Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 9263–9267, December 1988
Medical Sciences

Even transcriptionally competent proviruses are silent in bovine leukemia virus-induced sheep tumor cells

(leukemogenesis/RNA·RNA hybridization/trans-activation/deletion)

ANNE VAN DEN BROEKE*†, YVETTE CLEUTER*, GAO CHEN‡, DANIEL PORTETELLE‡, MARC MAMMERICKX§, DANIEL ZAGURY¶, MICHÈLE FOUCHARD¶, LAURE COULOMBEL||, RICHARD KETTMANN‡, AND ARSÈNE BURNY*‡

*Department of Molecular Biology, University of Brussels, 1640 Rhode St-Genèse, Belgium; †Faculty of Veterinary Medicine, University of Liège, 1070 Brussels, Belgium; ‡Faculty of Agronomy, 5800 Gembloux, Belgium; §National Institute for Veterinary Research, 1180 Brussels, Belgium; ¶Laboratory of Cellular Biology, University Pierre et Marie Curie, Paris, France; and ||Institut National de la Santé et de la Recherche Médicale, Hopital Bicêtre, Paris, France

*Communicated by J. Brachet**, August 16, 1988*

ABSTRACT **To investigate the role of proviral integration and expression in cellular transformation induced by bovine leukemia virus (BLV), three BLV-induced tumors harboring a single proviral copy were selected upon restriction and hybridization analysis. Tumors 344 and 395 were shown to contain a full-size proviral copy, whereas in tumor 1345 the provirus appeared to be heavily deleted. RNA gel blot hybridization with an antisense RNA probe showed no transcription of the viral sequences in the fresh tumors or in sheep tumor cells growing *in vitro*. The proviruses were cloned and transfected in mammalian cell lines. Transient-expression experiments revealed that the complete proviruses were still able to express the trans-activating protein (Tat) as well as structural proteins, demonstrating that the nonexpression of a provirus in a tumor cell does not necessarily imply a structural alteration of the viral information. In contrast, sequence analysis of the provirus with a large deletion and transient-expression assays proved that this truncated provirus, isolated from a tumor, was unable to code for viral proteins. These data indicate that expression of viral genes, including *tat*, is not required for the maintenance of the transformed state.**

Bovine leukemia virus (BLV) is an exogenous retrovirus that induces a chronic disease in cattle, often causing persistent lymphocytosis (PL), with lymphosarcomas developing in a small number of infected animals (for review, see refs. 1 and 2). The same virus infects sheep, where it induces tumors with very high frequency (3).

Structurally and functionally, BLV is a relative of human T-lymphotropic viruses I and II (HTLV-I and HTLV-II). These viruses lack typical oncogenes (4) and are characterized by a similar organization and strategy for gene expression. In addition to the genes involved in viral replication (*gag, pol, env*), the BLV genome contains an *X* region coding for an 18-kDa protein (5, 6) and a 34-kDa product, called Tat protein, that acts as a trans-activator of proviral transcription (7–13). It is now hypothesized that the BLV Tat protein trans-activates some cellular genes and that this process is the key to initiation of cell transformation.

All BLV-induced tumors are clonal and contain at least a portion of a provirus (14–16) integrated at many sites in the host genome (17, 18). All deleted proviral copies examined have shown preservation of the *X* region, stressing again its probable role in tumorigenesis.

Viral RNA was not detected in fresh lymphocytes isolated from animals with PL or in B-cell tumors (15, 19–21). When PL lymphocytes were isolated from their host and maintained in short-term cultures, viral expression took place (22, 23). In contrast, when tumor cells were cultivated *in vitro*, only a very low level of expression, if any, could be detected (24).

As a step toward understanding the molecular mechanisms controlling proviral expression and cellular transformation, we have isolated and characterized three different tumor proviruses. We show here that these proviruses are silent in the tumor cell. We also analyzed their ability to express viral functions, in particular the Tat protein, once cloned and isolated from their host tumor cell.

METHODS

Animal Tissues. Bovine material was collected from field cases of enzootic bovine leukosis (animal 1345). Ovine material was collected from sheep experimentally infected with a Belgian variant of BLV (animals 395 and 344). Peripheral blood leukocytes and lymphoid tumors, kept at −70°C, were used as source of DNA and RNA. Leukocytes from a normal animal were used as a source of control DNA and RNA.

Cellular RNA Extraction. RNA was extracted by direct treatment of the frozen samples with $NaDodSO_4$/proteinase K, followed by phenol extraction. Polyadenylylated mRNA was obtained by oligo(dT)-cellulose affinity chromatography.

Molecular Cloning of Proviral DNAs. BLV tumor proviruses were inserted into bacteriophage λ vectors, essentially according to Maniatis *et al.* (25). Proviruses 344 and 395 were cloned in λ Charon 4A (26). Provirus 1345 was inserted into λ 2001 (27).

