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The U3 portion of feline leukemia virus DNA identifies horizontally acquired proviruses in leukemic cats

(long terminal repeat/proviral integration/virus-negative leukemia/virus-positive leukemia/DNA-mediated transfection)

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ABSTRACT **The presence and location of DNA sequences related to the U3 and U5 portions of the infectious exogenous feline leukemia virus (FeLV) long terminal repeat (LTR) in various cat DNAs have been determined by hybridization experiments. In uninfected cat DNAs, the U5 LTR segment from the Gardner–Arnstein strain B virus is present at approximately 150 copies per cell. This level is approximately 10-fold greater than that of endogenous internal FeLV sequences. The U5 sequences differ in copy number and, to some extent, in location from one animal to another. For any one animal, the sequence organization of the U5 segments is the same among different tissues, showing that the pattern is inherited through the germ line. Most importantly, the viral U3 LTR probe hybridizes only very weakly with uninfected cat DNAs. Both the U3 and the U5 regions of the LTR from the Gardner–Arnstein strain of virus cross-hybridize with DNA derived from four other infectious FeLVs representing A, B, and C subtypes. Thus, the U3 region may be used as a probe for studying the number and location of exogenously acquired FeLV proviruses in infected cat tissues. In some cases exogenously acquired proviruses are present in unique sites in the genome of virus-positive cat lymphosarcomas, indicating a monoclonal origin for the tumor. In other tumors, the proviral sequences are randomly distributed over many sites. Lymphosarcomas of virus-negative cats have no exogenous U3 sequences despite epidemiological evidence of an association of virus-negative leukemia with exposure to FeLV.**

Feline leukemia virus (FeLV) is a replication-competent retrovirus found associated with a wide group of malignancies of hematopoietic origin in domestic cats (1). As with other mammalian leukemia retroviruses, FeLV does not appear to encode a transforming gene. Thus the induction of leukemia is due to a still undefined interaction between viral and host genetic information.

A central difficulty in studying virus–host interactions in cats is the presence of endogenous FeLV-related DNA sequences. Although these sequences are not inducible as virus (2), their presence at 8–10 copies per haploid genome hinders molecular analysis of exogenously acquired proviruses (3). We find that the U3 portion of the long terminal repeat (LTR) of infectious (exogenous) FeLV is not endogenous to domestic cats, and it may be used as a probe to determine the presence, number, and location of horizontally acquired FeLV proviruses. This report focuses on the organization of U3 and U5 portions of the LTR in uninfected and infected tissues, both normal and malignant, from domestic cats.

FeLV is present in, and is believed to be the infectious agent in, about two-thirds of all cases of leukemia in cats. However, about one-third of the naturally occurring lymphoid tumors in domestic cats do not contain infectious FeLV or viral structural proteins (1). Yet, epidemiological evidence suggests that virus-negative tumors are associated with exposure to FeLV (4). We have used the virus-specific U3 probe to survey the occurrence of exogenous FeLV DNA sequences in virus-positive and virus-negative lymphoid tumors.

MATERIALS AND METHODS

Source, Preparation, and Transfection of DNA. Tissues were obtained from infected and uninfected domestic cats. All animals were examined grossly and histologically for pathological changes. Blood samples were characterized as being virus-negative or virus-positive by fluorescent antibody (FA) assays directed to the FeLV p30 antigen. DNAs were transfected into dog D17, feline embryo fibroblasts (AH 927), and human RD4 cells as described (5). Isolation of DNA from tissues and tissue culture cell lines was carried out as described (6).

Construction of Hybridization Probes. An *Eco*RI fragment containing an entire infectious FeLV provirus (Gardner–Arnstein B) with human RD4 host flanking sequences was isolated from a λ Charon 4A clone designated HF60 (7), inserted into the plasmid vector pKC 7 (8), and subcloned. This plasmid, denoted pKHR-1, contains several *Kpn* I sites, all within the viral sequences (Fig. 1). There is a *Kpn* I site almost precisely in the middle of the R region of each of the two LTRs. Digestion of pKHR-1 with *Kpn* I followed by religation gives the plasmid pKHR-7 (Fig. 1). It contains one LTR, no other viral sequences, and human host flanking sequences. The lengths of the U3, R, and U5 segments of the FeLV LTR are about 337, 68, and 72 base pairs, respectively (J. Santon, personal communication). Thus, by digestion of pKHR-7 with *Kpn* I and *Eco*RI, we can use gel electrophoresis to isolate one fragment containing the 337-base pair U3 sequence, 34 base pairs of R, and 0.8 kilobase pairs (kb) of human flanking sequences; we refer to this as the U3-specific probe. The other fragment isolated by gel electrophoresis contains 34 base pairs of the R region, the 72-base pair U5 sequence, and 0.44 kb of human copy flanking sequences. We refer to these as the U5-specific probe. Nick-translation, DNA digestion, Southern gel filter transfer, and hybridization were performed as described (7, 9).

