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Retrovirus sequences in a leukemic gibbon and its contact: Evidence for partial provirus in the nonleukemic gibbon

(type C viruses/molecular hybridization/restriction enzymes)

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ABSTRACT **Integrated viral DNA sequences were detected in tissues from two gibbon apes, a leukemic gibbon (6G-1) from whose leukocytes a distinct strain of gibbon ape leukemia virus ($GaLV_H$) was isolated, and gibbon 6G-4, a contact of 6G-1 from the same colony that had uremia and cachexia of unknown origin. Although 6G-4 had no detectable neoplasia or viral proteins, its serum contained persistent antibody against GaLV antigens. Whereas DNA from most of the tissues of 6G-1 contained GaLV provirus, DNA from only three tissues (kidney, spleen, and liver) from 6G-4 showed detectable viral sequences, and the extent of hybridization in each case was lower than with 6G-1. After cleavage with *Bam*HI, two virus-specific DNA fragments were detected in tissues of 6G-1. Only one of these fragments was detected in the positive tissues of 6G-4. The results indicate that: (*i*) 6G-4 was exposed to and infected by GaLV; (*ii*) early target sites for infection of gibbon by GaLV may be limited to a few tissues; and (*iii*) infection can be contained by integration of only partial provirus in a few tissues.**

We recently reported the isolation of a type C virus from a gibbon with spontaneous malignant lymphoma and leukemia (1). This virus, designated $GaLV_H$, is in the same family that contains other gibbon ape leukemia virus isolates (2–5) and the simian sarcoma virus (SSV) and simian sarcoma-associated virus (SSAV) isolated from a fibrosarcoma of a pet woolly monkey (6, 7). Although closely related, the members of this group including $GaLV_H$ may be distinguished from each other by analysis of their RNA (8) or of the gp70 envelope protein (9). Proviral DNA and viral RNA and proteins were detected in most tissues of the leukemic gibbon (6G-1), and virus was recovered from all tissues, including tissues of the oral cavity (1). In addition to leukemic gibbon 6G-1, at least four of the eight animals of this colony were also exposed to $GaLV_H$ as indicated by the detection of serum antibodies (9, 10) even though none was viremic. For unknown reasons, one of the antibody-positive, nonleukemic, nonviremic animals, designated 6G-4, developed renal failure, became emaciated, and died. This provided an opportunity for search for proviral sequences in DNA from various tissues of this gibbon and to compare these sequences to those of 6G-1, the leukemic, viremic animal.

Here we report results of molecular hybridization experiments showing that proviral sequences were present in 6G-4 but only in DNA extracted from liver, kidney, and spleen, and the plateau hybridization values were much lower than those obtained with DNA from 6G-1. Using restriction enzyme cleavage of cellular DNA followed by hybridization of the fragments to ^{125}I-labeled viral RNA, we confirmed and extended our previous finding of proviral DNA in tissues from 6G-1. Two viral fragments were detected after cleavage with *Bam*HI. More interestingly, DNA from the positive tissues of 6G-4 was shown to contain only one of these viral fragments, indicating that only part of the GaLV provirus is integrated in the DNA of these tissues.

MATERIALS AND METHODS

Gibbon Apes. Both 6G-1 and 6G-4 were members of a gibbon ape colony from Bermuda and were obtained under the auspices of the Rockland Research Institute (Orangeburg, NY) (1). The gibbons were obtained from Southeast Asia and, to our knowledge, had not been in contact with other known gibbon ape colonies. As reported in detail elsewhere (1), 6G-1 was a 7-year-old male that died with acute lymphosarcoma and a lymphatic T-cell leukemia. It had been viremic at least 1 year prior to death, and the type C virus isolated from its tissues were closely related to, but distinguishable from, previous isolates of GaLV.

6G-4 was also a 7-year-old male that had been temporarily housed in a cage adjacent to 6G-1. It was emaciated on arrival at the quarantine facilities at Litton Bionetics, Inc. (Kensington, MD) and died shortly afterward. Clinical, laboratory, and postmortem examinations failed to disclose a definitive diagnosis. Blood chemistry values obtained several hours prior to death indicated the presence of severe metabolic disturbances with renal failure. The gross and microscopic postmortem findings were unremarkable except for extensive fatty infiltration and degeneration of many tissues, including the kidneys. Light microscopic and electron microscopic examination of the kidneys did not reveal any specific acute or chronic renal disease. There was no evidence of neoplasia. Tissues were collected from both gibbons at autopsy shortly after death.

