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Chromosome Integration Domain for Bovine Leukemia Provirus in Tumors

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The 3'-end host-virus junction fragments from two bovine leukemia virus (BLV)-induced lymphoid tumors (tumors 15-4 and 1351), each containing a single provirus, were used as probes to detect large restriction fragments flanking these proviruses. The DNAs from 28 other independent BLV-induced tumors were checked by Southern analysis of their restriction fragments for possible rearrangement due to the insertion of a BLV provirus in the cellular sequences corresponding to those flanking the proviruses in tumors 15-4 and 1351. In no case did proviral integration occur in cellular sequences corresponding to those implicated in the tumors of origin. According to the statistical analysis performed, if a preferential domain for BLV integration exists, it has a size of 1,304 kilobases when the probability of not observing an integration event in the cellular fragments considered in tumors 15-4 and 1351 is 0.50.

Bovine leukemia virus (BLV), an exogenous retrovirus, may induce B-cell lymphoid tumors in cattle after a long latency (1). At present, leukemogenesis by this virus is not understood. BLV does not appear to carry its own oncogene (3), and its provirus is not expressed in tumors (7). Tumors are clonal, containing one or a few proviral copies (7). The proviruses are not inserted in the same sites in different tumors, and no proximate downstream promotion has been observed (7). These data leave us confronted with two alternatives. (i) BLV provirus is expressed in the pretumorous phase of the target cell. A rare transforming event might be induced by a proviral product. Once this event has taken place, expression of the provirus stops; the cell is transformed and will invade the host. (ii) BLV acts through its position in the host genome and exerts a distant regional effect upon more or less proximate neighboring genes. The existence of a preferential integration domain has been recently reported for the mouse mammary tumor virus (MMTV) provirus (10). To investigate the latter possibility in the BLV system, we performed this study with two tumors, each containing a single provirus (which might be supposed to be integrated within a critical region of the host genome). The 3'-end host-virus junction fragments of these two tumors were used as probes to search for large restriction fragments flanking the proviruses in the tumors of origin. The identity and size of these cellular fragments were then compared with those of their counterparts in the DNAs of 28 other independent BLV-induced tumors.

MATERIALS AND METHODS

Bovine tissues. Bovine material was collected from field cases of enzootic bovine leukosis in Belgium, France, Japan, and the United States. Lymphoid tumors, kept at −70°C, were used as sources of DNA. Calf thymus from a normal animal was used as the source of control DNA.

Molecular cloning of DNA fragments and restriction enzyme mapping. *Eco*RI tumor DNA fragments containing the right-end *Eco*RI junction fragments of tumors 15-4 and 1351 were previously cloned in Charon 21 A λ phage (7), essentially as described by Maniatis et al. (8), and were subcloned in plasmid pBR322. A cellular *Msp*I-*Eco*RI fragment of 70 base pairs (bp) derived from the right-end junction fragment of T15-4 was subsequently cloned in M13 mp8 phage according to Messing et al. (9).

Cellular DNA extraction. Cellular DNA extraction was performed with sodium dodecyl sulfate plus phenol-chloroform, essentially as described by Kay et al. (5). The molecular weights of all DNAs were checked by agarose gel electrophoresis, using λ and λ *Eco*RI DNAs as markers; they were all found to be more than 50 kilobase pairs (kb) long.

Restriction enzyme analysis and electrophoresis of DNA. All restriction enzymes except one used in this study were purchased from Bethesda Research Laboratory, Neu Isenburg (West Germany). *Eco*RI was a product of Boehringer Mannheim (West Germany). DNA samples (10 μg) were digested—under conditions recommended for each restriction enzyme by the

TABLE 1. Probability of no BLV integration in the 32-kb fragment, investigated in 28 independent BLV-induced tumors[a]

P(0)	x (kb)
0.01	211
0.05	315
0.10	405
0.20	573
0.30	760
0.40	994
0.50	1,304
0.60	1,770
0.70	2,528
0.80	4,031
0.90	8,520
1.00	∞

[a] For the equation used to find P(0), see the text.

supplier—with an excess of enzyme (5 to 10 U per μg of DNA) for 3 h at 37°C. The digested chromosomal DNA was run in 0.6 or 0.8% agarose gels, using *Hin*dIII-digested λ DNA and *Hae*III-digested φX174 DNA as size markers. DNA was transferred from the gel to nitrocellulose filters (Millipore Corp.) by the method of Southern (12). In some cases (see figure legends), a depurination of the DNA fragments was performed (0.25 N HCl for 15 min) before denaturation of the DNA (14).

