Even Transcriptionally Competent Proviruses Are Silent in Bovine Leukemia Virus Induced Tumor Cells*

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A. Introduction

Bovine leukemia virus (BLV) is an exogenous retrovirus which induces a chronic disease in cattle, often causing persistent lymphocytosis (PL), with lymphosarcomas developing in a small number of infected animals (for review [1]). The same virus infects sheep, where it induces tumors with very high frequency [2]. In addition to the genes involved in viral replication (*gag, pol, env*), the BLV genome contains an "X" region coding for a 18-kDa protein [3, 4] and a 34-kDa product acting as a transactivator of transcription of the provirus, called Tat protein [5–7]. It is now hypothesized that the BLV Tat protein transactivates some cellular genes, and this is thought to be the key process to initiation of cell transformation. All BLV-induced tumors are clonal and contain at least a portion of a provirus [8, 9] integrated at many sites in the host genome [10, 11]. All deleted proviral copies examined have shown preservation of the X region, stressing again its probable role in the tumoral process. Viral RNA was not detected in fresh lymphocytes isolated from animals with PL or in tumors [9, 12, 13]. When tumor cells were cultivated in vitro, only a very low level of expression, if any, could be detected [14].

In order to investigate the role of proviral integration and expression in cellular transformation induced by BLV, we have isolated and characterized three different tumor proviruses. We show here that these proviruses are silent in the tumor cell. We also sought to determine whether they were able to express viral functions, in particular the Tat protein, once cloned and isolated from their host tumor cells.

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B. Results

I. Viral RNA Expression in Tumor Cells Containing Full-Length Proviruses

Two BLV-induced tumors carrying single full-length proviral copies were selected upon restriction and hybridization analysis (T344 and T395). YR2, which is an established cloned lymphoid cell line derived from T395 tumor cells, displayed the same restriction pattern as T395, confirming that the clone proliferating in culture was indeed the clone present in vivo.

To determine whether viral expression took place in tumors 344 and 395 in vivo as well as in the YR2 cell line in vitro, we looked for the presence of viral sequences in their mRNA, using a labeled antisense X RNA as a probe on Northern blots. No hybridization was detected with RNA from tumor 344, tumor 395, or YR2 cells (data not shown). Comparison of these data to results obtained with BLV-producing cell lines allowed us to assume that less than 0.002 copies of viral RNA were present per tumor cell.

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 infected each with 10^7 YR2 cells. After 5 months, no seroconversion occurred, whereas in a previous experiment carried out with PL lymphocytes [15], infection took place after 18 days with as little as 926 inoculated leukocytes.

II. Biological Activity of the Cloned Tumor-Derived Full-Length Proviruses

To establish whether proviruses 344 and 395 would be able to express viral genes once isolated from their natural host cell, the proviral sequences were cloned, generating the recombinant plasmids pV344 and pV395. We looked for viral proteins, in particular the Tat protein, in transient expression assays after transfection of the cloned proviruses in several cell lines. Plasmids pV395 and pV344 carrying the proviral DNA were cotransfected with plasmid pBLVCAT [5] in noninfected mammalian cells: ovine kidney (OVK), Chinese hamster ovary (CHO), and Raji cells. Levels of chloramphenicol acetyltransferase (CAT) enzymatic activity reflect the ability of the proviral DNA to transactivate the LTR sequences located 5′ to the CAT gene in pBLVCAT. As expected, no CAT activity was detected in CHO, OVK, or Raji cells transfected with pBLVCAT alone (Fig. 1, lanes 2–4) or with pBLVCAT and pSP18, a plasmid without any insert (lanes 8, 12, 16). However, in the presence of pV395, a low level of CAT activity was detected in CHO and OVK (lanes 5, 9) but not in Raji cells (lane 13). Upon cotransfection of pV344 and pBLVCAT, much higher levels were observed in CHO and OVK cells. Even in Raji cells an appreciable level was detected (lanes 6, 10, 14).

Production of CAT under control of the BLV long terminal repeats (LTR) in the cotransfected cells showed that the cloned complete tumor proviruses under consideration were able to express a functional protein and transactivate. 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.

