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Isolation and Identification of Lymphocytic and Myelogenous Leukemia-Specific Sequences in Genomes of Gibbon Oncornaviruses

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Five gibbon ape leukemia virus substrains (two from gibbons with lymphocytic leukemia and three from gibbons with myelogenous leukemia) were examined for unique genomic sequences specific for each form of leukemia. By using sequential adsorption procedures, the genome from each gibbon ape leukemia virus was fractionated into four sets of distinct nucleotide sequences. Based on their hybridization specificities toward DNAs of leukemic tissues, these sequences were designated as follows: (i) "COM," (ii) "LYM" or "MYE," (iii) "UNI," and (iv) "UND." The COM fraction represented sequences common to all of the viral genomes. The LYM fraction, which was isolated only from gibbon ape leukemia viruses associated with lymphocytic leukemia, represented genomic sequences associated with lymphocytic leukemia since the RNA hybridized at a 4- to 15-fold-higher rate to infected tissue DNA from lymphocytic leukemic gibbons than to infected tissue DNA from myelogenous leukemic gibbons. The MYE fraction, which was isolated only from gibbon ape leukemia viruses associated with myelogenous leukemia, represented genomic sequences associated with myelogenous leukemia since the RNA hybridized at a 5- to 15-fold-higher rate to infected tissue DNA from myelogenous leukemic gibbons than to infected tissue DNA from lymphocytic leukemic gibbons. The UNI fraction contained sequences unique to one virus substrain. The UND fraction contained sequences which varied depending upon the substrains involved in the adsorption procedures. These findings suggest that each gibbon ape leukemia virus examined in this study contains subgenomic sequences that are specifically identifiable only with the form of leukemia from which the virus was isolated.

Type C viruses have been isolated from gibbons with spontaneous lymphocytic (12, 19) and myelogenous (18; T. G. Kawakami, L. Sun, T. S. McDowell, and G. V. Kollias, Proc. Symp. Comp. Pathol. Zoo Anim., in press) leukemias. These viruses are similar in several respects. They share antigenic determinants on the reverse transcriptase (11, 36, 37) and on the p30 structural proteins (13, 27, 35) and they share nucleotide sequence homologies on the viral genomes (4, 12, 30, 41, 44). However, these viruses, which were isolated from different origins, are not identical, based on radioimmune assays of the p12 polypeptides (45) and molecular hybridization studies on the degree of genomic homology (32, 41, 44). Even though these viruses have subtle differences which are detectable by specific immunological and biochemical assays, these differences apparently are not related directly to specific forms of leukemia.

In our laboratory we have isolated five gibbon ape leukemia viruses (GaLV), including two from lymphocytic leukemia and three from myelogenous leukemia of gibbons (18, 19; Kawakami et al., in press). Based on molecular hybridization studies, the genomes of these viruses were shown to be distinct from each other and to share partial sequence homology (30, 41, 44). In addition, the genome of one virus remained essentially unchanged upon serial passages through several unrelated gibbons, each of which developed the same type of leukemia (41). These results demonstrated that the major portion of the viral genome is not identifiable with a specific form of leukemia and suggested the presence of stable subgenomic sequences which can determine the form of leukemia that can be induced by the virus. In this biochemical study we isolated and characterized nucleotide sequences which represented different portions of each viral genome and identified the nucleotide sequences specifically related to either lymphocytic or myelogenous leukemia in gibbons.

MATERIALS AND METHODS

Viruses and cells. The two type C viruses isolated from gibbons with lymphocytic leukemia were designated GaLV-1L and GaLV-2L. GaLV-1L was propa-

gated in the original lymphoid cell line UCD-144 (19). GaLV-2L was propagated in human rhadomyosarcoma cell line RD (23). The three viruses isolated from gibbons with myelogenous leukemia were designated GaLV-3M, GaLV-4M, and GaLV-6M. GaLV-3M was propagated in a CCL-88 bat lung cell culture (American Type Culture Collection). GaLV-4M and GaLV-6M were propagated in human lymphoid cell line NC-37 (Pfizer). All cells were grown in Leibovitz medium (Pacific Biological) supplemented with 15% (vol/vol) heat-inactivated fetal calf serum (Irvine Scientific). Viruses were concentrated from infected cell culture medium by differential centrifugation, as previously described (19). Viral 70S RNA was isolated from freshly prepared GaLV as described by Sun et al. (41). After being used for virus production, the tissue culture cells were harvested for DNA extractions.