Plasmids. The recombinant plasmids pV344 and pV395 contain the complete proviral information isolated from tumors 344 and 395, respectively. pV344 has 570 base pairs (bp) of cellular 5′ flanking sequence, 1470 bp of cellular 3′ flanking sequence, and is cloned into the *Eco*RI site of pSP64. In pV395, the provirus is flanked by 1040 bp of 5′ and 15 bp of 3′ cellular sequence and is inserted between the *Eco*RI and *Hin*dIII sites of pSP18. pV1345 carries the genome of the deleted provirus isolated from tumor 1345. It is cloned into the *Eco*RI site of pSP64 and is flanked by 1350 bp of 5′ and 50 bp of 3′ cellular sequence. Plasmid pAX2 is a subclone of the *X* region (3′ region) of pV344. The 1238-bp *Bam*HI–*Sac* I fragment was inserted between the *Bam*HI and *Sac* I sites of pSP65, giving rise to a recombinant plasmid containing the *X* region in the 3′ → 5′ orientation downstream of the SP6

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: BLV, bovine leukemia virus; HTLV, human T-lymphotropic virus; CAT, chloramphenicol acetyltransferase; CHO, Chinese hamster ovary; FLK, fetal lamb kidney; OVK, ovine kidney; PL, persistent lymphocytosis; LTR, long terminal repeat.
**Deceased.

promoter. Plasmids pSP18, pSP64, and pSP65 were obtained from Bethesda Research Laboratories. Plasmid pBLVCAT was constructed by Rosen *et al.* (9), and pSV2cat was described by Gorman *et al.* (28). Plasmid preparations to be used in transfection experiments were purified on two successive CsCl gradients.

Synthesis of an Antisense X RNA Probe. Plasmid pAX2 DNA was linearized with *Pvu* II and transcribed *in vitro* by using SP6 RNA polymerase and [α-^{32}P]UTP according to the manufacturer (Amersham).

DNA Sequencing. DNA sequence was determined by the dideoxy chain-termination method (29) using overlapping restriction fragments in M13mp18 and -mp19 and pEMBL8+ (30).

Cells and Cell Culture. YR2 is a cloned lymphoid cell line established from leukocytes of animal 395, which bears BLV-induced tumors (24). Clones were produced as follows: single cells from exponentially growing culture were isolated by micromanipulation and cultured individually in 200-μl flat-bottom wells of FB Falcon microplates containing a feeder monolayer formed by sheep bone marrow stromal cells. Culture medium was RPMI 1640 containing 10% (vol/vol) fetal bovine serum, supplemented with 30% (vol/vol) conditioned medium (filtered supernatant of YR2 mass culture). In these conditions, 10–20% of wells yielded growing YR2 cell clones. BLV-infected fetal lamb kidney (FLK) cells, a BLV-producer cell line, were obtained from M. J. Van der Maaten (31). Other cell lines included Chinese hamster ovary cells (CHO), uninfected ovine kidney cells (OVK), and a human B-lymphocyte line (Raji). All cells were grown in Optimem medium (GIBCO) supplemented with 10% heat-inactivated fetal bovine serum.

Transfections and Chloramphenicol Acetyltransferase (CAT) Assays. Plasmid DNA was transfected into fibroblasts as a calcium phosphate coprecipitate (28, 32). A DEAE-dextran method was employed for transfection of lymphocytes (33). Cells were incubated for 48 hr after transfection and assayed for CAT activity. Cell extracts were obtained and CAT assays were performed as described by Gorman *et al.* (28).

RESULTS

Restriction Analysis of Tumor Cell DNAs Containing Full-Length Proviruses. To investigate the role of proviral integration and expression in cellular transformation, two BLV-induced tumors carrying single proviral copies were selected (T344 and T395). Fig. 1 shows the restriction analysis of DNAs from T344, T395, and YR2, which is an established cloned lymphoid cell line derived from T395 cells.

To determine the number of BLV proviral copies, T344, T395, and YR2 DNAs were digested with *Hin*dIII restriction endonuclease. In some BLV variants (35), called Belgian variants, this restriction enzyme does not cleave the proviral DNA, generating a single fragment containing the proviral information per integrated copy. The *Hin*dIII digests were hybridized to a ^{32}P-labeled full-size BLV probe. Both *Hin*dIII-digested tumor DNAs displayed a single fragment containing proviral information, of 10.5 kb for T344 (Fig. 1*A*, lane 2) and 12 kb for T395 (lane 3). The YR2 digests (lane 4) displayed the same pattern as T395, confirming that the clone proliferating in culture was indeed the clone present *in vivo*. No hybridization occurred to the control ovine leukocyte DNA (lane 1). Since the size of the BLV provirus is 8.7 kb, these results provided strong evidence that these tumors had acquired only one proviral copy.