III. Analysis of Tumor Cells Containing a Single Heavily Deleted Provirus

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. The proviral sequences were cloned, generating the recombinant plasmid pV1345 and further characterized by sequence analysis. The sequence was compared with that of a complete BLV genome [16], as shown in Fig. 2. 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 and spans between nucleotides 1022 and 5332.

To determine whether expression took place in tumor 1345, Northern blots were performed as described for tumors 344 and 395. No viral expression could be detected. Transient expression CAT assays with the cloned provirus were negative (Fig. 1, lanes 7, 11, 15) as well as Gag p24 and Env gp51 detection. These results demonstrate that the truncated provirus, even isolated from the tumor, is unable to code for viral proteins including Tat.

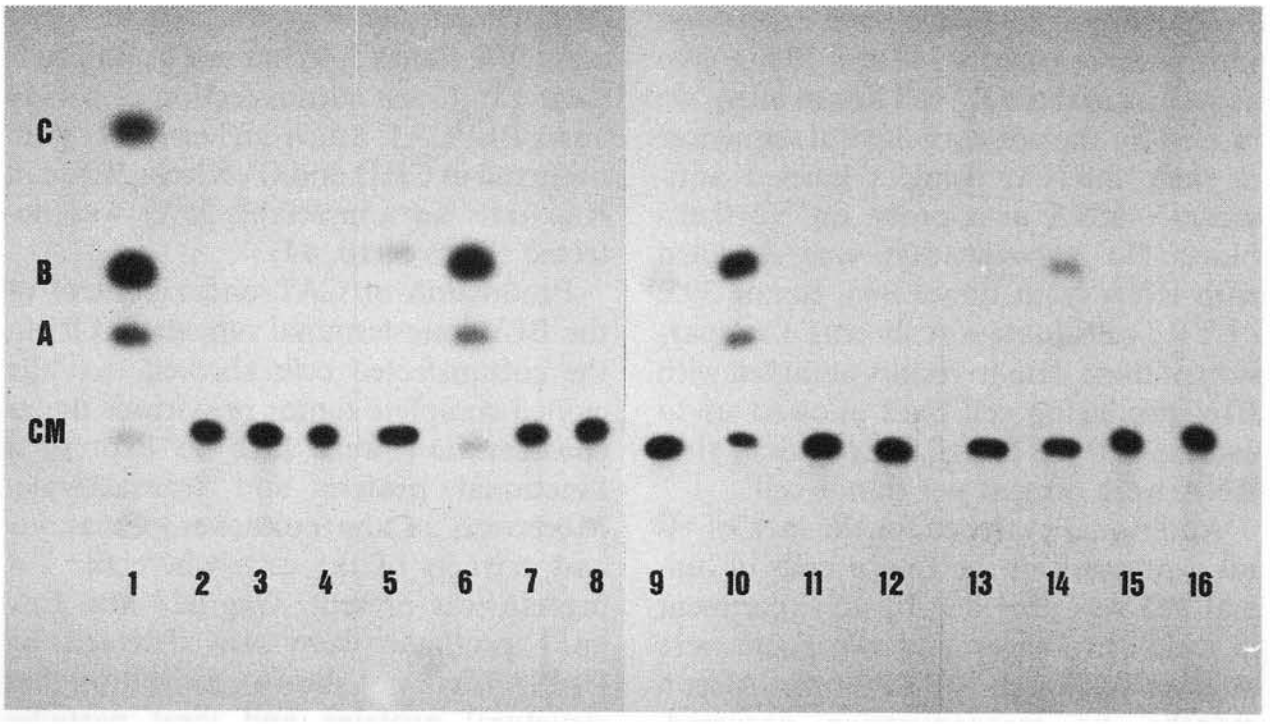

Fig. 1. Assay of CAT activity in fetal lamb kidney (FLK), Chinese hamster ovary (CHO), ovine kidney (OVK), and Raji cells. Transfections and CAT assays were performed as described by Gorman et al. [21]. Chloramphenicol (*CM*) and its acetylated forms (*A* and *B*, monoacetate forms; *C*, diacetate form) were detected by autoradiography. The different lanes show the products of chloramphenicol after incubation with extracts of FLK, a BLV-producing cell line, transfected with pBLVCAT (*lane 1*); CHO, OVK, and Raji transfected with pBLVCAT (*lanes 2–4*), CHO transfected with pBLVCAT and pV395 (*lane 5*), pBLVCAT and pV344 (*lane 6*), pBLVCAT and pV1345 (*lane 7*), pBLVCAT and pSP18 (*lane 8*); OVK transfected with pBLVCAT and pV395 (*lane 9*), pBLVCAT and pV344 (*lane 10*), pBLVCAT and pV1345 (*lane 11*), pBLVCAT and pSP18 (*lane 12*); and Raji transfected with pBLVCAT and pV395 (*lane 13*), pBLVCAT and pV344 (*lane 14*), pBLVCAT and pV1345 (*lane 15*), pBLVCAT and pSP18 (*lane 16*)

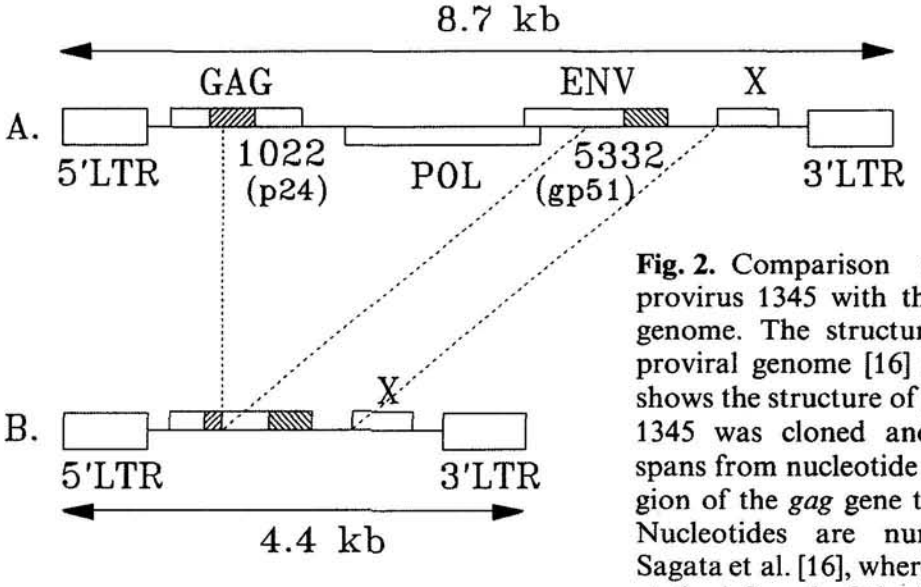

