Bovine Leukemia Virus: An Exogenous RNA Oncogenic Virus?

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Abstract

Short term cultures of bovine leukemic lymphocytes release virus particles with biochemical properties of RNA oncogenic viruses. These particles, tentatively called Bovine Leukemia Virus (BLV) have a high molecular weight-reverse transcriptase complex and a density averaging 1.155 g/ml in sucrose solutions. Molecular hybridizations between BLV-^{3}H cDNA and several viral RNAs show that BLV is not related to Mason-Pfizer Monkey Virus (MPMV) Simian Sarcoma Associated Virus (SSV-1) Feline Leukemia Virus (FeLV) or Avian Myeloblastosis Virus (AMV). Rauscher Leukemia Virus (RLV) exhibits a slight but reproducible relatednesse to BLV. The high preference of BLV reverse transcriptase for Mg^{++} as the divalent cation suggests that BLV might be an atypical mammalian leukemogenic type C virus. Hybridization studies using BLV ^{3}H cDNA as a probe suggest that the DNA of bovine leukemic cells contains viral sequences that cannot be detected in normal bovine DNA.

Abbreviations: Eagle MEM: Eagle minimum essential medium – SDS: sodium dodecyl sulfate – MMTV: mouse mammary tumor virus.

Introduction

Bovine leukemia is a lymphoproliferative disease appearing in cattle herds under several forms (1). The following observations lead to the conclusion that viruses are the most probable etiological agents of the onzootic form of the disease:

(a) Bovine leukemia often appears in geographically localized foci. It spreads by horizontal as well as by vertical transmission (Mostly from mother to offspring) (2, 3, 4).

(b) Infected animals develop antibodies directed against an antigen present in the virus fraction of leukemic lymphocyte cultures. This antigen can be detected by immunofluorescence, immunodiffusion or complement fixation (5–9).

(c) Virus particles are occasionally seen in milk and tissues of leukemic animals (10, 11).

(d) Cultures of bovine leukemic material produce virus particles generally considered as type C (11–15) although they are morphologically somewhat different from typical type C viruses (16–17).

(e) Whole blood from leukemic animals transfers the disease with high frequency when fed to newborn calves (18, 3) or sheep (18, 19, 20). Successful infections are also obtained with the viral concentrate from short term cultures (21, 22).

Considering all these observations, it seems to us of basic interest to identify biochemically Bovine Leukemia Virus, to determine by molecular hybridization to what extent it could be related to other known type C viruses and finally and mostly to characterize it as an exogenous or an endogenous bovine virus.

Materials and Methods

Animals.

Our experimental herd was established from animals diagnosed as leukemic by hematological test (key of Göttingen). A sample of leukocytes from each animal was submitted to short term culture (see below) and examined by electron microscopy for the presence of "C type" virus. Every culture derived from a leukemic animal produced virus particles while cultures made of normal leukocytes remained negative (4).

Cell cultures.

Blood was collected from the jugular vein; clotting was prevented by the use of 3 ml of 1 % heparine for each 150 ml of blood anticipated to be taken. Leukocytes were separated by the distilled water 1.7 % saline method, as applied by Stock and Ferrer (21). After the red blood cells were lysed by hypotonic shock, the cells were adjusted to 3.0×10^6/ml of Eagle MEM supplemented with 20 % inactivated (30 min at 56 °C) fetal calf serum and maintained as stationary cultures. Penicillin (100 units/ml) and streptomycin (100 µg/ml) were added to the media. These short term cultures were incubated for 72–96 hours at 37 °C.

Virus concentration.

A. – *From short term culture supernatants.*

The medium was clarified by centrifugation at 1500 x g for 45 min at 3 °C and the virus was purified according to Bishop et al. (24) except that TNE (0.01 M Tris-HCl, pH 8.3; 0.15 M NaCl; 0.001 M EDTA) was used instead of MEM or Tris-HCl pH 7.5.

B. – *From cells.*

3 g of cultured lymphocytes were homogenized with an ultra-turrax homogenizer (Janke and Kunkel, type ZF) at full speed for 3 x 20 seconds in 4 volumes of TNE at 4 °C. The homogenate was then processed as the virus suspension.

Assay of 60–70S RNA directed DNA polymerase : simultaneous detection test.

Pellets obtained after equilibrium density gradient centrifugation of BLV were resuspended in 0.01 M Tris-HCl, pH 8.3 at a protein concentration of 3 mg/ml. Triton X-100 was adjusted to a final concentration of 0.03 % and a simultaneous detection test was performed (25).

Preparation of BLV ^{3}H cDNA.