Viruses. $GaLV_H$ was obtained from blood of the leukemic gibbon (6G-1) and subsequently transmitted to bat cells (B88 cells). SSV(SSAV) grown in 71AP1, a marmoset tumor line, was obtained from Electronucleonics, Inc. (Bethesda, MD).

Purification of High Molecular Weight DNA. Tissues were minced with scissors and homogenized with 4 vol of TE buffer (50 mM Tris·HCl, pH 9/10 mM EDTA) for 15 sec in a Sorvall Omnimixer. The samples were adjusted to 1% in sodium dodecyl sulfate ($NaDodSO_4$) and extracted with phenol/chloroform. The purified DNA samples were stored at a concentration of 1–2 mg/ml. The average molecular weight of these DNA preparations was in excess of 20×10^6 as revealed by agarose gel electrophoresis.

Preparation of Nucleic Acid Probes. [^{3}H]cDNA of SSV(SSAV) grown in 71AP1 cells and of $GaLV_H$ produced by the original cultured leukemic blood lymphocyte cell line was synthesized as described (11). SSV(SSAV) cDNA protected 50% of labeled SSV(SSAV) RNA from RNase digestion at a cDNA/RNA ratio of 1:1 and about 90% at a cDNA/RNA ratio of 5:1. Viral 70S RNA was prepared by phenol extraction and

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Abbreviations: $GaLV_H$, strain of gibbon ape leukemia virus; SSV, simian sarcoma virus; SSAV, simian sarcoma-associated virus; $NaDodSO_4$, sodium dodecyl sulfate.

Table 1. Summary of evidence for GaLV and antibodies to GaLV in gibbons 6G-1 and 6G-4*

Test	Gibbon 6G-1	Gibbon 6G-4
Whole virus		
Electron microscopy	+	−
Virus isolation by cocultivation	+	−
Virus Proteins		
p30	+	−
Reverse transcriptase	+	−
Serum antibodies		
Immunofluorescence	−	+
Virus neutralization	−	+
Reverse transcriptase neutralization	−	+
Viral protein precipitation†	−	+

* Data from refs. 1 and 9.
† Viral proteins p12, p30, and gp70; data from ref. 9.

velocity sedimentation (11) and was iodinated *in vitro* (12) to a specific activity of 50–100 × 10^6 cpm/μg. ^{125}I-Labeled 18S and 28S ribosomal RNA from HeLa cells were gifts of W. Prensky.

Digestion of DNA with Restriction Endonucleases. *Bam*HI and *Eco*RI were purchased from Bethesda Research Laboratories (Rockville, MD). Cellular DNA was digested by using 2 units of enzyme per μg of DNA for 16 hr at 37°C. Reaction conditions followed published procedures (13).

Agarose Slab Gel Electrophoresis and Blotting Technique. Twenty micrograms of restricted cell DNA was placed in each of 10 wells of vertical slab gels (16 × 23 × 0.6 cm) of 0.8% agarose (Sigma), and electrophoresis was carried out at a constant voltage of 150 V for 20 hr. The gels were calibrated by adding 1 μg of *Eco*RI-digested λ DNA to alternate wells. After electrophoresis, the gels were stained with ethidium bromide (1 μg/ml in buffer) and photographed with 259-nm illumination from below. Denaturation of the DNA and transfer from gels to nitrocellulose paper was carried out according to the procedure of Southern (14) as modified by Ketner and Kelly (15). After transfer, the filters were rinsed in 0.45 M NaCl/0.45 M sodium citrate and baked at 80°C for 4 hr.

