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Leukemogenesis by bovine leukemia virus: Proviral DNA integration and lack of RNA expression of viral long terminal repeat and 3′ proximate cellular sequences

(RNA tumor virus/lymphoid tumor/restriction endonuclease/RNA dot blot)

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ABSTRACT **The DNA from 17 lymphoid tumors induced by bovine leukemia virus (BLV) was digested with the restriction endonuclease *Eco*RI. Filter hybridization analysis using radioactive probes specific for the BLV genome showed that all tumors contained at least one or a portion of one provirus. Digestion of these proviruses with *Sac* I demonstrated that deletions occurred in about 25% of the cases and involved sequences located in the 5′ half of the provirus. No sequence homology was observed between the cloned proximate cellular sequences flanking two different proviruses at their 3′ end and the corresponding sequences in 16 other tumor DNAs, thus showing that a wide range of genomic sites could accommodate BLV proviruses. Transcription of viral DNA including long terminal repeated sequences was not detected, strongly suggesting that viral gene expression is not required for maintenance of the tumor state. No expression of 3′-proximate cellular sequences was observed, indicating that no proximate downstream promotion took place in the cases examined.**

Bovine leukemia virus (BLV), an exogenous retrovirus of cattle (1, 2), induces B lymphocyte neoplasms (called enzootic bovine leukosis, EBL) after long latent periods (3). BLV does not contain any host cellular sequences and does not appear so far to bear genes directly inducing transformation. It follows that leukemogenesis by bovine leukemia virus might be due to (*i*) initiation of transcription of a cellular gene from a viral promoter as found in avian leukosis virus (ALV)-induced lymphoid leukosis (4); (*ii*) position effect of the provirus with, as a consequence, enhancement of expression of neighboring cellular information or extinction of previously expressed normal DNA; (*iii*) expression of viral information. The present study is aimed at characterizing the BLV provirus in tumor cell DNA and at providing some insight into the molecular events that lead to tumor development.

MATERIALS AND METHODS

Bovine Tissues and Cells. Bovine material was collected from field cases of enzootic bovine leukosis in Belgium (animals 12, 15, 82, 950, 2586, and 2587), France (animals 53, 56, 104, 106, 108, and 120), Japan (animals 119, 1345, 1347, and 1351), and the USA (animals 79-2, 3156, 3168, 3202, and 3261). Circulating leukocytes or lymphoid tumors, kept at −70°C, were used as sources of DNA or RNA. Leukocytes from a normal animal (animal 94) were used as a source of control DNA and RNA.

Molecular Cloning of DNA Fragments. The isolation of the 9.2-kilobase (kb) *Sac* I fragment containing all the BLV information has been described (2). *Eco*RI tumor DNA fragments containing the right viral long terminal repeat (LTR) together with flanking cellular sequences of various lengths (see Fig. 2) were cloned in Charon 21A λ phage (5) essentially as described by Maniatis *et al.* (6). The National Institutes of Health guidelines for recombinant DNA research were followed during the entire procedure.

3′-Enriched cDNA Synthesis, Cellular DNA Extraction, Restriction Endonuclease Digestion, DNA Blotting, and Hybridization. These procedures were performed as described in ref. 7.

Nick-Translation. Conditions were as described by Rigby *et al.* (8).

Cellular RNA Extraction. RNA was extracted from leukocytes and tumor cells as described (9).

RNA Dot Blots. Blots were prepared according to Thomas (10).

RESULTS

A Few Proviral Copies Are Integrated in the Tumor Cell Genome. To estimate the number of exogenous BLV proviral copies in tumor DNA, the DNA from 19 tumors was digested with restriction endonuclease *Eco*RI. This enzyme cleaves only once in BLV proviral DNA (7), so two fragments containing viral information are generated per integrated BLV copy. Furthermore, because the size of these fragments is determined by the location of the nearest *Eco*RI cleavage sites in the cellular sequences flanking the provirus, *Eco*RI digestion also provides information about the integration sites of the proviral DNA. The *Eco*RI tumor DNA fragments were hybridized to two types of probes: (*i*) a cDNA enriched in sequences copied from the 3′ end of the viral RNA (3′-enriched BLV cDNA) (7) and (*ii*) a probe obtained by nick-translation of a full-sized BLV proviral DNA cloned in λgt*WES*·λB (2). The results of this analysis are shown for four tumor DNAs in Fig. 1*A* and summarized in Table 1. As shown previously (7), *Eco*RI digestion of linear unintegrated viral DNA generates two viral fragments, 9.2 and 0.8 kb, the latter being located at the 3′ end of the viral genome (7). A comparison of the hybridization patterns with the 3′-enriched and the full-size probe allowed orientation of the restriction fragments of the provirus with respect to viral RNA. This type of analysis is illustrated for DNA 3168 (Fig. 1*A*, lanes 2 and 3). The same comparison was done for tumor DNAs 1345, 119, and 3261 (Fig. 1*A*, lanes 4–6; only patterns of hybridization with full-size probe are shown). All the strongly positive viral fragments

