JOURNAL OF VIROLOGY, Feb. 1984, p. 583–587
0022-538X/84/020583-05$02.00/0

Vol. 49, No. 2

Molecular Cloning of Covalently Closed Circular DNA of Bovine Leukemia Virus

S. V. S. KASHMIRI,* RAFIA MEHDI, AND JORGE F. FERRER

School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania 19348

Received 25 July 1983/Accepted 17 October 1983

The two species of covalently closed circular DNA molecules of bovine leukemia virus were cloned in the lambda phage vector λgtWES · λB. Of the nine independent recombinant λ-bovine leukemia virus clones that were analyzed, three were derived from the small and six were derived from the large circular molecules carrying, respectively, one and two copies of the long terminal repeat sequences. Comprehensive restriction endonuclease mapping of the unintegrated bovine leukemia virus and the cloned DNA molecules showed that eight of the nine clones carried viral information without any detectable deletions or insertions of more than ca. 50 base pairs. One of the nine clones, which carries a retroviral insert with one copy of the long terminal repeat, had a deletion of ca. 150 base pairs.

Bovine leukemia virus (BLV), an exogenous retrovirus of cattle, has been established as the causative agent of enzootic bovine leukemia and persistent lymphocytosis, an apparently benign condition which is frequently associated with the leukemia (4). Hybridization analyses indicate that one to several copies of the integrated BLV proviral DNA are present in bovine leukemic cells and in infected lymphocytes (10; Kashmiri et al., manuscript in preparation). The integrated proviral DNA of BLV resides in its target cell in a repressed state (5, 10). The molecular mechanism of BLV-induced leukemogenesis and the molecular basis for the covert nature of the virus are not known.

Extrachromosomal DNA isolated from cultured bat lung cells (CCL88) freshly infected with cell-free virus contains a linear viral DNA duplex carrying a copy of the long terminal repeat (LTR) sequence at each of its termini (8, 9). In addition, we have identified two species of covalently closed circular molecules of BLV DNA in extrachromosomal DNA isolated from cocultivated cultures of chronically infected and uninfected CCL88 cells. It has been shown that the small circle contains one copy of the LTR, whereas the large circle has two copies (8).

An understanding of the molecular organization of the BLV genome is important to gain an insight into the covert nature of BLV infection and the molecular mechanism of BLV-induced leukemogenesis. The limited quantity of viral DNA synthesized in infected cells has made it difficult to make a comprehensive study of the BLV genome. We therefore decided to molecularly clone the unintegrated BLV DNA. In this communication, we report the cloning of

TABLE 1. Restriction endonuclease cleavage products of the unintegrated BLV proviral DNA[a]

