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Restriction Endonuclease Mapping of Linear Unintegrated Proviral DNA of Bovine Leukemia Virus

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A detailed restriction map was deduced for the genome of the exogenous bovine leukemia virus. The cleavage sites for nine restriction enzymes were mapped. The unintegrated linear viral DNA intermediate that is produced by infection of permissive cells with bovine leukemia virus was isolated. The linear viral DNA had a unique restriction map, indicating that it is not a set of random circular permutations of the RNA genome. From hybridization with a 3'-enriched probe, the DNA restriction map was aligned relative to the 5'-to-3' orientation of the viral RNA. Restriction enzyme analysis of integrated bovine leukemia virus information present in animals with enzootic bovine leukosis provided evidence for the existence of genetic variants of the virus.

Bovine leukemia virus (BLV), an exogenous retrovirus (9) of cattle, is the pathogenic agent of enzootic bovine leukosis (see reference 1 for a review). The target cell is the B lymphocyte (11). BLV infection may induce persistent lymphocytosis (characterized by an increased number of apparently normal B lymphocytes in the peripheral blood of the infected animal) and, in a later stage, lymph node tumors.

Proviral copies were found to be integrated at several sites in the genomes of one-fourth to one-fifth of the leukocytes of animals with persistent lymphocytosis, but at only one or very few sites in the genomes of a larger fraction of leukocytes or tumor cells of animals in the tumor stage of the disease (7). A few (one to three) copies of proviral DNA are integrated per haploid genome (8; unpublished data).

We have isolated unintegrated duplex viral DNA from freshly infected permissive cells and have characterized it by restriction endonuclease digestion. We report here the first detailed restriction endonuclease mapping of a bovine retrovirus genome.

MATERIALS AND METHODS

Viruses and cells. BLV was produced by the BLV-infected fetal lamb kidney (FLK) cells established by van der Maaten and Miller (19). This cell line was generously provided by van der Maaten at passage 69. Bat cells (Tb_1Lu cell line), used for acute infection with BLV, were purchased from the American Type Culture Collection, Rockville, Md.

Isolation of viral DNA. BLV produced by the BLV-infected FLK cell line at passage 70 was used to infect bat cells (at 70% confluency) in roller bottles at a multiplicity of infection of about 10^4 virus particles per cell in the presence of 10 μg of Polybrene per ml (18). After 24 to 48 h of incubation at 37°C, the cells were treated by the method of Shoyab and Sen (16). In brief, bat cells were lysed with 8 M urea–1% sodium dodecyl sulfate–10 mM EDTA in 0.24 M phosphate buffer (pH 6.8), and the lysate was centrifuged at 80,000 × *g* for 60 min to sediment high-molecular-weight DNA. After reextraction of the pellet, the pooled supernatant fractions were applied to hydroxyapatite equilibrated with the lysing solution. After washing with 8 M urea–0.24 M phosphate buffer to remove protein and RNA, the column was sequentially eluted with 0.15 M phosphate and 0.50 M phosphate buffer, both at pH 6.8. Double-stranded extrachromosomal DNA, delivered in the 0.50 M phosphate elution, was reconcentrated by hydroxyapatite, dialyzed against 0.01 M Tris-hydrochloride–1 mM EDTA (pH 7.5), and concentrated under ethanol. In general, 5 to 20 μg of DNA was recovered by a 900-cm^2 roller bottle infected at about 70% confluency.

Cellular DNA extraction. Cellular DNA extraction was performed with sodium dodecyl sulfate plus phenol-chloroform essentially as described by Kay et al. (6).

Restriction endonuclease digestions. All restriction enzymes, except one, used in this study were purchased from New England Biolabs, Beverly, Mass.; *Eco*RI was a product from Boehringer Mannheim Corp., New York. About 5 μg of total extrachromosomal DNA (or 10 μg of FLK DNA) was digested for 2 h in 20 μl of the appropriate buffer with an excess of restriction endonuclease. The extent of digestion was usually monitored by including a marker DNA of a known cleavage pattern in the digestion mixture. Alternatively, we ascertained that the resulting pattern of viral DNA bands was not altered by a longer enzymatic digestion. When double-enzyme digests were performed, nucleic acid precipitation took place before the second digestion.

