Integration of bovine leukemia virus DNA in the bovine genome

|provirus/Cs₂SO₄/3,6-bis(acetatomercurimethyl)dioxane centrifugation/restriction endonucleases/agarose gels|

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ABSTRACT   DNA preparations from circulating leukocytes, lymph node tumors, and spleens of three bovine leukemia virus-infected cattle were fractionated by Cs₂SO₄/3,6-bis(acetatomercurimethyl)dioxane density gradient centrifugation. Bovine leukemia virus proviral sequences were found in large GC-rich fragments having a buoyant density in CsCl close to 1.708 g/cm³. Provirus integration, therefore, does not take place at random locations in the host genome, but in a specific class of DNA segments. Hybridization of cDNA synthesized on viral RNA to EcoRI and Xba I restriction fragments of the DNA from infected cells showed that: (i) only one copy of proviral DNA is integrated per haploid genome; (ii) different restriction patterns were found in the proviral DNAs present in the genomes of different animals, providing evidence for the existence of several viral strains or mutants; and (iii) different integration sites for the proviral DNA were found in the genome of different animals and of different infected cells in the same animal. The latter finding strongly suggests a polyclonal origin of bovine leukemia virus-infected cells.

Bovine leukemia virus (BLV), an exogenous retrovirus (1–3) of cattle, is the pathogenic agent of enzootic bovine leukosis. The target cell is the B lymphocyte (4). BLV infection may induce persistent lymphocytosis (characterized by an increased number of apparently normal B lymphocytes in the peripheral blood of the infected animal) and, in a later stage, lymph node tumors.

In the present work, we have tried to answer a number of questions concerning the integration process of proviral DNA into the host cell genome—namely: (i) whether integration occurs at random in the bovine genome or in one of the DNA components in which this genome can be resolved by density gradient centrifugation techniques (5–7); (ii) how many proviral copies are integrated in the genome; (iii) whether integration occurs at the same chromosomal location in the genomes of different infected animals and in the genomes of different infected cells in the same animal; and (iv) whether different viral strains or mutants can be detected by studying the proviral sequences.

MATERIALS AND METHODS

Bovine Tissues and Cells. Bovine material was collected from two field cases of enzootic bovine leukosis (animals 15 and 928) and from an experimentally infected animal (animal 92); in the latter case, the BLV was obtained from the infected herd to which animal 928 belonged. Animals 15 and 92 were at the tumoral stage of the disease with splenomegaly (8). Animal 928 was in persistent lymphocytosis without tumors (8). Leukocytes (W15 and W928), two lymph node tumors (T15 and T92), and spleens (S15 and S92) were used as sources of DNA. The thymus

(CT) from a normal calf was used as a source of control DNA.

Preparation of Virus and Viral RNAs and cDNA Synthesis. BLV production and purification and viral RNA extraction and purification were carried out as described (9). The two poly (A⁺) 38S RNAs present in each viral particle were prepared from 60–70S viral RNA by heat denaturation, chromatography on oligo(dT)-cellulose, and sedimentation in a denaturing sucrose gradient (9).

BLV [³²P]cDNA (4.2 × 10⁸ cpm/μg) was synthesized as in ref. 2, except that |α-³²P|dTTP (350 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ becquerels) (Amersham) was used as the labeled deoxytriphosphate at a final concentration of 0.057 mM. cDNAs synthesized on 60–70S RNA and 38S RNA will be called here cDNA-70 and cDNA-38, respectively. cDNA synthesized on 38S RNA protected, at cDNA-to-RNA molar ratios of 1 and 3, 78% and 100%, respectively, of the viral genome against a mixture of RNase A and RNase T1. This shows that the totality of the viral genome was represented in cDNA-38. The size of cDNAs was determined by gel electrophoresis by using native and denatured Hae III digests of φX174 DNA as M_r markers and was found to be 100–400 nucleotides long.

