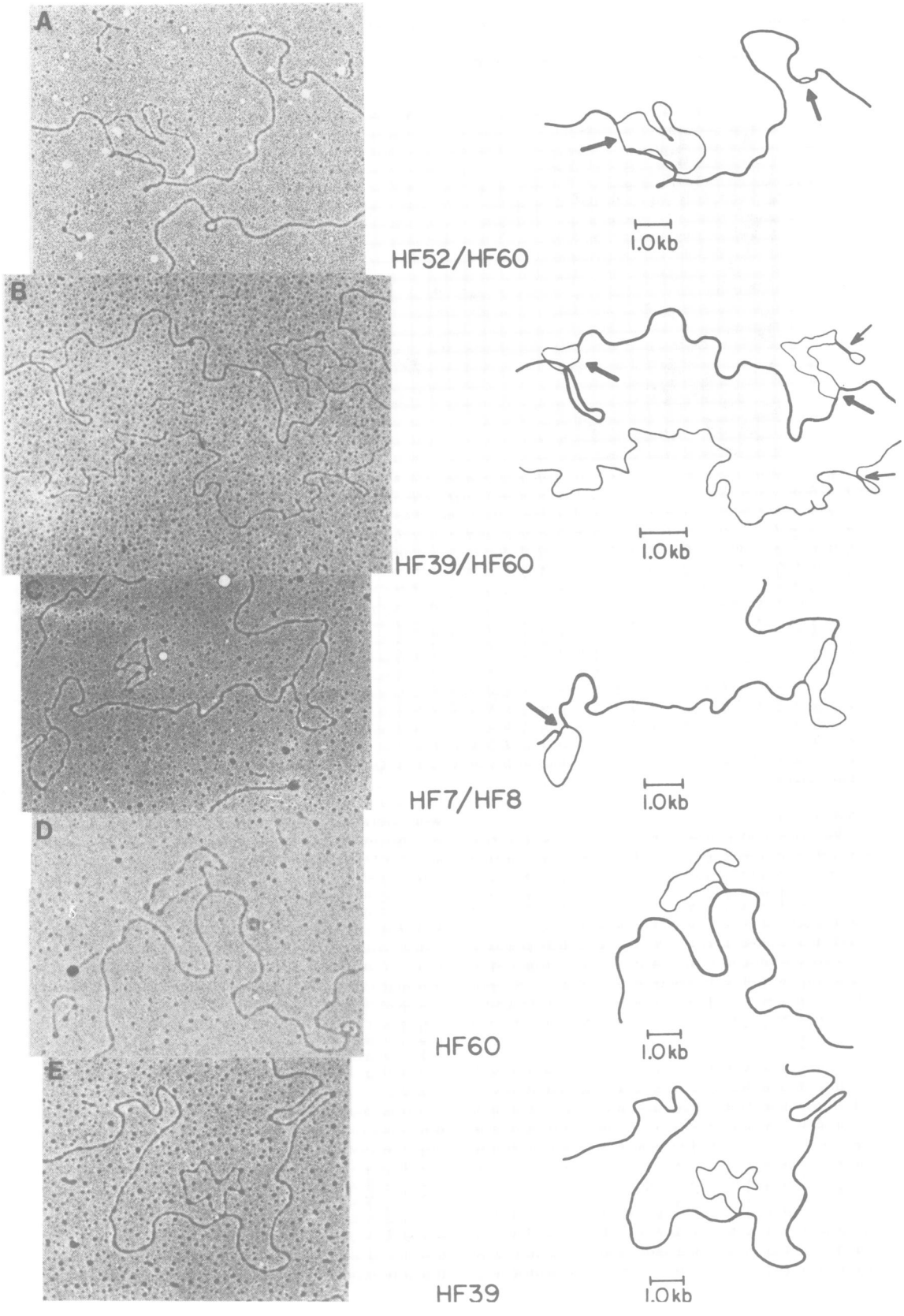
A
HF52/HF60
1.0kb
B
HF39/HF60
1.0kb
C
HF7/HF8
1.0kb
D
HF60
1.0kb
E
HF39
1.0kb

TABLE 5. *Contour lengths of heteroduplex molecules observed by electron microscopy*

