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Sequence organization of feline leukemia virus DNA in infected cells

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ABSTRACT

A restriction site map has been deduced of unintegrated and integrated FeLV viral DNA found in human RD cells after experimental infection with the Gardner-Arnstein strain of FeLV. Restriction fragments were ordered by single and double enzyme digests followed by Southern transfer (1) and hybridization with ^{32}P-labeled viral cDNA probes. The restriction map was oriented with respect to the 5' and 3' ends of viral RNA by using a 3' specific hybridization probe. The major form of unintegrated viral DNA found was a 8.7 kb linear DNA molecule bearing a 450 bp direct long terminal redundancy (LTR) derived from both 5' and 3' viral RNA sequences. Minor, circular forms, 8.7 kb and 8.2 kb in length were also detected, the larger one probably containing two adjacent copies of the LTR and the smaller one containing one copy of the LTR. Integrated copies of FeLV are colinear with the unintegrated linear form and contain the KpnI and SmaI sites found in each LTR.

INTRODUCTION

The feline leukemia virus (FeLV) is a Type C retrovirus which is horizontally transmitted among domestic cat populations (2-6) and is detectable in 60-70% of cats bearing leukemia or lymphoma (7). FeLV is found in high titer in the infected cat's saliva and at generally lower levels in the blood and lymphatic tissues (5,8). Although the yearly incidence of leukemia and lymphoma in cats is 44 per 100,000 in the USA (9), it is estimated that exposure to FeLV in natural cat populations may be as high as 50% (10). Several members of the genus *Felis*, including domestic cats, possess sequences which will cross hybridize with FeLV RNA (11,12) yet no spontaneous induction of FeLV has been reported. Evidence has been presented which suggests that a portion of the infectious FeLV is missing in the endogenous

cat genome (13). Studies in our laboratory indicate that endogenous FeLV sequences in domestic cat DNA show a considerable difference in sequence organization from that of the FeLV sequences in infected heterologous cells (Casey et al., manuscript in preparation).

FeLV will productively infect homologous and sensitive heterologous tissue culture cells; the latter include monkey, dog and human cell lines (14-16). In all cases, a number of DNA copies of the viral RNA genome are found integrated into the host genome. Purified DNA extracted from productively infected heterologous cells is itself infective for cat and sensitive heterologous tissue culture cells (15). DNA from normal cat tissues or cat cells in culture is not infective for recipient cells unless derived from a virus productive state (Nicolson, unpublished observations). Several different isolates and subgroups of FeLV have been described, distinguishable by host range and immunological characteristics (17, 18,19).

We wish to understand the relation between viral gene expression, leukemia and the sequence organization and site of integration of viral sequences. The appropriate initial study is of the viral sequences in infected heterologous cells, where there is no background of endogenous sequences. These problems can now be approached by recombinant DNA methodology. In preparation for such an approach, we have deduced a detailed restriction map of FeLV DNA found in RD(FeLV) cells, human RD cells infected with the Gardner-Arnstein strain of FeLV (20), by Southern blotting, restriction mapping methods.

In the several cases studied, Type C RNA viruses have been shown to replicate through double stranded DNA intermediates, linear and circular forms of which can be found independent of host chromosomal DNA within a few hours after infection (for review see Ref. 21). Subsequently, a variable number of viral DNA sequences integrate into host chromosomal DNA and new virus production can usually be detected within 48 hours after infection (15). In agreement with these findings we find that unintegrated viral DNA's can be observed in cells infected with FeLV. The predominant 8.7 kb linear form was mapped using

13 six base recognition site endonucleases. This map has been helpful in interpreting the restriction digest patterns of minor unintegrated forms and of integrated (proviral) FeLV sequences.

MATERIALS AND METHODS

Isolation of viral RNA. The Gardner-Arnstein strain of FeLV was harvested at 4 hour intervals from the cell culture medium of a subline (RD(FeLV)-2) of chronically infected human RD cells after an initial 12-hour label with 25 μC/ml of uridine-5-^{3}H in NTE, pH 7.2 (NTE is 10 mM Tris, 100 mM NaCL and 1 mM EDTA). Virus was twice centrifuged to equilibrium in 20 to 50% sucrose gradients, the first in a discontinuous gradient in a Ti 15 zonal rotor at 28 Krpm, 5°C, for 2 hours. The second centrifugation was on a linear gradient in a SW27 rotor at 27 Krpm and 2°C for a minimum of 5 hours. Virus containing fractions were pooled, diluted and pelleted by centrifugation in an SW27 rotor at 27 Krpm and 2°C for 2 hours. The concentrated virus was lysed with 0.5% SDS in NTE, pH 7.2, digested with 500 μg/ml proteinase K at 42°C for 40 minutes, and the viral RNA separated by velocity sedimentation in 10 to 30% linear sucrose gradients in an SW 50.1 rotor, 45 Krpm, for 2 hours at 6°C. The 70S RNA was located by radioactivity, appropriate fractions pooled, dialyzed against H_2O and used as a template for cDNA synthesis.