Iodination of viral RNA. Iodination of purified viral RNA was by a modified method, as described by Cumberford (7), Tereba and McCarthy (42), and Prensky (28). The reactions were carried out at 60°C for 12 min in 30 to 50 μl of 0.1 M sodium acetate buffer (pH 4.5) containing 2 to 10 μg of viral RNA, 1 to 2 mCi of carrier-free ^{125}I (Amersham), and 0.625 mM $TlCl_3$. Reactions were terminated by chilling to 0°C. Unstable intermediates were destroyed by heating at 60°C for 30 min in a solution containing 1.25 mM Na_2SO_3 and 150 μl of 0.4 M sodium phosphate buffer (pH 6.8). Iodinated RNA was separated from free iodide by Sephadex G25M (Pharmacia) column chromatography in 0.02 M Tris-hydrochloride (pH 8.1)–0.1 M NaCl–1 mM EDTA–0.5% sodium dodecyl sulfate and directly ethanol precipitated from the void volume of the column. The specific radioactivity of the [^{125}I]RNA was 5×10^7 cpm/μg; 80 to 88% of the [^{125}I]RNA was hybridizable to homologously infected cellular DNA.

DNA extraction. Approximately 10^8 tissue culture cells were suspended in 5 ml of 10 mM Tris-hydrochloride buffer (pH 7.4) containing 15 mM KCl, 3 mM $MgCl_2$, and 100 μg of sodium heparin (Calbiochem) per ml. Triton X-100 was added to a final concentration of 1%. After the mixture was shaken vigorously for 2 min, the nuclei were pelleted by centrifugation at 2,000 rpm for 5 min. DNA was extracted and purified from the nuclei by the method of Marmur (22), and it was sheared by sonication to fragments of 500 nucleotides (40). DNAs from gibbon tissues were extracted by the method of Walker and McLaren (47) and were similarly sheared for hybridization studies.

Fractionation of ^{125}I-labeled viral RNA. The ^{125}I-labeled viral RNA was fractionated according to the schemes shown in Fig. 1 and 2. RNA-DNA hybridizations were carried out with a 10^6-fold excess of cellular DNA (2 mg/ml) in 0.02 M Tris-hydrochloride, pH 7.2, containing 1 mM EDTA, 0.8 M NaCl, and 0.1% sodium dodecyl sulfate. Incubation was at 68°C until a C_0t (moles of nucleotides × seconds/liter; corrected for NaCl concentration) of 30,000. The hybridized RNA was separated from the unhybridized RNA by hydroxylapatite column chromatography (DNA grade; Bio-Gel HTP; Bio-Rad Laboratories) with sodium phosphate buffer (pH 6.8) at 60°C. The RNA from each stage of fractionation was freed of DNA by heat dissociation followed by Cs_2SO_4 density gradient centrifugation (ρ = 1.53 g/ml in 0.01 M Tris-hydrochloride [pH 7.2]–1 mM EDTA–0.1 M NaCl; centrifuged at 31,000 rpm and 20°C for 66 h in an SW50.1 rotor). After extensive dialysis to remove Cs_2SO_4, [^{125}I]RNA was concentrated by lyophilization.

Hybridization conditions. A total of 500 to 1,500 cpm of [^{125}I]RNA from each of the subgenomic fractions was mixed with 300 μg of denatured tissue DNA in 150 μl of 0.02 M Tris-hydrochloride (pH 7.2)–1 mM EDTA–0.8 M NaCl and incubated at 68°C to a C_0t of 20,000. Then the mixtures were diluted to a final concentration of 0.3 M NaCl and incubated with 50 μg of RNase (100°C, 5 min) per ml for 1 h at 37°C. Acid-insoluble material was collected on membrane filters (pore size, 0.45 μm; Millipore Corp.).