Restriction endonuclease	Fragments (kbp) generated by single digestion	Fragments (kbp) generated by subsequent digestion with:		
		*Eco*RI	*Sal*I	*Hin*dIII
*Pvu*I	8.20, 0.70			
*Eco*RI	8.10, 0.80			
*Hin*dIII	4.70, 4.20			
*Sal*I	7.70, 1.20			
*Xho*I	3.55, 2.35, 1.90, 1.10	3.55, 2.35, (1.10, 1.10),[b] 0.80	(2.35, 2.35), 1.90, 1.20, 1.10	3.55, 2.35, 1.90, 0.65
*Xba*I	3.35, 3.25, 1.95	3.35, 3.25, 1.15, 0.80	3.35, (2.05, 1.95), 1.20	3.25, 2.75, 1.95, 0.60
*Bgl*I	3.75, 2.10, (1.55, 1.50)	3.75, 2.10, 1.55, (0.80, 0.70)	2.55, 2.10, (1.55, 1.50), 1.20	3.75, 2.10, 1.50, 1.10
*Bam*HI	3.25, (2.05, 2.05), 1.55	3.25, (2.05, 2.05), (0.80, 0.75)	3.25, 2.05, 1.55, 1.20, 0.85	2.15, (2.05, 2.05), 1.55, 1.10
*Cla*I	4.30, 1.65, (1.50, 1.45)	4.30, 1.65, 1.50, 0.80, 0.65	4.30, (1.50, 1.45), 1.20	2.25, 2.05, 1.65, (1.50, 1.45)
*Bcl*I	4.85, 2.85, (0.60, 0.60)	4.85, 2.05, 0.80, (0.60, 0.60)	4.25, 2.85, (0.60, 0.60, 0.60)	3.60, 2.85, 1.25, (0.60, 0.60)
*Bgl*II	2.95, 2.45, 1.90, 1.30	2.95, 1.90, 1.65, 1.30, 0.80	2.45, 1.90, 1.75, 1.30, 1.20	2.95, 2.45, 1.30, (0.95, 0.95)
*Pst*I	5.35, 1.45, 1.15, (0.50, 0.45)	4.55, 1.45, 1.15, 0.80, (0.50, 0.45)	5.35, 1.45, 1.15, 0.50	
*Sst*I	7.00, 1.35	7.00, 0.75, 0.60	6.15, 1.35, 0.85	
*Pvu*II	3.55, 3.10, 1.70, 0.45[c]	3.55, 3.10, 1.35, 0.45	3.55, 2.00, 1.70, 1.10, 0.45	

[a] Small fragments not detected under our experimental conditions are not listed.
[b] Numbers in parentheses constitute a doublet or a triplet band.
[c] Diffused hybridization band was evident on prolonged exposure only.

* Corresponding author.

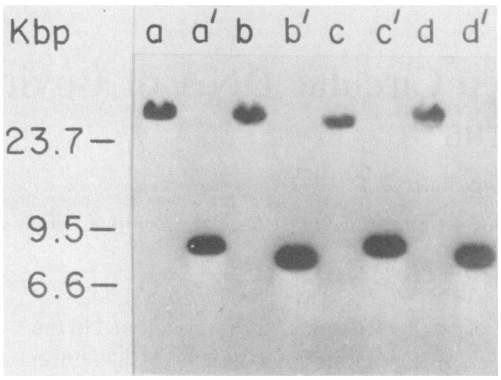

FIG. 1. Southern blot analysis of recombinant DNAs. Unintegrated BLV DNAs were produced by cocultivation of uninfected bat lung fibroblasts (CCL88) and BLV-producing bat cells (the BLV-bat clone) as described previously (8). Covalently closed circular DNAs were purified from the Hirt extract (7) by using the acid-phenol extraction procedure of Zasloff et al. (17). Purified *Eco*RI arms of λgtWES · λB (Amersham Corp., Arlington Heights, Il.) were mixed with *Eco*RI-digested covalently closed circular DNAs at a molar ratio of vector to substrate DNA of 3:1 and a final DNA concentration of 120 μg/ml. Ligation was carried out overnight at 12°C with 200 U of T4 ligase (P. L. Biochemicals, Inc., Milwaukee, Wis.) per ml of a reaction mixture containing 66 mM tris-hydrochloride (pH 7.6), 6.6 mM $MgCl_2$, 10 mM dithiothreitol, and 1.0 mM ATP. Ligated vector-substrate DNAs were packaged in vitro into lambda particles as described by Blattner et al. (2). The packaging extracts from *E. coli* N5428 and *E. coli* gd805 were purchased from Amersham Corp. The packaging reaction was plated on *E. coli* LE392. The plaques were screened for recombinants by the method of Benton and Davis (1), using the BLV [^{32}P]cDNA probe. Positive plaques were isolated and purified twice before their propagtion by lytic growth on *E. coli* LE392. Bacteriophages were purified by polyethylene glycol adsorption (16) and banded twice in CsCl gradients. Their DNAs were extracted as described by Sternberg et al. (15). Approximately 0.3 μg of untreated (lanes a to d) and *Eco*RI-digested (lanes a' to d') recombinant DNAs from λ-BLV clones 2, 5, 6, and 9, respectively, was subjected to electrophoresis in a 0.8% agarose gel. The DNA was transferred to a nitrocellulose filter and hybridized to the BLV [^{32}P]cDNA probe, as described previously (8). *Hin*dIII fragments of wild-type λ DNA were used as size markers. The numbers on the left represent the lengths of the marker DNA fragments.

both species of covalently closed circular BLV DNA in the λgtWES · λB vector. A detailed analysis of the structure of the cloned DNAs and their comparison with the linear unintegrated DNA are also presented.