Radioactive probes and molecular hybridizations. Conditions for nick translation of probe 2 in pBR322 were as described by Rigby et al. (11). Single-stranded M13 DNA containing a bovine cellular insert (probe 1) was transcribed in a standard reverse transcriptase reaction (6), but with M13 primer (Bethesda Research Laboratory) instead of partial DNase-digested calf thymus DNA.

The nitrocellulose filters with the transferred DNA were preincubated for 6 h in a plastic box with 40 ml of a mixture containing 3× SSC, 10× Denhardt medium (2), 0.1% sodium dodecyl sulfate, and 100 μg of denatured salmon sperm DNA per ml (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The filters were then hybridized for 48 h in the same mixture containing 5×10^6 to 7×10^6 cpm of ^{32}P-labeled probe per ml (see figure legends). Final washings of the filters were performed in 0.1% sodium dodecyl sulfate and 0.2× SSC at 68°C. After drying, they were exposed to Kodak XAR-5 films at −70°C in the presence of Siemens Special intensifying screens for 3 to 5 days.

Restriction enzyme mapping. The mapping of the several restriction sites on the chromosomal DNA was performed by single and double digestions, gel electrophoresis, transfer to nitrocellulose filters, and hybridization with viral probes. Restriction maps of BLV proviral DNAs were previously reported (3, 6).

Statistical calculations. Assuming a random integration for BLV provirus in a domain of x kb, the probability of BLV integration in the 32-kb observed fragment (15 kb + 17 kb) is $p = 32/x$. The $P(0)$ probability of no BLV integration in the 32-kb fragment, investigated in 28 independent BLV-induced tumors, is

$$P(0) = (1 - p)^{28} = \left(1 - \frac{32}{x}\right)^{28}$$

Some probability values in terms of x are presented in Table 1.

RESULTS

To determine whether BLV provirus preferentially integrates in a given chromosomal domain, we chose BLV-induced tumors bearing a single provirus. As previously observed, tumors 15-4 (T15-4, a Belgian case) and 1351 (T1351, a

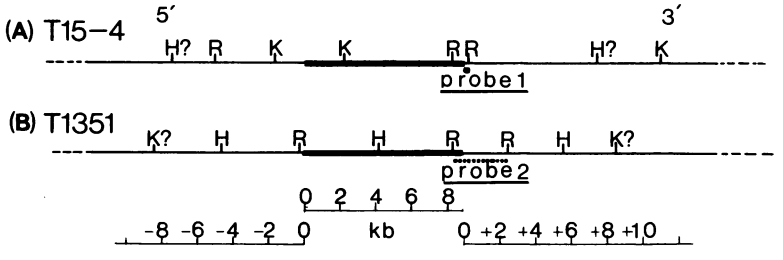

FIG. 1. (A) Restriction map of T15-4 proviral DNA and its flanking cellular sequences. The restriction enzyme sites were mapped with both the chromosomal DNA from Belgian tumor 15-4 and a proviral cloned DNA derived from tumor 15-2 (3). The orientation of the chromosomal DNA was arbitrarily chosen to be the same as the orientation of the provirus (5′ to 3′). Only sites of interest in the present study are shown. Abbreviations: R, *Eco*RI; H, *Hin*dIII; K, *Kpn*I. *Hin*dIII sites were not positioned due to the absence of such a site in that proviral DNA. Probe 1 is a cloned 70-bp *Msp*I-*Eco*RI cellular fragment derived from the cloned right-end *Eco*RI-*Eco*RI junction fragment. Symbols: ▬, proviral DNA; ——, cellular DNA; ■, probe 1. (B) Restriction map of T1351 proviral DNA and its flanking cellular sequences. The restriction sites were mapped with both the chromosomal DNA from tumor 1351 and linear unintegrated proviral DNA from the same BLV variant (6). The restriction enzymes used were the same as for T15-4. *Kpn*I sites were not positioned due to the absence of such a site in that proviral DNA. Probe 2 is the cloned 3.4-kb right-end *Eco*RI-*Eco*RI junction fragment. Symbols: ▬, proviral DNA; ——, cellular DNA; - - - -, probe 2.