Isolation of Cellular DNA's. DNA was extracted from near-confluent RD(FeLV)-2 cell cultures by a minor modification of the method described in Ref. 15. Cells were lysed with 0.5% SDS in 0.1 M NaCl, 0.01 M Tris, pH 8.2, 0.1 M EDTA, and incubated for 18 hr with 100 μg/ml proteinase K at 37°C. After extraction with chloroform:isoamyl alcohol (24:1), the precipitated DNA was redissolved in 0.01 M Tris, pH 7.2, 0.01 M NaCl, 0.01 M EDTA, incubated with 100 μg/ml boiled ribonuclease A, followed by proteinase K at 50 μg/ml. The final extraction and precipitation was as described.

Unintegrated linear FeLV DNA was isolated from a subline of RD cells (RD4) 12-32 hours after infection with a fresh pool of G.A. FeLV, derived from the subline RD(FeLV)-2, at a multiplicity of infection of approximately 1.0. The cells were fractionated according to the procedure of Hirt (Ref. 22) and DNA from the

soluble extrachromosomal fraction was purified by phenol extraction and ethanol precipitation, and examined for the presence of unintegrated FeLV DNA sequences.

Restriction endonuclease digestion, gel electrophoresis, and Southern blotting. Restriction endonucleases were obtained from New England Biolabs, Bethesda Research Labs and Boehringer Mannheim. Digests were carried out with a 2-8 fold enzyme excess for 1-4 hours. After digestion 1/10 volume of 50% Ficoll, 125 mM EDTA (pH 8.5), 0.1% xylene cyanole FF and 0.1% bromophenol blue was added and the samples loaded directly onto gels.

Electrophoresis was conducted in 0.4 cm x 14 cm x 18 cm vertical agarose gels in 40 mM Tris-acetate (pH 7.8), 5 mM sodium acetate, 1 mM EDTA. Gels were typically run at 40 V for 12-24 hours. The gels were then soaked in ethidium bromide (0.5 µg/ml) and photographed under short wave ultraviolet illumination.

DNA was transferred to nitrocellulose paper (0.45 µm; Schleicher and Schuell) according to the procedure of Southern (1). After transfer, filters were washed in 3XSSC (1XSSC = 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0), air dried and baked *in vacuo* for 2-4 hours at 75°C, then stored at room temperature.

Preparation of hybridization probes. 3' specific viral RNA was isolated by oligo-dT (Collaborative Research) selection of poly A containing fragmented RNA molecules according to the procedure of the manufacturers. Aprpoximately 20% of the viral RNA in our preparations adheres to oligo-dT cellulose in this procedure.

Representative and 3' specific cDNA's were synthesized in 30 µl reactions containing 1 µg of RNA, 30 units of AMV reverse transcriptase (provided by Dr. J. Beard), 40 µg of calf thymus primer DNA (23) and 0.5 mCi of α^{32}P-dCTP (20-40 µM; New England Nuclear), in the presence of 50 mM Tris-HCL (pH 8.1), 8 mM magnesium acetate, 40 mM KCL, 2 mM dithiothreitol, 0.01% noniodet P-40 and 200 µM each of dATP, dGTP and TTP. Reactions were carried out at 37°C for 3 hours. Unincorporated nucleotides were removed by chromatography on a 0.5 cm x 20 cm Sephadex G-50 column, eluted with 250 mM NaCl, 50 mM Tris-HCL (pH 8.0), 2 mM

EDTA, and 0.05% sodium dodecyl sulfate.