Gel electrophoresis. Restriction enzyme digests

were added directly to horizontal slab agarose (Sigma Chemical Co., St. Louis, Mo.) gels. The electrophoresis apparatus was purchased from Bethesda Research Laboratories, Inc., Bethesda, Md. We prepared 0.4-cm-thick slab gels of 0.8% agarose. Restriction fragments of λ DNA and φX 174 DNA were used as size standards.

Blotting. After electrophoresis, DNA fragments were denatured and transferred from agarose gels to a sheet of 0.45-μm cellulose nitrate (Millipore Corp., Bedford, Mass.) in 3 M NaCl, pH 11 (10). After rinsing in 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), the filter was dried in air and baked at 80°C for 3 h.

Viral RNA. BLV production and purification and viral RNA extraction and purification were carried out as previously described (4). In brief, polyadenylated RNAs present in each viral particle were prepared from 60 to 70S viral RNA by heat denaturation and chromatography on two successive oligodeoxythymidylic acid cellulose columns. Sedimentation of this polyadenylated RNA on a denaturing sucrose gradient showed that about 25 to 30% of the molecules were full-length 38S, the other molecules having lengths ranging from about 12 to 38S (data not shown). This material was a suitable template for the synthesis of 3′-enriched BLV complementary DNA (cDNA; see below).

cDNA synthesis. BLV [^{32}P]cDNA's (4.2×10^8 cpm/μg) were synthesized as described previously (8), using limit DNase-digested calf thymus DNA primer (1 mg/ml). Total 60 to 70S RNA was used as a template for the synthesis of DNA complementary to the entire BLV genome ($cDNA_{rep}$). To characterize the probe, cDNA (0.7 ng) was hybridized to ^{125}I-labeled BLV 60 to 70S RNA. At DNA/RNA molar ratios of 1 and 5, the viral RNA fraction protected from RNase A plus T_1 digestion was, respectively, 63 and 61%. Total polyadenylated RNAs (see above) were also used as templates for the synthesis of cDNA. Again, characterization of the probe was obtained by protection experiments. At DNA/total 60 to 70S RNA molar ratios of 1 and 5, the viral RNA fraction protected from RNase A plus T_1 digestion was, respectively, 29 and 61%. This is indicative of a heterogeneous population of cDNA molecules.

Since RNA templates were selected by two chromatographic runs on oligodeoxythymidylic acid columns, it must be concluded that cDNA transcripts are preferential representatives of the 3′ end of the viral genome. These were referred to as $cDNA_{3' \text{ enriched}}$ and were used for all data reported here except those mentioned in Table 1 under the column $cDNA_{rep}$.

Hybridization. Baked, blotted nitrocellulose filters were rinsed in 3× SSC and prehybridized in a plastic box with 40 ml of a mixture containing 3× SSC, 10× Denhardt medium (3), 0.1% sodium dodecyl sulfate, and 100 μg of denatured calf thymus DNA per ml. The [^{32}P]cDNA probe (about 5×10^6 cpm/ml) was prehybridized separately in the same mixture, except denatured calf thymus DNA was present at a concentration of 2 mg/ml to avoid ribosomal contaminants. After an overnight incubation at 65°C, the filters were removed from their prehybridization mixture and then hybridized with mixing for 24 h with the prehybridized [^{32}P]cDNA probe. Filters were washed for about 1 h at 65°C in several changes of 3 × SSC. This was repeated with 0.3× SSC for 30 min. Dried filters were exposed at −70°C to preflashed Kodak X-Omat-R films in the presence of Siemens Special intensifying screens for 1 to 3 days.

Quantitation of radiographs. When applicable, the relative intensities of autoradiographic bands were estimated with a densitometer (Joyce, Loebl and Co. Ltd., Gateshead, England) essentially as described by Sherr et al. (15).