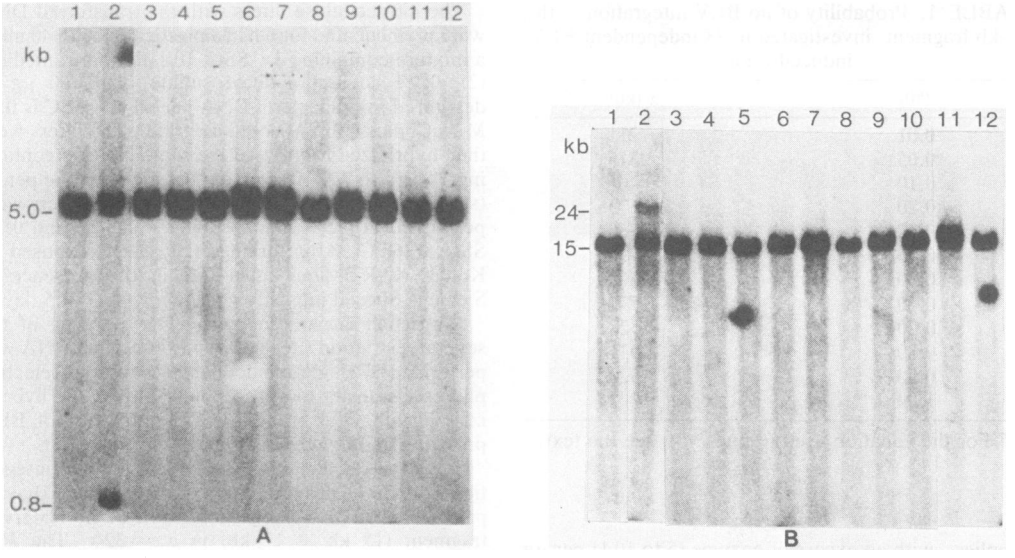

FIG. 2. DNA hybridizations using probe 1. (A) Ten micrograms each of normal calf thymus DNA (lane 1), bovine tumor 15-4 DNA (lane 2), and 10 other bovine tumor DNAs (lanes 3 to 12) were digested to completion by *Eco*RI and electrophoresed on a 0.8% agarose gel. The Southern blots of the DNA fragments were treated as described in the text. Hybridization to ^{32}P-labeled probe 1 (0.007 μg/ml; 10^9 cpm/μg) was for 48 h at 65°C. The autoradiography was a 5-day exposure. (B) Same as in A, but with *Hin*dIII. The agarose gel was 0.6%, and a depurination step was included (see the text).

Japanese case) each contained one provirus (7). The integration region in tumor 15-4 DNA was investigated by using a cellular subclone (70 bp) of the *Eco*RI right-hand junction fragment as a probe (probe 1) (Fig. 1). Hybridization of Southern blots (12) to this probe revealed cellular fragments of 5, 13, and 15 kb in (respectively) *Eco*RI (Fig. 2A, lane 1), *Kpn*I (not shown), and *Hin*dIII (Fig. 2B, lane 1) digests of calf thymus DNA. The restriction map of tumor 15-4 provirus and its surrounding cellular sequences was established and is shown in Fig. 1A. After hybridization to probe 1, the T15-4 *Eco*RI blot displayed a 0.8-kb fragment in addition to normal fragments of 5.0 kb derived from the uninterrupted locus (Fig. 2A, lane 2); T15-4 *Kpn*I digest hybridized to an 18-kb fragment in addition to the 13-kb fragment (not shown), and T15-4 *Hin*dIII digest hybridized to a 24-kb fragment in addition to the 15-kb fragment (Fig. 2B, lane 2). Thus, as expected, rearrangements were observed in T15-4 DNA as a consequence of proviral integration. Thus, it seems reasonable to assume that if a preferential domain of integration exists around the T15-4 proviral insertion site, we might expect to find the same cellular fragments rearranged in the DNAs of other tumors. The smaller this domain would be, the higher the probability would be of observing such rearrangements in other tumors.