DNA Preparation and Fractionation. DNA was purified as described by Kay et al. (10). The M_r of the DNA from the control animal was about 15×10^6 as estimated from sedimentation velocity; those of all other DNA preparations were higher than 20×10^6. The latter M_r estimates were obtained by electrophoresis on 0.5% agarose gel by using undigested λ DNA and EcoRI and Sal I digests of λ DNA as M_r markers. DNA was fractionated by centrifugation in Cs₂SO₄/3,6-bis(acetatomercurimethyl)dioxane (BAMD) density gradients as described by Cortadas et al. (6). Analytical CsCl density gradient centrifugations were done as described by Filipski et al. (5).

DNA Digestion with Restriction Endonucleases and Gel Electrophoresis. EcoRI (Boehringer Mannheim) digestions were carried out according to Polisky et al. (11); Xba I (BioLabs) digestions were done according to conditions given by the manufacturers. Vertical agarose (Sigma) slab gels (0.4 × 20 × 20 cm) were used for gel electrophoresis. Ethidium bromide staining and photography of the gel were done according to Prunell et al. (12). The EcoRI or HindIII fragments of phage λ DNA and Hae III fragments of simian virus 40 DNA were used routinely as M_r markers (13, 14).

Gel transfer and hybridization were carried out as described (15).

Ribosomal DNA probes. Two recombinant pBR313 plas-

Abbreviations: BAMD, 3,6-bis(acetatomercurimethyl)dioxane; BLV, bovine leukemia virus; CT, calf thymus.
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mids containing the two *Eco*RI fragments of *Xenopus laevis* ribosomal DNA (rDNA) were used as probes after ^{32}P-labeling by nick translation (16) to a specific activity of 4×10^6 cpm/μg. Recent work (15) has shown that *X. laevis* rDNA hybridizes with the ribosomal genes from the bovine genome and that some spurious hybridization also occurs with bovine satellite DNAs.

RESULTS

Hybridization of BLV cDNA with *Eco*RI Digests of Unfractionated Bovine DNA. The kinetics of annealing of BLV [^{3}H]cDNA-70 with bovine DNA had shown (2) that the DNA from tumor cells of animal 15 (T15 DNA) contained very few (probably one) BLV proviral DNA copies per haploid genome. The detection of proviral sequences in an *Eco*RI digest of T15 DNA was done here as follows. BLV [^{32}P]cDNA-70 was hybridized to *Eco*RI digests of T15 and CT DNAs. Both digests shared two strong and two weak hybridization bands (Fig. 1). The strong bands had the M_rs (16×10^6 and 5×10^6) of *Eco*RI fragments of bovine rDNA and the faint bands had M_rs (1.5×10^6 and 1.1×10^6) of bovine satellite DNAs (15). In addition, the T15 DNA digest showed two bands of M_r 8×10^6 and 0.6×10^6 which were absent in CT DNA and corresponded to fragments containing sequences of viral origin.

Hybridization of BLV cDNA with Restriction Digests of Fractionated Bovine DNA. DNA preparations from T15, W15, S15; T92, S92; W928; and CT were fractionated in preparative Cs$_2$SO$_4$/BAMD density gradients. Fig. 2A shows the absorbance profile obtained with T92 DNA. After analytical centrifugation in CsCl of every other fraction from the preparative gradients, fractions were pooled as shown in Fig. 2A. The analytical CsCl profiles of the pooled fractions so obtained from T92 DNA are shown in Fig. 2B. Similar results were obtained with all other DNA preparations.

BLV cDNA-38 and rDNA probes were hybridized on *Xba* I digests of each pooled fraction. Fig. 3 shows, as an example, the results obtained with CT and T92 DNA preparations. The rDNA probe strongly hybridized on a fragment of about M_r
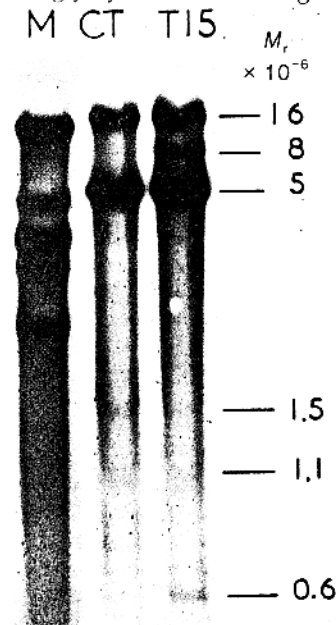