The 60–70S RNA-^{3}H DNA complex of a BLV simultaneous detection test was recovered by alcohol precipitation, treated with alkali to destroy RNA and chromatographed on hydroxyapatite to purify single stranded ^{3}H cDNA molecules (26, 27).

Preparation of ^{3}H cDNA probes of AMV, SSV-1, RL Vand FeLV.

The reaction conditions were those previously described (28) except for RLV and feLV where Mg^{++} was replaced by Mn^{++} at a final concentration of 0.001 M.

Preparation of viral RNAs.

Viral proteins were solubilized and digested by a mixture of SDS and proteinase K (Merck, Darmstadt) at final concentrations of 0.5 % and 0.2 mg/ml respectively. The digested mixture was extracted twice at room temperature with phenol-cresol-chloroform (6:1:7). 60–70S RNA was recovered from the aqueous phase by ethanol precipitation and purified by sedimentation in sucrose gradient.

Preparation of DNAs.

DNA from leukemic and normal cells was isolated according to Sweet et al. (29). All DNAs were reduced to a sedimentation constant of 6–8S by ultrasonic vibration. DNAs extrated by this method had an A_{260}/A_{280} nm ratio of 1.85–1.95.

Hybridization reactions.

^{3}H cDNA (2000 cpm) was added to indicated amounts of RNA (or DNA) in a final volume of 56µl of 0.4 M NaCl, 0.001 M EDTA, 0.1 % SDS and 0.01 M Tris-HCl, pH = 7.7. The mixture was incubated at 68 °C for various periods of time. The extent of hybrid formation was estimated either by Cs_2SO_4 equilibrium density gradient centrifugation (28) (for DNA-RNA hybrids) or S_1 nuclease digestion (30) (for DNA-DNA hybrids).

Results

Characterization of a 60–70S RNA and reverse transcriptase.

BLV particles released in the culture supernatant were concentrated as described in Methods and used as a source of template-primer and reverse transcriptase in an RNA dependent DNA synthesis reaction. The reaction proceeded linearly for at least 30 minutes. In some cases, linear incorporation lasted for as long as 2 hours. As a rule, reactions were stopped after 30 min. and analyzed by the simultaneous detection technique (25). We systematically searched for optimum conditions for cDNA recovery. Incubation of the reaction mixture with proteinase K (Merck, Darmstadt) before phenol-chloroform extraction improved the cDNA yield by at least 70 %.

Fig. 1 shows the outcome of a simultaneous detection test. Fractions 5 to 15 represent the region where 60–70S viral RNA-^{3}H cDNA complexes sediment. The presence of these complexes per se is a strong indication that BLV contains a high molecular weight RNA and reverse transcriptase, molecules characteristic of

