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Experiments with Cloned Complete Tumor-Derived Bovine Leukemia Virus Information Prove that the Virus Is Totally Exogenous to Its Target Animal Species

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Taking advantage of the existence of a unique *Sac*I restriction site in the long terminal repeats of the integrated bovine leukemia virus proviral DNA isolated from a bovine tumor, the total viral information (about 9.2 kilobases) was cloned in the lambdoid vector λWES.λB. Use of this cloned bovine leukemia virus DNA allowed us, for the first time, to definitely rule out the existence of any endogenous bovine leukemia virus sequence in the bovine, ovine, caprine, murine, feline, chicken, or human genomes. These data prove the absence of acquired cellular information in the provirus that has given rise to a tumor.

Bovine leukemia virus (BLV) is a type C retrovirus that was proven to be the etiological agent of a horizontally spreading lymphocytic leukemia in cattle. BLV induces tumors in infected animals after a latency period that varies between a few months to years (2). Liquid hybridization experiments with ^{3}H-labeled BLV cDNA recycled on normal calf thymus DNA have clearly established that the preleukemic stage of the disease and the overt tumoral phase are characterized by the presence of newly acquired exogenous proviral DNA in the target cell genome (9). Since BLV viral RNA still remained contaminated with some ribosomal RNA even after two chromatographic runs through oligodeoxythymidylic acid-cellulose columns, the cDNA probe as well as the filters were prehybridized with saturating amounts of calf thymus DNA to avoid strong ribosomal gene detection in the Southern blotting experiments. Nevertheless, faint ribosomal bands still remained to be seen on the autoradiograms (10, 12). We therefore were not able to rule out the presence of proviral sequences in the normal bovine genome because of a possible comigration with ribosomal bands. Moreover, the prehybridization step with saturating amounts of normal calf thymus DNA could have prevented the detection of some possible cellular information in the viral genome. To definitely settle this issue, we cloned the complete BLV proviral information in the bacteriophage λWES.λB. (13).

We started from tumor DNA (T15 DNA) that had previously been shown to harbor one proviral DNA copy inserted at a unique genomic site (10) and bearing only one *Sac*I cleavage site in each of the viral long terminal repeats (LTRs) (11). As shown in Fig. 1 (lane 2), ^{32}P-labeled BLV cDNA hybridized on two virus-positive *Sac*I fragments (9.2 and 1.7 kilobases [kb]) of T15 DNA. The 9.2-kb fragment was previously shown to be the viral internal fragment in that BLV variant (11), and the 1.7-kb fragment contained viral sequences linked to host sequences. A third fragment containing both viral and cellular information was not detected, probably because it was too short to be transferred.

Since the LTRs are direct repeats (11), the 9.2-kb proviral fragment contains all of the BLV genetic information; each LTR is thus approximately 0.75 kb long. (It should be recalled that the total length of unintegrated BLV DNA is about 10.0 kb [11].) The three additional hybridization bands (5.2, 3.3, and 1.8 kb) also shown in the control DNA (Fig. 1, lane 1), corresponded to ribosomal DNA, as deduced from hybridization to a specific ribosomal DNA probe (12). The proviral DNA to be cloned was partially purified in two successive steps. An approximately 10-fold enrichment in proviral sequences was achieved by centrifugation of the high-molecular-weight cellular DNA (about 25×10^{6}) in a preparative Cs_2SO_4-3,6-bis(acetatomercurimethyl)dioxane density gradient (12). The viral DNA-enriched fractions were identified by Southern blot analysis of *Sac*I digests using ^{32}P-labeled BLV cDNA as a probe (data not shown). These fractions were subsequently pooled, dialyzed, and extensively digested by *Sac*I before electrophoresis in a preparative agarose slab gel. The DNA fragments of about 9.2 kb were extracted from the gel by incubating the agarose