Molecular Hybridization. For filter hybridization, the nitrocellulose sheets containing DNA transferred from gels were preincubated overnight in hybridization buffer (50% formamide/0.45 M NaCl/0.045 M sodium citrate/0.02% Ficoll/0.02% bovine serum albumin/0.02% polyvinylpyrrolidone/0.5% $NaDodSO_4$ containing 4 μg of calf liver tRNA per ml. The vessels used for hybridization were similar to those described by Botchan *et al.* (16). The nitrocellulose sheets were first wetted with hybridization buffer and then rolled into cylinders and inserted into 50-ml test tubes with screw caps. Additional 0.4 ml of the same buffer was added and the tube was tightly capped and placed in a rotating wheel in a 37°C room. After overnight preincubation, the liquid was replaced by 0.4 ml of fresh hybridization buffer containing 0.5 × 10^6 cpm of ^{125}I-labeled viral RNA, and hybridization was conducted at 37°C for 7 days. After hybridization, the filters were rinsed with two changes of 0.30 M NaCl/0.03 M sodium citrate containing 0.5% $NaDodSO_4$ at 60°C for 3 hr each and then with the same saline/citrate alone, dried, mounted on a cassette with tungstate screens (Du Pont, Lightning Plus), and exposed to Kodak X-Omat R film for 3–10 days at −70°C.

Liquid hybridization was carried out at cellular DNA excess with either [^{3}H]cDNA or ^{125}I-labeled viral RNA. The conditions for hybrid formation and detection have been described (17).

RESULTS

Evidence for Expression of or Exposure to GaLV. As reported (1) and summarized in Table 1, there was abundant evidence of expression of complete GaLV in the tissue and blood of gibbon 6G-1. The levels of virus expression approximated the degree of infiltration by virus-shedding leukemic cells, suggesting that other (parenchymal) tissues were not sites of substantial virus production. By these same techniques, no evidence of virus expression was found either in the tissues or plasma of the nonleukemic gibbon, 6G-4. On the other hand, no free antibody to GaLV could be found in the serum of gibbon 6G-1, probably due to the binding of antibodies by the large quantity of circulating viral antigens, whereas antibodies to GaLV proteins were readily demonstrated in gibbon 6G-4 (10) by various tests utilizing either unfractionated serum or purified IgG. This indicates prior infectious exposure to $GaLV_H$. Detailed results related to the immunological testing of gibbons 6G-1 and 6G-4 are presented elsewhere (9).

Hybridization of Labeled Viral Probes to Excess Cell DNA. DNA from all tissues (except the muscle and brain) of 6G-1 contained $GaLV_H$ provirus (1). We examined DNA from several tissues of 6G-4 for the presence of proviral sequences, using different viral probes: RNA and cDNA from the homologous virus $GaLV_H$ and a highly related virus, SSV(SSAV). These two viruses are approximately 50% homologous in their genomes (1). DNAs from all tissues were negative except those from liver, kidney, and spleen, and the amount of [^{3}H]cDNA that hybridized to these positive samples was only 20–40% of the amount that hybridized to DNA from 6G-1 leukemic cells (Table 2). The ratio of cell DNA to [^{3}H]cDNA was varied from 10^7 to 10^8 without producing significant difference in the hybridization values (data not shown). DNA from the marrow did not contain detectable proviral sequences, in contrast to the

Table 2. Hybridization of labeled viral probes to DNA from gibbon ape tissues

Source of DNA	Virus probe* GaLV$_H$ [^{3}H]cDNA	GaLV$_H$ ^{125}I-RNA†	SSV(SSAV) [^{3}H]cDNA	SSV(SSAV) [^{3}H]cDNA$_{5'}$‡
6G-1 leukocytes§	100	100	50	98
6G-4				
Spleen	40	—	—	—
Liver	33	20	20	95
Kidney	28	12		
Heart	15	—		
Marrow	6	5		
Lung	8	8		10
Lymph nodes	5	—		
Appendix	1	—		
Cerebellum	3	—		
Esophagus	1	5		
Tongue	8	—		
Normal gibbon spleen	—	5		
Calf thymus	4	—		2

* Results shown as % hybridization normalized to the homologous DNA (i.e., 6G-1 leukocytes for $GaLV_H$ probes and the infected cells for the SSV(SSAV) probes. The numbers are plateau values. Increasing cell DNA-to-probe ratio did not increase hybridization yields.
† ^{125}I-Labeled RNA.
‡ [^{3}H]cDNA$_{5'}$ (strong stop cDNA) was a 135-nucleotide transcript extending from the primer binding site to the 5′ terminus of the virus genome (kindly provided by W. Haseltine).
§ Results with other tissues from this leukemic animal have been described (1).