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Abbreviations: ALV, avian leukosis virus; BLV, bovine leukemia virus; EBL, enzootic bovine leukosis; FLK, fetal lamb kidney cells; kb, kilobase(s); LTR, long terminal repeat; PL, persistent lymphocytosis.

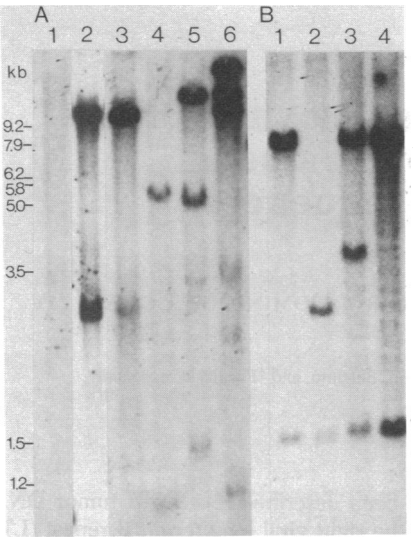

FIG. 1. DNA hybridization using BLV cDNA and nick-translated cloned BLV DNA as probes. (*A*) Ten micrograms each of normal bovine leukocyte DNA (animal 94, lane 1) and bovine tumor DNA (animal 3168, lanes 2 and 3; animal 1345, lane 4; animal 119, lane 5; animal 3261, lane 6) were digested to completion by *Eco*RI and electrophoresed on a 0.8% agarose gel. The Southern blots of the DNA fragments were soaked in the prehybridization mixture at 65°C (7) and hybridized for 24 hr with ^{32}P-labeled nick-translated cloned BLV DNA at 5×10^6 cpm/ml (specific activity 2×10^8 cpm/μg), except the nitrocellulose strip corresponding to lane 2, which was hybridized for 24 hr with 5×10^6 cpm of 3′-enriched BLV [^{32}P]cDNA per ml (specific activity 3×10^8 cpm/μg). The last washings were performed in 15 mM NaCl/1.5 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 3 days. Fragment lengths are given in kb. Only fragments larger than 0.5 kb were detected on these gels. *Eco*RI-digested λ DNA as well as *Hae* III-digested φX174 DNA were used as molecular weight markers. (*B*) Ten micrograms each of bovine tumor DNA (animal 3168, lane 1; animal 1345, lane 2; animal 119, lane 3; animal 3261, lane 4) were digested to completion by *Sac* I and treated as described for *A* with ^{32}P-labeled nick-translated cloned BLV DNA as a probe.

larger than 9.2 kb (one per BLV copy) were found to be located at the 5′ end of the provirus (Fig. 1*A*). All fragments smaller than 9.2 kb corresponded to the 3′ end or to deleted 5′ end junction fragments. Such deletions are illustrated in Fig. 1*A*, lanes 4 and 5. In the case of tumor 1345 DNA (lane 4) two *Eco*RI fragments smaller than 9.2 kb were detected, indicating the presence in this tissue of a provirus with a deletion; in the case of tumor 119 DNA (lane 5), four *Eco*RI fragments were observed but only one of these was longer than 9.2 kb, suggesting that one complete provirus and one with a deletion were present in this tumor. To sum up our observations, 11 tumors were found to harbor 1 proviral copy. Such was the case for tumor DNA 3168 and 1345, whose *Eco*RI digests displayed two BLV-positive fragments, of length 10.0 and 3.0 kb, and 5.4 and 1.0 kb, respectively (Fig. 1*A*, lanes 3 and 4). Four tumors contained two BLV proviral copies as in tumor DNA 119, for which four BLV-positive *Eco*RI fragments, of length 12.0, 5.0, 3.2, and 1.4 kb, were visualized (Fig. 1*A*, lane 5). Two tumors harbored three BLV copies, as was the case for tumor DNA 3261 (Fig. 1*A*, lane 6), whose *Eco*RI digest displayed six BLV-positive fragments, of length 17.0, 13.0, 10.0, 3.4, 2.4, and 1.1 kb. It should be mentioned that no hybridization occurred to control leukocyte DNA (Fig. 1*A*, lane 1). This analysis shows that each *Eco*RI-digested tumor DNA displayed at least two viral bands (Fig. 1*A*), providing strong evidence that all tumors acquired at least one or a portion of one BLV provirus.