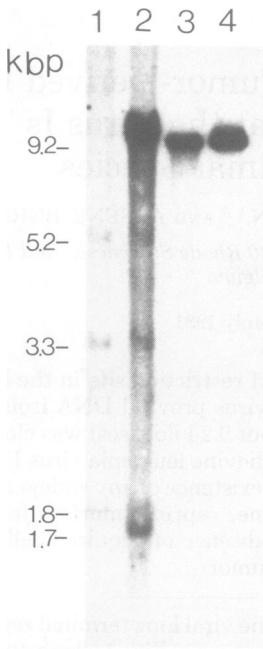

FIG. 1. *DNA hybridization with BLV cDNA as a probe. Ten micrograms each of DNAs from calf thymus (lane 1) and bovine tumor 15 (T15) (lane 2) were digested by SacI (Biolabs, Beverly, Mass.) according to the conditions suggested by the manufacturer. A sample (about 0.2 μg) of the viral DNA-enriched material obtained after Cs_2SO_4–3,6-bis(acetatomercurimethyl)dioxane gradient centrifugation and 0.8% preparative agarose slab gel electrophoresis (see text) was run in parallel (lane 3), as well as about 20 pg of the pure cloned BLV genome after excision from the λ vector (lane 4). All samples were electrophoresed in a 0.8% agarose gel. After the restriction fragments were transferred to nitrocellulose sheets, they were soaked in the prehybridization mixture at 65°C (10, 11) and hybridized for 24 h with 5×10^6 cpm of 3′-enriched, ^{32}P-labeled BLV cDNA per ml (11) (specific activity, 3×10^8 cpm/μg); this probe was separately prehybridized with 2 mg of calf thymus DNA per ml to reduce the ribosomal DNA detection (10, 12). The last washing was performed in 45 mM NaCl–4.5 mM sodium citrate. Filters were exposed at −70°C to preflashed Kodak X-Omat-R film in the presence of Siemens special intensifying screens for 2 days. Fragments length is given in kilobase pairs (kbp). Only fragments larger than 0.5 kbp were detected on these gels. EcoRI- and HindIII-digested λ DNA as well as HaeIII-digested φX174 DNA were used as molecular weight markers. It should be noted that the slight delay in the migration rate of the 9.2-kb BLV proviral fragment present in lane 2 versus that in lanes 3 and 4 is due to differences in the amount of DNA input: 10 μg in lane 2 and less than 1 μg in lanes 3 and 4.*

band cut out of the gel in presence of agarase (Calbiochem, La Jolla, Calif.) by the method of Finkelstein and Rownd (5). The agarase-treated material was engaged in the cloning reactions after one sample had been analyzed by the Southern technique (Fig. 1, lane 3).

λWES.λB phage DNA was prepared by standard methods (1) and digested with *Sac*I endonuclease. Inactivation of the restriction enzyme, reannealing of the λ cohesive ends, ligation of the vector with the exogenous DNA, in vitro packaging, library amplification, and screening of the recombinant phage population were performed by the methods of Maniatis et al. (14). Several positive clones were detected that were confirmed to contain BLV genetic information. As examplified in Fig. 1 (lane 4) for one of them, they contained the 9.2-kb BLV proviral fragment originally detected in T15 DNA *Sac*I digests (Fig. 1, lane 2). The restriction map of the cloned proviral DNA is presented in Fig. 2. This map of a Belgian BLV variant differs from that obtained for the BLV fetal lamb kidney isolate (11), an American variant, except for the unique *Eco*RI site which happens to be invariably present and located very close to the right LTR in the proviral molecule. This held true for all of the 25 tumors, originating from all over the world, examined so far (3). The *Sac*I site present inside the LTRs (Fig. 2) also appears to be well conserved in the various BLV isolates (11).

The 9.2-kb cloned proviral DNA, representative of the whole proviral information, was hybridized to *Eco*RI-digested normal bovine DNA (Fig. 3A, lanes 1 to 4). In no case was there the faintest positive hybridization band; neither did *Sac*I-digested DNA from six other species (namely, sheep, goats, mice, cats, chickens, and humans; Fig. 3A, lanes 5 through 10, respectively) show hybridization with the λBLV nick-translated probe. This probe was hybridized to *Eco*RI-digested tumor DNAs that contain one proviral copy (3) (Fig. 3A, lanes 11 and 12). As expected, two fragments containing viral information were observed in each case (10.0 and 3.0 kb, lane 11; 11.0 and 2.0 kb, lane 12), the 3.0- and 2.0-kb fragments being located at the 3′ side of the proviral molecule. Since the proviral contribution in the 3′ junction fragments was almost exclusively made of LTR sequences (Fig. 2), it appeared that the λBLV probe clearly detected LTR sequences. Consequently, it should be noted that LTR sequences would not have escaped detection in the forementioned analysis of the various normal genomes. Moreover, as shown in Fig. 3A, lane 13, the nick-translated probe not only identified the two internal viral fragments (7.7 and 1.5 kb) from a *Sac*I-digested tumor cell DNA (American variant [11]), but it also revealed the two virus-cell junction fragments (3.6 and 2.6 kb [3]), the proviral contribution of them being a portion of LTR (Fig. 2).