Since it would be necessary to check the fidelity of the cloned DNA with the BLV genome, we first developed a comprehensive restriction endonuclease map of the unintegrated linear DNA. The 8.9-kilobase-pair (kbp) linear DNA was purified free of the supercoiled and nicked circular forms of viral DNA as described previously (8). The DNA was digested with restriction endonucleases, subjected to agarose gel electrophoresis, and hybridized with the $cDNA_{rep}$ probe by using the Southern procedure (14). ($cDNA_{rep}$ is a probe which represents the entire BLV genome.) The conditions for cDNA synthesis and hybridization have been described previously (8). The results of this restriction analysis are presented in Table 1.

The viral DNA lacks sites for *Kpn*I and *Hpa*I, since the size of the DNA remained unaltered when it was digested with these enzymes (data not shown). Each of the enzymes *Puv*I, *Eco*RI, *Hin*dIII, and *Sal*I has a unique site on the BLV DNA, since digestion with each of these enzymes generated two fragments whose combined size equals the genome length in each case. To orient viral DNA ends with respect to the 3′ and 5′ ends of the viral RNA, the viral DNA fragments generated by cleavage with one-cut enzymes were hybridized to the 3′-enriched [^{32}P]cDNA probe. Polyadenylate-containing viral RNA used for the synthesis of the 3′-enriched [^{32}P]cDNA probe was prepared by the procedure described by Sherr et al. (13). Of the two fragments generated by each of the enzymes, the 0.7-kbp *Puv*I, 0.8-kbp *Eco*RI, 4.7-kbp *Hin*dIII, and 7.7-kbp *Sal*I fragments showed preferential hybridization with the 3′-enriched cDNA probe (data not shown). These fragments, therefore, are homologous to the 3′ ends of the viral RNA (see Fig. 4C). These results are consistent with an earlier report (9) defining the orientation of restriction fragments of the linear proviral DNA of BLV from a different cell line with respect to viral RNA.

Table 1 shows the cleavage products of the unintegrated linear DNA by single digestion with 10 other restriction endonucleases. Results of the double-digestion experiments, also presented in Table 1, helped to orient these fragments with respect to each other and to the 3′ and 5′ ends of the viral RNA (see Fig. 4C).

To molecularly clone the two species of covalently closed circular DNA molecules of BLV, the unintegrated DNAs were isolated by the Hirt procedure (7). The supercoiled BLV DNAs were purified by acid phenol extraction (17) and then further enriched on a 15 to 30% linear sucrose gradient.