RESULTS

Hybridization of the U5 LTR Sequence to DNAs from Various Cat Cell Lines. Preliminary hybridization studies with the U5 probe shown in Fig. 1 indicated that there were many copies

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Abbreviations: FeLV, feline leukemia virus; LTR, long terminal repeat; FA, fluorescent antibody; kb, kilobase pair(s).

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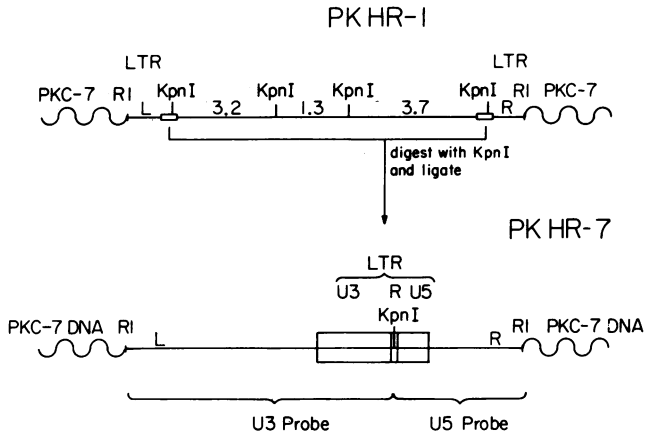

FIG. 1. Construction of the U3 and U5 FeLV LTR probes. L and R are the left and right human flanking DNA sequences for this particular integrated FeLV. Lengths are given in kilobase pairs. RI, *Eco*RI cleavage site.

of the U5 sequence in uninfected cat DNAs. To measure the copy number we compared the hybridization pattern of DNA obtained from a feline embryo fibroblast cell line AH 927 with that of a cloned FeLV-infected (Gardner–Arnstein B) human cell line termed RD FeLV-2 (Fig. 2), which contains about 20 provirus copies per cell (7). Digestion of RD FeLV-2 DNA with *Kpn* I and hybridization with the U5 probe yields an intense hybridization band at 3.2 kb, representing the 5′ internal portion of the provirus, and 20 faintly hybridizing bands of different

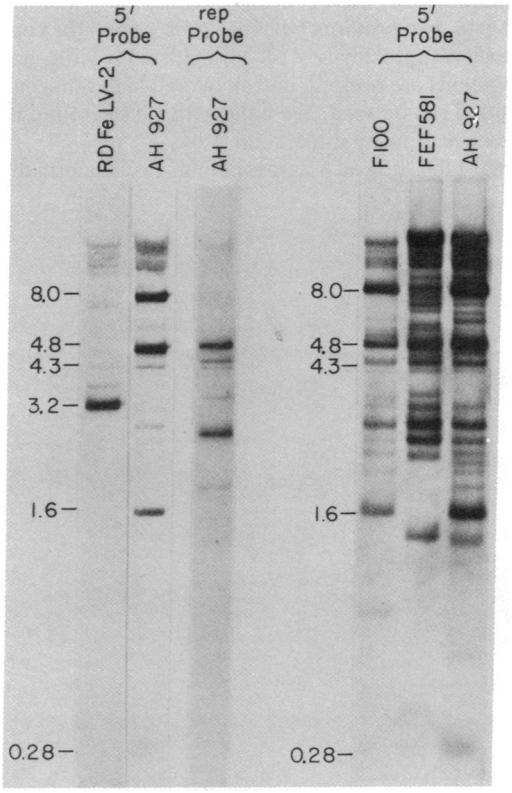

FIG. 2. Hybridization of the U5 probe and a representative probe (rep) to various cat DNAs. Each lane contained 5 μg of *Kpn* I-digested DNA. The specific activities of the U5 and rep probes were 1.6×10^8 cpm/μg and 3.1×10^8 cpm/μg, respectively. Fragment sizes in this and the following figures are given in kb.