FIG. 1. Hybridization of BLV cDNA-70 to *Eco*RI digests of CT and T15 DNAs. Total CT and T15 DNAs (30 μg) were digested by *Eco*RI and electrophoresed on a 1% agarose gel. Transfer and hybridization were carried out with BLV [^{32}P]cDNA-70. The last washing was performed in standard saline citrate. *Eco*RI fragments of phage λ ^{32}P-labeled DNA were used as M_r markers (M).
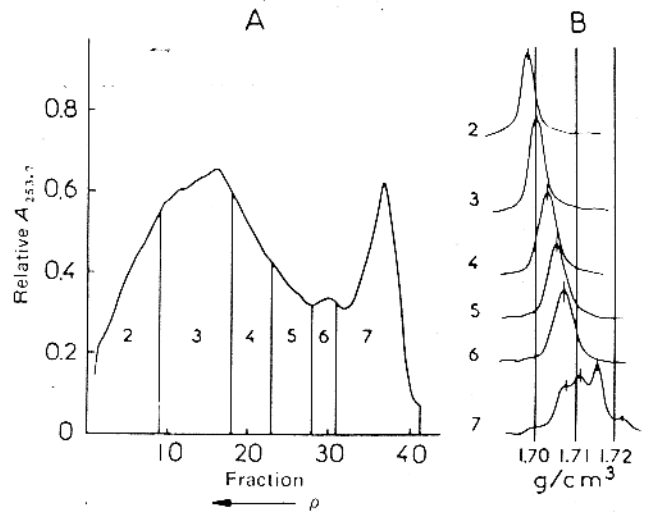

FIG. 2. Analysis of T92 DNA by density gradient centrifugation. (A) Fractionation of T92 DNA in Cs$_2$SO$_4$ density gradient in the presence of BAMD at a BAMD-to-nucleotide molar ratio of 0.1. T92 DNA (60 A_{260} units) in 100 mM Na$_2$SO$_4$/5 mM Na$_2$B$_4$O$_7$, pH 9.2, containing BAMD and Cs$_2$SO$_4$ (ρ = 1.47 g/cm^3) was centrifuged in a Beckman type 30 rotor at 25°C for 110 hr at 25,000 rpm. Fractions were collected and the relative absorbance was monitored at 253.7 nm with an LKB Uvicord (Bromma, Stockholm, Sweden). The arrow indicates the direction of density increase in the gradient. Vertical lines determine the limits of the pooled fractions which are numbered from 2 to 7, number 1 being the pellet. (B) Analytical CsCl density gradient centrifugation profiles of pools 2–7.

17×10^6, which was mainly found in pool 7 (Fig. 3 A and B). A faint hybridization band corresponded to a fragment of M_r 2.4×10^6 and was found in pool 6 (Fig. 3 A and B). This hybridization band corresponded to an ethidium bromide-stained band present in the *Xba* I digest (not shown) and is due, therefore, to a restriction fragment of one of the satellite DNAs present in the GC-rich fractions of bovine DNA (6). The viral probe showed, in addition to the hybridizations exhibited by the rDNA probe, hybridization on other DNA fragments present only in tumor DNA samples; among them were two fragments of M_r 5.9×10^6 and 2.4×10^6, which were present in pools 6 and 7 (Fig. 3D). Although the latter fragment coincided in size with the satellite fragment present in pool 6, its viral origin is clearly demonstrated by the fact that it was also present in pool 7, in which no satellite hybridization was found (Fig. 3C), and by its strong relative intensity. This latter point will be further commented upon below. In all other tumor DNAs, proviral sequences were mainly found in pool 6, except for T15 DNA, for which they were found mainly in pool 5. The modal buoyant densities in CsCl of DNA fractions present in pools 5 and 6 were close to 1.708 g/cm^3; pool 7 showed a multimodal profile comprised of a peak close to 1.708 g/cm^3.