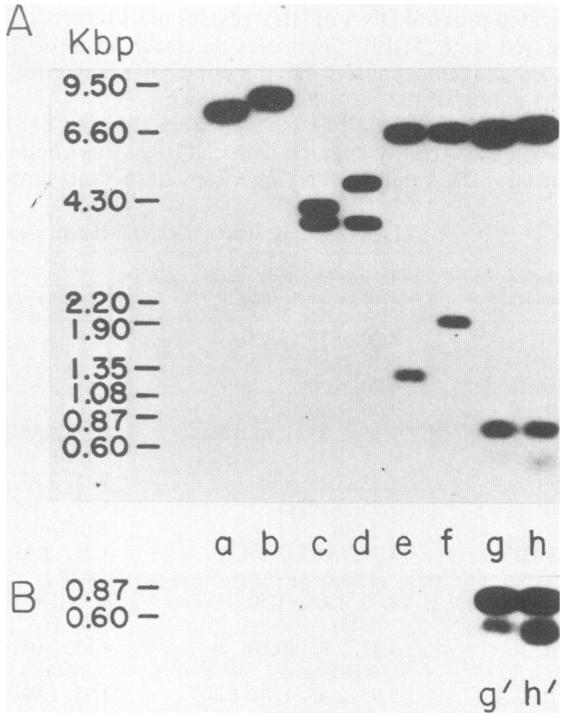

FIG. 2. Comparison of the large and small retroviral DNA inserts by restriction endonuclease cleavage. λ-BLV clone 9 and 6 DNAs were treated with *Eco*RI alone (lanes a and b) or treated with *Eco*RI and subsequently treated with *Hin*dIII (lanes c and d), *Sal*I (lanes e and f), or *Sst*I (lanes g and h). Enzyme-digested DNAs were electrophoresed, transferred to nitrocellulose filters, and hybridized to the BLV [^{32}P]cDNA probe, as described in the legend to Fig. 1. *Hin*dIII fragments of wild-type λ DNA and *Hae*III fragments of ϕX174 replicative-form DNA were used as size markers. The numbers on the left represent the lengths of the size markers. (B) Prolonged exposure of the lower portion of (A) for lanes a to h.