RESULTS

Distribution of subgenomic sequences. The genomes of five distinct virus substrains (GaLV-1L, GaLV-2L, GaLV-3M, GaLV-4M, and GaLV-6M) were fractionated into subgenomic nucleotide sequences by sequential adsorption procedures. The detailed procedures are shown in Fig. 1 and 2. Although the 70S RNA under study was purified from freshly prepared virus, it could contain a small amount of host cell sequences. To eliminate the possibility of carrying host cell sequences into subgenomic fractions, viruses involved in either fractionation scheme were each propagated in a cell line with a unique origin and phenotype. Any sequences of host cell origin were then collected in the unhybridized fraction after the initial adsorption step.

To demonstrate the feasibility of using DNA-driven hybridizations with radioactively labeled RNA to isolate subgenomic sequences, we determined the extent of hybrid formation between each ^{125}I-labeled viral genome and the DNA of the cells from which the virus was isolated. When the conditions described above were used, between 80 and 88% of the [^{125}I]RNA was found in the hybrid form. This indicates that the majority of the ^{125}I-labeled genomic RNA was adsorbed by proviral DNA sequences in homologously infected cell cultures. Figure 3 further demonstrates the gradual increase in adsorption with increasing amounts of cellular DNA. At a ratio of DNA to RNA of 10^6, maximum adsorption was obtained. Therefore, we used a ratio of DNA to RNA of more than 10^6 for the adsorption steps involved in the fractionation procedures and carried the hybridization reaction to a C_0t of 30,000. Under these conditions, adsorption was complete.

In each case, the initial adsorption was with DNA from infected cells carrying the complete proviral sequences of a distinct GaLV which was isolated from a similar type of leukemia. This step separated genomic sequences into homolo-