The DNAs from 29 other tumors containing one to three proviruses (some of them were analyzed in reference 7) were digested to completion with *Eco*RI, *Kpn*I, and *Hin*dIII, and their respective blots were hybridized to probe 1. As illustrated for some of the *Eco*RI (Fig. 2A, lanes 3 to 12) and *Hin*dIII (Fig. 2B, lanes 3 to 12) digests, no rearrangement was observed in the cellular sequences corresponding to those flanking at both 5′ and 3′ sides the provirus in tumor 15-4. For example, the 5-kb fragment was the only one present in *Eco*RI digests, and the 15-kb fragment was the unique fragment in *Hin*dIII digests.

To avoid deriving conclusions from only one set of experiments, we performed a similar analysis starting from another tumor bearing a single provirus, tumor 1351. The probe used for this investigation was the *Eco*RI right-hand junction fragment (probe 2) (Fig. 1). The cellular moiety of this probe hybridized to a 2.9-kb (7), a 10-kb (Fig. 3, lane 2), and a 17-kb (not shown) fragment in (respectively) *Eco*RI, *Hin*dIII, and *Kpn*I digests of calf thymus DNA. The restriction map of tumor 1351 provirus and its flanking cellular sequences is shown in Fig. 1. After annealing to probe 2, essentially due to its cellular sequences, the T1351 *Eco*RI digest showed a strong positive reaction with a 3.4-kb 3′ junction fragment in addition to the 2.9-kb fragment derived from the uninterrupted locus (7). The T1351 *Hin*dIII annealing pattern showed a 9.3-kb 3′ junction

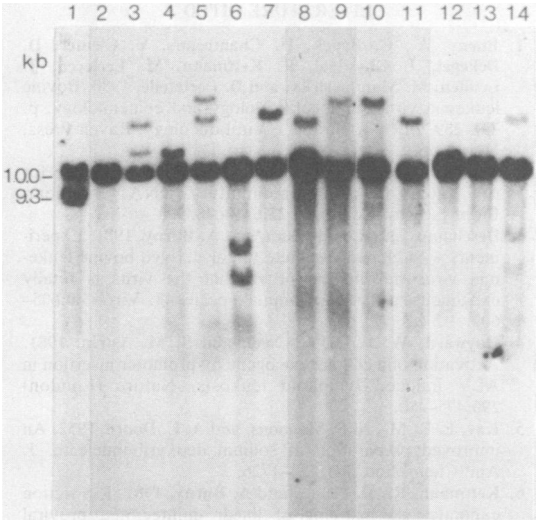

FIG. 3. DNA hybridizations using probe 2. Ten micrograms each of bovine tumor 1351 DNA (lane 1), calf thymus DNA (lane 2), and 12 other tumor DNAs (lanes 3 to 14) were digested to completion by *Hin*dIII, electrophoresed on a 0.6% agarose gel, and treated as described for Fig. 2B. Hybridization to ^{32}P-labeled probe 2 (0.010 μg/ml; 6×10^8 cpm/μg) was for 24 h at 65°C. The autoradiography was a 3-day exposure.

fragment in addition to the normal 10-kb fragment (Fig. 3, lane 1), and the T1351 *Kpn*I digest exhibited positive hybridization to a 26-kb 3′ junction fragment in addition to the normal 17-kb fragment (not shown). The DNAs from 29 other tumors containing one to three proviruses were digested with *Eco*RI, *Kpn*I, and *Hin*dIII, and their blots were hybridized to probe 2. As previously shown for some *Eco*RI digests (7) and illustrated here for some *Hin*dIII digestions (Fig. 3, lanes 3 and 4), again no rearrangement was observed in the cellular sequences corresponding to those flanking at both 5′ and 3′ sides the provirus in tumor 1351. For example, in addition to viral fragments weakly revealed by the long terminal repeat moiety of probe 2 (this was verified by annealing to a BLV *rep* probe), a unique *Hin*dIII cellular fragment of 10 kb was present in all DNAs tested. Indeed, data obtained with probe 2 were easily interpreted as the probe was made of about 700 bp of viral sequences and 2,700 bp of flanking cellular sequences. Thus, hybridization signals corresponding to cellular sequences were expected to be quite a bit stronger than those revealing viral fragments.

