JOURNAL OF VIROLOGY, June 1985, p. 899–901
0022-538X/85/060899-03$02.00/0

Vol. 54, No. 3

Role of the 3′ Long Open Reading Frame Region of Bovine Leukemia Virus in the Maintenance of Cell Transformation

RICHARD KETTMANN,[1,2]* YVETTE CLEUTER,[1] DIANE GREGOIRE,[1] AND ARSÈNE BURNY[1,2]

Department of Molecular Biology, University of Brussels, 1640 Rhode St-Genèse,[1] and Faculty of Agronomy, 5800 Gembloux,[2] Belgium

Received 14 November 1984/Accepted 11 March 1985

Viral RNA expression was studied by dot blot hybridization with polyadenylated RNAs extracted from a bovine (YR-1) and an ovine (YR-2) tumor cell clone. Both clones were derived from in vivo bovine leukemia virus-induced tumors. The probes used were either the bovine leukemia virus information or only the long open reading frame sequences. No viral RNA corresponding to the bovine leukemia virus long open reading frame region was detected in YR-2, and a very limited amount of bovine leukemia virus messages was unraveled in YR-1. These results strongly suggest that viral expression, even in the long open reading frame region, is not required to maintain transformation of at least some tumor cells.

Bovine leukemia virus (BLV) is a retrovirus distantly related to the human T-cell leukemia viruses (HTLVs) (2). It induces B-cell lymphomas in cattle after long latency periods. The BLV provirus has widely dispersed integration sites (6, 10), a finding also made for HTLVs (8, 17). Both BLV and HTLVs contain an unexpected sequence, the X gene, located between the *env* gene and the 3′ long terminal repeat (15, 18). One can hypothesize that the X gene plays a key role in inducing and maintaining cell transformation because it contains, in both BLV and HTLVs, long open reading frames (LOR) (15, 18). BLV-producing cell lines express three mRNAs (9.0, 5.0, and 2.1 kilobases) corresponding to the genomic RNA, the *env* LOR, and LOR messages, respectively (12). It has been repeatedly observed that fresh persistent lymphocytosis lymphocytes do not contain any BLV RNA or BLV proteins (2, 9, 11). However, when these cells are put in culture, they express virus particles within a few hours, and thus BLV provirus escapes repression (1, 7). Similarly, no viral messages could be detected in fresh cells of BLV-induced bovine (9, 11) or ovine tumors (R. Kettmann, unpublished data). In this paper, we report the establishment in culture of two BLV-induced tumor cell clones and indicate that, in contrast to persistent lymphocytosis lymphocytes, tumor cells either do not express (YR-2) or express at a very low level viral mRNAs.

Two lymphoid cell lines were established with peripheral leukocytes of animals bearing BLV-induced tumors. One, YR-1, was derived from a naturally BLV-infected cow (no. 158); the other, YR-2, was established from an experimentally BLV-infected sheep (no. 395). Leukocytes were recovered from peripheal blood after lysis of the erythrocytes by hypotonic shock and cultivated in suspension in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, 50 μM 2-mercaptoethanol, and 100 μg of penicillin-streptomycin per ml. These cells formed colonies in soft agar (data not shown). DNA was extracted twice with phenol-chloroform after a 1-h incubation at 37°C in TNE buffer (TNE = 0.01 M Tris-hydrochloride [pH 8.3], 0.15 M NaCl, 0.001 M EDTA) containing 200 μg of predigested pronase and 0.5% sodium dodecyl sulfate per ml (10). The DNA was then ethanol precipitated and dissolved in TE buffer (TE = 0.01 M Tris-hydrochloride [pH 7.5], 0.001 M EDTA). After 3 to 4 weeks, each culture was essentially a monoclonal population of tumor cells, as shown by the *Eco*RI restriction patterns of their DNAs (Fig. 1A, lanes 2 to 4; Fig. 1B, lanes 2 to 4).