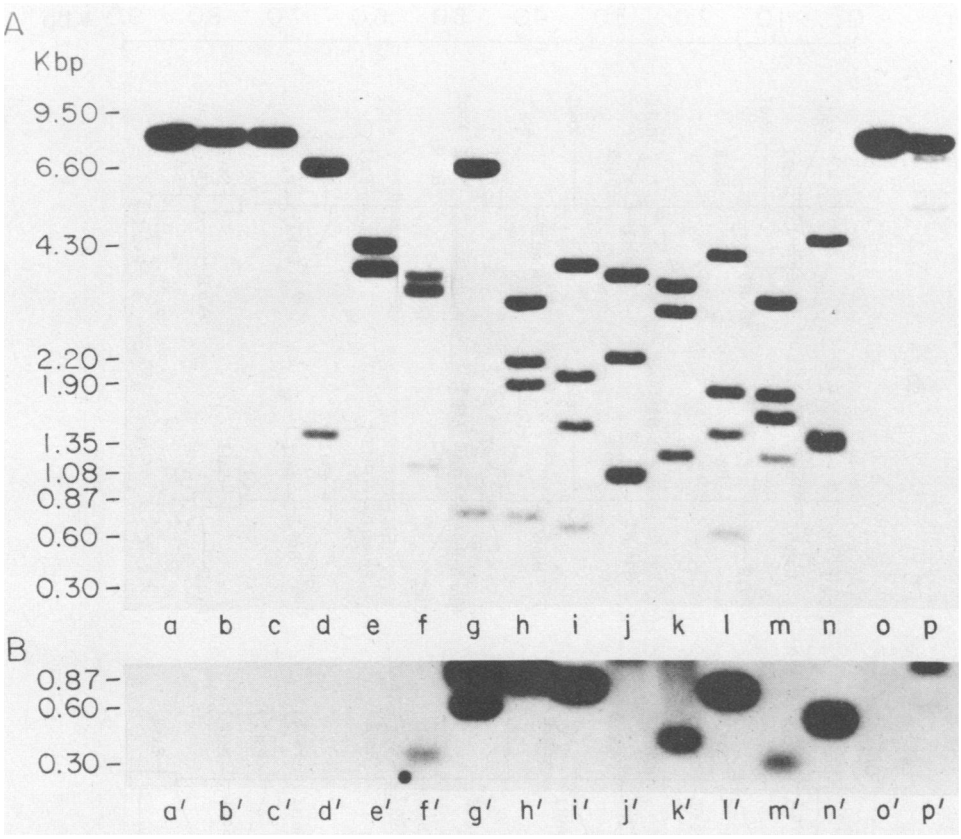

FIG. 3. Restriction endonuclease analysis of the retroviral DNA insert from λ-BLV clone 9. λ-BLV clone 9 DNA was digested with *Eco*RI alone (lane a) or treated with *Eco*RI and subsequently treated with *Kpn*I, *Pvu*I, *Sal*I, *Hin*dIII, *Xba*I, *Sst*I, *Bam*HI, *Bgl*I, *Xho*I, *Pvu*II, *Cla*I, *Bgl*II, *Pst*I, *Hpa*I, or *Bcl*I (lanes b to p, respectively). Approximately 0.25 μg of the enzyme-digested DNAs was loaded on an agarose gel and subjected to electrophoresis. BLV-specific sequences were detected by Southern blot hybridization with the BLV [^{32}P]cDNA probe as described in the legend to Fig. 1. The numbers on the left represent the lengths of the size markers as described in the legend to Fig. 2. (B) Prolonged exposure of the lower portion of (A) for lanes a to p.

The purified DNA was linearized by treatment with *Eco*RI and ligated to *Eco*RI arms of λgtWES · λB. Ligated molecules were then packaged into infectious particles. Approximately 3.5×10^4 plaques, generated by plating 20 μl of packaging reaction on *Escherichia coli* LE392, were screened for the presence of BLV-specific DNA by hybridization with the BLV [^{32}P]cDNA probe. Fifty-five recombinant plaques were identified and isolated. Of these, nine were further analyzed. Their DNAs were purified and subjected to agarose gel electrophoresis before and after cleavage with *Eco*RI. UV fluorescence of the ethidium bromide-stained gel showed that, on cleavage with *Eco*RI, each recombinant yielded λgtWES · λB arms and a single-insert DNA (data not shown). Southern hybridization analysis revealed that each of the DNA inserts generated by *Eco*RI cleavage of the recombinant DNAs hybridized with the BLV [^{32}P]cDNA probe. Southern analysis of four clones is presented in Fig. 1. The inserts essentially belonged to two different size categories. DNAs from six clones, including those of BLV clones 2 and 6 (lanes a and c), yielded large inserts (lanes a' and c'), whereas DNAs from three clones, including those of BLV clones 5 and 9 (lanes b and d), yielded small inserts (lanes b' and d'). The large inserts were ca. 8.9 kbp long. The λ-BLV clone 9 yielded an insert of 8.35 kbp, and the insert from the λ-BLV clone 5 (lane b') consistently showed slightly faster migration (see below).

The structural relationship of two different-sized inserts was investigated by comparing restriction endonuclease cleavage products of λ-BLV clones 6 and 9, which yield large and small inserts, respectively. The inserts were first cleaved from the DNAs of the two clones by *Eco*RI treatment. Subsequent digestion was done either with *Hin*dIII or *Sal*I, both of which have a site on the linear DNA, or with *Sst*I, which has a site on the BLV LTR and an internal site on the unintegrated linear DNA.

The autoradiogram in Fig. 2 is the result of such an examination. Digestion of the 8.35- and 8.90-kbp inserts from λ-BLV clone 9 and λ-BLV clone 6 DNAs (lanes a and b) with *Hin*dIII generated two fragments in each case (lanes c and d). The small fragments generated from the two inserts comigrated. In contrast, the larger fragments had different mobility, thus reflecting the difference in sizes of the inserts. *Sal*I digestion also generated two fragments from each insert (lanes e and f). Only the small fragments differed in size. These results suggested that the permuted molecules differ in the 5'-end fragments.