Evidence for the integrity of viral sequences was provided by experiments in which *Sac* I-cleaved tumor DNA was analyzed. In the Belgian variants, *Sac* I was found to cleave the BLV proviral DNA only in the long terminal repeats

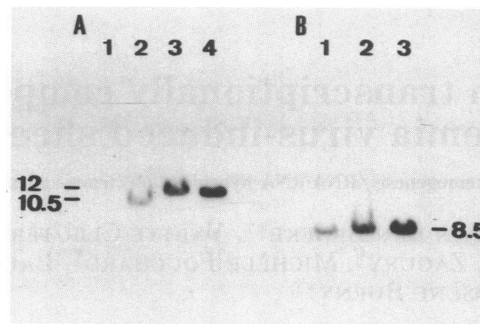

FIG. 1. Tumor DNA hybridization using nick-translated cloned BLV DNA as probe. (*A*) Ten micrograms of DNA from normal ovine leukocytes (lane 1), tumor T344 (lane 2) or T395 (lane 3), or cell line YR2 (lane 4) was digested to completion with *Hin*dIII, electrophoresed in a 0.8% agarose gel, and blotted onto nitrocellulose filters. DNA blots were hybridized with a probe prepared by nick-translating cloned total BLV DNA (4). The filters were preincubated for 6 hr at 65°C with 50 ml of a mixture containing 3× SSC, 10× Denhardt medium (34), 0.1% $NaDodSO_4$, and 100 μg of denatured salmon sperm DNA per ml (1× SSC is 0.15 M NaCl/0.015 M sodium citrate). The filters were hybridized for 16 hr in the same mixture containing 4×10^6 cpm of ^{32}P-labeled nick-translated BLV probe per ml (specific activity, 2×10^8 cpm/μg). Final washings of the filters were performed in 0.1% $NaDodSO_4$/0.2× SSC at 65°C. Filters were exposed to Kodak XAR-5 films for 48 hr. Fragment lengths are given in kilobases (kb). (*B*) Ten micrograms of DNA from tumor T344 (lane 1) or T395 (lane 2) or cell line YR2 (lane 3) was digested to completion with *Sac* I and treated as described in *A*.

(LTRs), thus generating a long internal fragment of about 8.5 kb. *Sac* I digestion of T344, T395, and YR2 DNAs (Fig. 1*B*, lanes 1–3, respectively) generated a single viral fragment of 8.5 kb, strongly suggesting that the integrated proviruses were complete.

Viral RNA Expression in Tumor Cells Containing Full-Length Proviruses. To determine whether viral expression took place in tumors T344 and T395 *in vivo* as well as in the YR2 cell line *in vitro*, we looked for the presence of viral sequences in their mRNAs. We took advantage of the stability of RNA·RNA hybrids by using an antisense X RNA to probe RNA gel (Northern) blots. Poly(A)$^+$ RNAs from tumors T344 and T395 and from YR2 cells (Fig. 2, lanes 3–5) were electrophoresed, transferred to nitrocellulose filter, and hybridized with the ^{32}P-labeled RNA probe. With FLK RNA (lane 2) used as positive control, the probe hybridized to an 8.6-kb RNA (the complete viral genomic RNA), to a 4.2-kb RNA (most probably the *env* subgenomic RNA), and to a subgenomic RNA of about 2 kb, representing the doubly spliced mRNA from which the 3′ portion of the BLV genome is translated (7, 37). After autoradiographic exposure for 1 hr (Fig. 2*A*) and 4 hr (Fig. 2*B*), no hybridization was detected with RNAs of OVK, the uninfected cell line used as negative control (lane 1), or with RNAs from T344, T395, and YR2 cells (lanes 3–5).

To quantitate the minimal amount of viral sequence detectable by the method used, serial dilutions of FLK RNA (1 μg to 100 pg) were analyzed by Northern blotting. Fig. 3 shows the signals obtained with FLK RNA amounts ranging from 10 ng to 100 pg (lanes 5–8) compared with those obtained with 10 μg of T344, T395, or YR2 RNA (lanes 2–4). The lowest amount of FLK RNA still yielding a detectable pattern after exposure for 48 hr was 500 pg (lane 7). The blots obtained with the highest quantities of FLK RNA yielded very strong signals after 48 hr of exposure (data not shown). For T344, T395, and YR2 RNAs analyzed in the same conditions, BLV-specific fragments were not detected, but a minor signal appeared at about 5 kb. Frequent nonspecific hybridization of RNA probes to 28S RNA has been reported