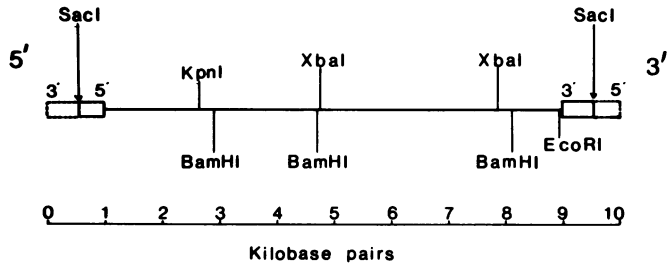

FIG. 2. *Restriction map of the cloned BLV provirus. The restriction sites of five enzymes (EcoRI, SacI, XbaI, KpnI, and BamHI) inside the cloned BLV proviral genome are shown schematically. Neither SalI nor HindIII cleaved the cloned proviral DNA molecule. The map was established as previously described for the unintegrated BLV DNA by using single and double digestions. Orientation with respect to viral RNA was performed using 3'-enriched cDNA as a probe (11).*

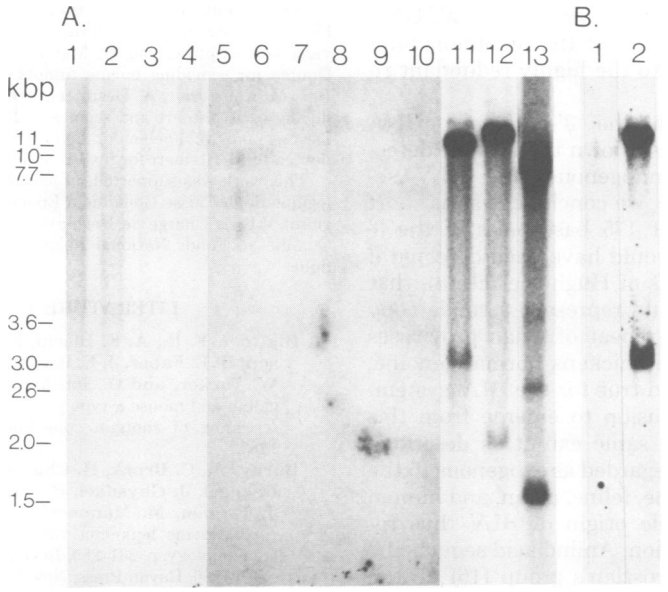

FIG. 3. *A, DNA hybridization with nick-translated cloned BLV DNA as a probe. Twenty micrograms each of normal bovine DNA from animals of different breeds (Holstein [lane 1], Belgian White Blue [lane 2], Friesian [lane 3], Normande [lane 4]) and 10 μg of bovine tumor DNA (animal 3168 [lane 11], animal 1347 [lane 12]) were digested to completion by EcoRI and electrophoresed on a 0.8% agarose gel. Samples (20 μg) of SacI-digested DNA from sheep kidney (lane 5), goat kidney (lane 6), RIII mouse kidney (lane 7) cat kidney ([lane 8]), chicken myoblast (lane 9), and human placenta (lane 10) and 20 μg of bovine tumor DNA (animal 3168 lane 13) were run in parallel. The Southern blot of the DNA fragments was treated as described in the legend to Fig. 1, except that calf thymus DNA was replaced by Escherichia coli DNA (at a concentration of 50 μg/ml) during the prehybridization and hybridization steps, and that 7×10^6 cpm of nick-translated λWES.λB recombinant DNA (specific activity, 4×10^8 cpm/μg; nick translation was carried out by the method of Rigby et al. [18]) per ml was used as a probe. Autoradiography was from a 4-day exposure. B, DNA hybridization with BLV cDNA as a probe. Ten micrograms of normal calf thymus DNA (lane 1) and bovine tumor cell DNA animal 3168 (lane 2) was exhaustively digested by EcoRI and treated as described in the legend to Fig. 1: 5×10^6 cpm of 3'-enriched, ^{32}P-labeled BLV cDNA per ml (11) (3×10^8 cpm/μg) was used as a probe. Both the filter containing the DNA and the probe were separately prehybridized with 2 mg of calf thymus DNA per ml to reduce the ribosomal DNA detection. The filter was exposed for 5 days. kbp, Kilobase pairs.*

