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Genomic integration of bovine leukemia provirus: Comparison of persistent lymphocytosis with lymph node tumor form of enzootic bovine leukosis

(restriction endonucleases/DNA·DNA hybridization)

RICHARD KETTMANN*†, YVETTE CLEUTER*, MARC MAMMERICKX‡, MICHÈLE MEUNIER-ROTIVAL§, GIORGIO BERNARDI§, ARSÈNE BURNY*†, AND H. CHANTRENNE*

*Département de Biologie Moléculaire, Université Libre de Bruxelles, 1640 Rhode St-Genèse, Belgium; †Faculté des Sciences Agronomiques de l'Etat, 5800 Gembloux, Belgium; ‡Institut National de Recherches Vétérinaires, 1180 Uccle, Belgium; and §Laboratoire de Génétique Moléculaire, Institut de Recherche en Biologie Moléculaire, Université Paris VII, 2, Place Jussieu, 75005 Paris, France

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ABSTRACT **Integration of bovine leukemia proviral DNA in the genome of infected cells was investigated in cattle affected by either the persistent lymphocytosis or the lymph node tumor form of enzootic bovine leukosis. In persistent lymphocytosis, proviral DNA was found to be integrated at a large number of genomic sites in one-fourth to one-third of circulating leukocytes. In the lymph node tumor form, in contrast, proviral DNA was found to be integrated at one or very few sites in the genomes of a larger fraction of both circulating leukocytes and lymph node tumor cells.**

Enzootic bovine leukosis (EBL) is a lymphoproliferative disease of cattle induced by an exogenous retrovirus, the bovine leukemia virus (BLV) (1–3), whose target cell is the B lymphocyte (4). Two forms of the disease are known: persistent lymphocytosis (PL), characterized by a permanent large number of peripheral lymphocytes (5–7), and a tumoral form, characterized by lymph node tumors. These two forms generally represent the early and the late stage of the disease, respectively. In some cases, however, these two pathological conditions do not affect the same cattle herds, suggesting that they may represent separate responses to BLV infection, both of them being under the control of genetic factors (8–10). In general, the fate of BLV-infected animals is variable and depends upon several factors, including age, genetic make-up, environmental factors, and immunological surveillance (see ref. 11 for a review).

In the present work, we have compared proviral integration into the genomes of host cells of naturally infected animals that were in either the PL or the tumor stage of the disease. The main result obtained in these investigations was that proviral copies were found to be integrated at several sites in the genomes of one-fourth to one-third of the leukocytes of animals in PL, but at only one or very few sites in the genomes of a larger fraction of leukocytes or tumor cells of animals in the tumor stage of the disease.

MATERIALS AND METHODS

Bovine tissues and cells were collected from seven animals with EBL. Four animals (nos. 928, 641, 2586, and 4) were in PL and three (nos. 15, 950, and 82) in the tumor stage of the disease. Leukocytes (W928, W641, W2586, W4, W15, W950, and W82), lymph node tumors (T15 and T950), and the thymus of a normal calf were used as sources of DNA.

Preparation of virus and viral DNA, cDNA synthesis, DNA extraction, digestion with restriction endonucleases, and gel electrophoresis were done as described (12, 13).

Gel transfer and filter hybridization were carried out as described (13, 14), except that BLV [^{32}P]cDNA was prehybridized for 16 hr with 3 mg of sonicated, denatured calf thymus DNA per ml; this largely prevented spurious hybridization of the probe with satellite and ribosomal DNA (13). Filters were exposed at −70°C to preflashed Kodak RP-Royal X-Omat film in the presence of Siemens "special" intensifying screens for 2–5 days.

Liquid hybridization was performed as described (2).

RESULTS

Persistent lymphocytosis

As shown in Fig. 1*A*, BLV [^{32}P]cDNA hybridized on a large number of *Eco*RI fragments from leukocyte DNA of animals in PL. Molecular weights of the fragments cover a wide range, varying between 5–6 × 10^6 and 10–12 × 10^6. In some samples, hybridization bands emerged over the continuous background, an indication that some integration sites were preferred. No fragment with M_r lower than 5 × 10^6 was apparent. The control, calf thymus DNA digest, showed a weak band at M_r 5 × 10^6, corresponding to ribosomal DNA (13, 14). The integrated BLV proviruses examined so far (13) showed none or at the most one *Eco*RI restriction site.