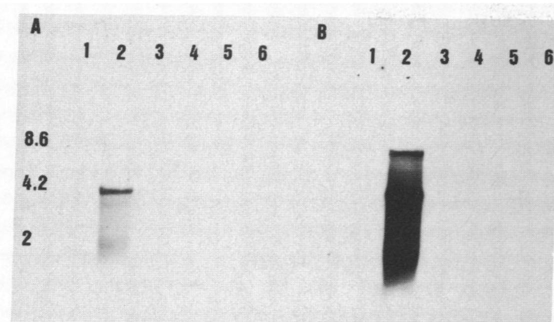

FIG. 2. Northern blot hybridization of RNAs from BLV-infected tumor cells with an antisense X RNA probe. Four micrograms of poly(A)$^+$ RNA from OVK cells (lane 1), FLK cells (lane 2), tumor T344 (lane 3) or T395 (lane 4), YR2 cells (lane 5), or tumor T1345 (lane 6) was glyoxylated, fractionated in 1% agarose gel, and transferred to nitrocellulose filter according to Williams and Mason (36). The RNA blots were preincubated for 4 hr at 60°C with 50 ml of a mixture containing 50% formamide, 5× SSC, 10× Denhardt solution, 0.1% $NaDodSO_4$, and 200 μg of salmon sperm DNA per ml. The filters were hybridized with an antisense X RNA probe (4×10^6 cpm/ml; specific activity, 10^8 cpm/μg) at 60°C for 20 hr in 25 ml of the same mixture. Final washings were performed at 65°C in 0.2× SSC. Fragment lengths are given in kb. Glyoxylated *Hin*dIII-digested λ DNA fragments were used as molecular size markers. The filter was exposed to Kodak X-Omat-S films for 1 hr (*A*) and 4 hr (*B*).

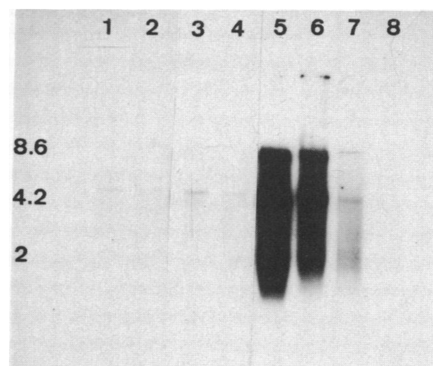

FIG. 3. Ten micrograms of OVK (lane 1), T344 (lane 2), T395 (lane 3), and YR2 (lane 4) poly(A)$^+$ RNA and increasing dilutions of FLK poly(A)$^+$ RNA (10 ng, lane 5; 2.5 ng, lane 6; 500 pg, lane 7; and 100 pg, lane 8) were treated as described for Fig. 2. Filters were exposed for 48 hr.

(38). With the OVK RNA used as negative control (lane 1), the same hybridization pattern was detected, confirming the nonspecificity of the 5-kb signal.

Considering that the BLV-infected cells contain about 40 molecules of BLV-specific RNA per cell (21) and that the dilution factor between the amount of FLK RNA and tumor cell RNA was 20,000, we could assume that less than the equivalent of 0.002 copy of viral RNA was present per cell in the tumor cell populations analyzed.

Additional evidence for the lack of viral expression in the tumor cells of animal 395 was provided by an experiment in which two sheep and two goats were each infected with 10^7 YR2 cells. After 5 months, no seroconversion occurred, whereas in a previous experiment carried out with PL lymphocytes (39), infection took place after 18 days with as few as 926 inoculated leukocytes.

No tumor cell line was derived from leukocytes of sheep 344. However, evidence for the lack of viral expression in cultured cells was provided by simultaneous detection experiments (40). Animal 344 died in tumor phase with severe lymphocytosis (860,000 leukocytes per mm^3, 96% B lymphocytes). At the time of the animal's death, leukocytes were collected and cultured for 3 days. Simultaneous detection tests were performed on 6 liters of culture supernatant. No viral RNA or reverse transcriptase activity was detected, whereas in similar conditions, lymphocytes from an animal with PL were induced to produce virus.

Biological Activity of the Cloned Tumor-Derived Full-Length Proviruses. To study the biological function of proviruses 344 and 395, the proviral sequences were cloned in bacteriophage λ Charon 4A and λ 2001, respectively, and subcloned in plasmids pSP64 and pSP18, respectively, generating the recombinant plasmids pV344 and pV395.

Data provided by the Northern blot experiments and simultaneous detection tests strongly suggested that the BLV information was silent in the tumor cells. This was true even for the Tat protein that is thought to play a key role in the transformation process. To establish whether proviruses 344 and 395 would be able to express viral genes once isolated from their natural host cell, we looked for viral proteins, and in particular the Tat protein, after transfection of the cloned proviruses in several cell lines.