molecular weights, originating from the 3′ end of the provirus (see Fig. 1). These faint hybridization signals are virus–host junction fragments, and their different molecular lengths are due to the various locations of the *Kpn* I sites on the flanking host DNA. The pattern for *Kpn* I-cleaved DNA from the AH 927 line hybridized on the same filter shows three very intense bands (1.6, 4.8, and 8.0 kb) along with many other less intense bands ranging from single copy to 40 copies per band (estimated from intensity). We estimate from densitometer tracings that this cell line contains a total of about 175 copies of the U5 sequence. The 3.2-kb Gardner–Arnstein type internal viral fragment is not present in this cat DNA. Hybridization of *Kpn* I-cleaved AH 927 DNA with a representative FeLV probe is included to show that the very intense U5 signal is due to reiteration of primarily the U5 sequence, because other FeLV sequences do not hybridize to either the 8.0-kb or the 1.6-kb bands with high intensity. Examination of the hybridization pattern of *Kpn* I-cleaved DNAs from two other cat cell lines is also shown in Fig. 2. In this case we exposed the blot for a longer period of time to increase the intensities of the weaker bands. Each cell line tested, including several not shown in Fig. 2, was derived from a separate uninfected cat. Each has some hybridizing bands that do not occur in AH 927 and some bands that are shared with those of AH 927 but are of different relative

Table 1. Characterization of tissue and DNA

Cat	FA	Tissue*	Co-cultivation	Trans-fection†	Virus‡
		Animals with virus-positive leukemia			
1449	+	Spleen	+	−	Foamy
		T-LNM	+	−	Foamy
		Liver	+	−	Foamy
1452	+	T-MLN	+	−	FeLV
		Kidney	+	+	FeLV
1423	+	T-ileum	−	+	Foamy and FeLV
		Spleen	−	+	FeLV
1417	+	T-MLN	NT	+	FeLV
		Animals with virus-negative leukemia			
1433	−	Spleen	−	+	Foamy
		T-MLN	NT	−	−
1435	−	T-MLN	NT	−	−
1436	−	T-intestine	−	−	−
1432	−	T-intestine	NT	−	−
1420	−	T-kidney	NT	+	Foamy
1409	−	T-kidney	NT	−	−
		Animals without leukemia			
1450	NT	Spleen	+	+	FeLV
		Liver	+	+	FeLV
1404	−	Spleen	NT	−	−
CHLA-1	NT	Liver	−	−	−
		Spleen	−	−	−
CHLA-2	NT	Liver	−	−	−
		Spleen	−	−	−
SPF§	NT	Kidney	NT	−	−
1446	−	T-SL	−	−	−
		Spleen	−	−	−
		Liver	−	−	−

NT, not tested.
* T, tumor; LNM, mediastinal lymph node; MLN, mesenteric lymph node; SL, sublingual.
† DNA-mediated transfection.
‡ Virus recovered from either cocultivation or DNA-mediated transfection.
§ Specific pathogen-free cat.

intensities. In each DNA, many of the hybridizing fragments contain multiple copies of the U5 sequence. Similar results were obtained with DNA from a variety of uninfected cat tissues (see Fig. 5).

The U3 Region Is Not Endogenous in Domestic Cats. Tissues from a number of cats were provided by local veterinarians and tested for infectious virus by FA assays, by cocultivation with virus-sensitive cells, and by transfection. [Many previous studies have shown that the DNA of cells expressing infectious virus is positive by the DNA-mediated transfection assay and conversely (5).] A number of these tissues did not contain expressed viral genomes, by at least two of these criteria (see Table 1).

Hybridization tests using high-sensitivity conditions were conducted with the U3 probe (Fig. 3). Control DNA (RD FeLV-2) gives an intense 3.7-kb internal proviral fragment originating from the 3′ end of the proviral genome and approximately 20 single-copy virus–host junction fragments of different molecular weights, originating from the 5′ portion of the viral genome (see Fig. 1). The hybridization patterns of the DNAs from the virus-negative nonleukemic animals show only very faint bands, each much less than single-copy in intensity. Thus we conclude that normal cat DNA does not contain any well-matched copies of the U3 sequence of infectious virus.