Table 1. Analysis of BLV proviral DNA in tumor DNA

Copy number per diploid* genome	Number of tumors tested	Distribution and description of proviruses†
1	11	10 with 1 complete provirus (15-2, 15-3, 82, 3168, 3156, 106, 108, 950, 1351, 1347) 1 with 1 defective provirus (1345)
2	4	1 with 2 complete proviruses (53) 1 with 1 complete and 1 defective provirus (119) 2 with 2 defective proviruses (56, 120)
3	2	2 with 3 complete proviruses (3261, 79-2).

* See text.
† Animal numbers are given in parentheses.

Deletion of the Provirus in Tumor DNA. Further evidence for the deletion of viral sequences from some of these proviruses was provided by experiments in which *Sac* I-cleaved tumor DNA was hybridized with 3′-enriched cDNA or cloned BLV DNA as a probe. In some BLV variants, *Sac* I was found to cleave the BLV proviral DNA only in the LTRs, thus generating a long internal fragment of about 9.2 kb (2). In other variants, the internal 9.2-kb fragment was split into two fragments, 7.7 and 1.5 kb, due to the presence of an additional *Sac* I site located close to the 3′ end of the proviral molecule (ref. 7 and Fig. 1*B*, lanes 1 and 4). The data obtained after hybridization of the *Sac* I digests of the 17 tumor DNAs with BLV-specific probes are illustrated in Fig. 1*B* and summarized in Table 1. Six out of 25 proviruses (some tumors harbored more than one provirus; see Table 1) examined were found to have undergone deletions. All the deletions observed were in BLV variants in which *Sac* I generated two internal fragments (7), the 1.5-kb fragment located at the 3′ side remaining at full size, and the 7.7-kb fragment located at the 5′ side being reduced in size. This is illustrated, for example, in the *Sac* I digest of tumor DNA 1345, in which the 7.7-kb fragment was reduced to 3.0 kb (Fig. 1*B*, lane 2). In tumor DNA 119, in addition to the 7.7-kb fragment corresponding to the complete proviral copy, a 4.0-kb fragment was observed (Fig. 1*B*, lane 3), substantiating the conclusions drawn from *Eco*RI restriction data and indicating that two proviral copies, one complete and one 5′-deleted, were present in this tumor. It should be added that, in the particular case of tumor 1345, the 3.0-kb fragment still hybridized with the cloned 15-4 DNA used as a probe to reveal LTR sequences (Fig. 2), implying that at least a portion of LTR was present at the 5′ end of the 5′-deleted provirus. The above data thus demonstrate that some of the BLV proviruses had undergone extensive structural alterations near or at their 5′ ends.

Multiple Integration Sites of the Proviruses in Tumor DNA. We previously reported that many sites of the B lymphocyte DNA could accommodate a BLV provirus. This has been particularly demonstrated in the condition named persistent lymphocytosis (PL) (11). We report here that the same holds true for the virus-induced neoplastic state. *Eco*RI digests of tumor cell DNA from a number of animals indicated that the 3′ viral fragment [almost exclusively made of LTR sequences (7)] was always linked to cellular sequences of variable length, suggesting that the integration sites were different from one animal to another. However, due to the outbred nature of the cattle pop-

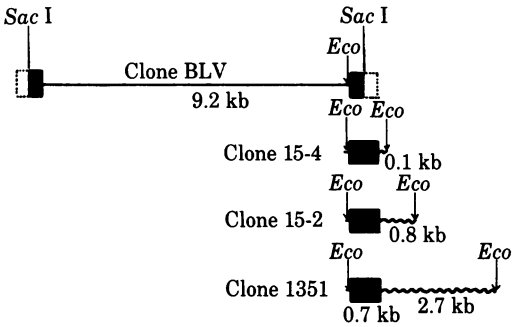

FIG. 2. Diagrams of the cloned DNAs. Dark box, LTR DNA; empty box, LTR DNA absent from cloned BLV DNA; straight line, viral DNA; wavy line, cellular DNA; *Eco*, *Eco*RI.

ulation considered, we could not rule out integration into allelic variants of the same integration region.