Filter hybridization and autoradiography. Baked filters were washed in 2 x SSC with light rubbing and then equilibrated in 50% formamide (Matheson, Coleman and Bell) in 2 x SSC. Prehybridizations (4-16 hours) and hybridizations (3 days in 50% formamide or 22 hours in 50% formamide plus 10% dextran sulfate (Pharmacia)) were carried out at 42°C with mild agitation in tape sealed plastic boxes with 10 ml per initial filter and 5 ml per additional filter. Prehybridization buffer was 50% formamide, 0.1 M PIPES, 0.8 M NaCl, 200 µg/ml of sonicated and denatured calf thymus DNA (500 µg/ml for dextran sulfate blots), 100 µg/ml HeLa RNA, 0.1% sodium n-lauroyl sarkosine and 0.1% each of Ficoll, polyvinylpyrollidone and bovine serum albumin (Pentax). After prehybridization the solution was drained and replaced with fresh prehybridization solution plus probe and where indicated, 10% dextran sulfate. Hybridization mix was heated to 65°C before each use. Probe concentrations varied from 5 x 10^5 dpm/ml (0.6 ng/ml) for dextran sulfate blots to 1-5 x 10^6 dpm/ml (1-6 ng/ml) in formamide blots.

After hybridization the solution was stored at -20°C for reuse and the filters washed twice at room temperature in 250 ml of 2 x SSC, 0.05% sodium n-lauroyl sarkosine, 0.02% sodium pyrophosphate for 10 min each. Subsequently, filters were washed in 4 changes (250 ml each) of 0.2 x SSC, 0.05% sodium n-lauroyl sarkosine, 0.01% sodium pyrophosphate at 50°C over a period of 1-2 hours. Excess fluid was blotted from filters which were then allowed to air dry. Dry filters were wrapped in Saran Wrap and sandwiched with one sheet of Kodak XR-5 film between two Cronex Lightning-Plus intensifying screens. Exposures were made at -70°C for one to several days.

RESULTS

Sequence Arrangement of Unintegrated DNA. We observe that infection of permissive cells with FeLV results in formation of a double stranded DNA copy derived from the single stranded viral RNA template. One to 10 copies per cell of this DNA can be isolated by the Hirt fractionation procedure (22) 12-32 hours after mass infection. DNA isolated by this method is largely

free of high molecular weight chromosomal DNA and can be analyzed without further purification after size separation on agarose gels, transfer to nitrocellulose paper (1), and hybridization using viral cDNA transcribed from viral RNA as probe. Typical loadings of ∿ 10 ug of total Hirt supernatant DNA (approximately 50 pg FeLV DNA) are sufficient to detect all hybridizable bands larger than ∿ 0.3 kb in 1-2 days of autoradiographic exposure.

(a) Linear unintegrated viral DNA. The major viral DNA species observed in Hirt supernatant preparations is one migrating with 8.7 kb linear DNA (Fig. 1). As shown below, all restriction enzymes which affect the mobility of this fragment result in two or more bands, the combined length of which equals 8.7 kb. This demonstrates that the 8.7 kb fragment is a linear molecule and that it possess unique, non-permuted termini. Five (minor) FeLV related bands are also observed in Hirt supernatant preparations (Fig. 1), their identities will be discussed below.

The restriction site maps shown in Fig. 3 were constructed by analyzing single and double enzyme digests of unintegrated viral DNA. These data are shown in Fig. 2.

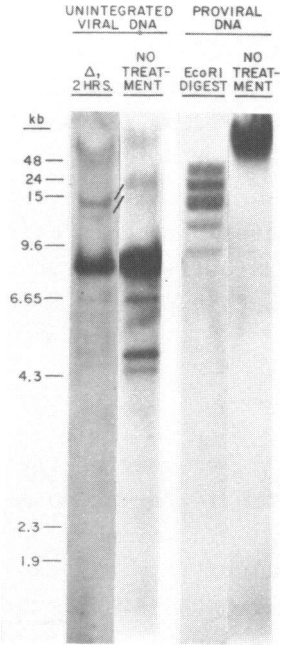

Figure 1. Analysis of unintegrated and integrated FeLV DNA. 10 ug of Hirt supernatant DNA (unintegrated viral DNA) obtained after 32 hours of infection of RD cells with FeLV, and 3 ug of chromosomal DNA from RD(FeLV)-2 cells (proviral DNA), was electrophoresed in 0.8% agarose gels after the treatment indicated (Δ refers to heat treating the DNA in EcoRI restriction buffer at 37°C). DNA was transferred to nitrocellulose paper and hybridized (the three lanes on the right were hybridized in the presence of 10% dextran sulfate) with representative ^{32}P-cDNA synthesized *in vitro* from an FeLV RNA template. Autoradiograms were exposed at -70°C. Molecular weight markers used were Hind III and Sal I digests of lambda DNA. Markers were visualized by ethidium bromide staining prior to blotting, and their positions marked on the nitrocellulose filter to correct for shrinkage of the filter during subsequent treatment.