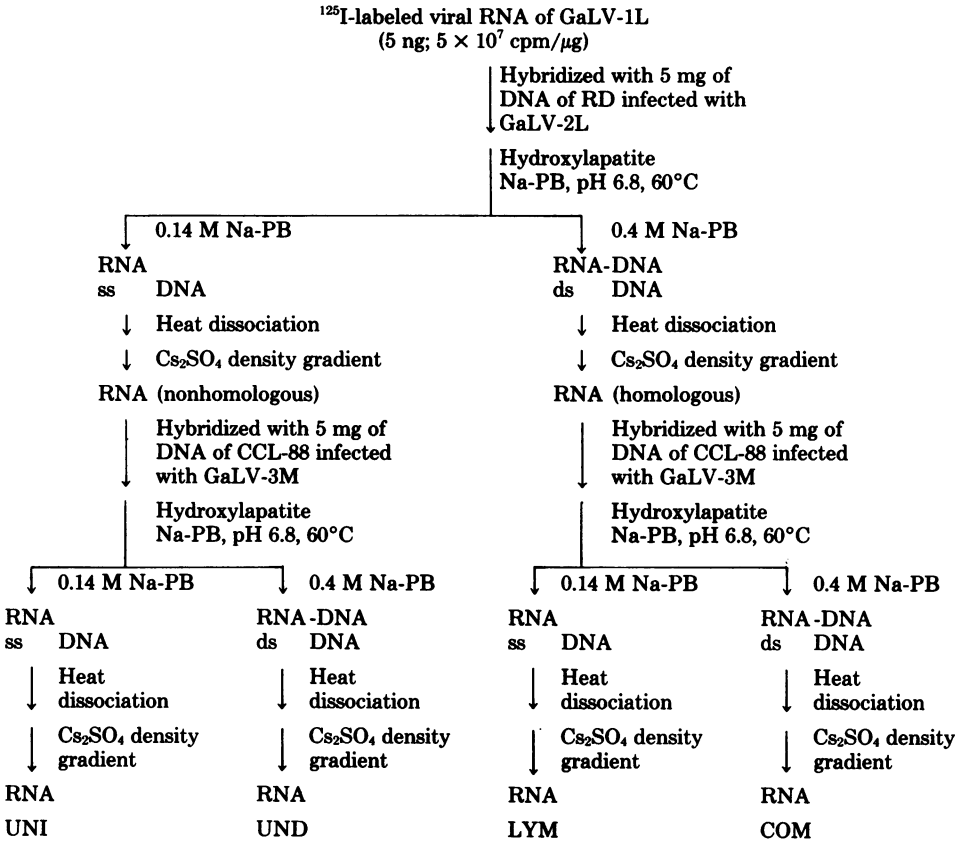

FIG. 1. *Fractionation procedures for genomic sequences of GaLV isolated from lymphocytic leukemia. The scheme shown here is for GaLV-1L. Similar procedures were used to fractionate genomic sequences of GaLV-2L, except that the initial hybridization was with 5 mg of DNA from UCD-144 infected with GaLV-1L. Na-PB, Sodium phosphate buffer; ss DNA, single-stranded DNA; ds DNA, double-stranded DNA.*

gous and nonhomologous fractions among GaLV's of similar types of leukemia. Each homologous fraction contained related genomic sequences of two GaLV's, and each nonhomologous fraction contained unrelated genomic sequences of two GaLV's. The second adsorption step was with DNA from infected cells carrying the complete proviral sequences of a GaLV which was isolated from a different type of leukemia. This step separated sequences of the homologous and nonhomologous fractions into sequences which represented smaller and more specific portions of the viral genomes. The designations adopted for each fraction are shown in Fig. 1 and 2. Experiments which demonstrated their hybridization specificities are described below. Table 1 summarizes the stepwise recovery of [^{125}I]RNA in each fraction. Based on the distributions of [^{125}I]RNA, the proportion of viral genome represented by the RNA in each final fraction was quantitated.

The recovery of [^{125}I]RNA after each adsorption step was between 18 and 33%. After the first adsorption, the amount of RNA was distributed between homologous and nonhomologous fractions essentially depending upon the degree of genomic homology among the substrains. After the second adsorption, the major portions of the homologous fractions were isolated as common (COM) sequences. The data indicate that 33 to 44% of the RNA was obtained as COM sequences, whereas only 12 to 28% of the RNA was obtained as lymphocytic leukemic (LYM) or myelogenous leukemic (MYE) sequences. On the other hand, the major portions of the nonhomologous fractions were isolated as unique (UNI) sequences. The data indicate that 17 to 49% of the RNA was obtained in the UNI fractions, whereas only 2 to 13% of the RNA was obtained in the undefined (UND) fractions. These results demonstrate that subgenomic nucleotide sequences which represent different portions of the GaLV genomes can be separated and quantitated.