Heteroduplex	Length (kb)					No. of molecules
	λ left	Single-stranded DNA	Provirus	Single-stranded DNA	λ right	
λHF39/λHF60	19.8 (0.6)[a]	0.9 (0.25)/0.34 (0.06)	9.0 (0.6)	6.3 (0.70)/0.4 (0.10)	11.0 (0.4)	23
λHF39/λHF52	19.7 (0.5)	1.7 (0.25)/6.2 (0.7)	8.7 (0.25)	8.2 (0.7)/0.6 (0.1)	10.9 (0.4)	38
λHF39/λHF8[b]			8.95 (0.04)			12
λHF7/λHF8	20.3 (0.6)	4.4 (0.25)/0.32 (0.06)	8.6 (0.1)	1.3 (0.2)/5.3 (0.45)	10.9 (0.3)	8
λHF8/λHF49	20.3 (0.6)	0.33 (0.06)/2.3 (0.2)	8.9 (0.1)	5.4 (0.5)/5.0 (0.25)	11.3 (0.3)	7
λHF60/λHF52	19.7 (0.5)	0.62 (0.08)/5.9 (0.5)	8.7 (0.5)	0.35 (0.05)/0.39 (0.05)	10.8 (0.4)	10

[a] The numbers in parentheses are each one standard deviation of the error.

[b] This heteroduplex was constructed between two clones with the provirus in opposite orientation relative to the λ arms. In the molecules measured, all of the sequences flanking the region of homology were single stranded.

a particular DNA sequence in the transformation procedure (48). Cotransfection with a number of noninfectious proviral clones could give rise to an infectious virus if the several clones contained mutations in viral genes which complemented one another. Cotransfection with 2 μg of each of four unique noninfectious clones failed to give rise to detectable virus (Table 4).

Transfection with sheared proviral DNAs and S1 nuclease-digested heteroduplexes. Cooper and Temin (7) have postulated that inactive proviruses result from *cis*-acting negative control elements in host flanking sequences. Cooper and Silverman (5) have reported that random shearing of genomic DNA containing a nontransfective avian provirus results in virus production when the sheared DNA is assayed by transfection. They attributed this phenomenon to the removal at a low frequency of a *cis*-acting negative control element close to the provirus in the genomic DNA. We performed a similar shearing experiment, using 4 μg of cloned proviral DNA (enriched in FeLV DNA by a factor of 6×10^4 compared with uncloned genomic DNA). Shearing so that there was a broad distribution of molecular lengths extending from less than 5 to more than 20 kb did not alter the infectivity status of any isolate, nor did *Eco*RI digestion or gel purification of the eucaryotic *Eco*RI fragment before transfection (Table 4).

If *cis*-acting negative control elements were present in the noninfectious clones, S1 nuclease digestion of a heteroduplex would be expected to remove them, unless they were homologous and present immediately adjacent to the provirus in the proximal few nucleotides. Consequently, we analyzed S1 nuclease-treated heteroduplex molecule preparations for infectivity (Table 4). Transfection with the digested heteroduplexes formed from 2 μg each of cloned DNA from two noninfectious clones remained noninfectious. A positive control experiment showing that S1 nuclease digestion did not suppress the transfection competence in a positive clone was difficult because the gel-isolated 8.7-kb bands of a heteroduplex between two infectious clones could be contaminated with the 40- to 49-kb homoduplex that was reformed during the hybridization and nonspecifically degraded by S1 nuclease. We observed that the gel-isolated 8.7-kb proviral band obtained after S1 nuclease treatment of a heteroduplex between two infectious clones was infectious, although at a level approximately 100-fold lower than untreated DNA. As a control for homoduplex contamination, we gel isolated the random, 8.7-kb fragments of DNA obtained after S1 nuclease treatment of 4 μg of a self-renatured preparation of an infectious clone. This DNA was noninfectious (Table 4). Therefore, these data support the hypothesis that the defect in transfection did not reside in a negative control element immediately proximal to the proviral genome.

FIG. 4. *Heteroduplexes between representative clones. Portions of representative heteroduplexes are shown, including the double-stranded provirus and the single-stranded host flanking sequences (A through C). Cloning vector arms are not shown. The molecules are oriented such that the left λ arm is to the left. Contour length measurements for the molecules in (A) through (C) are given in Table 5. In the interpretive tracings, thick and thin lines represent double- and single-stranded DNAs, respectively. (A) λHF52/λHF60 (infectious with infectious). The arrows indicate single-stranded host flanking sequences. (B) λHF39/λHF60 (infectious with noninfectious). The large arrows indicate host flanking sequences. The single strand below the heteroduplex was from λHF39 and shows the characteristic inverted repeat structure (small arrows). (C) λHF7/λHF8 (noninfectious with noninfectious). The arrow indicates a single-stranded host flanking sequence. (D) λHF60. Self-renatured sample showing a heteroduplex between DNA molecules with two and one copies of the FeLV provirus. (F) λHF39. Self-renatured sample showing heteroduplex between a molecule containing one complete copy of the insertion and a molecule with an 8.2-kb deletion in the FeLV proviral sequence.*