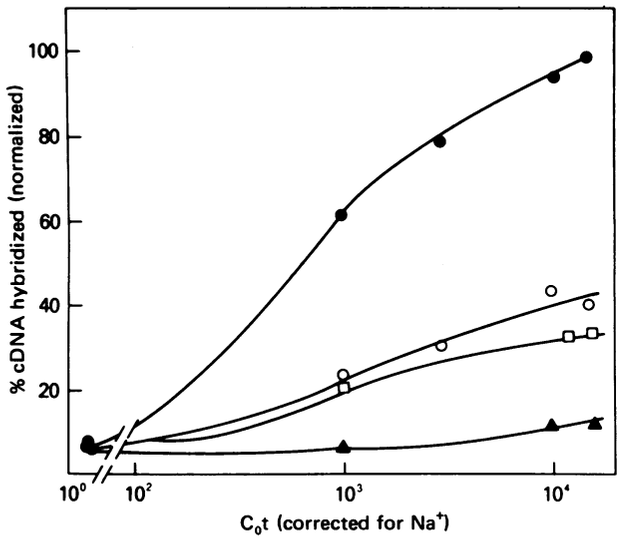

FIG. 1. Kinetics of hybridization of $GaLV_H$ [^{3}H]cDNA to gibbon tissue DNA. [^{3}H]cDNA was prepared from $GaLV_H$ by using whole disrupted virions. Hybridization was carried out using a 2×10^7-fold excess of cellular DNA to [^{3}H]cDNA; assay was by resistance to S1 nuclease. C_0t values (concentration in moles of nucleotide per liter times time in seconds) were corrected for Na^+ concentration. ●, 6G-1 leukocytes; ○, 6G-4 spleen; □, 6G-4 kidney; ▲, 6G-4 esophagus.

situation after infection of cats by feline leukemia virus which is apparently tropic for the marrow of exposed cats (18). ^{125}I-Labeled viral RNA hybridized even less efficiently to DNA of 6G-4 tissues, but the results also indicate that DNA from the same three tissues contained virus-related sequences not detectable in the DNA of other tissues from the same gibbon or in the DNA of a normal gibbon. Strong stop cDNA from SSV(SSAV), which is transcribed from the 5′ terminus of the virus genome (19), hybridized almost completely to 6G-4 liver DNA. This result again indicates that at least a portion of the GaLV genome was integrated in DNA of this tissue, but the lower hybridization plateau suggests that a complete provirus might be lacking.

Kinetics experiments performed with $GaLV_H$ cDNA and DNA from 6G-1 leukocytes and three 6G-4 tissues are presented in Fig. 1. Even at $C_0t > 10^4$ moles·sec/liter, hybridization to DNA from spleen and kidney of 6G-4 did not exceed 40%. DNA from the esophagus of 6G-4 is shown as an example of an apparently negative tissue. The results again suggest that only some tissues from 6G-4 contained integrated viral sequences, and even these may not constitute a complete provirus. However, by these experiments we could not rule out alternative possibilities for the lower hybridization values obtained with 6G-4—i.e., (*i*) detection of a related provirus that has only partial homology with the $GaLV_H$ provirus and is more distant to SSAV than to $GaLV_H$; and (*ii*) selection of one of two or more components in $GaLV_H$ and SSAV.

Hybridization of ^{125}I-Labeled rRNA to Gibbon and Human DNA Digested with *Bam*HI. In order to analyze further the integrated proviral sequences of 6G-1 and 6G-4, we hybridized ^{125}I-labeled viral RNA to restriction enzyme-cleaved DNA fragments, using the blotting technique (14). In the course of the experiments, the labeled viral RNA preparations were found to contain small amounts of rRNA (approximately 1% of the total RNA). Under the conditions of hybridization, this contamination is sufficient to reveal ribosomal DNA bands. Rather than eliminating this hybridization by competion with unlabeled rRNA, it is advantageous to have the rDNA bands to serve as internal markers for completeness of the nuclease digestion, efficiency of the transfer process, and position of the virus-specific DNA bands. Therefore, it was essential to establish which bands were ribosomal in origin. High molecular weight DNA was purified from isolated nuclei of several tissues of 6G-1 and 6G-4 and cleaved with *Bam*HI. The restricted fragments were separated on agarose slab gels and transferred to nitrocellulose filters. DNA from the human lymphoid cell line NC 37 infected with SSV(SSAV) was used as a positive control. DNA from normal gibbon spleen and normal human liver served as negative controls, and one of several replicate nitrocellulose filters containing DNA was hybridized to ^{125}I-labeled rRNA.