BLV [^{32}P]cDNA also hybridized on two *Bam*HI fragments, M_r 2.0 × 10^6 and 1.3 × 10^6, from the above DNAs. Weak hybridization bands were also observed in some cases on both higher and lower M_r fragments (Fig. 1*B*); the control DNA digest did not show any hybridization.

The sum of M_rs of *Bam*HI fragments was 2.0 × 10^6 plus 1.3 × 10^6, which equaled 3.3 × 10^6; unintegrated linear proviral DNA showed a M_r of 6.4 × 10^6 (data not shown). One would have expected to consistently find two additional fragments with a total M_r of 3.1 × 10^6 (6.4 × 10^6 minus 3.3 × 10^6) if unintegrated forms had been present in detectable amounts. Our results rule out this possibility.

Lymph node tumor stage

As shown in Fig. 2*A*, BLV [^{32}P]cDNA hybridized on two *Eco*RI fragments, M_r 6.0 × 10^6 and 3.7 × 10^6, of W950 DNA; two *EcoRI* fragments, M_r 6.0 × 10^6 and 1.0 × 10^6, of W82 DNA; and three *Eco*RI fragments, M_r 17 × 10^6, 9.6 × 10^6, and 1.2 × 10^6, of W15 DNA. W15 DNA showed, in addition, a spurious

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Abbreviations: EBL, enzootic bovine leukosis; BLV, bovine leukemia virus; PL, persistent lymphocytosis; C_0t, product of initial DNA concentration (mol of nucleotide/liter) and renaturation time (sec).

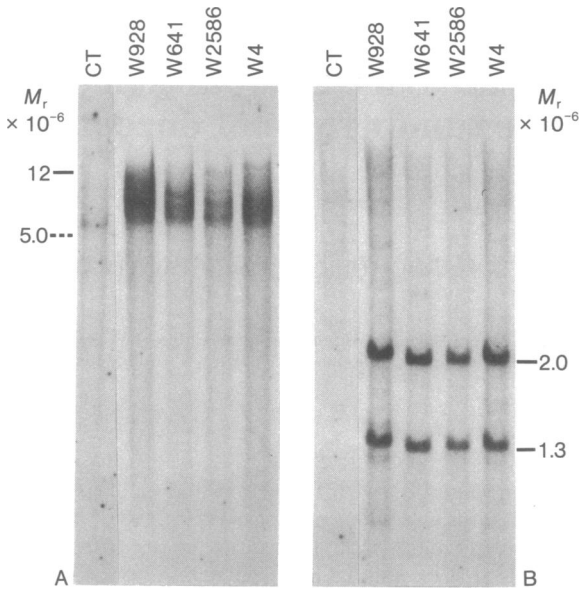

FIG. 1. Hybridization patterns of BLV [^{32}P]cDNA on DNA restriction fragments from leukocytes of animals in PL. Twenty micrograms each of leukocyte W928, W641, W2586, and W4 and calf thymus (CT) DNAs were exhaustively digested by either *Eco*RI (*A*) or *Bam*HI (*B*) and electrophoresed on a 1% agarose gel. Only fragments larger than 0.5×10^6 were detected on these gels. After the restriction fragments were transferred to nitrocellulose sheets, they were soaked in the prehybridation mixture at 65°C and hybridized for 24 hr with 5×10^6 cpm of BLV [^{32}P]cDNA per ml; this probe was separately prehybridized with 3 mg of calf thymus DNA per ml. The last washing was performed in 45 mM NaCl/4.5 mM Na citrate. Autoradiograms are shown.

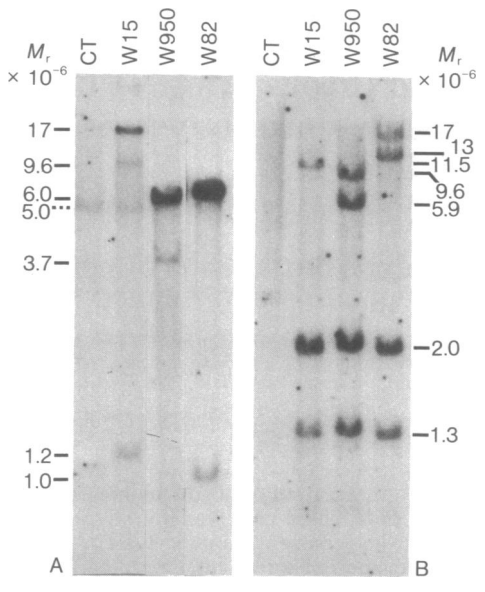

FIG. 2. Hybridization patterns of BLV [^{32}P]cDNA on DNA restriction fragments from leukocytes of animals with lymph node tumors. Twenty micrograms each of leukocyte W15, W950, and W82 and calf thymus (CT) DNAs were exhaustively digested by either *Eco*RI (*A*) or *Bam*HI (*B*) and electrophoresed on a 1% agarose gel. After the restriction fragments were transferred to nitrocellulose sheets, they were treated as described in the legend to Fig. 1. Autoradiograms are shown. Because a new calibration curve was used, some M_r values given here are slightly different from those of ref. 13.