Fig. 2. Comparison of the structure of provirus 1345 with that of a complete BLV genome. The structure of a complete BLV proviral genome [16] is illustrated in (*A*). *B* shows the structure of provirus 1345. Provirus 1345 was cloned and sequenced. Deletion spans from nucleotide 1022 within the p24 region of the *gag* gene to 5332 in the *env* gene. Nucleotides are numbered according to Sagata et al. [16], where nucleotide 1 is the first at the left end of the 5'LTR

C. Discussion

Previous experimental data [9, 12–14] 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 unique provirus was apparently intact or carried extensive deletions. Two cases showing integration of a unique apparently intact provirus were studied (tumor 395 and tumor 344). In both cases, the cloned full provirus was transfected and expressed; BLV Gag and Env proteins were detected in culture supernatants, and Tat expression was easily demonstrated in CAT assays. These results demonstrate that the nonexpression of a provirus in a tumor cell does not necessarily imply a structural alteration of the viral information.

Another tumor case (bovine 1345) harbored a single heavily deleted integrated provirus. Cloning and sequencing data showed an extended deletion (4310 nucleotides) expanding from the middle of p24, in the *gag* gene, to the middle of the *env* gene, in the gp51 region. No functional mRNA, even the *tat* gene message, could be transcribed from that unique proviral genome, as the spliced-in segment corresponding to the end of *pol* was missing [17–20]. 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 points 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.

Experiments are under way to unravel biochemical mechanisms that lock BLV tumor proviruses in the silent stage. The identification of critical cellular genes activated or repressed by transient expression of Tat should further our understanding of BLV-induced leukemogenesis.

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