The U3 Region Is Common to the FeLV Subgroups A, B, and C. The three known FeLV subtypes, A, B, and C (10), are related in sequence but differ in restriction endonuclease patterns (7, 11). In order to obtain integrated DNA copies of these viral genomes, viruses of the three subtypes were used to infect dog or human cells. DNAs were extracted from the cells 6 weeks after infection and examined by restriction endonuclease blotting analysis with the U3 probe. Under these circumstances, only internal viral bands were visible because of the great heterogeneity of junction fragments after a mass infection. The results in Fig. 4 show that the Gardner–Arnstein U3 probe hybridizes with all of the DNAs. Some of the hybridization intensity differences seen in Fig. 4 are due to differences in virus copy number in the different DNAs (data not shown). The A subtype, derived from an original Rickard isolate, contains two species with hybridizing bands of 3.7 kb and 1.0 kb, whereas the A Glasgow isolate contains only a 3.7-kb species.

Presence of the U3 Sequence Correlates with Virus-Positive Tumors. The data reported above show that the U3 probe may be used to test for the presence in cat DNAs of provirus acquired by infection. Accordingly, we have studied the DNAs from tumors and other tissues of animals with diagnosed leukemia (Table 1). We first consider animals characterized as having leukemia and testing FA^+ for FeLV p30 (Fig. 3).

Cat 1449 had a mediastinal lymph node tumor. When hybridized with the U3 probe, the *Kpn* I digest of this DNA gives an intense 3.7-kb internal viral band like those of Gardner–Arnstein, Sarma, and Glasgow provirus, with a copy number of approximately 10. There are also many distinct but less intensely hybridizing bands with intensities similar to those observed for the single-copy virus–host junction bands of the control RD FeLV-2. We therefore believe that this tumor is monoclonal in origin. The DNAs isolated from spleen and liver of this animal also contain proviral sequences. However, the single-copy bands present in the tumor are not present in the spleen and liver DNA samples. We suggest that these latter tissues have received a secondary infection of virus and that the integration sites therein are heterogeneous. Cat 1452 gave a 5.1-kb restriction fragment with an intensity that is approximately single copy and no 3.7-kb fragment. Thus, its restriction endonuclease pattern is unlike that of any known FeLV provirus. Cat 1423 had a possible mixture of two provirus types, one having a 3.7-kb fragment and a second having a 1-kb U3 hybridizing band. From intensity data, there are approximately 7 proviruses per cell and no observable single-copy virus–host junction fragments. Thus this tumor and the spleen have FeLV proviruses inserted at many different sites in different cells. The most convincing example of heterogeneous provirus integration is animal 1417. Again, two provirus types are present and the copy number of each is approximately 10. No single-copy virus–host junction fragments are present, and an overall high semicontinuous background can be seen. We believe this background is due to the presence of many integration sites.