RESULTS

It was assumed that BLV infection of permissive cells would result in the synthesis of linear and circular viral duplexes of unintegrated and integrated forms (5, 14). To isolate the unintegrated viral DNAs for mapping by restriction analysis, we used the procedure described by Shoyab and Sen (16). The DNAs were analyzed by agarose gel electrophoresis, Southern blotting (17), and hybridization to a [^{32}P]cDNA probe (7, 8). Figure 1 is a map of restriction endonuclease sites on the linear BLV genome for nine restriction enzymes. The data leading to these assignments and to the orientation of the map relative to the RNA genome are presented below.

This method of analysis showed that extrachromosomal DNA after Shoyab and Sen extraction (16) usually contained only one major viral band, corresponding to DNA molecules with lengths of about 10.0 kilobases (kb) (Fig. 2, lane A). This linear duplex DNA is approximately the same length as a subunit of viral RNA (3.10^6 daltons) (4). Occasionally, some very faint bands with lengths of less than 10 kb were observed after a long exposure time. They may have been circular viral DNA forms and were not characterized further. When the extrachromosomal DNA containing the 10.0-kb linear viral DNA was digested by restriction endonu-

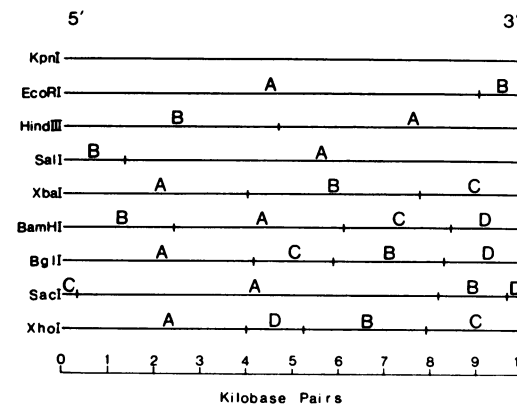

FIG. 1. *Restriction map of BLV (BLV FLK isolate) unintegrated viral DNA.*

cleases *Eco*RI and *Hin*dIII (Fig. 2, lanes B and E), two smaller viral DNA fragments were detected; in each case their sum was 10.0 kb, within experimental error.

As shown in Fig. 1, *Kpn*I did not cleave the proviral genome. Digestion by this enzyme of DNA extracted from BLV FLK cells showed three hybridization bands with lengths of 16, 18, and 21 kb, an observation compatible with the existence of three viral copies in this DNA. Digestion of the same DNA material by *Eco*RI and *Hin*dIII generated, in each case, six virus-positive fragments, thus establishing the presence of one *Eco*RI and one *Hin*dIII cleavage site (data not shown).

These results indicate that: (i) the 10.0-kb unintegrated DNAs are linear molecules; and (ii) the unintegrated linear DNA consists primarily of a single population having specific termini (that is, it did not represent a set of differently permuted molecules with respect to the viral RNA). Therefore, it was a suitable substrate for analysis with other restriction endonucleases.

Orientation of the 3′ end of linear BLV DNA with respect to viral RNA. We attempted to orient BLV DNA by using 3′-enriched cDNA transcripts prepared with a 12 to 38S poly(A)-containing BLV template and calf thymus primer (see above). Figure 2 and Table 1 show the results obtained when 3′-enriched cDNA was hybridized to various BLV DNA restriction fragments. Table 1 also is a comparison of the above results and data obtained with $cDNA_{rep}$. When transcripts representing the entire BLV genome were used, the intensities of bands corresponding to different restriction fragments were primarily a function of fragment length. In contrast, 3′-enriched cDNA hybridized preferentially to the 3′-end DNA fragments; 5′-end DNA fragments were visualized less efficiently. Since *Eco*RI removes a sequence of 0.92 kb from the 3′ end of linear BLV molecules, BLV DNA was, in part, oriented with respect to viral RNA by codigestion experiments performed with *Eco*RI.

***Kpn*I, *Eco*RI, *Sal*I, and *Hin*dIII digestions (no or one cleavage site).** Among the nine restriction endonucleases tested, one, *Kpn*I, was found that did not cleave the genome (see above).