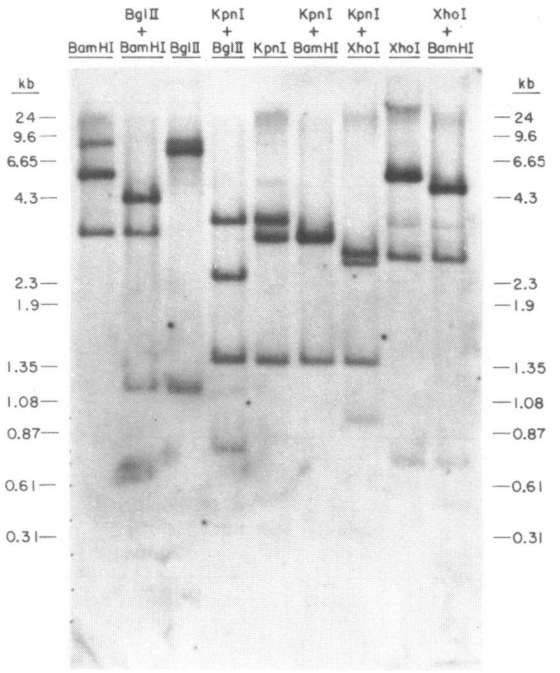

Figure 2. Mapping unintegrated viral DNA. 10 ug of Hirt supernatant DNA was digested with the restriction endonucleases indicated. The products were electrophoresed in a 1.2% agarose gel and visualized as described in the legend to Fig. 1. Markers were a Hind III digest of lambda DNA and a Hae III digest of ØX174 DNA.

Digestion with Bam HI results in two bands, 5.45 and 3.25 kb in length. The 5.45 kb Bam HI fragment is positioned on the left side of the map shown in Fig. 3, line A, in accordance with the 5' to 3' polarity determination described below. Digestion with Bgl II results in two bands of 1.2 and 7.5 kb in length. A Bam HI/Bgl II double digest results in 3 fragments, 1.2, 4.3 and 3.25 kb. Therefore the Bgl II site lies within the 5.45 kb Bam HI fragment. These relative positions are shown in Fig. 3, line B.

Digestion with Kpn I results in fragments of 3.65, 3.15, 1.35 and 0.35 kb. Bgl II cleaves the 3.15 kb Kpn fragment, resulting in five fragments, 3.65, 2.45, 1.35, 0.75 and 0.35 kb in length. Since 1.2 kb of viral DNA is found to the left of

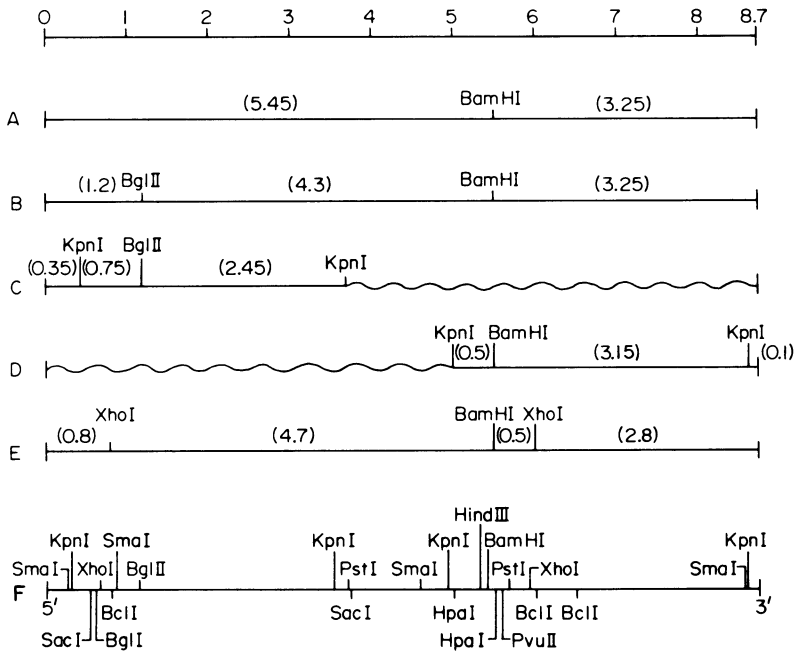

Figure 3. Restriction maps of unintegrated linear FeLV DNA. Physical maps of cleavage sites derived from the data shown in Fig. 2 are shown in lines A-E. The complete map depicting cleavage sites made by all thirteen enzymes found to cut within viral sequences is shown in line F. Two enzymes, Eco RI and Xba I do not cleave FeLV DNA. The orientation of maps relative to the 5' and 3' ends of viral RNA was determined as described in the text.