Analysis of Virus-Specific Sequences in Restriction Digests of Fractionated DNA. The results of hybridization of cDNA-38 with *Eco*RI and *Xba* I fragments from viral-enriched DNA fractions obtained from T15 (pool 5) and W15, S15, T92, S92, and W928 DNAs (pool 6) are shown in Fig. 4 A and C. Among the fragments detected by cDNA-38, some were proven to be of ribosomal or satellite origin by incubation of the filters with [^{32}P]rDNA probes. These fragments had M_rs of 5.0×10^6, 1.5×10^6, and 1.1×10^6 in *Eco*RI digests (Fig. 1) and 17×10^6 and 2.4×10^6 in *Xba* I digests (Fig. 3A and B) for all DNAs tested. It is of interest that no hybridization of cDNA-38 was found on the *Eco*RI 16×10^6-M_r fragment of rDNA (see Fig. 1); the reason for this is that this fragment contains only the 18S rRNA gene, (15), which was absent from the 38S viral RNA; the 28S rRNA contamination of the 38S viral RNA, on the other hand,
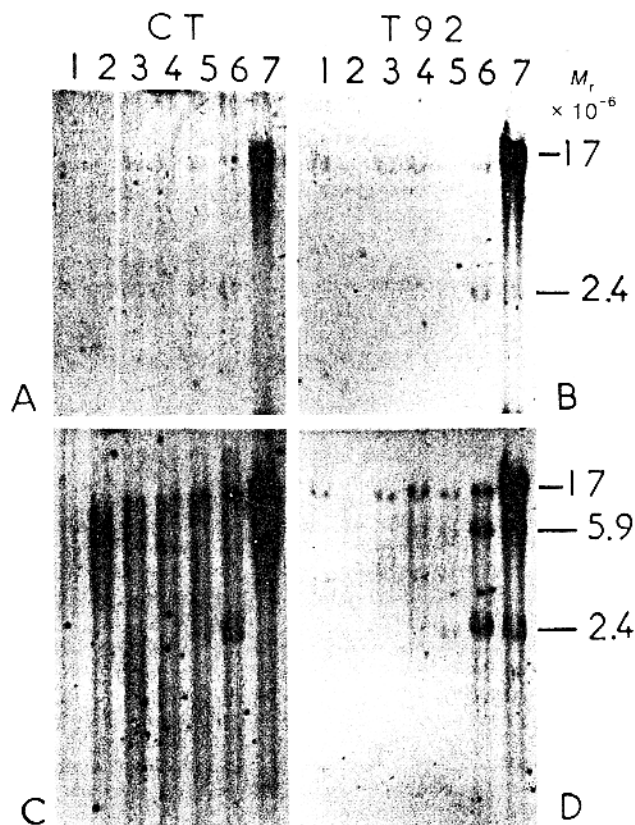
FIG. 3. Detection of BLV proviral sequences and ribosomal DNA in *Xba* I digests from fractionated CT and T92 DNAs. Five micrograms of CT (*A* and *C*) or T92 (*B* and *D*) DNAs of the seven pools from Cs₂SO₄/BAMD density gradient centrifugation (see Fig. 2A) were digested with *Xba* I and electrophoresed on a 1% agarose slab gel. After transfer on nitrocellulose sheets, hybridization was carried out with BLV [^{32}P]cDNA-38. The final washing was performed in one-tenth concentrated standard saline citrate. Autoradiograms are shown in *C* and *D*. The same nitrocellulose sheets were reused as described in ref. 15 for hybridization with both recombinant [^{32}P]rDNA plasmids and the autoradiograms are shown in *A* and *B*.