hybridization band, M_r 5.0×10^6, also shown by the control DNA and corresponding to ribosomal DNA (refs. 13 and 14; see also above). This band might also be present in the DNAs of W950 and W82. The results obtained with W15 are similar to those already reported (13) except that in previous work only the strong M_r 17×10^6 and 1.2×10^6 bands were seen. We will show that these correspond to the provirus-positive segments which predominate in these cells; the other provirus-positive segments originate the M_r 9.6×10^6 band and another band, M_r 0.6×10^6, which is too weak to be seen in Fig. 2*A* (see also Fig. 3*A*). Previous work showed that spleen cells from animal 15 showed the same predominant provirus-positive bands as leukocytes (13).

BLV [^{32}P]cDNA also hybridized on two common *Bam*HI fragments, M_r 2.0×10^6 and 1.3×10^6, of DNAs from W15, W950, and W82, and on two different *Bam*HI fragments of the DNAs from W82 (M_r 17×10^6 and 13×10^6), W950 (M_r 9.6×10^6 and 5.9×10^6), and W15 (M_r 11.5×10^6 and, possibly, 2.0×10^6; the latter suggestion is derived from the strong relative intensity of the M_r 2.0×10^6 band) (Fig. 2*B*).

BLV [^{32}P]cDNA also hybridized on the same *Xba* I fragments (M_r 6.3×10^6, 2.6×10^6, and 2.3×10^6) from the DNAs of five lymph node tumors of animal 950 (Fig. 3*B*). A faint M_r 1.0×10^6 band, present in all *Xba* I digests including that of the control DNA, corresponds to contaminating DNA (ribosomal DNA or satellite DNA; see ref. 13). The situation in animal 15 was more complex than that in animal 950. Hybridization of the viral probe took place (Fig. 3*B*) on four fragments (M_r 7.1×10^6, 3.8×10^6, 1.7×10^6, and 0.7×10^6) from the DNAs of three tumors, T15-3, T15-4, and T15-5 and on four fragments (M_r 3.1×10^6, 2.0×10^6, 1.7×10^7, and 0.7×10^6) from the DNA of tumor T15-2. Interestingly, the two smallest fragments from tumor T15-2 were those already found in tumors T15-3, T15-4, and T15-5; these fragments, probably corresponding to internal proviral fragments, were not present in the DNA from tumors of animal 950. The sum of M_rs of BLV-positive fragments encountered in tumor T15-2 DNA was 7.5×10^6, a good indication that a single proviral copy was integrated per haploid genome. Hybridization of the viral probe for animal 15 also occurred on six fragments from the DNA of tumor T15-1; this pattern was equal to the sum of the other two patterns, the bands corresponding to the DNA of tumors T15-3, T15-4, and T15-5 being predominant. The hybridization pattern exhibited by the *Eco*RI digest of the T15-1 DNA (Fig. 3*A*) was similar to that shown by the leukocyte DNA of the same animal (Fig. 2*A*) except that the M_r 17×10^6 and 1.2×10^6 bands were weaker than the M_r 9.6×10^6 and 0.6×10^6 bands, another indication of the existence of two sets of provirus-positive segments in animal 15. The faintness of the M_r 17×10^6 band and the smear under the M_r 9.6×10^6 band are probably due to the fact that the particular DNA preparation used had a relatively low M_r.

In order to further compare PL and the tumor phase of EBL, BLV [^{3}H]cDNA was hybridized in liquid solutions to DNAs of infected cells from animals with PL and those with tumors. Fig. 4*A* displays the results obtained when the labeled BLV probe was annealed to DNAs from circulating leukocytes of the four animals with PL studied in Fig. 1*A*. DNA from FLK cells infected by BLV, a 1:5 dilution of this DNA with normal bovine DNA, and normal calf thymus DNA were taken as references. In Fig. 4*B*, the rates of annealing of the same BLV cDNA probe with T15-2, T950-1, W15, and W82 DNAs are represented. The same DNAs as in Fig. 4*A* were used as references.