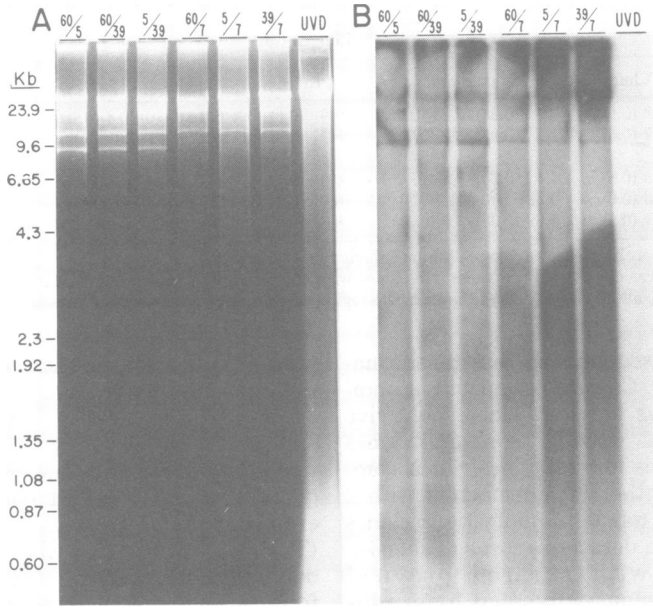

FIG. 5. *S1 nuclease analysis of heteroduplexes. Heteroduplexes were formed between the recombinant phage indicated and treated with S1 nuclease as described in the text. The amount of S1 nuclease and the conditions of digestion used were such that a similar quantity of closed circular φX174 DNA was converted into a 1:1 mixture of nicked circular and linear molecules. S1 nuclease digests and unintegrated viral DNA (UVD) were loaded directly onto 1.0% agarose gels and electrophoresed for 650 V·h. Molecular weight markers were an HindIII digest of λ DNA and an HaeIII digest of φX147 DNA. (A) Ethidium bromide staining pattern. In addition to material at the origin, the following four bands are visible in each lane: 40- to 49-kb homoduplex DNA, 19.8- and 10.9-kb Charon 4A vector fragments, and a 8.7-kb FeLV provirus band. The slight difference in mobility observed between unintegrated linear viral DNA and proviral DNA from S1 nuclease-treated heteroduplex DNA could be attributed to salt concentration differences in the gel loading buffer. Heteroduplexes were loaded in 0.2 M sodium chloride, whereas unintegrated DNA was loaded in 0.006 M sodium chloride. These differences were not observed in other experiments (data not shown), in which samples were loaded in comparable salt concentrations. (B) Autoradiogram of a blot of the gel shown in (A) hybridized with ^{32}P-labeled FeLV complementary DNA. In addition to the heteroduplexes shown, similar results were obtained with all heteroduplexes constructed and examined by S1 nuclease analysis (λHF52/λHF60, λHF7/λHF8, λHF7/λHF49, and λHF8/λHF39).*

DISCUSSION

Provirus structure and integration. With the exception of one absent site and one new *Kpn*I site in the left LTR of λHF62, all of the restriction sites that have been mapped are conserved in all of the FeLV proviruses which we studied and are the same as those found in unintegrated linear viral DNA. Furthermore, there have been no detectable insertions or deletions of sequences, as judged by the electrophoretic mobilities of the restriction fragments.

Restriction endonuclease mapping indicated that there are a number of sites in the host genome at which proviruses have integrated. Restriction mapping and heteroduplex analysis provided no evidence for any sequence homology among these several sites. However, it should be noted that none of the analyses performed (restriction mapping, S1 nuclease digestion, and electron microscopic studies of heteroduplexes) would have detected regions of homology in the sequences immediately adjacent to the provirus that were 100 nucleotides long or shorter. Thus, our data do not prove whether or not there are short regions of sequence homology among the various integration sites.

Members of the Alu family and one other repeat occur in the host sequences flanking five of the seven independent proviral isolates studied. The positions of these repeats are variable and do not correlate with the infectivity of the proviral DNA.