We are well aware that the detection of hybridizing sequences within this range of magnitude approaches the limit of the method; nevertheless, we estimate that the results obtained under our stringent hybridization conditions allow us to rule out the presence of endogenous true BLV sequences larger than about half an LTR (i.e., 350 base pairs) in the target and nontarget ani-

mal species tested so far. Moreover, previous studies (3, 11) with a BLV cDNA probe highly enriched in 3′ sequences showed a very strong hybridization in both the 3′ and the 5′ proviral fragments from *Eco*RI digests of tumor DNAs (3). This is exemplified in Fig. 3B (lane 2) for tumor DNA 3168 containing one proviral copy. Although this BLV cDNA probe had been exhaustively prehybridized with normal bovine DNA, it still strongly recognized BLV unique sequences, one of which represented almost exclusively the viral 3′ LTR. We therefore estimate that such a 3′-enriched BLV cDNA probe would never fail to detect even half of the 3′ moiety of an LTR sequence (about 175 base pairs) present in any cellular DNA. It obviously did not reveal any hybridization fragment in normal calf DNA (Fig. 3B, lane 1), except for the remaining faint band corresponding to the highly redundant ribosomal genes (12).

Experiments with the 3′-enriched cDNA probe allow us to push down the limit of detection of putative endogenous true BLV sequences. In this case, we conclude that as short a fragment as about 175 base pairs of the 3′ moiety of an LTR would have been detected if present. The findings of Hughes et al. (8), that sequences which could represent a single copy of the 3′5′ terminal repeat of avian proviruses can be unravelled in chickens normal genome, do not appear to hold true for the BLV system.

The second conclusion to emerge from this study is that to the same extent as described above, BLV can be regarded as exogenous to the ovine, caprine, murine, feline, avian, and human species. The possibile origin of BLV thus remains an open question. Amino acid sequencing data obtained by Oroszlan's group (16) on the NH_2 terminus of BLV p24 pointed to a very distant relatedness between BLV and a murine ancestor. Our data do not contradict this conclusion but call for use of very relaxed annealing conditions and subcloned parts of the BLV proviral DNA as probes when studies on the distant relatedness between BLV and a variety of animal genomes are to be performed.

Our third conclusion is that no cellular information (longer than 350 base pairs of DNA) is to be found in the integrated proviral genome which has given rise to a tumor, as is the case for sarcoma viruses (4), for the defective avian acute leukemia viruses (19), and for Abelson murine leukemia virus (20). It is clear also that there is no endogenous sequence the involvement of which might have generated a highly oncogenic virus through a recombination event with the long-latency BLV, as is the case for the AKR-murine leukemia virus system (6). This situation was expected since it was known from previous studies (2) that virus rescued from tumor-bearing animals was neither more acutely oncogenic than nor distinguishable from the initially infecting BLV.

It therefore appears that leukemogenesis by bovine leukemia virus might be due to (i) expression of some virus information; (ii) promoter insertion, as has been described for the avian leukosis virus system (7, 15); (iii) a still unknown mechanism enhancing the expression of some cellular information as a result of viral integration (17); or (iv) blockage of a normal cellular function critical for cell growth and differentiation.

