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Bovine Leukemia Virus Gene Expression In Vivo

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The in vivo transcriptional status of bovine leukemia virus was assessed at three stages of infection during the progression of the disease: aleukemic stage, persistent lymphocytosis, and leukemia/lymphosarcoma. Bovine leukemia virus transcripts could be amplified from total or cytoplasmic enriched lymphocyte RNA by reverse transcription polymerase chain reaction in cells from all but a few aleukemic animals. With primer pairs diagnostic for differentially spliced transcripts (full length-genomic, envelope, *tax/rex*, and alternatively spliced), a trend toward exclusion of both full-length and envelope RNAs, with retention of the *tax/rex* message, appears as leukemia/lymphosarcoma develops.

Bovine leukemia virus (BLV), a member of the *Oncovirinae* subfamily of *Retroviridae*, is the etiologic agent of enzootic bovine leukosis (17). The virus is predominantly B-cell lymphotropic and persists in the animals in spite of a vigorous immune response. The outcome of infection is variable. Animals may seroconvert and remain clinically and hematologically normal (aleukemic) or develop persistent lymphocytosis (PL), a nonneoplastic proliferation of B lymphocytes. In less than 5% of infections, leukemia/lymphosarcoma appears, with neoplastic lymphocytes having characteristics of mature B cells (6), with or without preceding PL. The pathogenetic mechanisms leading to PL or to tumor formation are unknown. BLV is structurally related and biologically similar to the human T-cell leukemia virus types I and II (HTLV-I and HTLV-II). Like BLV, these viruses lack a known oncogene and do not integrate into preferred sites in host cell genomes. Besides the structural genes, these cognate viruses encode additional products termed *tax* and *rex*, which serve as *trans*-acting regulatory proteins (16). Thus, these viruses can be regarded as complex retroviruses (3). In vivo, the expression of BLV (and HTLV-I and -II) is repressed. With conventional Northern (RNA) blot analysis or immunofluorescent-antibody procedures, neither viral RNAs nor proteins are detected in either freshly isolated lymphocytes or tumor cells. The molecular basis of this restriction is unresolved but may be attributed in part to the complex interaction of *cis*- and *trans*-acting factors in infected cells. By using the sensitive polymerase chain reaction after reverse transcription (RT-PCR), the presence of the *tax/rex* mRNA both in peripheral blood mononuclear cells of patients with HTLV-I-associated diseases (5, 9) and in normal and neoplastic lymphocytes of BLV-positive sheep and cattle (8) was demonstrated.

In this study, we extended these findings by investigating the transcriptional pattern of BLV during different stages of infection by using ex vivo RT-PCR amplification of mRNA. Figure 1 shows the genomic organization, the known transcripts of BLV, and the locations of the primers used for PCR. An unspliced full-length RNA serves as the message for *gag* and *pol* proteins as well as the precursor for the singly spliced *env* mRNA directing the synthesis of the envelope proteins and for a doubly spliced mRNA for the translation of *tax* and *rex*. Primers for the *env* and *tax/rex* messages included the first and second splice junction sites, respectively. These amplified products could be unambiguously identified by Southern blot hybridization using oligonucleotides as probes which spanned the respective splice junction site. The unspliced full-length message was detected by using an internal oligonucleotide as a probe (Fig. 1).

We were also interested to learn whether an alternatively spliced mRNA, generated by use of the first splice donor and the second splice acceptor, would be synthesized. A splice junction-specific oligonucleotide was utilized as a probe to detect this transcript. Labeling of probes was carried out with nonradioactive digoxigenin-dUTP (Boehringer Mannheim Corp.) by tailing with terminal transferase (15). Positive hybrids were identified by immunochemiluminescent detection (Lumi-Phos 530; Boehringer Mannheim Corp.) by following the methods recommended by the supplier.