*Eco*RI cleaved the linear DNA once, resulting in fragments of 9.08 and 0.92 kb (Fig. 2, lane B), the latter fragment being oriented at the 3′ end of the molecule (Table 1). The *Eco*RI digest of FLK DNA (which contains three BLV proviral copies) confirmed the presence of one cleavage site in the BLV provirus in that three large bands (12.3, 10.8, and 9.33 kb) and three faint

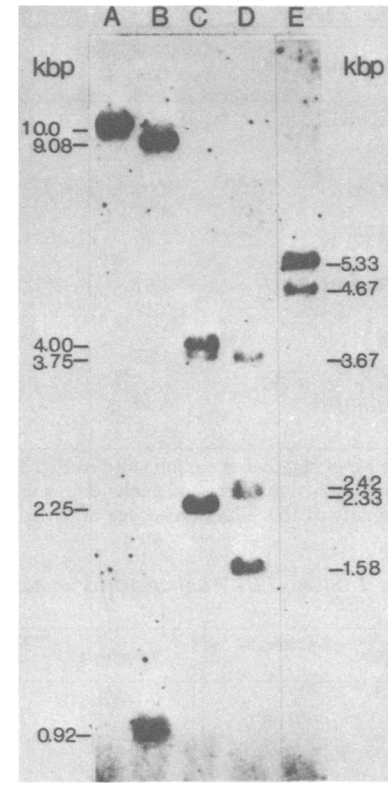

FIG. 2. *Detection of BLV linear DNA restriction enzyme fragments with a 3′-enriched cDNA probe. Approximately 5 μg of undigested total extrachromosomal DNA (A) or DNA digested with restriction enzyme EcoRI (B), XbaI (C), BamHI (D), or HindIII (E) was electrophoresed in an 0.8% agarose gel at 2 V/cm for 16 h. The DNA was transferred to a 0.45-μm nitrocellulose sheet, baked, prehybridized, and hybridized as described in the text. Hybridization to a 3′-enriched cDNA probe (0.012 μg/ml; 4×10^6 cpm/μg) was for 24 h at 65°C. The autoradiogram is a 1-day exposure. Lengths are given in kilobase pairs (kbp).*

bands (3.33, 2.83, and 2.66 kb) were detected (data not shown).

*Sal*I digestion gave two fragments with lengths of 8.66 and 1.34 kb (Fig. 1). Since *Eco*RI cut the 8.66-kb fragment, the *Sal*I site resides at about 1.34 kb from the left end of the linear map (Table 2).

*Hin*dIII produced a single cut in BLV DNA near the center of the molecule, generating two fragments with lengths of 5.33 and 4.67 kb (Fig. 1). Since *Eco*RI cut the 5.33-kb fragment, this positioned the *Hin*dIII site at 5.33 kb from the 3′ end of the linear molecular (Table 2).

The *Hin*dIII digest of FLK DNA was also compatible with the presence of one restriction site in the BLV provirus. In fact, six fragments

TABLE 1. *Orientation of the 3' end of linear BLV DNA with respect to viral RNA*

Restriction enzyme used	Size of fragment (kb)	% of genome	Fragment[a]	Avg intensity (mg%)[b]	
				$cDNA_{rep}$	$cDNA_{3'\ enriched}$
*Eco*RI	9.08	90.8	A	86	68
	0.92	9.2	B	14	32
*Xba*I	4.00	40.0	A	39	30
	3.75	37.5	B	33	15
	2.25	22.5	C	28	55
*Bam*HI	3.67	36.7	A	32	26
	2.42	24.2	B	24	13
	2.33	23.3	C	26	19
	1.58	15.8	D	18	42
*Hin*dIII	5.33	53.3	A	52	73
	4.67	46.7	B	48	27

[a] Position of fragments as indicated in Fig. 1.
[b] Areas under densitometric peaks determined by weight measurement were summed and normalized to a total intensity of 100. The percentage of the total intensity recorded for each fragment was then calculated.