Cleavage patterns of different DNAs after hybridization to rRNA are presented in Fig. 2. Different mammalian DNAs yielded similar but distinct patterns of rDNA bands upon cleavage with *Bam*HI. Gibbon DNA yielded three fragments of molecular weights 4.1, 3.4, and 0.9×10^6 (lanes a and b). Human DNA displayed two major bands at 3.4 and 0.9×10^6 and two fainter bands at 4.5 and 5.0×10^6 (lanes c and d). These values are slightly different from those reported for mouse rDNA bands (20).

Hybridization of ^{125}I-Labeled SSV(SSAV) RNA to Control DNA Samples Digested with *Bam*HI. Preliminary experiments of hybridization of *Bam*HI-digested 6G-1 DNA to either SSV(SSAV) RNA or $GaLV_H$ RNA yielded identical patterns, indicating that all the viral fragments generated by this enzyme contained regions of homology between the two virus genomes (data not shown). Because SSV(SSAV) was produced at a much higher titer than $GaLV_H$, this virus was used as probe for subsequent experiments. Fig. 3 presents results obtained with the control samples. The bands observed with rRNA were present in all DNA samples. DNA from the spleen of a normal gibbon did not contain any virus-related fragment (compare lanes b and a). Uninfected human DNA revealed an additional fragment at 8.0×10^6 daltons (lane c) (21). In addition, DNA from human tissue culture cells (NC37) infected with SSAV yielded four viral bands, of 2.0, 1.5, 1.1, and 0.7×10^6 daltons, respectively (lane d).

Hybridization of ^{125}I-Labeled SSV(SSAV) RNA to *Bam*HI-Digested DNA from Tissues of 6G-1 and 6G-4. Results obtained with DNA from tissues of gibbons 6G-1 and 6G-4

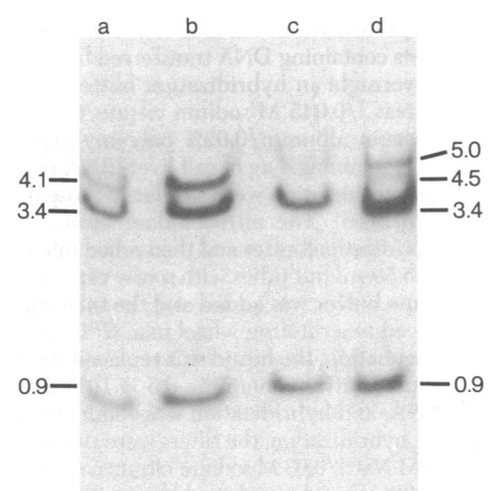

FIG. 2. Cleavage patterns of human and gibbon ribosomal DNA sequences with *Bam*HI. High molecular weight DNA was extracted from human and gibbon tissues and digested with *Bam*HI. Electrophoresis of the products and transfer onto nitrocellulose filters for hybridization are carried out. ^{125}I-Labeled 18S and 28S HeLa ribosomal RNA were used as probes. Lanes: a, 6G-1 heart; b, 6G-4 kidney; c, human spleen; d, human lymphoid cells (NC37) infected with SSAV. Numerals are molecular weights $\times 10^{-6}$.