To determine whether the cloned proviruses were competent to direct trans-activation of the BLV LTR regulatory sequences, they were examined in transient-expression assays. Plasmids pV395 and pV344, carrying proviral DNA, were cotransfected with plasmid pBLVCAT in noninfected mammalian cells and levels of CAT enzymatic activity were measured (28). In pBLVCAT, the BLV LTR is placed 5′ to the CAT gene. The inability of the BLV LTR sequences to function as transcriptional elements in uninfected cells was reported previously (9). Levels of CAT activity in the cotransfected cells provide a measure of the ability of the proviral DNA to trans-activate the LTR sequences located 5′ to the CAT gene. In addition to pBLVCAT, pV344, and pV395, two plasmids were used for control purposes: pSV2-cat, which contains the simian virus SV40 promoter 5′ to the CAT gene, and pSP18, a plasmid without any insert.

We tested the expression of the CAT gene after introduction of the plasmids into FLK, CHO, OVK, and Raji cells. Forty-eight hours after transfection, the cells were harvested and extracts were prepared and assayed for CAT activity. The autoradiograms (Fig. 4) were developed after 16 hr. Upon transfection into BLV-infected FLK cells, the pBLVCAT plasmid yielded high levels of CAT activity (lane 1) and appreciable levels were obtained with pSV2cat in CHO, OVK, and Raji cells (data not shown), indicating that the transfection procedure used resulted in efficient uptake and expression of DNA. As expected, no CAT activity was detected in CHO, OVK, and Raji cells transfected with pBLVCAT alone (lanes 2–4). However, in the presence of pV395, a low level of CAT activity was detected in CHO (lane 5) and OVK (lane 9) cells but not in Raji cells (lane 13). Upon cotransfection of pV344 and pBLVCAT, much higher levels were observed in CHO and OVK cells (lanes 6 and 10). Even in Raji cells (lane 14) an appreciable level was detected. When pSP18 (no insert) was used together with pBLVCAT (CHO, lane 8; OVK, lane 12; Raji, lane 16), no CAT activity was induced.

Production of CAT under control of the BLV LTR in the cotransfected cells showed that the cloned complete tumor proviruses under consideration were able to express a functional protein and trans-activate. Moreover, in the culture supernatants and extracts of the cells where the Tat protein was present, Gag p24 and Env gp51 products were also detected by ELISA (data not shown), indicating that structural proteins and viral particles were indeed produced.

Sequence Analysis and Biological Activity of Provirus 1345 with a Large Deletion. Whether unique or multiple in the tumor, proviral copies are complete or harbor deletions. Tumor 1345 was shown to harbor a single provirus with a large 5′ deletion of about 4.4 kb probably preserving the *tat* region (16). For analysis of the structure and the biological

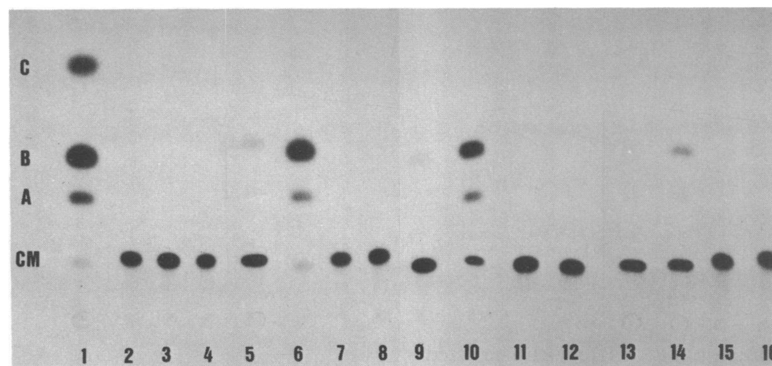

FIG. 4. Assay of CAT activity in FLK, CHO, OVK, and Raji cells. Transfections and preparation of the cell extracts for the assay were performed as described in *Methods*. Ten micrograms of plasmid DNA was used per transfection. Reaction mixtures contained 40 μl of cell extract and were incubated for 90 min at 37°C except for the Raji cell extract, which was incubated for 4 hr. Chloramphenicol (CM) and its acetylated forms (A and B, monoacetate forms; C, diacetate form) were detected by autoradiography. The lanes show the products of chloramphenicol after incubation with extracts of FLK cells transfected with pBLVCAT (lanes 2–4); CHO cells transfected with pBLVCAT and pV395 (lane 5), pBLVCAT and pV344 (lane 6), pBLVCAT and pV1345 (lane 7), or pBLVCAT and pSP18 (lane 8); OVK cells transfected with pBLVCAT and pV395 (lane 9), pBLVCAT and pV344 (lane 10), pBLVCAT and pV1345 (lane 11), or pBLVCAT and pSP18 (lane 12); and Raji cells transfected with pBLVCAT and pV395 (lane 13), pBLVCAT and pV344 (lane 14), pBLVCAT and pV1345 (lane 15), or pBLVCAT and pSP18 (lane 16).