the Bgl II site, a Kpn site must be found about 0.75 kb to the left, and another 2.45 kb to the right (Fig. 3, line C). Bam HI cleaves the largest Kpn fragment resulting in two fragments at 3.15 kb, and fragments 1.35, 0.5 and 0.35 kb in length. This positions one Kpn site 0.5 kb to the left of the Bam HI site and suggests that a fourth Kpn site is found $\sim$ 0.1 kb from the right end of the map (Fig. 3, line D). A 0.1 kb fragment would not be observed under these conditions due to diminished retention of small DNA fragments on nitrocellulose (Mullins, unpublished observations), and reduced sensitivity for the detection of short fragments.

Xho digestion results in three fragments, 5.2, 2.8, and

0.8 kb in length. Bam Hl cleaves the largest Xho fragment resulting in fragments 4.7, 2.7, 0.8 and 0.5 kb in length. These data indicate that the Xho sites are found 0.8 kb and 2.7 kb from the left and right ends of the map, respectively (Fig. 3, line E). Kpn I digestion shortens the 2.8 kb Xho fragment to 2.7 kb, positioning the Kpn site 0.1 kb from the right end of the map. Subsequent double digests with Bcl I (shown in Fig. 6) as well as Pvu II (data not shown) confirm the position of this Kpn I site. Restriction sites of 8 additional enzymes were mapped in a similar manner by double digestion with Kpn I or Bam HI (data not shown). The resulting complete map is shown in Fig. 3, line F. Two enzymes, Eco Rl and Xba I do not cut unintegrated FeLV DNA.

(b) The long terminal repetition. Both unintegrated and integrated forms of Type C retrovirus DNA's have been found to contain long terminal redundancies 300-1200 bp in length (24-27). These redundancies result from retrovirus replication and are composed of sequences homologous to both the 3' and 5' ends of viral RNA in the order 3'|5'—//—3'|5' (24,25,27). Long terminal redundancies (LTR's) are detected by hybridization of 3' and 5' specific probes to both ends of viral DNA and by identical ordering of restriction sites near the ends of the DNA.

Figure 3F shows that there are Sma I sites very close to the Kpn I sites about 350 bp from the left end and 100 bp from the right end of the linear duplex. The 350 bp band produced by Sma I, Kpn I and Sma I plus Kpn I are indistinguishable (Fig. 4). Studies of the cloned integrated FeLV sequences (manuscript in preparation) suggest that the Sma I sites are about 10 bp to the left of the Kpn I sites at both the left and right ends. It is quite improbable that this arrangement is due to random chance, and strongly suggests the existence of a long terminal repetition of length 350 + 100 = 450 bp.

A hybridization probe enriched for 3' sequences was prepared by using oligo-dT chromatography to select poly A containing (3' end) viral molecules of average length 2 kb $\pm$ 1 kb from randomly degraded viral RNA. Representative cDNA of this RNA was then synthesized using DNase I digested calf thymus DNA as primer. The use of this probe resulted in intense hybridi-

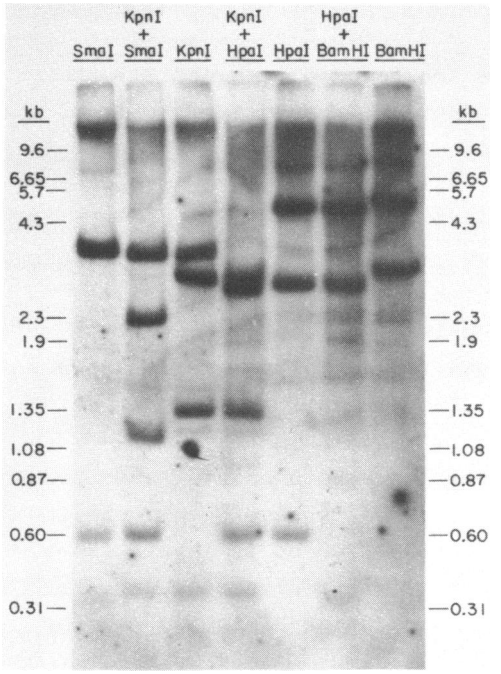

Figure 4. Mapping unintegrated viral DNA. 10 ug of Hirt supernatant DNA was digested, electrophoresed in a 1.2% agarose gel and visualized as described in the legend to Fig. 1. Molecular weight markers were the same as described in Fig. 2.

zation to restriction fragments from both ends of the viral DNA, demonstrating that 3' sequences are found on both ends. (c.f. the 3.15 and 3.45 kb Kpn I fragments and the 1.0 and 1.7 kb Bcl I fragments in Fig. 5.) This is additional evidence for the presence of LTR's. Futhermore, with this probe there is relatively greater intensity of hybridization of internal fragments derived from the right side of the restriction map (Fig. 5). Thus the orientation of viral DNA relative to the 5' and 3' ends of viral RNA was determined, and the map shown in Fig. 3 is drawn with the 3' end of the viral RNA on the right.