One microgram of heat-denatured total RNA, isolated from peripheral blood mononuclear cells or tumor tissues (1), was used for PCR. All RNAs described in this report were pretreated with RNase-free DNase (Worthington Laboratories) before RT-PCR. Reverse transcription was accomplished in a 20-μl volume containing 50 mM KCl, 5 mM MgCl, 10 mM Tris-HCl (pH 8.3), 10 mM dithiothreitol, 1 mM each deoxynucleoside triphosphate, 20 U of RNase inhibitor, 2.5 μM random hexamers, and 50 U of Moloney murine leukemia virus reverse transcriptase. The reaction mixture was incubated at 37°C for 15 min, 42°C for 30 min, and 99°C for 5 min. After the addition of 80 μl of 1× PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$), a 0.5 μM concentration of each primer, and 2.5 U of AmpliTaq DNA polymerase (Cetus Corp.), the mixture was subjected to 35 cycles, each consisting of a denaturation step (94°C, 1 min) followed by a primer annealing step (55°C, 1 min) and a polymerization step (72°C, 2 min). An aliquot of this reaction mixture was electrophoresed on a 2% agarose gel, stained, photographed, and subsequently transferred to a nylon membrane (Boehringer Mannheim Corp.) for Southern blot analysis.

Viral RNAs could always be demonstrated in lymphocytes of PL animals and in tumor tissues. In the case of serologically positive but hematologically normal animals, however, this was not always achieved, most likely because of the low number of BLV-infected cells in the early stage of disease. Figure 2 shows a comparative Southern blot analysis of the amplification products from animals in different stages of the

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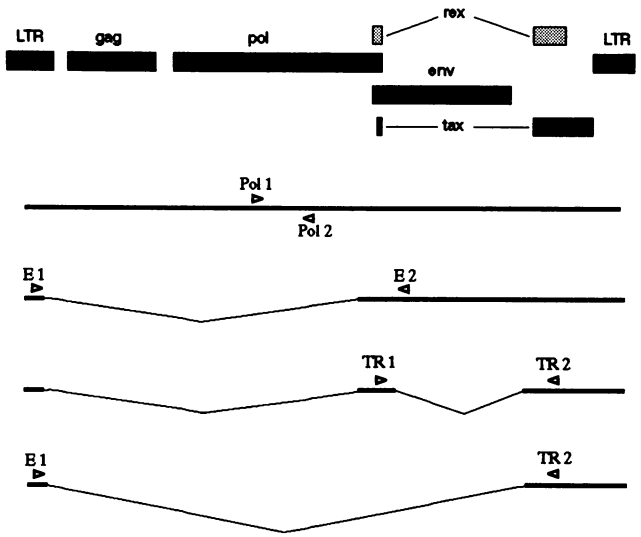

FIG. 1. BLV transcripts. A diagram of the BLV genome is given (top). The approximate locations of the primers on the transcripts used for RT-PCR are shown by arrowheads. The locations of splices within the primers are indicated by diagonal lines. Full-length RNA was amplified with primers Pol 1 (5′-ATTCCAGTCGAAGACC GCTT-3′) and Pol 2 (5′-TCCCAAGACCGCCTGTAATT-3′). Hybridization of the amplified product was performed with an internal oligonucleotide (5′-TCTGGTGTCCTATATGGACG-3′). Primers E 1 (5′-TCTCTTGCTCCCGAGACCTT-3′) and E 2 (5′-TTTCCTAGG GACAGGGAGCA-3′) for the RT-PCR of the envelope (*env*)-specific transcript encompassed the first splice junction site, and amplified products were identified by hybridization with a splice junction-specific oligonucleotide (5′-AGCGGTCAGAGGGCGGAG AA-3′). By analogy, amplified cDNA of the transcript for Tax and Rex (*tax/rex*) with primers TR 1 (5′-CAAAACAATCGTCGGTGG CT-3′) and TR 2 (5′-GATGGTGACATCATTGGACA-3′) was hybridized with an oligonucleotide complementary to the second splice junction site (5′-CATCAGATGGCAAGTGTTGT-3′). By using primers E 1 and TR 2, an alternatively spliced PCR product (*alt*) was demonstrated with an oligonucleotide as a probe which was specific for a first splice donor and second splice acceptor junction sequence (5′-CAGCGGTCAGCAAGTGTTGT-3′). LTR, long terminal repeat.