To address this issue, *Eco*RI tumor DNA junction fragments containing the 0.75-kb 3′ viral DNA fragment and its cellular flanking sequences were cloned in Charon 21A (5). These clones were referred to as clone 15-4 and clone 1351; they are schematically represented in Fig. 2. *Eco*RI digests of DNA from normal leukocytes and from tumor cells were hybridized to a probe obtained by nick-translation of these cloned sequences (8). Fig. 3 illustrates, for 8 out of 17 DNAs tested, the results obtained with cloned 1351 DNA as a probe. Two lines of evidence indicate that the weak hybridization bands, present only in the tumor cell DNA, corresponded to the viral fragments revealed by the LTR moiety of the probe. First, their number (two per complete provirus) varied according to the number of proviruses present in the DNAs under investigation and, second, the viral patterns were strictly identical (except in the intensity of the bands) with those obtained when BLV cDNA or cloned BLV DNA was used as a probe (data not shown). In all DNAs tested, including the control leukocyte DNA, the cellular moiety of the probe strongly hybridized to a 2.9-kb fragment (not revealed by a viral probe) (Fig. 3, lanes 1–8). In addition, cloned 1351 DNA hybridized to a 3.4-kb fragment in the homologous 1351 tumor cell DNA (Fig. 3, lane 2), this fragment being revealed also by a viral probe (see discussion). Hybridization of the same DNA samples with cloned T15-4 DNA as a probe allowed identification of the same virus-specific fragments as detected by probe 1351. With probe T15-4, however, a 5.0-kb fragment was consistently revealed in all samples, including normal bovine leukocyte DNA. These results indicate that the cellular sequences flanking the provirus at its 3′ side in tumors T15-4 and 1351 are different and differ from the cellular sequences adjacent to the 3′ side of the proviruses in 16 other tumors tested.

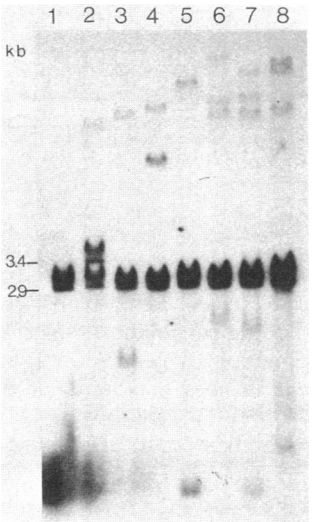

FIG. 3. DNA hybridization using cloned 1351 DNA as a probe. Ten micrograms each of normal bovine leukocyte DNA (lane 1) and bovine tumor DNA (animal 1351, lane 2; animal 104, lane 3; animal 106, lane 4; animal 15, lane 5; animal 3202, lane 6; animal 3261, lane 7; and animal 79-2, lane 8) were digested to completion by *Eco*RI and electrophoresed on a 0.8% agarose gel. The Southern blots of the DNA fragments were treated as described in the legend to Fig. 1 except that nick-translated cloned 1351 BLV DNA (see Fig. 2) at 0.5×10^6 cpm/ml (specific activity 3×10^7 cpm/μg) was used as a probe. Autoradiography after a 5-day exposure.

Lack of Viral RNA Expression. From liquid hybridization studies performed with BLV [^{3}H]cDNA, it appeared that no viral RNA expression was detected in total RNA isolated from circulating leukocytes from animals in PL or from tumor cells (9). However, the viral probe used at that time was not a uniform representation of the viral genome: the viral RNA template used for the preparation of the cDNA had been prepared by oligo(dT)-cellulose affinity chromatography and was thus enriched in the 3′ poly(A)-containing region of the viral RNA.

Using as a probe the complete cloned BLV proviral DNA ^{32}P-labeled by nick-translation, we could extend these previous studies; we also took advantage of the sensitive dot blot hybridization technique (10).

As described in the legend of Fig. 4, total RNA from circulating leukocytes of animals in PL (lanes 3, 4, and 5) and from tumors of several other animals (lanes 6–13) was spotted on a nitrocellulose filter and hybridized with the complete ^{32}P-labeled BLV DNA probe.