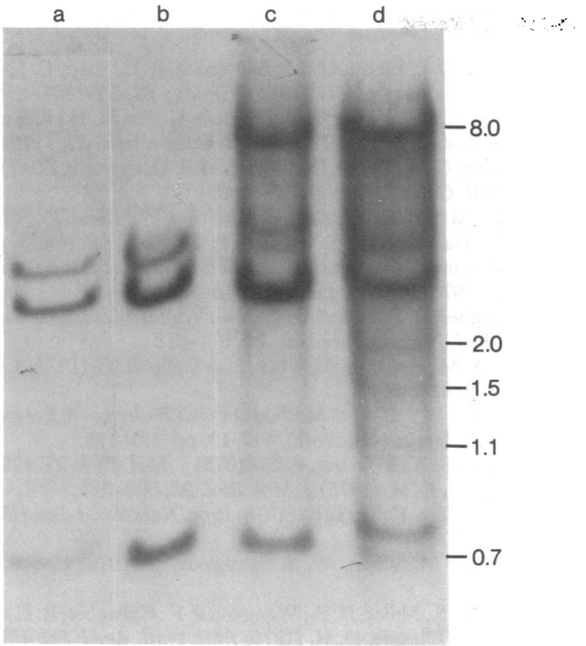

FIG. 3. Hybridization of ^{125}I-labeled SSV(SSAV) RNA to control DNA samples digested with *Bam*HI. Cellular DNA was digested with *Bam*HI and processed. Lanes: a, normal gibbon spleen × ^{125}I-labeled 18S and 28S RNA; b, normal gibbon spleen × ^{125}I-labeled SSV(SSAV); c, normal human spleen × ^{125}I-labeled SSV(SSAV); d, NC37 (SSAV) × ^{125}I-labeled SSV(SSAV).

are presented in Fig. 4. DNA from two tissues of 6G-1, heart and abdominal lymph nodes, contained two dominant bands of 2.0 and 1.5 × 10^6 daltons (lanes c and d). Because the sum of these two fragments is less than one provirus equivalent and because they are the only dominant bands, they probably represent "internal" fragments—i.e., generated by three cuts of the enzyme within the integrated virus genome. A faint band at 1.1 × 10^6 daltons and many other faint bands in the high molecular weight region are visible. These faint bands are likely to be fragments containing viral and flanking cellular sequences. Their presence suggests that integration of the provirus occurs at many sites, so one cannot determine whether the integration sites are the same for the two tissues. However, the results suggest a unique rather than permuted orientation of the provirus after integration because the internal fragments are identical.

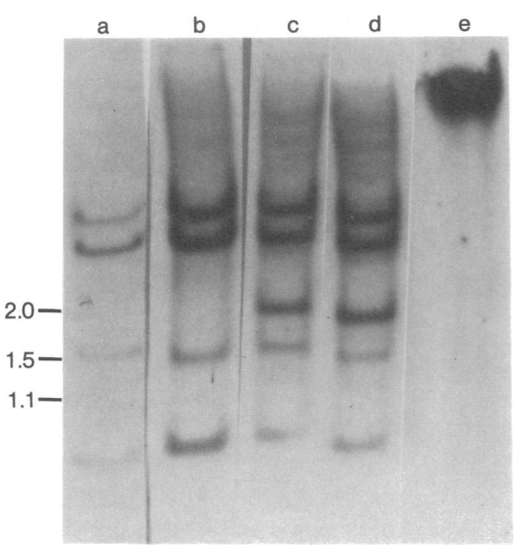

FIG. 4. Hybridization of ^{125}I-labeled SSV(SSAV) RNA to *Bam*HI digested DNA of tissues from 6G-1 and 6G-4. Lanes: a, 6G-4 liver; b, 6G-4 kidney; c, 6G-1 heart; d, 6G-1 abdominal nodes; e, 6G-4 kidney DNA, undigested.

DNA from tissue of gibbon 6G-4 were also analyzed (lanes a and b). Two of these tissues, liver and kidney, which showed increased hybridization to viral cDNA and RNA, contained a fragment of 1.5 × 10^6 daltons that hybridized SSV(SSAV) RNA. This corresponds to only one of the two viral fragments observed in 6G-1. Because both viral DNA fragments found in DNA from 6G-1 are subgenomic in size and the most dominant band at 2.0 × 10^6 daltons found in 6G-1 is missing in 6G-4, the result clearly indicates that a complete provirus is absent from 6G-4. The 1.5 × 10^6 dalton viral fragment is probably integrated because unrestricted DNA only showed hybridization associated with high molecular weight DNA at the top of the gel (lane e). Trapping of an unintegrated fragment by the high molecular weight cellular DNA is unlikely because migration of fragments from *Eco*RI-digested λ DNA in the same sample well was not affected (not shown).