disease (aleukemic stage, PL, tumor-positive stage). The major mRNA species in all our samples was the *tax/rex* transcript. Messenger RNA for the envelope proteins was likewise consistently present, although to a much lesser degree in the tumor samples. Full-length mRNA was barely detectable in aleukemic and PL samples. Moreover, all samples contained small amounts of the alternatively spliced mRNA, and to our knowledge this is the first description of this phenomenon in BLV-infected cells. An alternative splice acceptor utilization in the genesis of P21x message has also been observed during HTLV-I infection in vitro (10). The analogous BLV message could give rise to a protein with 154 residues if it was initiated from the only methionine located centrally in the message (13). In summary, whereas in the early (polyclonal) phase of the disease all known mRNAs were displayed, there appeared to be a decrease in the transcription of the structural messages relative to the regulatory transcripts towards the final (monoclonal) tumor stage.

Since the presence of these mRNAs does not necessarily equate with translational usage, we sought to obtain additional suggestive evidence for the presence of functional messages by assaying for the viral RNAs in the cytoplasm of infected cells. Cytoplasmic RNA was prepared (14) from

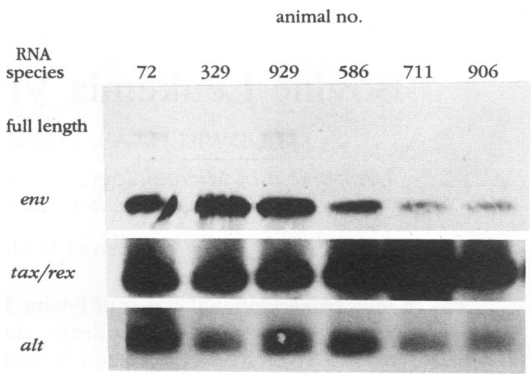

FIG. 2. Southern blot analysis of RT-PCR products with total RNA. cDNAs in lane 1 (animal 72, aleukemic stage) and lanes 2 and 3 (animals 329 and 929, respectively, PL) originated from peripheral blood mononuclear cells; lanes 4 to 6 (animals 586, 711, and 906) contain the RNAs of neoplasms.

freshly isolated lymphocytes of seropositive aleukemic and PL animals. Again, BLV-specific transcripts were not found in cells from all aleukemic animals. When they were detected, the pattern shown in Fig. 3A (animals 18, 80, and 94) was characteristic and all three major transcripts could be detected. When blood samples from PL animals with distinctly increased leukocyte counts (>9,000 lymphocytes per ml) from an unrelated herd were compared, only the *tax/rex*

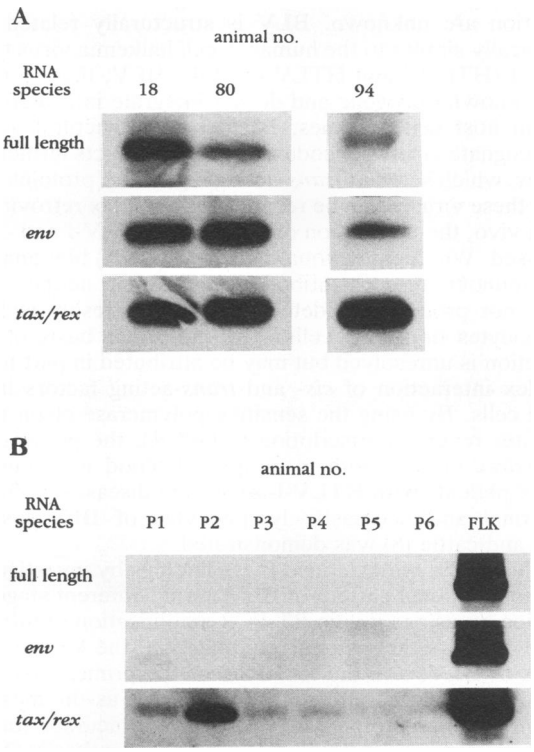

FIG. 3. Southern blot analyses of RT-PCR products with cytoplasmic RNA. (A) Comparison of amplified cDNAs with cytoplasmic RNA from peripheral blood mononuclear cells of aleukemic (no. 18, 80, and 94) animals. (B) Amplified cDNAs from PL animals (lanes P1 to P6) from an unrelated herd. Lane 7, cDNA of cytoplasmic transcripts from the BLV persistently infected FLK cell line.