FIG. 4. Restriction endonuclease pattern and hybridization of BLV cDNA to bovine DNAs. Pool 6 of S15, W15, T92, S92, CT, and W928 DNAs (15 µg) and pool 5 of T15 DNA (15 µg) were digested by *Eco*RI or *Xba* I before electrophoresis on 1% agarose gels. Only fragments larger than M_r 0.5 × 10⁶ were detected on these gels. (*A* and *C*) Autoradiograms of DNA restriction fragments after hybridization with BLV cDNA-38. After transfer of the restriction fragments (*A*, *Eco*RI; *C*, *Xba* I), the nitrocellulose sheets were soaked in the prehybridization mixture at 65°C and hybridized with BLV [^{32}P]-cDNA-38 in the same mixture. The last washes were performed in three-tenths concentrated standard saline citrate. (*B* and *D*) Schematic representation of *Eco*RI (*B*) and *Xba* I (*D*) restriction fragments containing BLV proviral sequences.

is responsible for the hybridization of cDNA-38 on the *Eco*RI 5.0 × 10⁶-M_r fragment of rDNA, which contains almost all of the 28S rRNA gene (15).

The fragments containing viral sequences (Fig. 4 *A* and *C*) are represented in the scheme of Fig. 4 *B* and *D*. As far as animal 15 is concerned, the *Eco*RI digest of T15 DNA exhibited two virus-specific fragments of M_r 8.0 × 10⁶ and 0.6 × 10⁶ (Fig. 1; Fig. 4 *A* and *B*). In contrast, the *Eco*RI digests of W15 and S15 DNAs both contained two virus-specific fragments of M_r 16 × 10⁶ and 1.2 × 10⁶. Therefore, although in all these three DNAs the proviral DNA contained only one *Eco*RI site, the *Eco*RI sites on the cellular flanking sequences were at different locations in the DNAs from leukocytes and spleen on the one hand and in the tumor DNA on the other. These conclusions were further supported by *Xba* I digests. In this case, T15 DNA exhibited four virus-specific fragments of M_r 5.9 × 10⁶, 3.7 × 10⁶, 1.7 × 10⁶ and 0.8 × 10⁶, a finding showing the existence of three *Xba* I sites inside the proviral DNA. W15 and S15 DNAs exhibited four virus-specific fragments of M_r 3.1 × 10⁶, 2.2 × 10⁶, 1.7 × 10⁶, and 0.8 × 10⁶. Two of these fragments, M_r 1.7 × 10⁶ and 0.8 × 10⁶, were common to the *Xba* I digest of T15 DNA and can, therefore, be assumed to represent internal sequences of the integrated provirus. The other two *Xba* I fragments contained sequences of both viral and cellular origin; these were of different length in T15 DNA on the one hand and in W15 and S15 DNAs on the other. These results indicate that
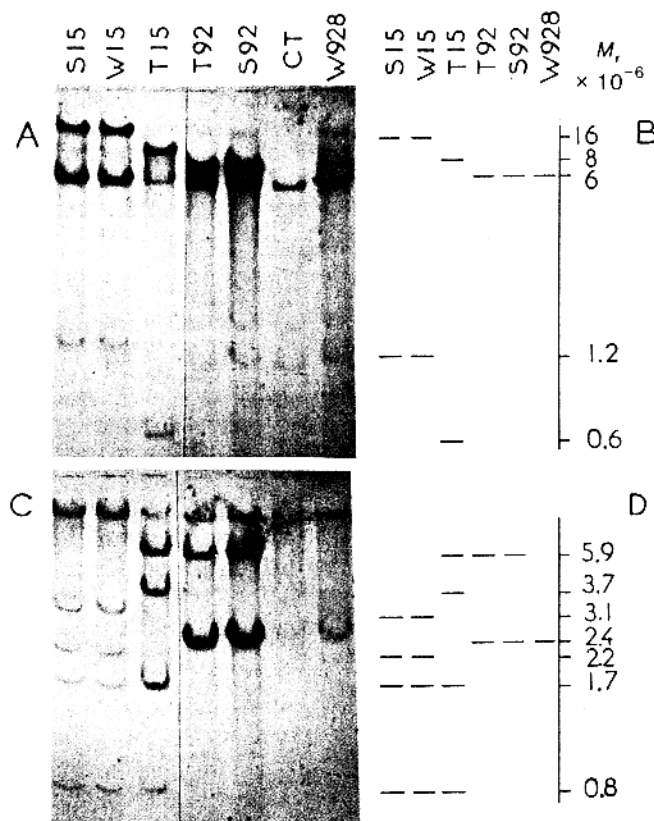
proviral sequences are integrated at different locations in the DNA of different infected cells of the same animal 15.