TABLE 2. *Sizes of BLV unintegrated linear DNA restriction enzyme fragments from single- and double-enzyme digests*

Fragment	Length (kb)	Fragment	Length (kb)	Fragment	Length (kb)	Fragment	Length (kb)
*Eco*RI		*Xba*I-*Hin*dIII		*Bgl*I		*Bam*HI-*Sac*I	
A	9.08	A	3.75	A	4.16	A	3.67
B	0.92	B	3.16	B	2.42	B	2.18
		C	2.25	C	1.75	C	2.09
*Hin*dIII		D	0.84	D	1.67	D	1.25
A	5.33					E, F	0.33[a]
B	4.67	*Bam*HI		*Bgl*I-*Eco*RI		G	0.15[a]
		A	3.67	A	4.16		
*Hin*dIII-*Eco*RI		B	2.42	B	2.42	*Xho*I	
A	4.67	C	2.33	C	1.75	A	4.00
B	4.41	D	1.58	D	0.92	B	2.67
C	0.92			E	0.75[a]	C	2.08
		*Bam*HI-*Eco*RI				D	1.25
*Sal*I		A	3.67				
A	8.66	B	2.42	*Bgl*I-*Hin*dIII		*Xho*I-*Eco*RI	
B	1.34	C	2.33	A	4.16	A	4.00
		D	0.92	B	2.42	B	2.67
*Sal*I-*Eco*RI		E	0.66[a]	C	1.67	C	1.25
A	7.74			D	1.42	D	1.16
B	1.34	*Bam*HI-*Hin*dIII		E	0.33[a]	E	0.92
C	0.92	A	2.42				
		B	2.33	*Sac*I		*Xho*I-*Hin*dIII	
*Xba*I		C	1.58	A	7.83	A	4.00
A	4.00	D	1.25	B	1.50	B	2.67
B	3.75			C, D	0.33[a]	C	2.08
C	2.25	*Bam*HI-*Sal*I				D	0.83
		A	3.67			E	0.42[a]
*Xba*I-*Eco*RI		B	2.33	*Sac*I-*Eco*RI			
A	4.00	C	1.58	A	7.83	*Xho*I-*Sal*I	
B	3.75	D	1.34	B	0.92	A	2.67[b]
C	1.34	E	1.08	C	0.58[a]	B	2.08
D	0.92			D, E	0.33[a]	C	1.34
						D	1.25

[a] Not detectable under these experimental conditions (0.8% agarose). Their length derives from calculation.
[b] Comigration with fragment B from *Xho*I digestion.

(two per proviral copy) containing viral information were detected (data not shown).

XbaI digestion (two cleavage sites). *Xba*I digestion of the linear BLV provirus yielded three fragments with lengths of 4.00, 3.75, and 2.25 kb (Fig. 1). *Eco*RI cleaved the 2.25-kb fragment, thus positioning it at the 3′ end of the linear molecule. *Hin*dIII, which produced a single cut in BLV DNA near the center of the genome, cleaved the 4.00-kb *Xba*I fragment (Table 2). Thus, this 4.00-kb fragment was the internal viral fragment. *Xba*I digestion of FLK DNA was also in agreement with that conclusion. In fact, *Xba*I digestion produced seven fragments in FLK DNA, one of them, the 4.00-kb fragment, being preferentially revealed (data not shown). Since FLK DNA contains three BLV proviral copies with different cellular flanking sequences, internal fragments will comigrate and, thus, will be preferentially detected. Therefore, the *Xba*I BLV restriction map is: 5′-3.75, 4.00, 2.25-3′.

BamHI, BglI, SacI, and XhoI digestions (three cleavage sites). *Bam*HI cleaved the linear DNA three times, producing fragments with lengths of 3.67, 2.42, 2.33, and 1.58 kb (Fig. 1). The 1.58-kb fragment can be placed at the 3′ end of the linear map because it disappeared upon cleavage with *Eco*RI in the double digest. In the *Bam*HI-*Hin*dIII double digest, the 3.67-kb fragment was cut, indicating the internal position of that fragment. The *Bam*HI digest of FLK DNA confirmed the internal position of the 3.67-kb fragment and suggested the internal position of the 2.33-kb fragment (data not shown). In fact, these two fragments were preferentially revealed. In a *Bam*HI-*Xba*I double digest, the 2.42-kb fragment was not reduced, whereas the 2.33-kb fragment disappeared. In a *Bam*HI-*Sal*I double digest, the 2.42-kb fragment was split into 1.34- and 1.08-kb pieces (Table 2). Taken together, these data imply a *Bam*HI map oriented as follows: 5′-2.42, 3.67, 2.33, 1.58-3′.