C_0t curves for BLV-infected FLK cells and T15-2 were superimposed, indicating that the frequency of BLV proviral sequences was identical in both DNAs (Fig. 4*B*). [C_0t is the product of the initial DNA concentration (mol of nucleotide/liter) and renaturation time (sec).] In a series of cytotoxicity experiments, it has been shown (15) that every BLV-infected

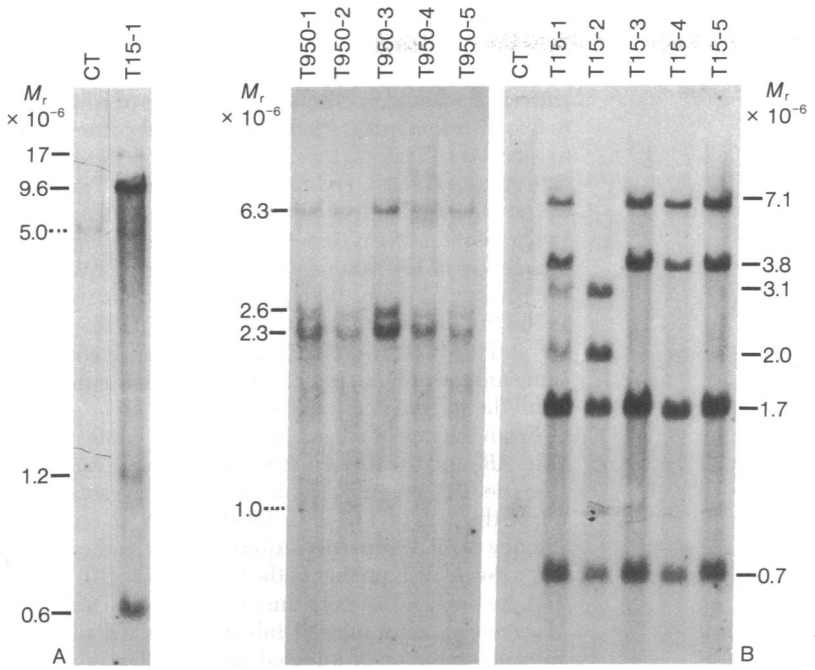

FIG. 3. Hybridization patterns of BLV [^{32}P]cDNA on DNA restriction fragments from lymph node tumors. Twenty micrograms each of DNAs from calf thymus (CT) and tumor T15-1 were digested by *Eco*RI; 20 μg each of DNAs from CT, tumors T15-1 to T15-5, and tumors T950-1 to T950-5 were digested by *Xba* I. All samples were electrophoresed on 1% agarose gels. After the restriction fragments were transferred, the nitrocellulose sheets were treated as described in the legend to Fig. 1. Autoradiograms are shown. Because a new calibration curve was used, some M_r values given here are slightly different from those of ref. 13.

FLK cell expressed BLV glycoprotein antigens and thus carried the BLV genome. Taken together with the restriction pattern of Fig. 3*B*, showing that BLV provirus was present in T15-2 as a single copy per haploid genome, the results imply that the vast majority, if not all, of the cells of T15-2 harbored one copy of BLV provirus per haploid genome. The rates of annealing of BLV [^{3}H]cDNA to W15, W82, and T950-1 DNAs were lower, indicating that less than one proviral copy per cell was present in these tissues, a conclusion also valid for the circulating lymphocytes of animals with PL (Fig. 4*A*). From the relative rates of annealing measured in these experiments, we roughly concluded that one-fourth to one-third of leukocytes were BLV carriers in both PL and tumor stages of EBL. Interestingly, W15 and W82 C_0t curves (Fig. 4*B*) were quite similar even with leukocyte counts of 16×10^3/mm^3 and 170×10^3/mm^3, respectively. The leukocyte counts of the animals with PL were 21×10^3, 24×10^3, 23×10^3, and 55×10^3 per mm^3 in animals 928, 641, 2586, and 4, respectively.