Cleavage of inserts with *Sst*I, which has one site on BLV LTR and a unique internal site on BLV DNA, confirmed this suggestion and defined the size difference between the inserts precisely. *Sst*I digestion of the 8.35-kbp insert generated three fragments of ca. 6.9, 0.85, and 0.60 kbp in length (Fig. 2A, lane g, and Fig. 2B, lane g'). *Sst*I cleavage products of the 8.9-kbp insert included, in addition to the three fragments generated from the small insert, a new fragment of

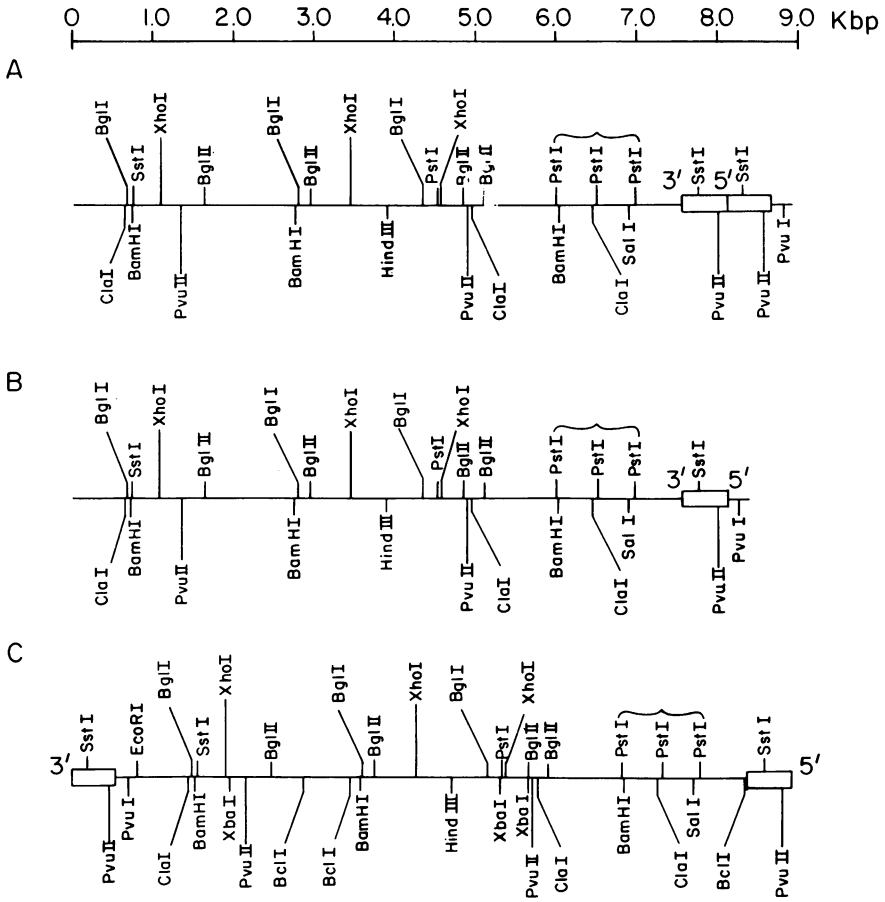

FIG. 4. Restriction endonuclease map of BLV DNA. (A) Retriction map of the 8.9-kbp permuted viral DNA generated by Southern analysis of the cleavage fragments of λ-BLV clone 6 DNA (data not shown). A similar analysis of λ-BLV clone 9 DNA is shown in Fig. 3. (B) Restriction map of the 8.35-kbp viral DNA insert derived by Southern analysis of the cleavage fragments of λ-BLV clone 9 DNA as shown in Fig. 3. (C) Restriction endonuclease cleavage sites in linear BLV DNA mapped from its cleavage fragments presented in Table 1. The open blocks represent the LTR.

ca. 550 base pairs that was missing from the small-insert digest (Fig. 2A, lane h, and Fig. 2B, lane h'). The 550-base-pair fragment could only be derived from the contiguous copies of the LTR in the large insert. These data are compatible with the suggestion that small inserts were derived from the BLV circular DNA molecules carrying one copy of LTR, whereas the large inserts were permuted molecules of circular DNA carrying two LTR copies placed in tandem.