Characterization of subgenomic sequences. To determine which subgenomic frac-

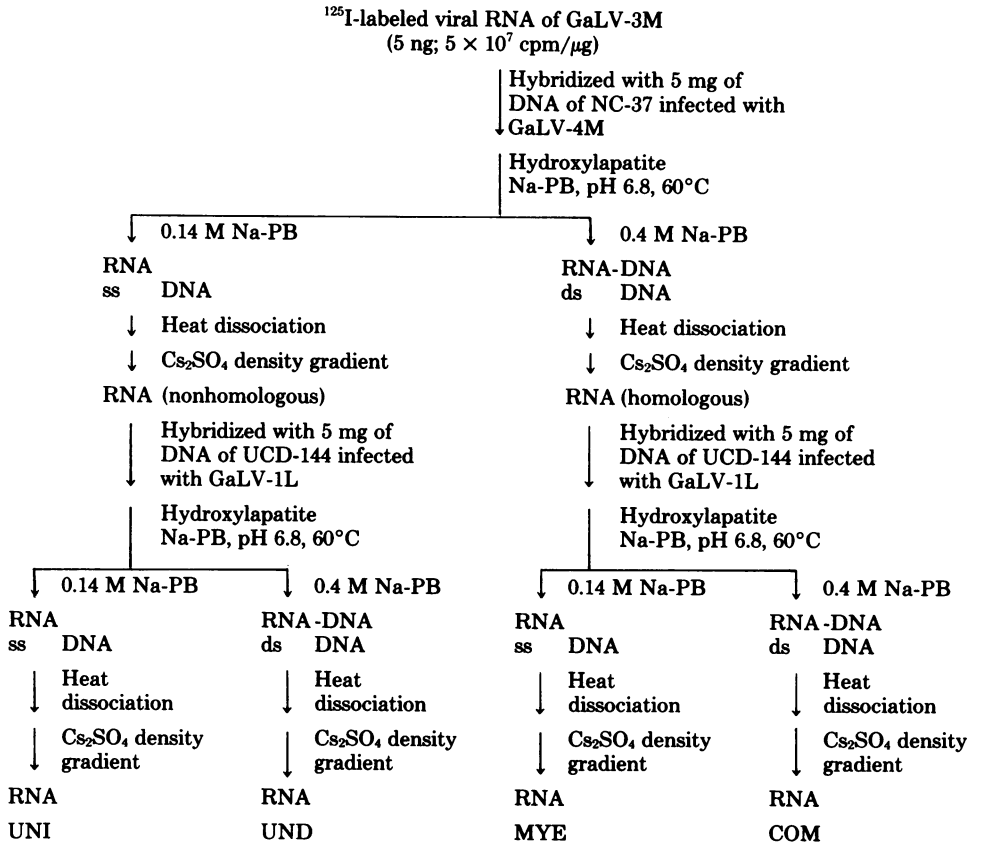

FIG. 2. *Fractionation procedures for genomic sequences of GaLV isolated from myelogenous leukemia. The scheme shown here is for GaLV-3M. Similar procedures were used to fractionate genomic sequences of GaLV-4M and GaLV-6M, except that the initial hybridization was with 5 mg of DNA from CCL-88 infected with GaLV-3M. Na-PB, sodium phosphate buffer; ss DNA, single-stranded DNA; ds DNA, double-stranded DNA.*

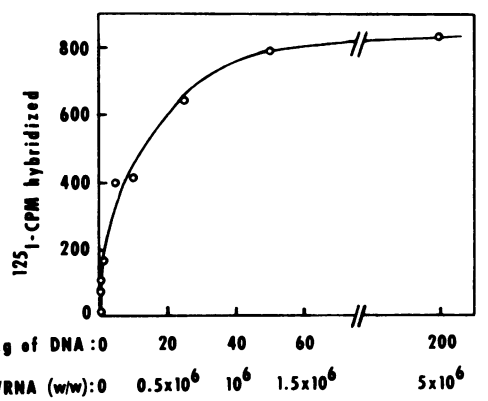

FIG. 3. *Hybridization of ^{125}I-labeled genomic RNA of GaLV-1L with increasing amounts of DNA from UCD-144. A total of 1,000 cpm of [^{125}I]RNA (2.5 × 10^7 cpm/μg) was hybridized with increasing amounts of DNA as indicated by using the conditions described in the text. Hybrid formation was measured after RNase digestion. The amounts of RNA hybridized are given as the actual acid-insoluble counts per minute collected on 0.45-μm Millipore filters.*

tion contained nucleotide sequences identifiable with a specific leukemia, each fraction was analyzed for sequences homologous to leukemia-specific DNA by molecular hybridization. Since the DNA of virus-infected tumor tissue was found to contain complete proviral sequences, the source of leukemia-specific DNA was the virus-infected liver or spleen tissue of gibbons with a known type of leukemia. HLA-30 was a gibbon with lymphocytic leukemia; hence, its liver and spleen contained lymphocytic-specific DNA sequences. Similarly, the DNAs from the livers of HLA-21, HLA-25, HLA-62, and S-76 contained myelogenous-specific sequences since each animal had myelogenous leukemia. [^{125}I]-RNA from each subgenomic fraction was hybridized to a 1×10^7 to 3×10^7 excess (by weight) of DNA from these tissues. Each fraction was defined according to its hybridization specificity. The fraction that possessed the highest homology to leukemia-specific DNA was defined as the leukemia-specific fraction. Background hybridization was established with liver DNA of a