According to the statistical analysis presented above and in Table 1, if a preferential domain for BLV integration exists, it must be very large. Indeed, the probability of detecting no provirus in the 15- and 17-kb sequences surrounding the T15-4 and T1351 proviruses in 28 other independent tumors is only 5% for a 315-kb domain and reaches 50% for a 1,304-kb domain.

DISCUSSION

Nondefective avian leukosis viruses (ALVs) (13), MMTV (13), and BLV (1) are oncogenic viruses that induce cell transformation after long latent periods. ALVs and MMTV seem to integrate without a small chromosomal domain of their host genomes. Indeed, ALV was found next to the c-*myc* locus in 31 of 37 cases (4), and MMTV integrated inside a 35-kb region in 18 of 26 tumor cases (10). In the latter case, no specifically activated oncogene has been identified so far. In both MMTV and ALV systems, integration at highly preferential genomic sites was readily observed from restriction profiles of tumor DNAs after annealing to a viral probe. The latter approach was applied to the BLV system and indicated that BLV-induced tumors (i) are monoclonal, (ii) contain at least a piece of the BLV provirus, and (iii) accommodate the proviral information at many different locations with no obvious relationship to each other.

However, restriction mapping with virus-specific hybridization reagents (7) or even with a cellular fragment adjacent to one provirus (7) might have failed to demonstrate that proviruses in tumors were at different positions within the same chromosomal domain. We wanted to clarify this point. Therefore, we investigated in DNAs from 28 independent BLV-induced tumors whether proviral integration induced rearrangements of the cellular sequences homologous to those flanking single proviruses present in two lymphoid tumors (tumors 15-4 and 1351). The host sequences under investigation were one of 15 kb starting from tumor 15-4 and one of 17 kb starting from tumor 1351. In no case did proviral integration occur in cellular sequences corresponding to those implicated in the tumors of origin. Assuming the existence of a preferential region of integration for BLV provirus, statistical analysis based on the data obtained for T15-4 and T1351 proviruses points to the existence of a large domain [x = 1,304 kb, $P(0)$ = 50%] rather than a small one [x = 211 kb, $P(0)$ = 1%]. In other words, if this domain were very small, we might expect to find, with a high probability, a provirus within the 32 (15 + 17)-kb region. This was not observed. In contrast, if the domain were very large, we might expect to detect, with a low probability, a provirus in the 32-kb region. This latter possibility was found to be correct according to our experimental data. These results are still compatible with the following phenomena.

Integration into a given chromosome or part of it (one bovine chromosome = ca. 6×10^4 kb). In this case, it might be postulated that the provirus acts through its position by a long distance effect on a particular gene(s). At any rate, overexpression of oncogenes (*myc*, *erb*, *myb*, *src*, *abl*, *ras*, *fes*, *sis*) has been tested and found to be negative (R. Kettmann, E. H. Westin, G. Marbaix, J. Deschamps, F. Wong-Staal, R. C. Gallo, and A. Burny, Haematol. Bluttransfus., in press). Experiments aiming at identification of chromosomes harboring a BLV provirus are in progress in our laboratory, using cell fusion techniques.

Random integration. It might well be that BLV provirus does not integrate into a preferential chromosomal domain. If this turns out to be true, it must be postulated that the virus does not act through its position but rather through a virus-coded protein in cells that allow BLV expression (tumor cells do not). Mechanisms involving retrotranscription of potentially oncogenic mRNA or cell transformation by viral products—their continuous production not being required for the maintenance of the leukemic stage (7)—are possible but hypothetical issues at present.

The observation that BLV provirus did not integrate in a narrow domain of the chromosomal DNA is in agreement with observations that no proximate downstream promotion takes place in BLV tumor cells (7). Maintenance of the tumorous stage by a provirus-induced regional effect on a crucial gene(s) remained, however, an attractive hypothesis.

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ADDENDUM

Recent data obtained from analysis of hamster-bovine cell hybrids indeed indicated that the BLV provirus was not present in the same chromosome in tumors 15-4 and 1351.

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