*Bgl*I cleaved the linear BLV provirus three times, resulting in fragments with lengths of 4.16, 2.42, 1.75, and 1.67 kb (Fig. 1). In a *Bgl*I-*Eco*RI double digest, the 1.67-kb fragment was cleaved. These data allowed the placement of the 1.67-kb fragment at the 3′ end of the map. *Bgl*I digestion of FLK DNA allowed the preferential detection of the 2.42- and 1.75-kb fragments (data not shown).

In a *Bgl*I-*Hin*dIII digest, the 1.75-kb fragment was cut (Table 2). Based on a consideration of the above data and the sizes of the different fragments, the *Bgl*I restriction map is: 5′-4.16, 1.75, 2.42, 1.67-3′.

*Sac*I digestion yielded two major fragments (7.83 and 1.50 kb), the sum of which was 0.67 kb less than the size of full-length linear BLV DNA (Fig. 1). This suggested that *Sac*I cut within terminal sequences at both ends of BLV DNA but also cleaved at another, internal site. *Sac*I digestion of FLK DNA allowed the preferential detection of the 7.83- and 1.50-kb fragments, confirming that these fragments were internal fragments, the six other ones containing both viral and cellular sequences being only weakly revealed (data not shown). Since *Eco*RI cleaved the 1.50-kb fragment in double digestion, the 1.50-kb fragment was localized to the right side of the linear map. It should be noted that the 0.92-kb fragment appearing after *Sac*I-*Eco*RI double digestion (Table 2) is not identical in sequence to that observed with *Eco*RI only. Codigestion with *Bam*HI and *Sac*I did not alter the 3.67-kb internal *Bam*HI fragment, but reduced the 2.33-kb internal fragment of about 0.15 kb and the two external *Bam*HI fragments of about 0.25 to 0.33 kb (Table 2). The above data imply the following *Sac*I restriction map: 5′-0.33, 7.83, 1.50, 0.33-3′.

*Xho*I digestion yielded four fragments (4.00, 2.67, 2.08, and 1.25 kb). In an *Xho*I-*Eco*RI double digest, it was the 2.08-kb fragment that was cleaved, indicating its 3′-end position on the linear map. The 1.25-kb fragment was cut in *Xho*I-*Hin*dIII double digestion, and the 4.00-kb fragment was cleaved in *Xho*I-*Sal*I double digestion. These data are consistent with only a single order for the fragments: 5′-4.00, 1.25, 2.67, 2.08-3′. Due to methylation, cleavage of FLK DNA by *Xho*I gave incomplete digestions even with a vast enzyme excess, thus preventing preferential detection of internal fragments of integrated proviral information.

Summary. The physical map of BLV DNA restriction enzyme sites for nine enzymes is shown in Fig. 1. The lengths of single- and double-digestion products are given in Table 2. One enzyme, *Kpn*I, was found that did not cleave the genome.

Differentiation of genetic mutants of BLV by restriction enzyme analysis. Integrated BLV proviruses present in the DNA of circulating leukocytes from animals with persistent lymphocytosis or in the DNA from tumors from animals with lymphosarcoma were compared by restriction endonuclease digestion, agarose gel electrophoresis, Southern blotting (4), and hybridization with a BLV [^{32}P] cDNA probe. The results of these investigations are summarized in Table 3. From these data, five BLV genetic mutants were unraveled on the basis of differences in internal proviral fragments. It should be noted that only one BLV variant was found in the U.S. cattle tested and that this variant was found to be identical to