TABLE 1. *Recovery and distribution of subgenomic sequences among GaLV substrains*

Animal	Origin[a]	Type of leukemia	Virus isolated	Recovery and distribution of [^{125}I]RNA[b]					
				First adsorption			Second adsorption		
				Fraction[c]	Amt recovered (cpm)[d]	Distribution (%)[e]	Fraction[c]	Amt recovered (cpm)[d]	Distribution (%)[e]
UCD-144	S.F.	Lymphocytic	GaLV-1L	Homologous	41,700	56	COM	8,100	33
							LYM	5,700	23
				Nonhomologous	33,300	44	UND	2,900	13
							UNI	6,800	31
HLA-30	Pet	Lymphocytic	GaLV-2L	Homologous	52,000	72	COM	9,500	44
							LYM	6,100	28
				Nonhomologous	20,700	28	UND	2,400	11
							UNI	3,600	17
S-74	SEATO	Myelogenous	GaLV-3M	Homologous	30,500	68	COM	3,900	44
							MYE	2,150	24
				Nonhomologous	14,500	32	UND	250	2
							UNI	3,500	30
S-76	SEATO	Myelogenous	GaLV-4M	Homologous	32,000	54	COM	4,600	39
							MYE	1,800	15
				Nonhomologous	27,500	46	UND	800	7
							UNI	4,400	39
HLA-21	SEATO	Myelogenous	GaLV-6M	Homologous	22,200	46	COM	4,900	34
							MYE	1,760	12
				Nonhomologous	25,800	54	UND	600	5
							UNI	5,600	49

[a] S.F., San Francisco; SEATO, Southeast Asia Treaty Organization.
[b] The amount of [^{125}I]RNA before the beginning of fractionation was 250,000 cpm.
[c] Fractions are designated as shown in Fig. 1 and 2.
[d] Values are actual amounts of RNA finally recovered for each fraction.
[e] Calculated percent distribution.

normal gibbon, HLA-4. Maximum hybridization was to the DNA of the tissue from which each GaLV was originally isolated (Table 2).

One fraction common only to lymphocytic leukemia viruses, which was isolated from GaLV-1L and GaLV-2L, was defined as LYM since the RNA of this fraction hybridized much more to DNAs from lymphocytic leukemic tissues than to DNAs from myelogenous leukemic tissues. More than 93% of the RNA hybridized to liver and spleen DNAs of the lymphocytic leukemic gibbon HLA-30, whereas less than 28% hybridized to liver DNAs of the four myelogenous leukemic gibbons. The low hybridization of this RNA to the DNAs from myelogenous leukemic tissues of HLA-25 and HLA-62 was expected since these animals were infected by GaLV-3M and the LYM fraction was obtained after the homologous RNA fraction was absorbed with proviral sequences of GaLV-3M. Hybridization of the same RNA to the DNAs from myelogenous leukemic tissues of two other animals infected by viruses distinct from GaLV-3M (HLA-21 and S-76) was similarly low. Even though the specificity of the LYM sequences appeared to vary depending upon the virus substrains used to prepare the subgenomic fraction, these LYM sequences could readily detect high levels of complementary DNA sequences in tissues of a lymphocytic leukemic gibbon but only low levels of similar sequences in tissues of four myelogenous leukemic gibbons. This clearly demonstrates that the LYM fraction contains sequences that are disease specific and identifiable with lymphocytic leukemia.