We have shown previously that the *Sma*I site 4.7 kb from the 5′ end of the viral genome is refractory to cleavage in about one-third of the proviruses in RD(FeLV)-2 cells (29). Furthermore, we have found that some of the *Hpa*II sites in the region of this *Sma*I site are methylated (28a). The *Sma*I recognition site (5′-CCCGGG-3′) is a subset of the *Hpa*II sites (5′-CCGG-3′), and neither of these is cleaved if

the underlined cytosine is methylated (16, 46). The host bacterium in which we propagated the recombinant phage (DP50 SupF) does not methylate cytosine at these sites. In this study we found that none of the cloned proviruses is missing any of the four *Sma*I sites in the viral genome. Thus, about one-third of the proviruses may have a *Sma*I-resistant site 4.7 kb from the 5′ end in RD(FeLV)-2 DNA because this site is methylated. The role of 5-methylcytosine in gene expression has not been established definitely. The available experimental data have demonstrated that 5-methylcytosine in the dinucleotide sequence CpG occurs more frequently in transcriptionally inactive DNA than in transcriptionally active DNA (34). Thus, our data suggest that approximately one-third of the proviruses in RD(FeLV)-2 cells are transcriptionally inactive. However, we do not suggest that methylation dictates infectivity of purified DNAs since the cloned DNAs used for transfection do not contain 5-methylcytosine. It remains to be determined whether noninfectious proviruses are transcriptionally inactive or specifically methylated or both in RD(FeLV)-2 cells.

Transfection. The virus produced by transfection with recombinant DNA was identified as belonging to subgroup B (Tables 1 and 2). This was as expected, since of the two FeLV subgroups present (subgroups A and B) in GA-FeLV, only subgroup B is thought to grow in human cells (23, 38). However, recent data indicate that at least two FeLV subgroup A isolates grow in human RD cells (FeLV-A Glasgow-7 and FeLV-A Rickard; M. Nicolson and O. Jarrett, unpublished data).

The minimum infective dose of cloned DNA for an infectious clone is about 100 pg/dish (about 20 pg of FeLV DNA) or 2×10^6 viral genome copies per dish. About 1 μg of RD(FeLV)-2 chromosomal DNA has a comparable infectivity and corresponds to approximately 3×10^6 proviral genomes. Therefore, the infectivity per viral genome of the active cloned DNA and the infectivity of the RD(FeLV)-2 chromosomal DNA are about the same. It is noteworthy that 10-fold-lower doses of cloned DNA (2×10^5 viral genome copies) have never given a positive transfection. The reason for this is not known.

Approximately two-thirds of the proviruses isolated are not transfective at all, even at doses 10^4 times higher than the dose found for the typical positive clones; thus, they appear to be totally defective.

None of our experiments revealed any sequence difference between infective and noninfective clones. We estimate that a region of nonhomology, insertion, or deletion 35 nucleotides long or longer or possibly shorter would have been detected by the S1 nuclease heteroduplex study.

The considerable background observed in the S1 nuclease experiments tended to obscure bands formed by inefficient cleavage of short regions of nonhomology. The use of purer preparations of S1 nuclease, substrates with fewer single-stranded nicks, alkaline gel analysis, and model substrates with known sequences of nonhomology should allow closer examination and definition of mutations within the viral genome, if they exist.

We observed that the 8.7-kb provirus band produced by S1 nuclease treatment of a heteroduplex between two infectious clones was infectious, but at a level about 100-fold lower than the level for untreated cloned DNA. One possible explanation for this is that a host sequence which promotes virus expression may be adjacent to the provirus and in some molecules may be removed by S1 nuclease digestion of a heteroduplex. Alternatively, the decrease in specific infectivity may be due to nonspecific degradation of proviral DNA by S1 nuclease.

The loss of infectivity is not an artifact of cloning, since independent isolates for any one provirus, as demonstrated by opposite orientations with respect to the vector and isolation without library amplification, are either all infectious or all noninfectious.

A possible mechanism of loss of infectivity is one or several single-base changes in the viral genome due to errors in reverse transcription before integration or due to mutation of proviral DNA. Alternatively, Cooper and Temin (7) have suggested that in some cases there are *cis*-acting negative control elements. Our observation that S1 nuclease digestion of heteroduplexes of two noninfectious clones does not render the heteroduplexes infectious argues against the latter hypothesis in this instance. However, as described above, we cannot exclude the possibility that there are short regions (less than about 100 nucleotides) of sequence homology at the integration sites for the several integrated proviruses. If there were two such preferred sites, one permitting virus expression and one a negative control element, the S1 nuclease experiment would not succeed in converting noninfectious DNA to infectious DNA.

At present, our data do not rule out any small differences in the LTRs found in infectious and noninfectious clones. Obviously, further experiments to determine whether the defect in virus infection is localized to one region of the genome and to define this defect at the sequence level are needed.

If the defect in the provirus clones which are

noninfectious is in some sort of a control element and not in a structural gene, it may be that infectivity of cloned DNA correlates with expression at the transcriptional level of the proviral genomes in RD(FeLV) cells. These hypotheses will be tested in future studies.

If there are proviral sequences which are not expressed to produce virus in an exogenously infected human cell line, there may be similar nonexpressed sequences in infected cat cells. Such sequences may play a role in the observed lymphomas of cats that are not virus producers.

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