TABLE 3. *Differences in cleavage sites within various variants of integrated BLV proviruses*

Variant	Country of origin	No. of animals tested	No. of restriction endonuclease cleavage sites and length of proviral internal fragments (kb)				
			*Eco*RI	*Xba*I	*Bam*HI	*Hin*dIII	*Sac*I
1 (BLV FLK)	United States (Wisconsin and Iowa)	5	1	2 (3.75)	3 (3.67 and 2.33)	1	3 (7.83 and 1.50)
2	Belgium (south) and France	4 and 2	1	2 (4.33)	3 (3.67 and 2.45)	0	2 (9.33)
3	Belgium (north)	4	1	3 (2.91 and 1.42)	3 (3.67 and 2.45)	0	2 (9.33)
4	France	2	1	2 (3.75)	3 (2.33 and 1.33)	0	NT[a]
5	Japan	2	1	2 (2.91)	3 (3.67 and 2.33)	1	NT[a]

[a] NT, Not tested.

that produced by the BLV-infected FLK cell line used in this study.

It should be also noted that animals with persistent lymphocytosis (one response to BLV infection) were of particular interest for the detection of internal proviral fragments in the DNA of circulating leukocytes. In fact, persistent lymphocytosis is characterized by a polyclonal population of BLV-infected cells. Only proviral internal fragments will be visualized as well-defined bands; the other ones containing proviral information plus cellular sequences of variable length are diluted out and thus poorly detected as a smear (7).

DISCUSSION

An analysis of extrachromosomal DNA from bat cells acutely infected with BLV (produced by the BLV-infected FLK cell line) and extracted by the method of Shoyab and Sen (16) defined only one major species of unintegrated proviral DNA. Several lines of evidence show that the 10.0-kb DNA form corresponds to the linear BLV provirus. We have mapped a number of restriction sites on this in vivo DNA. The map is unique and appears to be colinear with that of the viral RNA.

The use of a 3'-enriched cDNA was of particular interest in that it allowed, by itself, a correct orientation of various proviral fragments. For example, the quantitation of the autoradiogram obtained with a *Bam*HI digest hybridized with a 3'-enriched cDNA gave an orientation of the linear map (Fig. 2 and Table 1) as follows: 5'-B, A, C, D-3'. This orientation was confirmed by codigestion experiments (Table 2).

One enzyme (*Sac*I) was found to cut at both sides of the BLV provirus at distances of between 0.25 and 0.33 kb from the ends. This suggests that the *Sac*I site must occur at or near the middle of the repeated sequence, the length of which being in the range of 0.50 to 0.70 kb. That *Sac*I has two sites located in the long terminal repeat sequences was confirmed by restriction enzyme analysis of leukocyte DNA from Belgian cattle with persistent lymphocytosis. In Belgian BLV variants lacking the internal *Sac*I site, a unique 5.40-kb fragment was generated in all cases examined (Table 3). Such long terminal repeats were previously described in the avian (13), baboon (2), murine (12, 20), and feline (15) proviruses.

Restriction enzyme analysis was performed on integrated proviruses of cattle from distant geographic origins (Belgium, France, Japan, and United States). Some restriction sites were shared among the DNAs (e.g., the *Eco*RI site). Some enzymes (e.g., *Xba*I), however, allowed the detection of BLV genetic variants. We have as yet mapped too few sites to permit an analysis of genetic stability with this approach. The variations do, however, have practical implications since they illustrate the danger of assuming that the restriction map for any mutant of BLV will be identical to that of previously mapped mutants.

This study provides the first detailed mapping data on the genome of a BLV mutant. The results for the BLV linear DNA are generally similar to those reported for other retroviruses. These mapping data will be useful in our ongoing studies of the integration site of BLV provirus in tumor cells of cattle with enzootic bovine leukosis.

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ADDENDUM IN PROOF

*Eco*RI and *Sac*I digests of a Japanese tumor DNA showed that only one proviral copy lacking about 4,800 kb at its 5′ end was present. The existence of such deleted variants might be helpful for understanding the mechanism by which BLV exerts its oncogenic effect.

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