A second fraction common only to myelogenous leukemia viruses, which was isolated from GaLV-3M, GaLV-4M, and GaLV-6M, was defined as MYE since the RNA of this fraction hybridized much more to DNAs from myelogenous leukemic tissues than to DNAs from lymphocytic leukemic tissues. More than 85% of the RNA hybridized to the DNAs of all of the myelogenous leukemic gibbons, whereas less than 22% hybridized to DNAs of the lymphocytic leukemic gibbons. Each MYE fraction contained sequences in tumor tissues from myelogenous leukemic gibbons, regardless of the virus substrains involved in the fractionation process. However, attempts to detect similar sequences

TABLE 2. *Hybridization of ^{125}I-labeled subgenomic RNA to DNA from tissues of normal and leukemic gibbons*

Class	Animal	Tissue	Virus isolated	% Hybridization[a]										
				LYM		MYE			UND		COM		UNI	
				GaLV-1L	GaLV-2L	GaLV-3M	GaLV-4M	GaLV-6M	GaLV-2L	GaLV-3M	GaLV-2L	GaLV-3M	GaLV-2L	GaLV-3M
Background hybridization[b]	HLA-4	Liver	None	10	11	7	12	12	18	12	18	15	4	9
Maximum hybridization[c]	HLA-30	Liver	GaLV-2L	47[d]	51				43		48		40	
	HLA-25	Liver	GaLV-3M			64				41		50		34
	S-76	Liver	GaLV-4M				69							
	HLA-21	Liver	GaLV-6M					66						
Myelogenous leukemia	HLA-25	Liver	GaLV-3M	0	17	100	94	104	109	100	99	100	27	100
	HLA-62	Liver	GaLV-3M	5	20	85	89	107	99	119	100	90	13	84
	S-76	Liver	GaLV-4M	7	28	99	100	116	100	20	102	96	23	16
	HLA-21	Liver	GaLV-6M	0	14	90	113	100	84	61	97	76	0	39
Lymphocytic leukemia	HLA-30	Liver	GaLV-2L	100	100	15	9	0	100	77	100	98	100	32
	HLA-30	Spleen	GaLV-2L	107	93	22	14	7	95	77	99	89	99	20

[a] Normalized values were calculated as follows: [(hybridization to DNA of a tissue − background hybridization) × 100]/(maximum hybridization − background hybridization).

[b] The background hybridization of each subgenomic RNA was taken as the percentage of input counts per minute annealed to the liver DNA of HLA-4.

[c] The maximum hybridization of each subgenomic RNA was taken as the percentage of input counts per minute annealed to the DNA of the tissue from which the virus was originally isolated.

[d] The original tissue from which GaLV-1L was isolated was not available. Normalization was with respect to liver DNA of HLA-30 since the RNA fraction was initially isolated as sequences homologous to GaLV-2L.

in the DNAs of lymphocytic leukemic tissues resulted in only 0 to 22% hybridization. These results indicate that the MYE fraction contains sequences that are disease specific and identifiable with myelogenous leukemia.

The degree of cross-hybridization between the RNAs of the LYM and MYE fractions indicates the presence of a small quantity of common sequences between these fractions. The contamination may be due to the RNA isolated as loops and unhybridized chains but attached in the RNA-DNA hybrids since enzyme hydrolysis was not employed in the purification process after the hydroxylapatite fractionation of homologous RNA-DNA hybrids. By using the fractionation procedures described above, the majority of disease-specific sequences were isolated either in the LYM or the MYE fractions, although a small amount of common or nonhomologous sequences may have been present.

One fraction from each genome was defined as common sequences (COM), since the RNAs of these fractions hybridized readily to all of the tissue DNAs tested. For GaLV-2L and GaLV-3M, each COM fraction contained sequences common to all viral genomes. In addition, one fraction from each genome was defined as containing unique sequences (UNI) since the RNAs of these fractions could hybridize only with the DNAs of tissues infected by the same virus. Data obtained for GaLV-2L and GaLV-3M are shown in Table 2. Whereas the RNAs of the LYM, MYE, COM, and UNI fractions were demonstrated to have hybridization specificities, the RNAs of the remaining fractions were undefined (UND), since their hybridization patterns varied depending upon the virus substrains involved in the fractionation procedure.