The *Eco*RI digests of T92 and S92 DNAs gave a single fragment of M_r 6 × 10⁶ containing viral sequences. After digestion of these DNA by *Xba* I, two virus-positive bands of M_r 5.9 × 10⁶ and 2.4 × 10⁶ were found. The strong relative intensity of the latter band suggests that this may be a doublet; if so, one fragment should be purely proviral and the other one should contain both proviral and flanking cellular sequences, like the M_r 5.9 × 10⁶ fragment. The identical restriction patterns of T92 and S92 DNAs indicate that the proviral DNA has the same location in the DNAs of the two tissues of animal 92. The single integrated provirus appeared to have no *Eco*RI-specific site (as far as no small fragments were lost) and to have two *Xba* I restriction sites. The different number of *Eco*RI and *Xba* I sites present in the proviral sequences of animal 92 compared to animal 15 indicates that these sequences are different.

W928 DNA from an animal in persistent lymphocytosis, digested by *Eco*RI and annealed to BLV [^{32}P]cDNA-38, showed one positive fragment M_r 6.0 × 10⁶. Some additional very weak bands of higher molecular weight were also present. After digestion with *Xba* I, a weak, virus-specific fragment of M_r 2.4 × 10⁶ was detected.

Nonspecific Hybridization. In addition to virus-specific

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fragments, other fragments hybridizing to the viral probe were found in all DNAs. These fragments hybridized with the cDNA synthesized on rRNA contaminating the viral RNA. Some of these fragments were derived from rDNA; others were from satellite DNAs. The origin of the former was shown by: (*i*) their M_rs after *Eco*RI digestion (15); (*ii*) their presence in the highest GC-rich fraction of the density gradient (15); and (*iii*) their hybridization with rDNA probes. In agreement with this explanation, a cDNA synthesized on a more extensively purified viral RNA, such as cDNA-38, showed a much lower hybridization which was limited to the 28S gene fragment (compare Figs. 4 and 1). rDNA contamination was also observed by others (17, 18) in other systems. It should be noted that a very limited contamination (0.1%) by ribosomal cDNA is sufficient to cause this spurious hybridization of the viral probe because of the high reiteration of ribosomal cistrons. These represent approximately 0.2% of the bovine genome (15), whereas the integrated proviral sequence represents only about 0.0001%.

In addition to fragments derived from rDNA, other fragments derived from satellite DNAs also showed a weak hybridization of the viral probe, which was due to the same reason. It has already been shown elsewhere (15) that there is, in fact, a slight sequence homology between some satellite DNAs and rDNA in the bovine genome. These satellite-containing bands were clearly identified when gels of DNA fractions (pool 6) enriched in satellite DNA were stained with ethidium bromide (not shown).

DISCUSSION

All DNAs from infected cells showed restriction fragments containing viral sequences not found in the control DNA from a normal animal, a confirmation that BLV is an exogenous virus (2). Only one copy of proviral DNA [M_r 5.8×10^6 (9)] appeared to be integrated, because the sum of the M_rs of the virus-specific restriction fragments was as low as 6×10^6 in the DNA of animal 92 and as low as 8×10^6 in the other DNAs. This finding is in agreement with the small copy number (1–3) found in all systems in which the virus is strictly exogenous (2, 19, 20); in contrast, multiple DNA copies are generally found with endogenous type C viruses (19). It should be noted that no unintegrated proviral DNA appeared to be present in the bovine DNAs tested, as observed by the hybridization patterns of undegraded DNA samples (data not shown).