For a comparison of the cloned DNAs with the unintegrated BLV DNA, a detailed restriction endonuclease analysis of the permuted DNA molecules was undertaken. Figure 3 shows the cleavage pattern of the insert from λ-BLV clone 9 (with one LTR copy). Similarly, DNA from λ-BLV clone 6 (with two LTR copies) was analyzed (data not shown), using all of the restriction endonucleases that were used for mapping the unintegrated linear DNA. For 14 of the 16 restriction endonucleases, the cleavage patterns of the cloned DNAs were consistent with the map of the unintegrated linear DNA. The small *Bgl*II fragment of ca. 300 base pairs which remained undetected among the cleavage products of the unintegrated linear DNA was easily detected among *Bgl*II digestion fragments of the cloned DNA (Fig. 3B, lane m'). However, the cleavage patterns of the cloned DNAs with *Xba*I and *Bcl*I were not consistent with the recognition sites of these enzymes on the linear DNA. Cleavage with *Xba*I yielded an extra fragment in addition to the fragments expected from the linear map, including a 350-base-pair fragment which remained undetected among the cleavage products of the linear DNA (Fig. 3A, lane f, and Fig. 3B, lane f'). The extra fragment, which had slower mobility than the expected doublet of 3.35- and 3.50-kbp fragments, was seemingly the product of partial digestion. Treatment of the cloned DNA with *Bcl*I left most of the DNA uncut and generated several partially digested fragments (Fig. 3A, lane p). *Bcl*I and *Xba*I are known to be sensitive to adenine methylation (11, 12). The inconsistency between the cleavage pattern of the unintegrated linear and the cloned DNAs with *Bcl*I and *Xba*I can be readily attributed to adenine methylation of BLV DNA by the *dam* methylase of *E. coli* (6) during cloning. Restriction endonuclease maps of the permuted DNA molecules with one and two copies of LTR are shown in Fig. 4B and A, respectively.

As mentioned earlier, the insert generated by the λ-BLV clone 5 DNA by *Eco*RI digestion consistently showed slightly faster migration than the inserts from λ-BLV clone 9, carrying one copy of retroviral LTR. *Sal*I cleavage of λ-BLV clone 5 and 9 inserts showed that the size difference between the inserts was confined to the small *Sal*I fragment. A comparison of the *Sal*I-*Sst*I fragments of λ-BLV clone 5

and 9 DNAs showed that a 0.85-kbp fragment, present among the digestion products of the λ-BLV clone 9 DNA, was replaced by a 0.7-kbp fragment among the cleavage products of the λ-BLV clone 5 DNA (data not shown). Thus, it seems apparent that λ-BLV clone 5 is derived from the BLV circular DNA carrying one copy of LTR and that its DNA has a deletion of ca. 150 base pairs mapping between 0.35 and 1.2 kbp from the 5′ end on the linear map. This clone, which is the first reported well-characterized deletion of BLV, could be important because the deletion is located either within the LTR sequence or near it.

Molecular cloning of the two species of covalently closed circular DNA of BLV reported here represents the first successful attempt at cloning the unintegrated BLV DNA. Deschamps et al. (3) have obtained a molecular clone of the BLV genome integrated in a bovine tumor. The cloning was accomplished by taking advantage of the existence of an *Sst*I site in the BLV LTR and the lack of an internal *Sst*I site in this BLV variant. A preliminary restriction endonuclease map of the BLV DNA cloned from the bovine tumor (3) shows differences from the restriction endonuclease maps of the linear DNAs of BLV isolates of fetal lamb kidney BLV (9) and of BLV-bat reported here.

Molecular cloning of the unintegrated BLV DNA has enabled us to undertake a comprehensive study of the structural organization and functional activity of the BLV genome. We have been able to subclone the BLV genomes and the subgenomic segments in *E. coli* plasmid vector pBR322. These well-defined probes are being used in our attempts to understand the complexity, sites, and number of integrations of the BLV genomes in BLV-induced bovine tumors. These studies should help gain an insight into the molecular mechanism of BLV-induced leukemogenesis and the repressed state of the BLV genome in vivo.