Animal 1450 showed no gross pathological abnormalities but

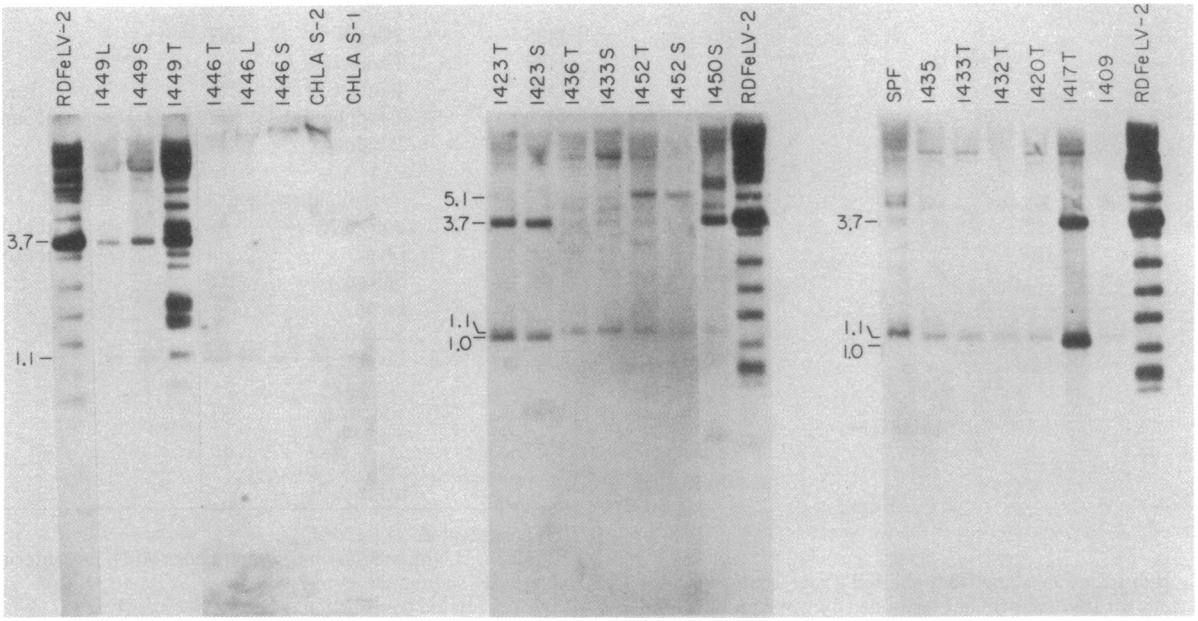

FIG. 3. Hybridization of the U3 probe to various cat DNAs. Each lane contained 25 μg of *Kpn* I-digested DNA. The probe was synthesized with [α-^{32}P]dCTP (specific activity 3200 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) to a final specific activity of 8.7×10^8 cpm/μg.

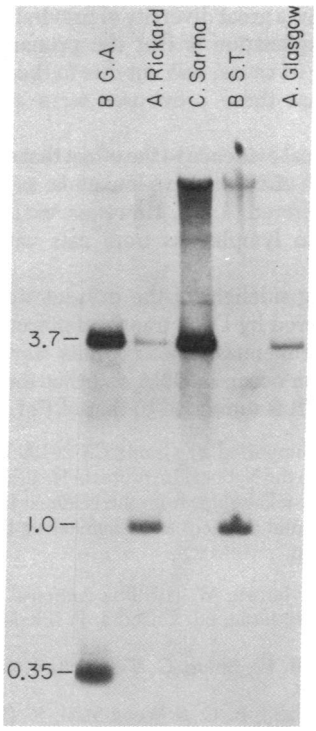

FIG. 4. Hybridization of the U3 probe to different subtypes of FeLV. Each lane contained 5 μg of *Kpn* I-digested DNA. The specific activity of the U3 probe was the same as given for Fig. 3.

was FeLV positive. About 5% of urban domestic cats are virus positive, and of these about 1% later develop leukemia (1). It was surprising to find that this animal's spleen was composed primarily of a clonal cell population of virus-infected cells, as evidenced by the four virus–host junction fragments of approximately single-copy intensity in addition to the intensely hybridizing 3.7-kb internal viral band. The absence of solid tumors and the fact that the spleen contains a high concentration of B cells suggests that this clonal population was due either to an early, undiagnosed virus-positive leukemia of single-cell origin or to the infection of a B lymphocyte stem cell.

Finally, we have assayed the tumors from six cats with virus-negative leukemias. Just as for virus-negative cat and cat cell line DNAs, there are a number of faint bands, but none of intensity comparable to those of the single-copy control, showing that there are no exogenously acquired U3 sequences in these tumors.

Table 1 summarizes the results of cocultivating tissues or transfecting DNA isolated from cats with and without leukemia. By one or both criteria, infectious FeLV was isolated from three of the four samples from animals with virus-positive leukemia, in agreement with all expectations. From the fourth animal, cat 1449, we were unable to isolate FeLV despite a high provirus copy number by hybridization. This suggests that the proviruses were defective. Animals with virus-negative leukemias were negative for FeLV by DNA-mediated transfection. This provides additional evidence that an exogenous FeLV provirus is not present in any of the tissues from these animals. It is interesting that in several cases of animals with virus-negative leukemias, and with the cat 1449 discussed above, foamy virus was recovered both by cocultivation and by transfection. In animals without leukemia, we were unsuccessful in isolating FeLV except from cat 1450. This is in accordance with the hybridization studies.

Hybridization of the U5 LTR Sequence to DNAs from Cats With and Without Leukemia. The U5 sequence is endogenous to normal cat DNAs, but the blotting patterns for different cell lines are somewhat different (Fig. 2). As shown in Fig. 5, variability is also seen among DNAs from different animals; however, the patterns of different tissues from any one animal are identical. This is most notable in the variation in intensity of the 8- and 1.6-kb hybridizing bands. For example, for cat 1446 (FA and leukemia negative), the DNAs from a sublingual (nonleukemia) tumor, liver, and spleen show identical hybridization pattern with the U5 probe. Cat CHLA1, with a somewhat different pattern than that of 1446, shows no differences between spleen and liver tissues.