Each cell line DNA displayed the same restriction profile as its corresponding tumor DNA when the Southern blots (20) were hybridized to ^{32}P-labeled cloned BLV DNA (4) as a probe (Fig. 1A, lanes 2 and f; Fig. 1B, lanes 2 and 4). This result indicated that the clones proliferating in the cultures were indeed the tumorous clones present in vivo. One and four proviral copies were present in YR-2 and YR-1 DNAs,

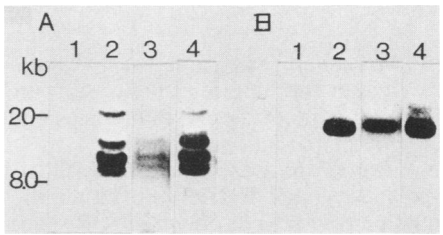

FIG. 1. DNA hybridization with nick-translated cloned BLV DNA as a probe. (A) Ten micrograms each of normal bovine leukocyte DNA (lane 1); cow (no. 158) tumor DNA (lane 2), cow (no. 158) peripheral uncultivated leukocyte DNA (lane 3), and cow (no. 158) cultivated leukocyte (YR-1 clone) DNA (lane 4) were digested to completion with *Eco*RI and electrophoresed in an 0.8% agarose gel. Southern blots (20) were preincubated for 3 h in a plastic box with 40 ml of a mixture containing 3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 10× Denhardt medium (3), 0.01% sodium dodecyl sulfate, and 200 μg of denatured salmon sperm DNA per ml. The filter was then hybridized for 18 h in the same mixture containing 5 × 10^6 cpm per μg. Final washings were performed in 0.1% sodium dodecyl sulfate–0.2× SSC at 65°C. After drying, the filter was exposed to a Kodak XAR-5 film at −70°C in the presence of a Siemens intensifying screen for 1 day. (B) Ten micrograms each of normal sheep leukocyte DNA (lane 1), sheep (no. 395) tumor DNA (lane 2), sheep (no. 395) peripheral uncultivated leukocyte DNA (lane 3), and sheep (no. 395) cultivated leukocyte (YR-2 clone) DNA (lane 4) were treated as described in (A). Only the high-molecular-weight regions of the Southern blots are shown.

* Corresponding author.

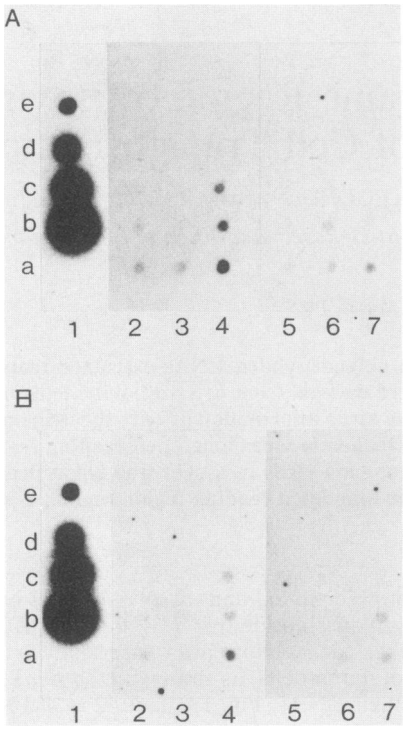

FIG. 2. Dot blot assay of polyadenylated RNAs. In lanes 2 to 7, 1:5 dilutions containing from 2 μg (a) to 3.2 ng (e) of polyadenylated RNA were tested. Polyadenylated RNA from BLV-infected fetal lamb kidney cells was taken as the positive control (lane 1, b to e). The dot blot preparations were hybridized with (A) the ^{32}P-labeled cloned BLV DNA fragment (specific activity was 4×10^8 cpm/μg) and (B) the ^{32}P-labeled 815-base-pair LOR *Cla*I-*Pvu*II (10) subcloned fragment (specific activity was 2×10^8 cpm/μg) and processed as described in the legend to Fig. 1. Autoradiography was for 1 day in (A) and 2 days in (B). Lane 2, Normal bovine leukocytes; lane 3, tumor of cow (no. 158); lane 4, YR-1 clone; lane 5, normal sheep leukocytes; lane 6, tumor of sheep (no. 395); and lane 7, YR-2 clone.

respectively. Moreover, restriction digests with *Sac*I (which cleaves only within the long terminal repeats in this BLV variant) showed that all of the proviral copies under consideration were complete (data not shown).

Viral RNA expression was studied by dot blot hybridization with polyadenylated RNAs with either the complete BLV information (Fig. 2A) or only the LOR sequences (Fig. 2B) as probes. RNA was extracted from leukocytes or tumor cells with a modified guanidine hydrochloride extraction technique and, after ethanol precipitation, chromatographed on oligodeoxythymidylic acid-cellulose (21). BLV-infected fetal lamb kidney cells were used as positive controls (Fig. 2A and B, lanes 1).