function of this particular provirus, the proviral sequences were cloned in bacteriophage λ Charon 4A and subcloned in pSP18, generating the recombinant plasmid pV1345. The provirus was further characterized by sequence analysis. The sequence was compared with that of a complete BLV genome (41), as shown in Fig. 5. The deletion starts within the gene coding for the major internal Gag protein p24 and ends in the middle of the gene coding for the surface glycoprotein gp51. The deletion is 4310 bp long (nucleotides 1022–5332). It should be noted that the 3′ long open reading frame (*lor*) has undergone no deletion. However, the region containing the end of the *pol* gene and the beginning of the *env* gene (which overlap) is missing. The BLV Tat mRNA, as shown by analysis of RNA isolated from BLV-producing cells, has a dually spliced structure: 5′ LTR-*pol/env* junction–*lor* (7, 37, 42, 43), with the *pol/env* junction providing the ATG initiation codon. In the case of provirus 1345, this predicted doubly spliced mRNA cannot be made. However, this provirus might be transcribed in a different way—for example, by using other splice donors/acceptors or by making a fusion protein, still yielding a functional trans-activator protein.

To determine whether expression took place in tumor T1345, RNAs were analyzed by Northern blot hybridization as described for Fig. 2. No viral expression was detected (Fig. 2, lane 6). To establish whether the cloned deleted provirus was competent to direct trans-activation, plasmid

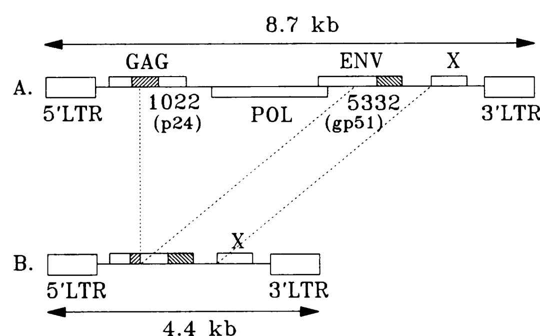

FIG. 5. Comparison of the structure of provirus 1345 (*B*) with that of a complete BLV proviral genome (41) (*A*). Provirus 1345 was cloned and sequenced as described in *Methods*. Deletion spans from nucleotide 1022 within the p24 region of the *gag* gene to nucleotide 5332 in the *env* gene. Nucleotides are numbered according to Sagata *et al.* (41): nucleotide 1 is the first at the left end of the 5′ LTR.

pV1345 was used in transient-expression assays. Upon transfection of pBLVCAT and pV1345 into CHO (Fig. 4, lane 7), OVK (lane 11), and Raji (lane 15) cells, no CAT activity was induced. In the same cell extracts, Gag p24 and Env gp51 products were undetectable (data not shown). These results demonstrate that the truncated provirus, even isolated from the tumor, is unable to code for viral proteins, including Tat.

DISCUSSION

BLV, a trans-activating retrovirus, induces tumors of the B-cell lineage in all infected sheep and in 5% of infected cattle. The disease has a long-lasting course, infection evolving for years with or without PL before the onset of the tumor. PL is a subclinical manifestation of BLV infection; it is a polyclonal proliferation of nontransformed B cells in which 30% of the cell population harbor BLV proviruses while 70% of the cells are reactive B cells with no proviral information in the cell DNA. The BLV-carrying PL lymphocytes can be induced to produce virus when transferred from the peripheral blood of PL animals to a tissue culture medium or into naive animal recipients. PL lymphocytes survive for 2–3 days *in vitro*, releasing virus particles in the medium, and then degenerate. In contrast BLV-induced tumors are monoclonal proliferations of pre-B cells. Most tumor cells can be grown in culture and established as tumor lines. None of our sheep lines, however, expressed viral information either *in vitro* or *in vivo* after injection into naive recipient sheep of a heavy load of cultured tumor cells. The BLV provirus integrated in sheep pre-B transformed cells is silent.

Having demonstrated that no part—including the *tat* gene—of a tumor provirus is expressed *in vivo* or *in vitro*, we turned our attention to the following basic questions. (*i*) Can a silent tumor provirus be expressed if cloned and transfected into a BLV-permissive cell? (*ii*) How can one explain the mode of action of a virus that acts as an inducer in the transformation process but is not required for the maintenance of the transformed state?

Previous experimental data (15, 19–21, 24) and the results presented here establish that no part of the BLV information is expressed in fresh tumors, in sheep tumor cell lines, or in sheep tumor lines injected into naive recipient sheep. The same conclusion held true whether the single provirus was apparently intact or carried extensive deletions. Two cases showing integration of a single copy of an apparently intact provirus were studied (tumors T395 and T344). In both cases,