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LITERATURE CITED

1. **Blattner, F. R., A. E. Blechl, K. Denniston-Thompson, H. E. Faber, J. E. Richards, J. L. Slightom, P. W. Tucker, and O. Smithies.** 1978. Cloning fetal γ-globin and mouse α-type globin DNA: preparation and screening of shotgun collections. Science **202**:1279–1283.
2. **Burny, A., C. Bruck, H. Chantrenne, Y. Cleuter, D. Dekegel, J. Ghysdael, R. Kettmann, M. Leclercq, J. Leunen, M. Mammerickx, and D. Portetelle.** 1980. Bovine leukemia virus: molecular biology and epidemiology. p. 231–289. *In* G. Klein (ed.), Viral oncology—1980, Raven Press, New York.
3. **Couez, D., M. Mammerickx, C. Olson, M. Onuma, A. L. Parodi, M. J. Van der Maaten, A. Burny, and R. Kettmann.** 1981. Base sequence variations in the bovine leukemia provirus. *In* G. Gentile and O. C. Straub (ed.), Proceedings of the Fourth International Symposium on Bovine Leukosis. Commission of the European Communities.
4. **Erikson, R. L.** 1980. Avian sarcoma viruses: molecular biology, p. 39–53. *In* G. Klein (ed.), Viral oncology—1980. Raven Press, New York.
5. **Finkelstein, M., and R. H. Rownd.** 1978. A rapid method for extracting DNA from agarose gels. Plasmid **1**:557–562.
6. **Hartley, J. W., N. K. Wolford, L. J. Old, and W. P. Rowe.** 1977. A new class of murine leukemia viruses associated with the development of spontaneous lymphomas. Proc. Natl. Acad. Sci. U.S.A. **74**:789–792.
7. **Hayward, W. S., B. G. Neel, and S. M. Astrin.** 1981. Activation of a cellular *onc* gene by promoter insertion in ALV-induced lymphoid leukosis. Nature (London) **290**:475–480.
8. **Hughes, S. H., K. Toyoshima, J. M. Bishop, and H. E. Varmus.** 1981. Organization of the endogenous proviruses of chickens: implications for origin and expression. Virology **108**:189–207.

9. **Kettmann, R., A. Burny, Y. Cleuter, J. Ghysdael, and M. Mammerickx.** 1978. Distribution of bovine leukemia virus proviral DNA sequences in tissues of animals with enzootic bovine leukosis. Leukemia Res. **2**:23-32.
10. **Kettmann, R., Y. Cleuter, M. Mammerickx, M. Meunier-Rotival, G. Bernardi, A. Burny, and H. Chantrenne.** 1980. Genomic integration of bovine leukemia provirus: comparison of persistent lymphocytosis with lymph node tumor form of enzootic bovine leukosis. Proc. Natl. Acad. Sci. U.S.A. **77**:2577-2581.
11. **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.
12. **Kettmann, R., M. Meunier-Rotival, J. Cortadas, G. Cuny, J. Ghysdael, M. Mammerickx, A. Burny, and G. Bernardi.** 1979. Integration of bovine leukemia virus DNA in the bovine genome. Proc. Natl. Acad. Sci. U.S.A. **76**:4822-4826.
13. **Leder, P., D. Tiemeier, and L. Enquist.** 1977. EK2 Derivatives of bacteriophage lambda useful in the cloning of DNA from higher organisms. The λgt WES system. Science **196**:175-177.
14. **Maniatis, T., R. C. Hardison, E. Lacy, J. Lauer, C. O'Connell, D. Quon, G. K. Sim, and A. Efstratiadis.** 1978. The isolation of structural genes from libraries of eucaryotic DNA. Cell **15**:687-701.
15. **Neel, B. G., W. S. Hayward, H. L. Robinson, J. Fang, and S. M. Astrin.** 1981. Avian leukosis virus-induced tumors have common proviral integration sites and synthesize discrete new RNAs: oncogenesis by promoter insertion. Cell **23**:323-334.
16. **Oroszlan, S., T. D. Copeland, L. E. Henderson, J. R. Stephenson, and R. V. Gilden.** 1979. Amino-terminal sequence of bovine leukemia Virus major internal protein. Homology with mammalian type C virus p30 structural proteins. Proc. Natl. Acad. Sci. U.S.A. **76**:2996-3000.
17. **Payne, G. S., S. A. Courtneidge, L. B. Crittenden, A. M. Fadly, J. M. Bishop, and H. E. Varmus.** 1981. Analysis of avian leukosis virus DNA and RNA in bursal tumors: viral gene expression is not required for maintenance of the tumor state. Cell **23**:311-322.
18. **Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labeling deoxyribonucleic acid to high specific activity in vitro by nick-translation with DNA polymerase I. J. Mol. Biol. **113**:237-251.
19. **Roussel, M., S. Saule, C. Lagrou, C. Rommens, H. Beug, T. Graf, and D. Stehelin.** 1979. Three new types of viral oncogenes of cellular origin specific for hematopoietic cell transformation. Nature (London) **281**:452-455.
20. **Shields, A., S. Goff, M. Pasking, G. Otto, and D. Baltimore.** 1979. Structure of the Abelson murine leukemia virus genome. Cell **18**:955-962.