YR-1 RNA showed a weak hybridization signal with both probes (Fig. 2A and B, lanes 4). To estimate the low amount of viral RNA present in the YR-1 cell, the signal obtained with 3.2 ng of fetal lamb kidney cell RNA (Fig. 1A, lane 4) was compared with that obtained with 2 μg of YR-1 RNA (Fig. 1A, lane 1). Considering that (i) the BLV-infected cells contain ca. 40 molecules of BLV 35S genomic RNA per cell (13), (ii) the dilution factor between the amount of fetal lamb kidney cell and RV-1 RNAs under consideration was 625, and (iii) the ratio of intensities of the signals as measured by densitometry was 2:1 for fetal lamb kidney cells versus YR-1, it appeared that 0.03 molecule of BLV 35S was present per cell in the YR-1 population. Similar data were obtained with LOR sequences as the probe (Fig. 1B, lanes 1 and 4). We are thus led to the conclusion that the expression of viral messages is extremely low in YR-1.

In YR-2, the situation seemed quite clear-cut. YR-2 RNA did not show any hybridization above background. It should be added that treatment of these cells with various concentrations of 5-iodo- or bromodeoxyuridine failed to induce synthesis of BLV viral proteins (data not shown).

We can summarize the present knowledge concerning leukemogenesis by BLV as follows. (i) All tumors contain at least one copy or a portion of a provirus, its 3′ side, including the LOR region, being always present (9). (ii) The provirus has widely dispersed integration sites in tumors, ruling out a *cis*-acting function to explain cell transformation (9, 10). (iii) No viral RNA has been found so far in the population of polyadenylated mRNAs extracted from BLV-induced tumors (9, 11). Similar findings were also reported for four or five samples of fresh human cells infected with HTLV-I (5). As BLV information is expressed to a very limited extent (YR-1) or is even silent (YR-2) in cultured tumor cells, we conclude that some tumor clones at least do not require LOR mRNA to maintain their transformed stage. Requirement or not of the LOR product may depend on the cell type, differentiation stage reached by the tumor cell, or both. Rosen et al. have recently shown (16) that factors present only in BLV-producing cells mediate transcriptional transactivation of the BLV long terminal repeat. Such a factor could be the LOR product (19). If so, we can imagine that transactivation of other crucial genes is mediated by the LOR product during the establishment of transformation. Once such genes are turned on, they drive the cell to proceed from one stage to the next in the series of discontinuous events toward cell transformation. From then on, expression of any part of BLV provirus becomes useless. Moreover, expression of cell membrane-associated viral antigens is detrimental to the transformed cell survival. In fact, cells expressing viral antigens are destroyed by the anti-BLV cytolytic antibodies present in high titers in the serum of BLV-infected animals (14). According to this model, only cells transformed by the LOR product of the virus and expressing no membrane-associated viral antigens will escape immune surveillance and proliferate as tumorous clones.

We warmly thank M. Mammerickx and A. L. Parodi for generously providing animal tissues and H. Morse and D. Portetelle for help in B-lymphocyte culture.

This project was supported in part by funds provided by the International Cancer Research Data Bank, the National Cancer Institute, National Institutes of Health, under contract no. NO1-CO-65341 (International Cancer Research Technology Transfer), and the International Union Against Cancer. This work was also supported by the Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite, Belgium. R.K. is Chercheur Qualifié du Fonds National Belge de la Recherche Scientifique.

LITERATURE CITED

1. **Baliga, V., and J. F. Ferrer.** 1977. Expression of the bovine leukemia virus and its internal antigen in blood lymphocytes. Proc. Soc. Exp. Biol. Med. **156**:388–391.
2. **Burny, A., C. Bruck, D. Couez, J. Deschamps, J. Ghysdael, R. Kettmann, M. Mammerickx, G. Marbaix, and D. Portetelle.** 1984. Bovine leukemia virus: a new mode of leukemogenesis, p. 17–24. *In* R. C. Gallo (ed.), Human T-cell leukemia-lymphoma viruses. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
3. **Denhardt, D. T.** 1966. A membrane-filter technique for the