(c) Origin of the minor bands. Five minor bands are seen in Southern blots of undigested FeLV Hirt supernatant DNA, migrating at positions corresponding to lengths of 21, 17.5, 6.65, 5.2, and 4.8 kb for linear DNA (Fig. 1). Limited nicking of this DNA by incubation at 37°C (Fig. 1) or by mild DNase I digestion (data not shown) results in the loss of the two lowermost bands, a corresponding increase in intensity of the two uppermost bands and no effect on the predominant linear form.

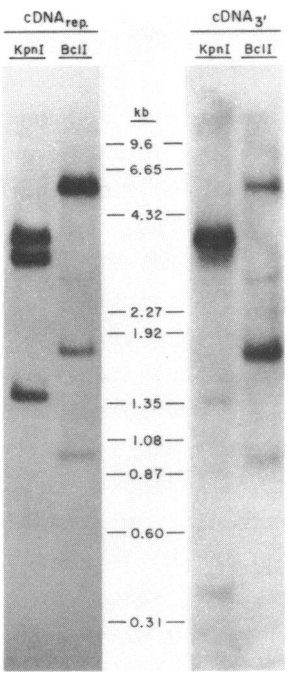

Figure 5. Orientation of the restriction map relative to the 5' and 3' ends of viral RNA. Oligo-dT cellulose chromatography was used to select poly A containing 3' viral RNA molecules in preparations of degraded viral RNA. Representative cDNA was synthesized from total RNA ($cDNA_{rep}$) and selected RNA ($cDNA_{3'}$) using DNase I digested calf thymus DNA as primer. The DNA's employed and procedures used for visualization were the same as described in the legend to Fig. 1. Molecular weight markers were the same as described in Fig. 2. Both blots were hybridized in the presence of 10% of dextran sulfate.

This suggests that two circular forms are present in these preparations. The two lowermost bands probably correspond to supercoiled closed circular forms and the two uppermost bands probably correspond to nicked circular forms. The mobility of the 6.8 kb minor band is not affected by single strand nicking therefore it is probably a linear form. Its origin is presently unknown. The presence of circular forms have been documented previously in similar preparations of the unintegrated DNA of avian sarcoma virus and murine leukemia virus (24,28,29). In these cases, the larger form corresponds to a molecule containing two adjacent copies of LTR's and therefore resembles a circularized linear form, whereas the smaller form contains only one LTR.

Digestion with any of the 5 restriction endonucleases making a single cut in linear viral DNA reproducibly results in two minor bands, one 8.7 kb, the same length as the uncut linear form, and another 8.2 kb in length. This occurs along with the corresponding loss of the circular bands seen without enzyme digestion (c.f., Bam HI and Bgl II digests patterns, Fig. 2; in

the reproductions shown here, only the more predominant 8.7 kb band is clearly visible). The intensities of these bands are about the same as those of the circular forms seen without enzyme digestion. These observations suggest that these minor forms represent linearized circles and that the circular forms have the same sequence organization as the predominant linear form and that the shorter form may lack one copy of the 450 bp LTR.

If circularized forms of the major, linear viral DNA are found as minor components, then digests of FeLV unintegrated DNA should reveal minor bands with an apparent molecular weight equal to the fusion product of the left and right terminal fragments of the linear molecule. In all cases in which the predicted fragments are sufficiently large to be detected, they are found.

The junction fragment from the longer form, which is more abundant, is observed when the predicted fragment length is 3 kb or greater; both the longer and shorter (by 0.45 kb) fragments are observed for fragment lengths 5 kb or greater. Examples are shown in Figs. 2 and 4. Xho digestion results in a minor band 3.6 kb in length (Fig. 2), the predicted length of a fusion product between the 2.8 and 0.8 kb end fragments of the linear molecule. Bam HI digestion does not cleave within the terminal Xho fragments and does not alter the mobility of the 3.6 kb Xho I fragment. Figure 4 shows a Hpa I digest of Hirt supernatant DNA, Hpa I makes two cuts, 0.6 kb apart, flanking the Bam HI site in the center of the genome. The minor bands detected after Hpa I digestion are 8.1 and 7.6 kb, 0.6 kb smaller than the minor bands in the Bam HI digest and the exact length predicted for fusion fragments. Furthermore, as expected Bam HI digestion cleaves the smallest Hpa I fragment derived from linear DNA and does not affect the mobility of the minor forms. The data indicate the presence of two circular forms, one probably containing two adjacent copies, and the other one copy of the LTR.