We thank Betsy Altland for her excellent technical assistance and Betty M. Thompson for preparation of the manuscript.

This research was supported in part by a grant from the Edgewater Corp.

LITERATURE CITED

1. **Benton, W. D., and R. W. Davis.** 1977. Screening λgt recombinant clones by hybridization to single plaques *in situ*. Science **196**:180–182.
2. **Blattner, F. R., B. G. Williams, A. E. Biechl, K. Denniston-Thompson, H. E. Faber, L.-A. Furlong, D. G. Grunwald, D. O. Kiefer, D. D. Moore, E. L. Sheldon, and O. Smithies.** 1977. Charon phages: safer derivatives of bacteriophage lambda for DNA cloning. Science **196**:161–169.
3. **Deschamps, J., R. Kettmann, and A. Burny.** 1981. Experiments with cloned complete tumor-derived bovine leukemia virus information prove that the virus is totally exogenous to its target animal species. J. Virol. **40**:605–609.
4. **Ferrer, J. F.** 1980. Bovine lymphosarcoma. Adv. Vet. Sci. Comp. Med. **24**:1–68.
5. **Ferrer, J. F., C. Cabradilla, and P. Gupta.** 1980. Bovine leukemia: a model for viral carcinogenesis. Cold Spring Harbor Conf. Cell Proliferation **7**:887–899.
6. **Hattman, S., J. E. Brooks, and M. Masurekar.** 1978. Sequence specificity of the P1 modification methylase (M. *EcoP1*) and the DNA methylase (M. *Eco dam*) controlled by the *Escherichia coli dam* gene. J. Mol. Biol. **126**:367–380.
7. **Hirt, B.** 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. J. Mol. Biol. **26**:365–369.
8. **Kashmiri, S. V. S., R. Mehdi, and J. F. Ferrer.** 1983. Detection, purification, and characterization of two species of covalently closed circular proviral DNA molecules of bovine leukemia virus. J. Virol. **45**:1172–1176.
9. **Kettmann, R., D. Couez, and A. Burny.** 1981. Restriction endonuclease mapping of linear unintegrated proviral DNA of bovine leukemia virus. J. Virol. **38**:27–33.
10. **Kettmann, R., J. Deschamps, Y. Cleuter, D. Couez, A. Burny, and G. Marbaix.** 1982. Leukemogenesis by bovine leukemia virus: proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3′ proximate cellular sequences. Proc. Natl. Acad. Sci. U.S.A. **79**:2465–2469.
11. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 102–103. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
12. **McClelland, M.** 1983. The effect of site specific methylation on restriction endonuclease cleavage (update). Nucleic Acids Res. **11**:r169–r173.
13. **Sherr, C. J., L. A. Fedele, L. Donner, and L. P. Turek.** 1979. Restriction endonuclease mapping of unintegrated proviral DNA of Snyder-Theilen feline sarcoma virus: localization of sarcoma-specific sequences. J. Virol. **32**:860–875.
14. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **38**:503–517.
15. **Sternberg, N., D. Tiemeier, and L. Enquist.** 1977. *In vitro* packaging of a λ dam vector containing *EcoRI* DNA fragments of *Escherichia coli* and phage P1. Gene **1**:255–280.
16. **Yamamoto, K. R., B. M. Alberts, R. Benzinger, L. Lawhorne, and G. Treiber.** 1970. Rapid bacteriophage sedimentation in the presence of polyethylene glycol and its application to large-scale virus purification. Virology **40**:734–744.
17. **Zasloff, M., G. D. Ginder, and G. Felsenfeld.** 1978. A new method for the purification and identification of covalently closed circular DNA molecules. Nucleic Acids Res. **5**:1139–1152.