As shown in Fig. 4, a strong hybridization was found with 35S BLV RNA (lane 1) and FLK RNA (lane 14) used as positive controls, whereas no hybridization was detected with leukocyte RNA from a normal animal (lane 2). A slight hybridization was seen with the highest RNA concentration from several of the tumors. However, these positive signals were due to DNA contamination of the RNA samples, because they were completely resistant to an intensive treatment of the filter with pancreatic ribonuclease before hybridization or a preliminary treatment of the RNA samples with alkali. Both treatments were indeed

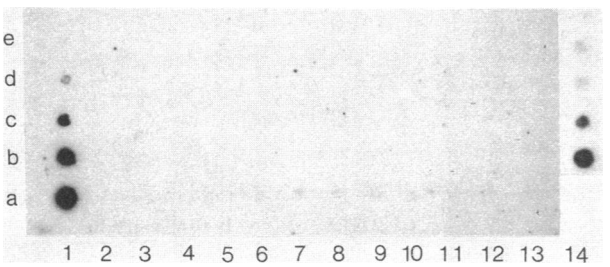

FIG. 4. Dot blot assay of total RNA from leukocytes of a normal animal (lane 2) or animals in PL (lanes 3, 4, and 5) and from tumors of several other animals (lanes 6–13). In lanes 2–13, 1:5 dilutions were tested, from 2 μg (a) to 3.2 ng (e) of total RNA. BLV 35S genomic RNA (lane 1) and total RNA from BLV-infected FLK cells [a BLV-producing fetal lamb kidney cell line used in previous studies (7)] (lane 14) were taken as positive controls. Also, 1:5 dilutions were tested, from 2 ng (a) to 3.2 pg (e) of viral RNA in lane 1 and from 360 ng (b) to 3 ng (e) of FLK total RNA in lane 14. The dot blot preparation was hybridized with the complete cloned ^{32}P-labeled BLV proviral DNA and processed as described in the legend to Fig. 1. Lane 3 corresponds to animal 12, lane 4 to animal 2586, lane 5 to animal 2587, lane 6 to animal 120, lane 7 to animal 96, lane 8 to animal 106, lane 9 to animal 104, lane 10 to animal 3168, and lanes 11, 12, and 13 to three tumors of animal 15.

effective for RNA degradation, because they abolished the signals due to a positive control of 35S BLV RNA (results not shown). It should be noted that the RNAs analyzed in Fig. 4 came from tumors containing one to three BLV provirus copies in their DNA.

From these results, it may be concluded that, assuming a RNA content of 10 pg per cell, which is certainly an overestimate, less than 3 copies of viral RNA are present in each tumor cell or in each leukocyte of an animal in PL. Other studies, using poly(A)$^+$ RNA from the same tumors, indicate that the viral expression might even be lower than one copy of viral BLV RNA per cell (results not shown). It should be added that the same cellular RNAs, at higher dilutions (from 6 ng to 2 pg), gave strong positive signals after hybridization to a labeled ribosomal cDNA probe (data not shown).

The absence or the very low level of viral RNA in all tumors tested strongly suggests that intensive or even moderate expression of the proviral gene(s) in all cells is not required for maintenance of the tumor state.

Absence of Proximal Promoter Activity of the Right LTR of the Provirus. It has been reported that the ALV provirus often integrates adjacent to the *c-myc* gene and that transcription, initiating at a viral LTR promoter, causes enhanced expression of *c-myc* information, possibly leading to leukemogenesis (4, 12). Although the variety of BLV integration sites in bovine tumors (see above) does not favor the existence of a similar mechanism in the case of bovine leukemia, we looked for the possible occurrence of 3′ virus-initiated transcription of cellular DNA sequences. Availability of cloned cellular sequences flanking the integrated provirus on its 3′ side made possible the search for complementary RNA transcripts.

Nitrocellulose filters containing the same RNA samples as those described in Fig. 4 were hybridized to a labeled cloned DNA essentially made of LTR sequences (clone 15-4, Fig. 2) and to cloned DNAs containing the 3′ viral LTR plus cellular flanking sequences of two different lengths (clones 15-2 and 1351, Fig. 2). For each of the three probes used, the hybridization results (not shown) were identical to those obtained with cloned BLV DNA as a probe (Fig. 4). Again, only the 35S viral RNA was clearly detected, although less intensively because only about 1/13th of the viral genome was present in the cloned DNAs.

We can again conclude that no expression or only very little expression of cellular sequences flanking the 3′ end of the BLV provirus occurs in BLV-induced tumors or in leukocytes of animals in PL.