Sequence organization of FeLV DNA after integration into host chromosomal DNA. RD(FeLV)-2 is a cell line derived by infection of RD cells with the Gardner-Arnstein strain of FeLV (30; Nicolson, unpublished), and cloning in soft agar.

Digestion of RD(FeLV)-2 chromosomal DNA with enzymes which do not cleave within the viral genome (c.f., Eco RI digest shown in

Fig. 1; Xba I and Xba I + Eco RI digests are not shown) results in eight or more FeLV containing bands, each greater than the viral genomic length of 8.7 kb. Some of the bands are broad and consist of several unresolved or partially resolved components, therefore we cannot make a precise statement as to the number of integrated FeLV sequences. Recombinant DNA studies of this cell line indicate at least 15 provirus copies are present (Mullins et al., manuscript in preparation).

These data are therefore consistent with the model that RD(FeLV)-2 cells contain 15 or more copies of full length FeLV proviral DNA, with each copy integrated at a different site within the host chromosome. The lengths of the fragments containing FeLV sequences are then determined by the positions of the restriction sites in the host sequences flanking the viral DNA.

According to this model, if the integrated FeLV sequences are not permuted relative to unintegrated FeLV, digests with enzymes that make two or more cuts within the viral sequences should give bands of about 15-copy intensity for the internal fragments, with single copy intensity for fragments defined by one cut within the viral DNA and one in the flanking sequences.

Digestion of unintegrated DNA with Kpn or Bcl reveals 4 major bands each, detectable in Southern blots (Fig. 6, panel A). Double digestion with these enzymes reveals six bands (Fig. 6, panel A). The lengths of these fragments can be predicted from the map in Fig. 3. Digestion of RD(FeLV)-2 DNA with either of these enzymes or combination reveals the same bands except for the left and right terminal fragments (Fig. 6, panel A). These observations support the model that FeLV DNA integrates as a complete molecule, colinear with unintegrated DNA. Colinearity is detectable out to the terminal Kpn and Sma sites. The intensities of these bands suggest that most, if not all, of the integrated copies contain the same internal arrangement of restriction sites. Fainter bands are visible which may plausibly be attributed to single copies of terminal viral sequences attached to host flanking sequences. This supports the model that viral DNA is integrated at different sites within the host genome and is not present in a tandem array. If viral genomes were present in a tandem array, fragments

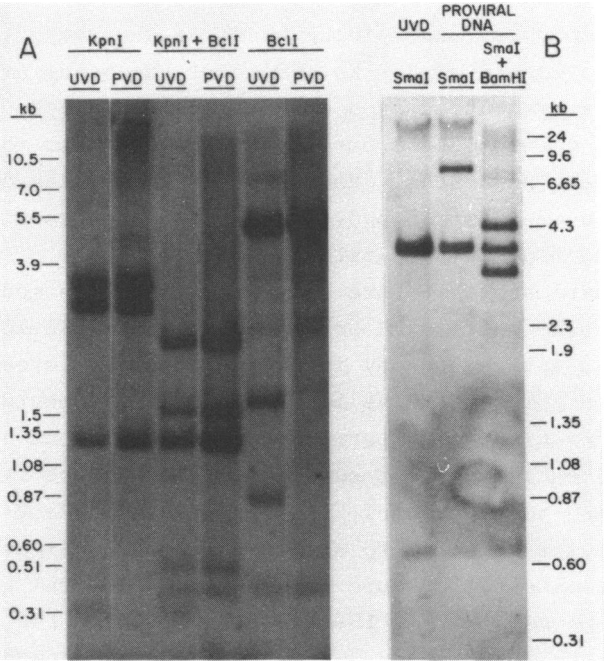

Figure 6. Sequence arrangement of FeLV proviral DNA. The restriction patterns of FeLV related DNA obtained after digestion of 10 ug of Hirt supernatant DNA (UVD) was compared to that of 2 ug of chromosomal DNA (PVD) from RD(FeLV)-2 cells. Protocols for visualization of bands are as indicated in the legend to Fig. 1, except that a 1.2% agarose gel was used. Molecular weight markers used were Bam HI digests of Charon 4 DNA, Hind III digests of lambda DNA and Hae III digests of ØX174 DNA.

of length equal to the fusion of left and right ends would be prominent. Such bands have not been detected. Additional mapping of integrated viral sequences using the enzymes Xho I, Pst I, Hind III, Pvu II and Sac I (not shown) give consistent results.