The proviral sequences from animals 15 and 92 have slightly different sequences because they contain a different number of restriction sites (one *Eco*RI and three *Xba* I sites in the case of animal 15; no *Eco*RI and two *Xba* I sites in the case of animal 92). In all likelihood, these differences are due to genetic variation in the viral genome, a situation already well known in other systems (21–23). Alternative explanations for these differences might be the use of different regions of the proviral sequence in the integration into the genome of the two animals or the insertion of cellular DNA sequences into the provirus. The first explanation would, however, lead to noncolinearity of proviral DNA and viral RNA (17), a possibility recently disproven in other retrovirus systems (21, 24). In contrast, the proviral sequences found in the DNAs of animals 92 and 928 might be identical, because they seem to share an internal proviral fragment of M_r 2.4 and 10^6. In connection with this possibility, it should be recalled that animal 92 was infected by BLV derived from the infection center of animal 928, and that animal 15 was infected by BLV derived from a different infection center.

Concerning the sites of the host genome into which provirus integration occurred, an important point shown here is that provirus integration does not occur at random in the host genome. This is indicated by the finding of proviral sequences in large (>M_r 20×10^6) GC-rich fragments having a buoyant density close to 1.708 g/cm³ and representing only a small fraction (less than 20%) of the host genome. No attempt was made in the present work to decide which of the components of the bovine genome (6) is responsible for previous integration. The candidates are a satellite DNA (buoyant density 1.709) and the major component (buoyant density 1.708). The former represents 4.5% and the latter 9% of the bovine genome (5–7).

A more detailed analysis of provirus integration into the host genome of different animals is of great interest. Tumor and spleen DNAs from animal 92 contained proviral sequences that were present in the same GC-rich fraction (pool 6) of the Cs₂SO₄/BAMD density gradient (ρ = 1.708 g/cm³ in CsCl). These two DNAs gave identical *Eco*RI and *Xba* I restriction patterns. Consequently, in animal 92, the BLV-infected cells proliferating in the tumor and in the spleen contained the proviral DNA integrated at the same chromosomal site, which is evidence for a common origin for these cells.

The DNA from the leukocytes of animal 928 showed a weak hybridization to the BLV probe. The reason for this phenomenon was that only 20% of the cells appeared to have proviral sequences in animal 928, as indicated by the kinetics of reassociation between BLV [³H]cDNA and host cellular DNA (data not shown). Interestingly, low reassociation levels were found to be a property of all DNAs from the leukocytes tested, whether obtained from animals in the initial or in the final stage of leukosis. Another result deserving to be commented on here was that W928 DNA showed a number of faint hybridization bands in addition to the main one (Fig. 4A). Such bands were also obtained with DNAs from leukocytes of other animals in persistent lymphocytosis, an eventual initial phase of leukosis, but not with those of animals in the advanced stage of leukosis with tumors. These faint hybridization bands indicate that the infected leukocytes of animals in the initial phase of leukosis are heterogeneous in the chromosomal location of the integration site of proviral sequences.

In animal 15, the proviral sequences present in leukocytes and spleen DNAs were found mainly in fragments (pool 6) having a buoyant density of 1.708 g/cm³, whereas those present in tumor DNA were found mainly in fragments (pool 5) having a slightly lighter buoyant density (1.706–1.707 g/cm³). This indication of a difference in integration sites in tumor DNA versus spleen and leukocyte DNA was unequivocally confirmed by the different restriction patterns shown by these DNAs. In fact, although two *Xba* I fragments containing proviral sequences were common to the three DNAs and corresponded in all likelihood to proviral internal fragments, the other two were different in size in the tumor DNA on the one hand and in spleen and leukocytes on the other. These results indicate that, in animal 15, the same provirus is located at one chromosomal site in the cells of the tumor examined and at another site in spleen and leukocytes. Interestingly, the DNA from a different tumor of animal 15 has shown in recent experiments (not presented here) a hybridization pattern identical to those of spleen and leukocyte DNAs.

The finding that proviral sequences have different chromosomal locations in different infected cells of animal 15 is another important result of the present work. We explain it as due to the fact that the infected cells studied here had a polyclonal origin from an initial, possibly small, cell population that was heterogeneous in the chromosomal location of the proviral insertion. This situation appears to exist in the early phase of leukosis (animal 928; see above). The insertion of proviral sequences in different chromosomal sites of the initially infected

cell population was apparently followed by a selection process that led to the preferential multiplication of some clones relative to other ones. This sequence of events is known to take place in the viral infection of cells in culture (25).

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