The U5 patterns due to exogenously acquired virus are different from the endogenous ones. For example, for cat 1449

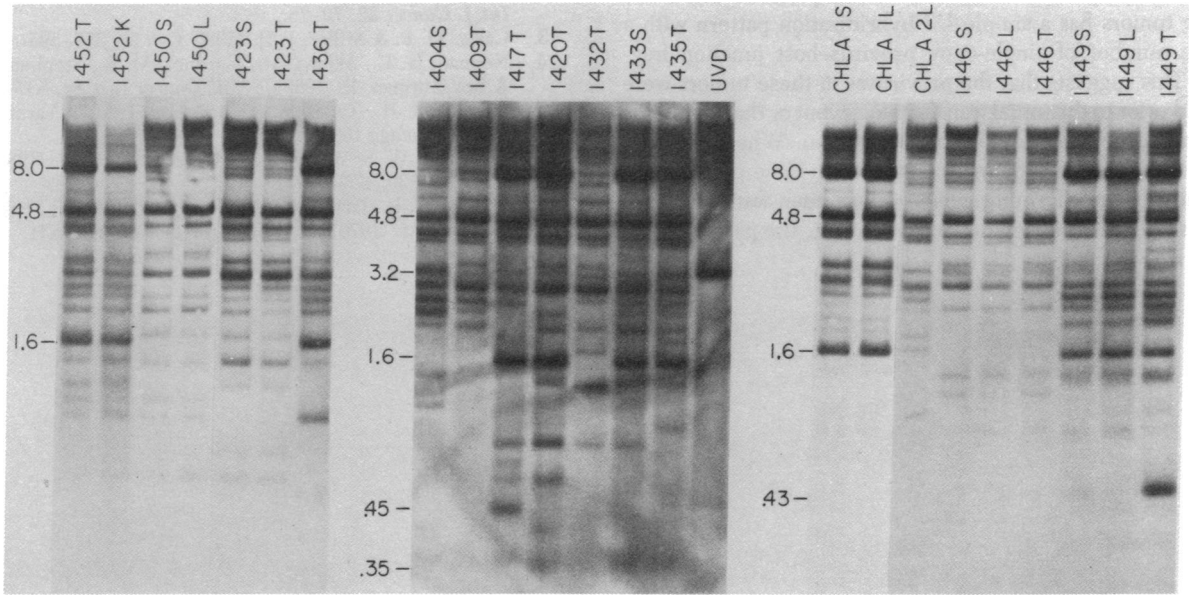

FIG. 5. Hybridization of the U5 probe with DNA from various cats and different tissues from individual cats. Each lane contained 5 μg of *Kpn* I-digested DNA. The specific activity of the U5 probe was the same as in Fig. 2.

there is an intense U5 band, due to an acquired internal viral sequence in the tumor, which is only barely visible in liver or spleen DNAs. This band corresponds to type A isolates, in agreement with the U3 hybridization pattern.

DISCUSSION

We find that sequences homologous to the U5 region of the LTR of infectious FeLV occur at a copy number of approximately 150 per cell in normal cat DNAs. Only a few are associated with the 8–10 endogenous copies of the main body of the FeLV genome (12). Comparison of the sequence organization of the endogenous U5 segment from different cell lines and from tissues of uninfected cats shows that the major hybridizing bands are conserved but that the relative amount of U5 DNA in a given band varies significantly. Furthermore, many minor bands are absent in some samples and new bands appear with different molecular lengths. Thus, every animal and cell line examined displays some unique fragments, but the locations of the U5 sequences in different tissues of the same animal are identical. We conclude that these sequences are stably inherited through the germ line. It is possible that the U5 elements and whatever other adjoining viral sequences are associated with them are mobile in the genome, similar to *copia*-like elements of *Drosophila* or δ sequences of yeast (13).