The recognition site for Sma I is 5' CCCGGG-3'. The underlined C is sometimes methylated in eukaryotic DNA, which renders the site resistant to Sma I cleavage (31).

Digestion of RD(FeLV)-2 DNA with Sma I reproducibly results in a 7.5 kb band in addition to the 3.7, 3.8, 0.6 and 0.35 kb internal bands expected (Fig. 6, panel B). The 7.5 kb band is

cleaved by Bam HI and a 4.5 kb fragment is observed as well as the expected fragments. This suggests that the Sma site between the 3.7 and 3.8 kb fragment is either missing or refractory to cleavage in about one third of the FeLV proviruses. However, examination of cloned FeLV proviral DNA (Mullins et al., manuscript in preparation) reveals that no SMA I sites are missing in at least 15 different copies of integrated DNA. This result is not due to partial digestion, since no evidence for partial digestion at other sites is observed and marker DNA's included in the digests were digested to completion in each reaction (data not shown). We suggest that the correct interpretation is that in some, but not all, of the copies of FeLV, the Sma I site in the center of the genome (Fig. 3F) is methylated.

Comparison of digests by Hpa II and Msp I can distinguish between methylated and unmethylated CCGG sequences and the Sma I site constitutes a subset of these sites (32). Our studies of FeLV digests by these enzymes demonstrate that some, but not all, of these sites in the region of the Sma site in the center of the FeLV genome are methylated (33).

DISCUSSION

We have presented restriction mapping data of FeLV related DNA sequences present in both unintegrated and integrated forms in virus infected human RD cells. Mapping was accomplished by single and double cleavage of the DNA of interest with restriction endonucleases, separation of fragments according to size in agarose gels, transfer to nitrocellulose paper and hybridization using ^{32}P cDNA made from purified FeLV RNA.

Both unintegrated and integrated FeLV DNA's were obtained after infection of RD cells with the Gardner-Arnstein strain of FeLV. This strain contains no detectable level of transforming FeSV (Nicolson, unpublished observations). Of the two FeLV subtypes present in this strain (A and B), only the B subtype is thought to infect human cells (14,19). Therefore the abundant DNA species seen in these preparations is probably FeLV-B.

The predominant form of unintegrated FeLV DNA observed is a linear 8.7 kb duplex DNA molecule. A 450 bp long terminal repetition of DNA derived from both ends of viral RNA is found in

preparations of unintegrated and integrated DNA. This compares with the 300-1200 bp repetitions found in other retroviruses (24-27). Two minor, probably circular forms of unintegrated viral DNA are detected in addition to the prominent 8.7 kb linear form. These minor forms are 8.7 and 8.2 kb in length and probably correspond to circular molecules containing 2 and 1 copies of the LTR, respectively. Such molecules have been detected in avian and murine systems as well. The minor 6.65 kb band seen in unintegrated DNA preparations may correspond to FeLV subtype A DNA which could have been pseudotyped by B particles, or to a deleted form of the predominant linear molecule. Experiments are currently underway to serotype virus derived from recombinant cloned FeLV DNA obtained from these cells.

Our results demonstrate that integrated FeLV sequences in RD(FeLV)-2 are colinear with the predominant unintegrated form. On the basis of restriction fragments produced by digestion of integrated DNA with six-base recognition enzymes, FeLV DNA appears to integrate at different sites in human DNA, since many, apparently single copy virus-cell junction fragments, are observed. Digestion of chromosomal RD(FeLV)-2 DNA with enzymes which make a single cut within the viral genome results in bands of variable lengths and no prominent bands of viral genome length (data not shown). In addition, no fragments of length equal to the fusion of right and left ends of the viral genome are seen in integrated DNA in contrast to unintegrated DNA preparations. This indicates that in these cells, viral genomes have not integrated in a tandem array. And while we have not ruled out the presence of linear concatenates in our unintegrated viral preparations, no evidence is found for the integration of these forms or permuted forms of circular molecules. Our data do not rule out short, specific, or preferred sites of integration at a repeated sequence in the human genome, undetectable by the restriction mapping carried out thus far. More detailed analyses of the sequence organization, integration specificity and biological activity of integrated FeLV DNA cloned in procaryotic vectors are currently underway in our laboratories.

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