mRNA was observed and it was found at different levels in individual animals (Fig. 3B). Transcripts for the envelope and the full-length RNAs were present in trace levels. The pattern of proviral integration in lymphocytes from animals with PL has been reported to be very similar to that in lymphocytes from seropositive animals without PL (2). Thus, it seems that the differences between aleukemic and PL animals observed here reflect differential transcriptional processing. Although we were unable to investigate the actual cytoplasmic partitioning of BLV transcripts in tumor cells because of the unavailability of fresh samples, it is evident from our first analysis (Fig. 2) that *tax/rex* mRNA is the major transcript.

In conclusion, only the *tax/rex* mRNA was consistently present in the cytoplasm of lymphocytes in cows regardless of the stage of infection, suggesting that persistent low-level expression of either of these gene products may be important in the pathogenesis of BLV infection. The *tax* protein of the related retrovirus HTLV-I has been implicated in cell proliferation and in the neoplastic process by effecting an increase in expression of viral and cellular genes through different host transcription factor pathways (3). It is interesting that the HTLV-I *tax* protein has been reported to induce proliferation of lymphocytes (4), intimating that even uninfected cells might be activated through a paracrine mechanism. It is this line of evidence that suggests that BLV *tax* may also play a role in initiating and maintaining a proliferative or neoplastic state. On the other hand, it has been shown that the second regulatory factor of HTLV-I, the *rex* protein, can down-regulate viral transcription (7). In the case of human immunodeficiency virus type 1, it was suggested that the level of the functionally analogous protein Rev could be a primary determinant in the establishment of a latent infection (11). An episodic cycling of antibodies against the BLV *rex* protein in BLV-infected sheep has been reported recently (12). Since the same BLV transcript is used for the synthesis of *tax* and *rex*, our approach could not provide further information regarding the respective extent of translation. Interestingly, in all of our transcript-positive samples, we could detect a cDNA copy of the *tax/rex* mRNA in the cellular DNAs (8) by using primers TR 1 and TR 2 (data not shown). The function of this cDNA in relation to tumorigenesis is currently obscure.

The generally low level of full-length mRNA in our samples is consistent with the lack of an overt viremia in infected cattle. The *env* mRNA seems to be expressed, at least in the early phases of the disease, which may account for the high titers of antibody against this surface protein during infection. The obvious low-level expression of *env* mRNA in the case of some PL animals (Fig. 3B) suggests a nonessential role for this protein in the maintenance of PL. Taken as a whole, this minimal in vivo expression of structural genes could be viewed as a unique processing strategy designed to evade immunosurveillance and to allow for persistent and low-level infection. Finally, even though BLV *tax* appears to have oncogenic potential (18), *tax/rex* mRNA expression was found in all stages of infection regardless of disease. If it is supposed that Tax is uniformly translated from this message in all stages of infection, then additional, yet-to-be-identified cellular events would appear to be necessary for the development of a full malignant cellular phenotype.