the cloned full-size provirus was transfected into CHO cells. Clone 344 was the best expressed; BLV Gag and Env proteins were easily detected in culture supernatants and Tat expression was easily demonstrated in CAT assays. Clone 395 showed a lower expression rate. The low expression could be due to a negative influence of sheep DNA sequences flanking the cloned provirus or to an alteration induced during the cloning procedure. For both clones, the relative expression efficiency in transfected cells was the highest in CHO and the lowest in Raji cells. Another tumor (bovine T1345) appeared to harbor a single heavily deleted integrated provirus. Cloning and sequencing data showed an extended deletion (4310 nucleotides) expanding from the middle of the p24 coding sequence, in the *gag* gene, to the middle of the *env* gene, in the gp51 coding region. No functional mRNA, even the *tat* message, could be transcribed from that single proviral genome, as the spliced-in segment corresponding to the end of *pol* was missing. As expected, the transfected 1345 provirus did not exhibit any Tat activity in the CAT assays. The inescapable conclusion of these experiments is that no viral function is required to maintain the transformed state, even if ample epidemiological and experimental evidence point to BLV as the etiological agent of bovine and ovine leukemia. Whether the provirus is complete or deleted, it is fully repressed in the transformed cell. It could even be suggested that switching of the proviral expression is a mandatory step along the cascade of events that leads to full transformation. A virus-expressing tumor cell could probably not survive in an animal exhibiting a high titer of neutralizing antibodies as is systematically observed.

Methylation can profoundly affect the transcription activity of DNA. Passage in bacteria would eliminate any methylation patterns that the provirus had taken on in the tumor cell. Leukemic peripheral blood lymphocytes induced by HTLV-I have been shown to carry extensively methylated proviruses (44). Preliminary results show that in our system, the proviral DNA is methylated to some extent. However, treatment of the sheep tumor cells with 5-azacytidine does not induce any viral expression. Therefore, it seems that methylation alone does not account for the complete shutoff of the proviral DNA.

Whatever the mechanisms set to work to keep the provirus silent, it remains to be explained how a transient viral function, probably Tat, helps push the cell across barriers beyond which an irreversible state leading to full malignancy is reached. Two possibilities can be considered: (*i*) the transformation process rests entirely upon regulatory mechanisms or (*ii*) alterations of the genetic material of the cell make that cell susceptible to progressing toward the neoplastic state. The observation of karyotypic alterations in BLV-induced tumors argues in favor of the second alternative. As in practically all systems, however, it is hard to distinguish whether chromosomal abnormalities cause the neoplastic process or whether they reflect adaptation of the cell to a newly created physiological environment. The identification of critical cellular genes activated or repressed by transient expression of Tat should further our understanding of BLV-induced leukemogenesis.

We thank Dr. M. Onuma for providing bovine tissues. This work was supported by the Fonds Cancérologique de la Caisse Générale d'Epargne et de Retraite, Belgium. R.K. is Maître de recherches au Fonds National de la Recherche Scientifique. A.V.d.B. was supported by fellowships from the Lady Tata Memorial Trust and the Fondation Rose et Jean Hoguet.