DISCUSSION

The results obtained in the present work bear essentially on the sites of integration of bovine leukemia provirus in the two different forms of EBL. The data obtained upon hybridization of the BLV probe with DNAs from leukocytes of animals in PL (Fig. 1) are consistent with the presence in these genomes of a large number of integration sites for the provirus. Hybridization of the probe with DNA degraded by *Eco*RI in fact shows a smear covering a wide M_r range, $5–6 \times 10^6$ to $10–12 \times 10^6$, with some bands emerging above the continuous background. The latter finding suggests that a number of identical integration sites are used by the genomes of the host cells. Most of the *Eco*RI fragments were large enough to contain one full-length or almost full-length copy of the provirus; they might well do so because in the two previously explored cases (13) one proviral genome (animal 92) contained no *Eco*RI site and the other one (animal 15) contained one *Eco*RI site near one end of the provirus. Some large fragments may, however, contain only a segment of provirus; some other fragments are just too short to contain more than that. In these cases, the other cellular DNA fragment containing a proviral segment may not be detected because either it is too short (our experiments did not detect fragments smaller than M_r 0.5×10^6) or the proviral segment is too short or because hybridization does not detect proviral fragments that are not sufficiently represented in cellular DNA (see below). These different reasons are not mutually exclusive.

The multiplicity of integration sites in the cells of animals in PL is confirmed by the experiments carried out on *Bam*HI fragments. The viral probe hybridized mainly on two restriction fragments having identical M_rs, 2.0×10^6 and 1.3×10^6, in the DNAs from leukocytes of four different animals. This indicates that the fragments may be internal proviral fragments and suggests that the same viral variant may be involved in the PL of the four animals. Because the sum of the M_rs of the two fragments is lower than that of the provirus (6.4×10^6), other chromosomal fragments possibly contain proviral segments. A few faint bands of different M_r are in fact seen in the restriction digests of different DNAs. Clearly, however, these correspond to some predominant flanking sequences and indicate the use of some identical integration sites, such as the strong *Eco*RI bands emerging above the continuous hybridization background. The majority of individual provirus-positive flanking sequences are probably present in one copy per many cell genomes and are therefore not detected.

The pattern of proviral integration in the leukocytes of animals carrying lymph node tumors is completely different from that described above in that a well-defined number of provirus-positive bands are present in both *Eco*RI and *Bam*HI digests (Fig. 2). The latter digests show two common hybridizing bands, M_r 2.0×10^6 and 1.3×10^6, in all DNAs. These bands, in all likelihood corresponding to internal proviral fragments, were also found in the four animals in PL (Fig. 1), a preliminary indication that the same viral variant was present in all cases. In addition, *Bam*HI digests revealed two additional provirus-positive cellular DNA fragments, which differed from animal to animal and corresponded to flanking sequences that were different in different animals. The results obtained with *Eco*RI digests agree with the interpretation; W950 and W82 appear