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REFERENCES

1. **Chomczynski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
2. **Coulston, J., R. C. W. Daniel, and M. F. Lavin.** 1991. Integration of bovine leukaemia virus at all stages of enzootic bovine leukosis. Arch. Virol. **119:**13–23.
3. **Cullen, B. R.** 1991. Human immunodeficiency virus as a prototypic complex retrovirus. J. Virol. **65:**1053–1056.
4. **Gartenhaus, R. B., Z. N. Bernemann, M. S. Reitz, R. C. Gallo, and M. Klotman.** 1991. Presented at the Fourth International Retrovirus Conference. Current Issues in Human Retrovirology: HTLV. Montego Bay, Jamaica.
5. **Gessain, A., A. Louie, O. Gout, R. C. Gallo, and G. Franchini.** 1991. Human T-cell leukemia-lymphoma virus type I (HTLV-I) expression in fresh peripheral blood mononuclear cells from patients with tropical spastic paraparesis/HTLV-I-associated myelopathy. J. Virol. **65:**1628–1633.
6. **Heeney, J. L., and V. E. O. Valli.** 1990. Transformed phenotype of enzootic bovine lymphoma reflects differentiation-linked leukemogenesis. Lab. Invest. **62:**339–346.
7. **Hikada, M., J. Inoue, M. Yoshida, and M. Seiki.** 1988. Post-transcriptional regulator (rex) of HTLV-1 initiates expression of viral structural proteins but suppresses expression of regulatory proteins. EMBO J. **7:**519–523.
8. **Jensen, W. A., J. Rovnak, and G. L. Cockerell.** 1991. In vivo transcription of the bovine leukemia virus *tax/rex* region in normal and neoplastic lymphocytes of cattle and sheep. J. Virol. **65:**2484–2490.
9. **Kinoshita, T., M. Shimoyama, K. Tobinai, M. Ito, S. Ito, S. Ikeda, K. Tajima, K. Shimotohmo, and T. Sugimura.** 1989. Detection of mRNA for the tax_1/rex_1 gene of human T-cell leukemia virus type I in fresh peripheral blood mononuclear cells of adult T-cell leukemia patients and viral carriers by using the polymerase chain reaction. Proc. Natl. Acad. Sci. USA **86:**5620–5624.
10. **Orita, S., A. Saiga, S. Takagi, T. Tanaka, K. Okumura, Y. Aono, Y. Hinuma, and H. Igarashi.** 1991. A novel alternatively-spliced viral m-RNA transcribed in cells infected with human T-cell leukemia virus type 1 is mainly responsible for expressing p-21X protein. FEBS Lett. **295:**127–134.
11. **Pomerantz, R. J., D. Trono, M. B. Feinberg, and D. Baltimore.** 1990. Cells nonproductively infected with HIV-1 exhibit an aberrant pattern of viral RNA expression: a molecular model for latency. Cell **61:**1271–1276.
12. **Powers, M. A., D. Grossman, L. C. Kidd, and K. Radke.** 1991. Episodic occurrence of antibodies against the bovine leukemia virus rex protein during the course of infection in sheep. J. Virol. **65:**4959–4965.
13. **Rice, N. R., R. M. Stephens, and R. V. Gilden.** 1987. Sequence analysis of the bovine leukemia virus genome, p. 115–144. *In* A. Burny and M. Mammericky (ed.), Enzootic and bovine leukosis and bovine leukemia virus. Martinus Nijhoff Publishing, Boston, Mass.
14. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Schmitz, G. G., T. Walter, R. Seibl, and C. Kessler.** 1991. Nonradioactive labeling of oligonucleotides in vitro with the hapten digoxigenin by tailing with terminal transferase. Anal. Biochem. **192:**222–231.
16. **Smith, M. R., and W. C. Greene.** 1991. Molecular biology of type 1 human T-cell leukemia virus (HTLV-1) and adult T-cell leukemia. J. Clin. Invest. **87:**761–766.
17. **Van der Maaten, M. J., and J. M. Miller.** 1990. Bovine leukosis virus, p. 419–429. *In* Z. Dinter and B. Morein (ed.), Virus infections of ruminants. Elsevier Science Publishers, Amsterdam.
18. **Willems, L., H. Heremans, G. Chen, D. Portetelle, A. Billiau, A. Burny, and R. Kettmann.** 1990. Cooperation between bovine leukaemia virus transactivator protein and Ha-*ras* oncogene product in cellular transformation. EMBO J. **9:**1577–1581.