1. Burny, A., Bruck, C., Chantrenne, H., Cleuter, Y., Dekegel, D., Ghysdael, J., Kettmann, R., Leclercq, M., Leunen, J., Mammerickx, M. & Portetelle, D. (1980) in *Viral Oncology*, ed. Klein, G. (Raven, New York), pp. 231–289.
2. Burny, A., Cleuter, Y., Kettmann, R., Mammerickx, R., Marbaix, G., Portetelle, D., Van den Broeke, A., Willems, L. & Thomas, R. (1987) *Cancer Surv.* **6**, 139–159.
3. Mammerickx, M., Dekegel, D., Burny, A. & Portetelle, D. (1976) *Vet. Microbiol.* **1**, 347–350.
4. Deschamps, J., Kettmann, R. & Burny, A. (1981) *J. Virol.* **40**, 605–609.
5. Rice, N. R., Simek, S. L., Dubois, G. C., Showalter, S. D., Gilden, R. V. & Stephens, R. M. (1987) *J. Virol.* **61**, 1577–1585.
6. Derse, D. (1988) *J. Virol.* **62**, 1115–1119.
7. Derse, D., Caradonna, S. J. & Casey, J. W. (1985) *Science* **227**, 317–320.
8. Rosen, C. A., Sodroski, J. G., Kettmann, R., Burny, A. & Haseltine, W. A. (1985) *Science* **227**, 321–323.
9. Rosen, C. A., Sodroski, J. G., Kettmann, R. & Haseltine, W. A. (1986) *J. Virol.* **57**, 738–744.
10. Rosen, C. A., Sodroski, J. G., Willems, L., Kettmann, R., Campbell, K., Zaya, R., Burny, A. & Haseltine, W. A. (1986) *EMBO J.* **5**, 2585–2589.
11. Derse, D. & Casey, J. W. (1986) *Science* **231**, 1437–1440.
12. Derse, D. (1987) *J. Virol.* **61**, 2462–2471.
13. Willems, L., Gegonne, A., Chen, G., Burny, A., Kettmann, R. & Ghysdael, J. (1987) *EMBO J.* **6**, 3385–3389.
14. Kettmann, R., Cleuter, Y., Mammerickx, M., Meunier-Rotival, M., Bernardi, G., Burny, A. & Chantrenne, H. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2577–2581.
15. Kettmann, R., Deschamps, J., Cleuter, Y., Couez, D., Burny, A. & Marbaix, G. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 2465–2469.
16. Kettmann, R., Mammerickx, M., Portetelle, D., Gregoire, D. & Burny, A. (1984) *Leuk. Res.* **8**, 937–944.
17. Kettmann, R., Deschamps, J., Couez, D., Claustriaux, J. J., Palm, R. & Burny, A. (1983) *J. Virol.* **47**, 146–150.
18. Gregoire, D., Couez, D., Deschamps, J., Heuertz, S., Hors-Cayla, M. C., Szpirer, J., Szpirer, C., Burny, A., Huez, G. & Kettmann, R. (1984) *J. Virol.* **50**, 275–279.
19. Gupta, P., Kashmiri, S. V. & Ferrer, J. F. (1984) *J. Virol.* **50**, 267–270.
20. Kettmann, R., Marbaix, G., Cleuter, Y., Portetelle, D., Mammerickx, M. & Burny, A. (1980) *Leuk. Res.* **4**, 509–519.
21. Marbaix, G., Kettmann, R., Cleuter, Y. & Burny, A. (1981) *Mol. Biol. Rep.* **7**, 135–138.
22. Driscoll, D. M. & Olson, C. (1977) *Am. J. Vet. Res.* **38**, 1897–1898.
23. Baliga, V. & Ferrer, J. F. (1977) *Proc. Soc. Exp. Biol. Med.* **156**, 388–391.
24. Kettmann, R., Cleuter, Y., Gregoire, D. & Burny, A. (1985) *J. Virol.* **54**, 899–901.
25. Maniatis, T., Fritsch, E. F. & Sambrook, J. V. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
26. Williams, B. G. & Blattner, F. R. (1979) *J. Virol.* **29**, 555–575.
27. Karn, J., Matthes, W. D. H., Gait, M. J. & Brenner, S. (1984) *Gene* **32**, 217–224.
28. Gorman, C. M., Moffat, L. F. & Howard, B. H. (1982) *Mol. Cell. Biol.* **2**, 1044–1051.
29. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74**, 5463–5467.
30. Dente, L., Cesareni, G. & Cortese, R. (1983) *Nucleic Acids Res.* **11**, 1645–1655.
31. Van der Maaten, M. J. & Miller, J. M. (1976) *Bibl. Haematol. (Pavia)* **43**, 360–362.
32. Graham, F. L. & Van der Eb, A. J. (1973) *Virology* **52**, 456–467.
33. Short, M. K., Okenquist, S. A. & Lenz, J. (1987) *J. Virol.* **61**, 1067–1072.
34. Denhardt, D. T. (1966) *Biochem. Biophys. Res. Commun.* **23**, 641–646.
35. Rice, N. R., Stephens, R. M. & Gilden, R. V. (1987) in *Enzootic Bovine Leukosis and Bovine Leukaemia Virus*, eds. Burny, A. & Mammerickx, M. (Nijhoff, Boston), pp. 115–144.
36. Williams, J. G. & Mason, P. J. (1985) in *Nucleic Acid Hybridization*, eds. Hames, B. D. & Higgins, S. J. (IRL, Oxford), pp. 139–160.
37. Mamoun, R. Z., Astier-Gin, T., Kettmann, R., Deschamps, J., Rebeyrotte, N. & Guillemain, B. J. (1985) *J. Virol.* **54**, 625–629.
38. Little, P. F. & Jackson, I. J. (1987) in *DNA Cloning*, ed. Glover, D. M. (IRL, Oxford), pp. 1–18.
39. Mammerickx, M., Palm, R., Portetelle, D. & Burny, A. (1988) *Leukemia* **2**, 103–107.
40. Kettmann, R., Mammerickx, M., Dekegel, D., Ghysdael, J., Portetelle, D. & Burny, A. (1975) *Acta Haematol.* **54**, 201–209.
41. Sagata, N., Yasunaga, T., Tsuzuku-Kawamura, J., Ohishi, K., Ogawa, Y. & Ikawa, Y. (1985) *Proc. Natl. Acad. Sci. USA* **82**, 677–681.
42. Sagata, N., Yasunaga, T. & Ikawa, Y. (1985) *FEBS Lett.* **192**, 37–42.
43. Willems, L., Bruck, C., Portetelle, D., Burny, A. & Kettmann, R. (1987) *Virology* **160**, 55–59.
44. Clarke, M. F., Trainor, C. D., Mann, D. L., Gallo, R. C. & Reitz, M. S. (1984) *Virology* **135**, 97–104.