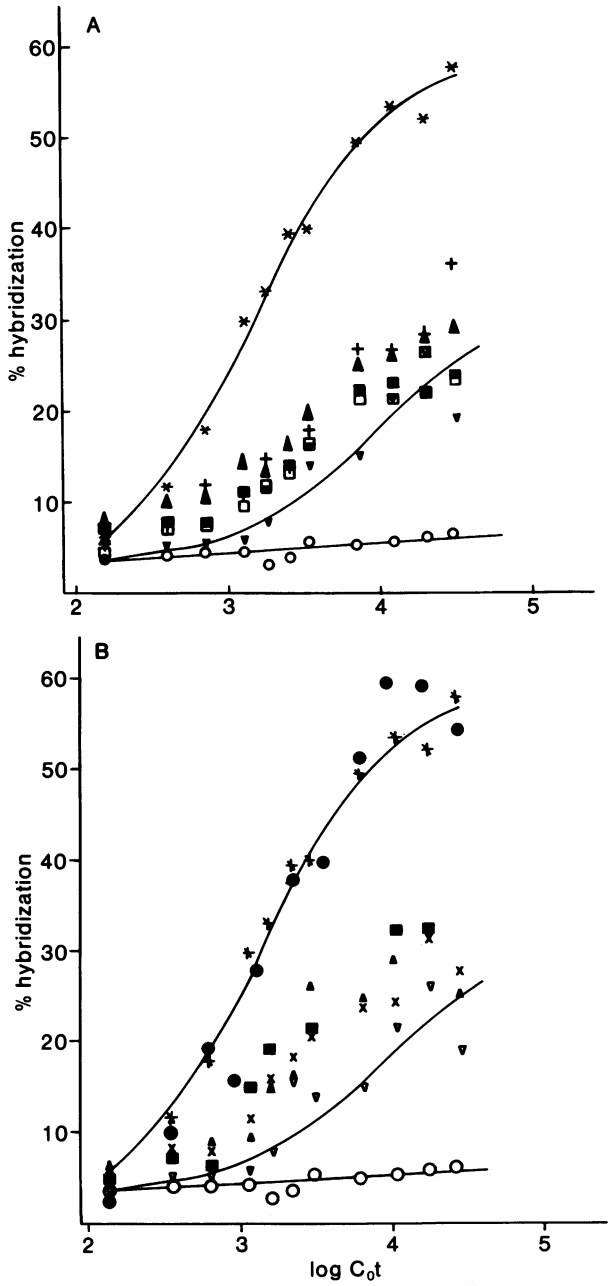

FIG. 4. Kinetics of hybridization of BLV [^{3}H]cDNA and cellular DNAs. BLV [^{3}H]cDNA (2500 cpm) and 500 μg of cellular DNA were hybridized in 0.4 M sodium phosphate, pH 6.8/0.05% NaDodSO$_4$. At various C_0t values, samples were assayed for resistance to nuclease S1. (*A*) DNAs from: *, BLV-infected FLK cell line; ▽, BLV-infected FLK diluted 1:5 with normal bovine DNA; ○, calf thymus; +, W4; ▲, W641; ■, W2586; and □, W928. (*B*) DNAs from: *, BLV-infected FLK cell line; ●, T15-2; ▽, BLV-infected FLK cell line diluted 1:5 with normal bovine DNA; ○, calf thymus; ■, W82; ▲, T950; and X, W15.

to hybridize the probe on one fragment (M_r about 6×10^6) containing most of the provirus and on one flanking fragment of different M_r. W15 is interesting in that two different sets of provirus-positive fragments appear to be present: M_r 17×10^6 and 1.2×10^6 and M_r 9.6×10^6 and 0.6×10^6. The first set is largely predominant over the other one and was the only one to be detected in earlier experiments (13). This set of fragments corresponds to the set seen only by hybridization on *Bam*HI fragments.

The results obtained with lymph node tumor DNA are similar to those just described in that they indicate well-defined hybridization patterns. The pattern is unique for five tumors in animal 950. Animal 15 is interesting because three sorts of patterns are found in different tumors, two of which correspond to two different integration sites; the third pattern corresponds to a mixed cell population in which both different integration sites are represented. Hybridization patterns on an *Eco*RI digest of the tumor DNA from animal 15 (Fig. 3*A*) indicate that the two proviral patterns present are the same as those found in the leukocytes of the same animal, but their relative proportions are reversed. Finally, both proviral restriction patterns present in the tumors of animal 15 shared two *Xba* I bands (M_r 1.7×10^6 and 0.7×10^6) already found in leukocyte and spleen of the same animal (13), an indication of a common proviral variant in all the infected cells of this animal.

From the rate of annealing of the BLV probe to T15-2 DNA (Fig. 4*B*) and the *Xba* I restriction pattern (Fig. 3*B*) it appears that one BLV proviral copy is present in the vast majority, if not all, of the cells of T15-2. In T950-1 DNA, in contrast, the frequency of BLV provirus is quite smaller, indicating that only a fraction of cells present in the tumor carries BLV information. Despite the fact that both tumors are of comparable size, T15-2 is a homogeneous mass of infected cells whereas T950-1 is a mixed population of infected and noninfected cells. That the concentration of BLV sequences was lower in any T950 than in any T15 was indeed already suggested by the relatively dimmer bands of hybridization obtained in T950 restriction gels (Fig. 3*B*).

The C_0t curves obtained with W15 and W82 DNAs (Fig. 4*B*) and with W928, W641, W2586, and W4 DNAs (Fig. 4*A*) indicate that, in all cases examined, circulating leukocytes are mixed populations consisting of roughly one-fourth to one-third of infected cells. This observation holds true for animals with PL or tumors, seems to be independent of the total leukocyte counts (16×10^3/mm^3 in animal 15; 170×10^3/mm^3 in animal 82), and correlates well with reports of others (16, 17) using different techniques.

In conclusion, the main result of this investigation is that the PL and the tumor stage of EBL are characterized by a polyclonality of infected cells in PL and a mono- or oligoclonality in the tumor stage, these terms referring to the BLV integration. Under these circumstances, it is possible that a selection of BLV-positive cells takes place during EBL. In principle, selection of a given preexisting clone may be favored by the genetic composition of the provirus or by its integration site or it might be the result of an event independent of the virus. It may also be that the continuous process of infection would lead to integration of the provirus at some critical site for control of cell proliferation.

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