DISCUSSION

Proviral sequences present in tissues from two gibbon apes derived from a colony from Hall's Island, Bermuda, were examined. Gibbon 6G-1 was viremic and leukemic and had yielded a new and distinct virus, $GaLV_H$ (1). Gibbon 6G-4 was one of four gibbons in the total colony of eight that showed significantly high titers of antibody against $GaLV_H$-specific proteins in their serum (10). However, there was no evidence of expression of virus or subviral components in tissues of 6G-4. As reported (1), tissues from 6G-1 contained integrated provirus as measured by liquid hybridization. When DNA from a number of tissues of 6G-4 was analyzed, we found that most samples did not hybridize significant amounts of radiolabeled probes of $GaLV_H$ or SSV(SSAV). Although three tissues showed considerable hybridization (liver, kidney, and spleen), the level of hybridization plateaued at 20–40% relative to DNA from 6G-1. These results suggested the possibility of integration of only a portion of the provirus, and this was shown by the restriction enzyme-blotting procedure. DNA from tissues of 6G-1 contained two dominant viral specific fragments after cleavage with *Bam*HI (2.0 and 1.5 × 10^6 daltons), whereas DNA from two "positive" tissues (liver and kidney) of 6G-4 had only the 1.5 × 10^6 dalton viral specific fragment. Because this fragment is subgenomic in size, it clearly argues for partial provirus integration.

It may be significant that the spleen, liver, and kidney were the only tissues positive for proviral sequences in gibbon 6G-4. These may be the initial target sites for virus infection. The absence of detectable proviral sequences in other tissues from the same animal argues against an early germ-line infection, but the homogeneity in the restriction patterns within the same tissues and among at least two different tissues raises the possibility that cells harboring proviral fragments are clonal. The apparent contradiction may be resolved if infection occurs at a specific time during development as previously hypothesized for leukemias that might be viral but do not exhibit replicating virus (22).

Because the animal expressed persistent antibody against the major structural viral proteins (p30, p12, gp70, and reverse transcriptase), there must be sufficient expression of these antigens to induce this immune response. The size of the 1.5 × 10^6 dalton fragment (≈2000 base pairs) is not sufficient to code for all these viral proteins; therefore, it is likely that some tissue(s) other than those examined contain a more extensive or complete virus genome or even serve as sites for limited virus replication.

Also, with the sensitivity of our technique, we cannot rule out the possibility that a small cell population of the tissues examined contains a complete virus genome.

Integration of subgenomic viral fragments has been well documented for DNA tumor viruses (23) but not for RNA retroviruses. Svoboda *et al.* (24) obtained evidence consistent with integration of only a partial provirus in a mammalian cell line nonproductively transformed with Rous sarcoma virus because they observed lower plateau hybridization values for these cells than with a producer cell line. However, other possibilities—e.g., only a small population of the cells being infected with whole provirus—are alternative interpretations of these results.

The evidence of partial provirus shown here in a naturally infected gibbon ape might serve as a model for other animal systems in which viruses appear to be etiologically involved in a disease but detection of extra viral nucleic acid sequences is difficult. One such example is feline leukemia among virus-negative cats. All leukemic cats have FOCMA, a tumor-specific membrane antigen apparently coded for by feline leukemia virus and feline sarcoma virus (25), yet a sizable proportion of these cats are virus negative (26) and their DNA does not hybridize detectably more feline leukemia virus probes than does DNA of normal cat (ref. 27; unpublished data). This dilemma might be explained if integration of only a small viral fragment of provirus were necessary to maintain transformation. Similar situations may exist in humans in whom there are only sporadic findings of viral markers in leukemic blood samples including proviral DNA related to known type C viruses (28, 29). More sensitive techniques or more specific probes may permit a more frequent detection of subgenomic viral sequences in the DNA of leukemic cells.

We are indebted to Dr. W. Prensky for helpful discussions and for a gift of ^{125}I-labeled rRNA, to Dr. W. Haseltine for a gift of strong stop cDNA of SSV(SSAV), and to S. Josephs, J. Luczak, and M. Voltin for excellent technical help.

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