detection of complementary DNA. Biochem. Biophys. Res. Commun. **23**:641–646.
4. **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.
5. **Franchini, G., F. Wong-Staal, and R. C. Gallo.** 1984. Human T-cell leukemia virus (HTLV-1) transcripts in fresh and cultured cells of patients with adult T-cell leukemia. Proc. Natl. Acad. Sci. U.S.A. **81**:6207–6211.
6. **Grégoire, D., D. Couez, J. Deschamps, S. Heuertz, M.-C. Hors-Cayla, J. Szpirer, C. Szpirer, A. Burny, G. Huez, and R. Kettmann.** 1984. Different bovine leukemia virus-induced tumors harbor the provirus in different chromosomes. J. Virol. **50**:275–279.
7. **Gupta, P., and J. F. Ferrer.** 1982. Expression of bovine leukemia virus genome is blocked by a nonimmunoglobulin protein in plasma from infected cattle. Science **215**:405–407.
8. **Hahn, B., V. Manzari, S. Colombini, G. Franchini, R. C. Gallo, and F. Wong-Staal.** 1983. A common site of integration of HTLV in cells of three patients with mature T-cell leukaemia-lymphoma: a retraction. Nature (London) **305**:340.
9. **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.
10. **Kettmann, R., J. Deschamps, D. Couez, J.-J. Claustriaux, R. Palm, and A. Burny.** 1983. Chromosome integration domain for bovine leukemia provirus in tumors. J. Virol. **47**:146–150.
11. **Kettmann, R., G. Marbaix, Y. Cleuter, D. Portetelle, M. Mammerickx, and A. Burny.** 1980. Genomic integration of bovine leukemia provirus and lack of viral RNA expression in the target cells of cattle with different response to BLV infection. Leuk. Res. **4**:509–519.
12. **Mamoun, R. Z., T. Astier-Gin, R. Kettman, J. Deschamps, N. Rebeyrotte, and B. J. Guillemain.** The pX region of the bovine leukemia virus is transcribed as a 2.1-kilobase mRNA. J. Virol. **54**:625–629.
13. **Marbaix, G., R. Kettmann, Y. Cleuter, and A. Burny.** 1981. Viral RNA content of bovine leukemia virus-infected cells. Mol. Biol. Rep. **7**:135–138.
14. **Portetelle, D., C. Bruck, A. Burny, D. Dekegel, M. Mammerickx, and J. Urbain.** 1978. Detection of complement-dependent lytic antibodies in sera from bovine leukemia virus-infected animals. Ann. Rech. Vet. **9**:667–674.
15. **Rice, N. R., R. M. Stephens, D. Couez, J. Deschamps, R. Kettmann, A. Burny, and R. V. Gilden.** 1984. The nucleotide sequence of the env gene and post-env region of bovine leukemia virus. Virology **138**:82–93.
16. **Rosen, C. A., J. G. Sodroski, R. Kettmann, A. Burny, and W. A. Haseltine.** 1985. Trans activation of the bovine leukemia virus long terminal repeat in BLV-infected cells. Science **227**:320–322.
17. **Seiki, M., R. Eddy, T. B. Shows, and M. Yoshida.** 1984. Nonspecific integration of the HTLV provirus genome into adult T-cell leukaemia cells. Nature (London) **309**:640–642.
18. **Seiki, M., S. Hattori, Y. Hirayama, and M. Yoshida.** 1983. Human adult T-cell leukaemia virus: complete nucleotide sequence of the provirus genome integrated in leukemia cell DNA. Proc. Natl. Acad. Sci. U.S.A. **80**:3618–3622.
19. **Sodroski, J. G., C. A. Rosen, and W. A. Haseltine.** 1984. Trans-acting transcriptional activation of the long terminal repeat of human T lymphotropic viruses in infected cells. Science **225**:381–385.
20. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
21. **Westin, E. H., F. Wong-Staal, E. P. Gelmann, R. Dalla Favera, T. S. Papas, J. A. Lautenberger, A. Eva, E. P. Reddy, S. R. Tronick, S. A. Aaronson, and R. C. Gallo.** 1982. Expression of cellular homologues of retroviral onc genes in human hematopoietic cells. Proc. Natl. Acad. Sci. U.S.A. **79**:2490–2494.