DISCUSSION

Previous hybridization experiments using BLV cDNA as a probe have shown that BLV-induced tumors are made of a clone harboring the provirus (11). The present analysis of other tumor DNAs with *Eco*RI and *Sac* I restriction enzymes and cloned BLV proviral DNA as a probe shows that the lymphoid cell clones (of the B cell lineage) proliferating in a tumor can harbor one to three copies of BLV provirus, these copies being either complete or having partial deletions. Among the 17 tumors screened, 2 harbored only defective proviral copies. The deletions being located in the 5′ half of the proviral molecule, it is assumed that expression of the viral information corresponding to the deleted sequences is not required for maintenance of the neoplastic process, whereas the 3′ half of the proviral molecule could be of importance.

Experiments involving 17 tumor DNA samples annealed with a labeled probe containing cellular sequences flanking the provirus at its 3′ side showed that these cellular sequences revealed a single DNA fragment, identical in all 17 samples examined irrespective of their normal or neoplastic origin. In addition, they revealed a second band corresponding to the 3′ viral junction fragment only in the tumor cell DNA from which the probe had been cloned (DNA 1351). In this tumor DNA, the two positive fragments appeared to be of equal intensity as determined by densitometry. The sum of these signals corresponded to the darkness of the single band found in the control as well as in all other tumor DNAs tested. Moreover, liquid hybridization experiments (data not shown) indicated that most of the 1351 tumor cells harbored the BLV provirus, as was the case for tumor 15-2 (11). Taken together, these results suggest that integration of the provirus in the tumor DNA can take place at many different sites into only one of the chromosomes of a given pair. (*In situ* hybridizations may allow us to determine if BLV provirus integrates preferentially into a given chromosome.) This event would be sufficient to switch on the tumorigenic process.

Search for viral RNA in bovine tumors has so far yielded negative results, irrespective of the method of detection used (ref. 9 and see below). Moreover, taking into account the absence of normal cellular sequences from the BLV provirus (2) as opposed to defective avian acute leukemia viruses (13) and Abelson leukemia virus (14), we are left with three possible hypotheses regarding the BLV mode of action—namely (*i*) downstream promotion of a gene implicated in a leukemogenesis, (*ii*) changes in chromatin conformation leading to (over)expression of the same gene, (*iii*) block of expression of a gene that is normally expressed. In the present study, the first hypothesis (downstream promotion) was tested by using pieces of 3′-end proviral sequences linked to normal cellular DNA as probes. Advantage was taken of the viral *Eco*RI restriction site located very close to the right LTR. The latter was cloned together with three different lengths of bovine cellular sequences originating from three different tumors as shown in Fig. 2. Results of dot blot hybridizations of those probes to RNAs prepared from leukocytes of normal and leukemic animals and to tumor cells RNAs were all negative, although clear positive results were obtained by annealing the same probes with BLV 35S RNA or with total RNA extracted from FLK, a BLV-producing cell line (Fig. 4). It follows that downstream promotion using the right LTR structure as a promoter (4, 12, 15, 16) has to be regarded as an infrequent—if existing at all—possibility in the BLV system.

To summarize our observations in the BLV system, it appears that:

(*i*) There is no evidence indicating that the virus codes for an oncogenic product (2, 17).

(*ii*) There is no detectable viral RNA expression in tumor cells. These data are, however, still compatible with either one or both of the following possibilities: tumor cells express, at a low rate, a region of the BLV genome; or a small proportion of BLV-carrying cells express the entire or part of the viral information.

(*iii*) Proviral integration occurs at multiple sites within the tumor cell genome, indicating that the activation (or inactivation) of many different cellular genes could be responsible for the tumor growth or that the expression of a unique "*onc*" gene could be affected by a long-distance effect.

(*iv*) The right-end LTR does not promote transcription of proximate downstream cellular sequences. However, the data presented here cannot rule out expression of sequences upstream to the left-end LTR or further downstream from the right-end LTR. But it is also possible that, as proposed by Payne *et al.* (16) for some ALV-induced tumors, viral activation of cellular oncogenes could be the result of a less straightforward and still unknown mechanism in which the provirus does not act as

a simple promoter of transcription but has a "regional chromosome activation" role.

Furthermore, it should be stressed here that we are looking only at the maintenance of the tumor in advanced neoplasms. Chromosomal changes (such as translocations) could have been induced by initial events in which the provirus played a key role. [It should be noted that chromosome changes were frequently observed in BLV-induced tumors (18).] It might then be hypothesized that the provirus, by itself, is not required for the maintenance of the tumor state in BLV-induced neoplasms.

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