DISCUSSION

The availability of several distinct GaLV substrains associated with lymphocytic and myelogenous leukemias in our laboratory has provided an unique opportunity to investigate the relatedness between viral genomic sequences and two forms of leukemia. We prepared four subgenomic RNA fractions from each of five GaLV substrains. The subgenomic fractions, each representing a different portion of the viral genome, were labeled with ^{125}I and analyzed for hybridization specificity toward DNAs of leukemic tissues. The major finding of this study is that two of the subgenomic fractions, LYM and MYE, each representing less than one-fourth of the viral genome, contain RNA sequences identifiable with lymphocytic and myelogenous leukemias, respectively.

Molecular hybridization techniques have been used for the isolation and purification of sarcoma virus-specific complementary sequences (complementary DNA) from avian and murine sarcoma viruses (8, 34, 38). By using the same technique, complementary DNAs specific for the new types of viral oncogenes in avian and murine defective leukemia viruses have been prepared (33, 46). Although the specificity of these complementary DNAs has been demonstrated, whether they represent the entire *src* gene of sarcoma viruses or the oncogene of defective leukemia viruses depends on how uniform the complementary DNA transcripts are. This report describes biochemical methods which allowed the isolation of disease-specific RNA sequences directly from the purified viral genome of GaLV. The basic techniques were essentially similar to those used by other investigators. These techniques involved the selection of viral sequences by using a large quantity of cellular DNA (15) and the separation of single-stranded DNA and RNA from DNA-RNA and DNA-DNA hybrids on hydroxylapatite column. Specific RNA sequences selected by this method are therefore more uniformly representative of a portion of the viral genome.

Although the approach used in this study does not distinguish whether LYM and MYE RNAs are related to functional oncogenes (2, 43) in GaLV, these RNAs are significant in several respects. They differentiate GaLV substrains associated with lymphocytic leukemia from those associated with myelogenous leukemia. They provide additional criteria for classifying new GaLV isolates. Since GaLV's have been isolated frequently from healthy viremic gibbons (20, 44), detection of either leukemia-specific sequence in the genomes of these GaLV's may provide an indication of the type of leukemia that these animals may develop. Preliminary studies indicate that GaLV isolated from nonleukemic gibbons can lack the leukemia-specific sequences. This indicates that the leukemia-specific sequences isolated from various GaLV's are related to the pathogenicities of the viruses (L. Sun and T. G. Kawakami, Abstr. J. Supramol. Struct. Suppl. 4, Abstr. 633, 1980). Further studies are now being undertaken with a cloned viral population to help identify the specific sequences and their origins.

Since RNA viruses have been established as the causal agents of leukemia in avian (3), murine, (14, 24), feline (16, 21), and primate (Kawakami et al., in press) species, it was postulated that similar viruses are involved in human leukemia. So far, conflicting results have been reported in this area of research. Several laboratories have reported a failure to detect viruses associated with human leukemia (5, 6, 39). In contrast, other reports have indicated that infec-

tious virus related to primate RNA virus can be isolated from cultured human cells grown under special conditions (9, 10, 17, 25, 26). Studies involving fresh leukemic tissues were directed toward detecting the presence of virus-related components, such as virus-related DNA sequences or a provirus. When molecular probes synthesized from murine (1) and primate (29, 31, 48) viruses were used, only a fraction of the tissues examined were found to contain proviral DNA sequences. This infrequent detection could be attributed to the existence of nonspecific nucleotide sequences in the detection probes (11), the integration of a partial provirus (49) whose sequences were not well represented in the probes used, or the presence of provirus in only a small population of cells in leukemic tissues. This study identifies disease-specific GaLV genomic sequences which may represent a more sensitive probe for detection of similar leukemogenic DNA sequences in human tissues. Further studies in this area with probes having similar disease specificities may be useful in helping to resolve the discrepancy in detecting oncogenic sequences in human leukemia.

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