A surprising and very useful finding is that the U3 portion of the exogenous LTR is not endogenous to domestic cats. Very minor hybridization is observed with the U3 probe, indicating the occurrence of an undetermined number of highly mismatched sequences. Therefore, the endogenous U3 sequences have either diverged or originally were quite different from the exogenous proviral U3. The transcriptional inactivity of endogenous proviruses (14) may be due to these sequence differences in the U3 region, where the promoter sequences for viral transcription occur. Differences between the endogenous and exogenous U3 region and conservation of the U5 region have been observed also in the avian system (15); however, the level of U3 divergence appears to be much less than with FeLV.

We have shown that the exogenous U3 probe is useful for identifying exogenously acquired FeLV proviruses of all three subtypes. Exogenously acquired proviruses are present in virus-positive lymphomas in one of two basic patterns. The DNA of some tumors has a simple U3 hybridization pattern with a discrete number of single-copy provirus–host junction fragments. This suggests that the proviruses in these tumors were acquired prior to the initial transforming event or that a provirus itself was responsible for the transformation. Whether any of these exogenous U3 sequences is located in close proximity to host-encoded transforming genes, as has been found in avian leukosis (16), remains unknown. In contrast, the patterns from other tumors show a great diversity of proviral integration sites. One possible explanation is that the primary transformation event, whatever its cause, was not due to the FeLV proviruses present, and that these proviruses were acquired by later infection.

Epidemiological evidence to the effect that exposure to FeLV is associated with virus-negative leukemia, as well as virus-positive, has been offered (4, 17). However, we find no exogenous U3 sequences in lymphomas from cats with virus-negative leukemia.

An interesting sidelight of the present study is that foamy virus was recovered by DNA transfection from several cases of virus-negative leukemias. These results show that the foamy virus genome can occur as DNA and that the U3 region of its presumptive LTR is unrelated to that of FeLV.

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1. Jarrett, O. & Jarrett, W. (1979) in *Antiviral Mechanisms in the Control of Neoplasia*, ed. Chandra, P. (Plenum, New York), pp. 111–124.
2. Benveniste, R. E., Sherr, C. F. & Todaro, G. T. (1975) *Science* **190**, 886–888.
3. Koshy, R., Gallo, R. C. & Wong-Staal, F. (1980) *Virology* **103**, 434–445.
4. Hardy, W. D., McClelland, H. A., Zuckerman, E. E., Snyder, W. H., MacEwen, E. G., Francis, D. & Essex, M. (1980) *Nature (London)* **288**, 90–92.
5. Nicolson, M. O., McAllister, R. M., Okabe, H. & Gilden, R. V. (1978) in *Advances in Comparative Leukemia Research*, eds. Bentvelzen, P., Hilgers, J. & Yohn, D. S. (Elsevier/North-Holland, Amsterdam), pp. 139–140.
6. Nicolson, M. O., Hariri, F., Krempin, H. M., McAllister, R. M. & Gilden, R. V. (1976) *Virology* **70**, 301–312.
7. Mullins, J. I., Casey, J. W., Nicolson, M. O., Burck, K. B. & Davidson, N. (1981) *J. Virol.* **38**, 688–703.
8. Nagaraja-Rao, R. & Rogers, S. G. (1979) *Gene* **7**, 79–82.
9. Mullins, J. I., Casey, J. W., Nicolson, M. O. & Davidson, N. (1980) *Nucleic Acids Res.* **8**, 3287–3305.
10. Sarma, P. S. & Log, T. (1971) *Virology* **44**, 352–358.
11. Sherr, C. J., Fedele, L. A., Oskarsson, M., Maizel, J. & Vande Woude, G. (1980) *J. Virol.* **34**, 200–212.
12. Okabe, H., Dubuy, F., Gilden, R. V. & Gardner, M. B. (1978) *Int. J. Cancer* **22**, 70–78.
13. Calos, M. P. & Miller, J. H. (1980) *Cell* **20**, 579–595.
14. Neiman, H. L., Akhavi, M., Gardner, M. B., Stephenson, J. R. & Roy-Burman, P. (1980) *J. Natl. Cancer Inst.* **64**, 587–594.
15. Hughes, S. H., Toyoshima, K., Bishop, J. M. & Varmus, H. E. (1981) *Virology* **108**, 189–207.
16. Haywood, W. S. W., Weel, B. G. & Astrin, S. M. (1981) *Nature (London)* **290**, 475–480.
17. Jarrett, W. F., Jarrett, O., Mackey, L., Laird, W., Hardy, W. P. & Essex, M. (1973) *J. Natl. Cancer Inst.* **51**, 833–841.