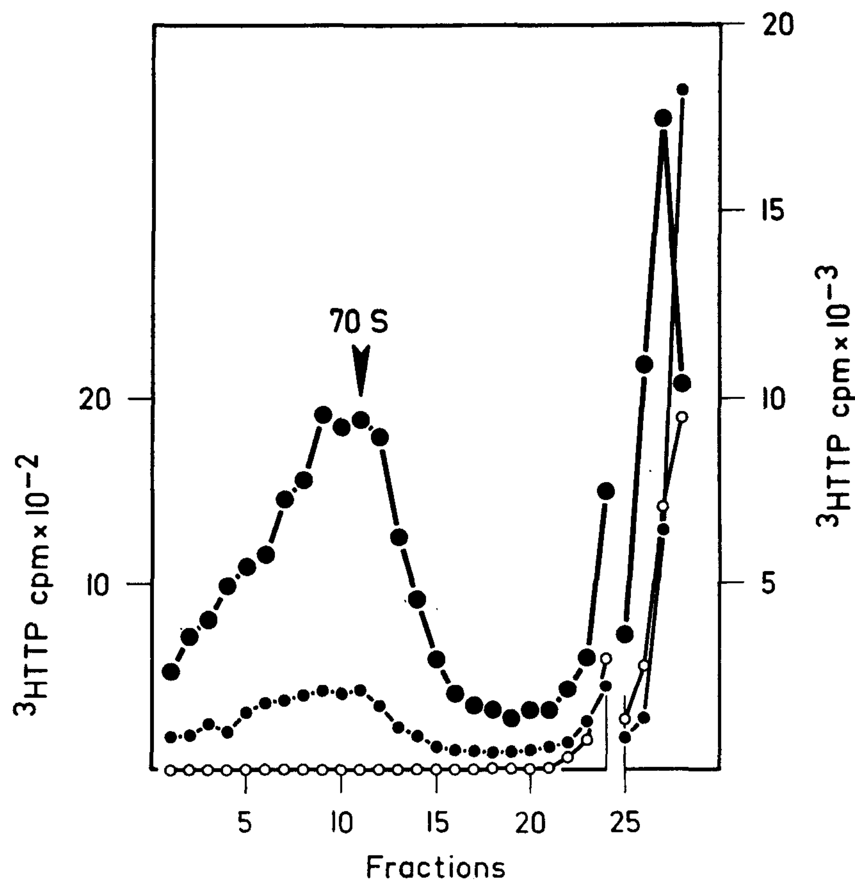


Fig. 1: Detection of the 60–70S RNA-^{3}H cDNA complex of BLV from culture supernatants. Twelve hundred ml bovine lymphocyte culture supernatant were processed as described under "virus concentrations" and "simultaneous detection" (see Methods). The virus concentrate was divided into three equal parts. A standard RNA-directed DNA polymerase reaction was performed on one part. The RNA-^{3}H cDNA product was sized on sucrose gradient (●——●). A second part of the virus concentrate was used in a reaction mixture lacking dATP (•——•). The third part of the virus concentrate was incubated in the complete reaction mixture supplemented with 100 µg/ml of RNase A (○——○).

RNA oncogenic viruses. The synthesis of 60–70S RNA-^{3}H cDNAhybrids is dependent on the presence of the four deoxytriphosphates in the incubation medium. Leaving out dATP reduced the ^{3}H TMP incorporation to about 20 % of the control value.

In the presence of RNAse A, ^{3}H TMP incorporation is reduced to a background level.

The same experimental technology was applied to virus detection in cultured leukemic lymphocytes. The same positive outcome of the simultaneous detection test was obtained. Similar results were also obtained when leukemic lymphocytes from sheep infected by bovine leukemic blood were examined (data not shown). This indicates that the observed endogenous DNA polymerase activity is both RNA dependent and not due to an end addition enzyme activity.

Requirements of the BLV-reverse transcriptase reaction:
1/ Non ionic detergent.

In contrast to avian RNA tumor viruses (28, 31, 32) mammalian oncornaviruses (25, 26) require no detergent (33) or very limited concentrations of detergents for optimal rate of DNA synthesis. BLV endogenous synthesis of DNA was performed in the presence of various concentrations of NP40 or Triton X-100. At

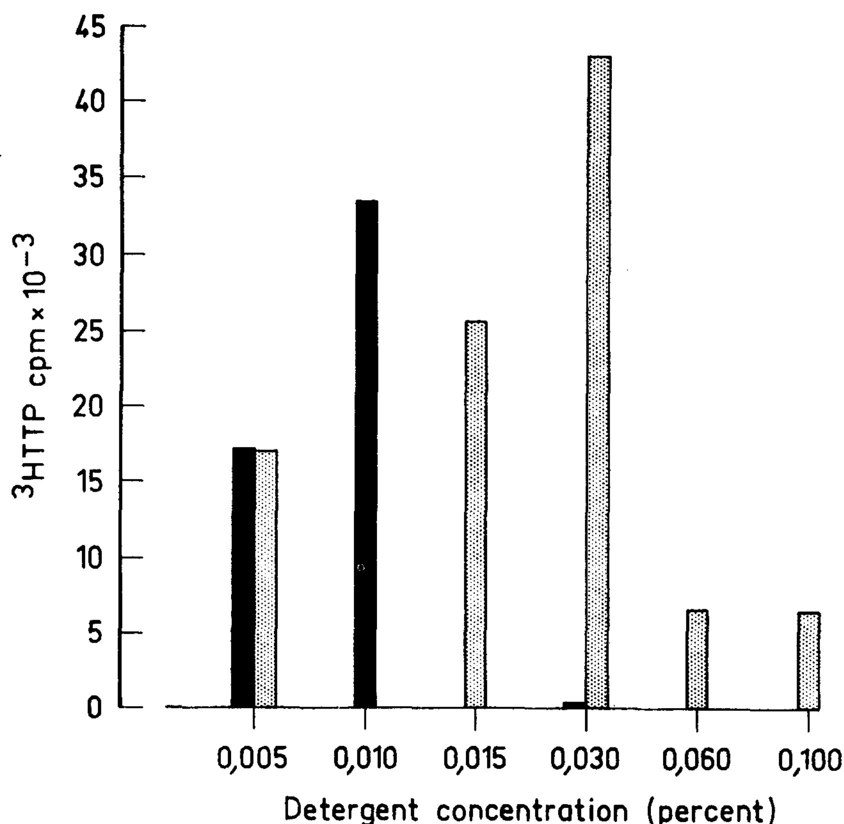
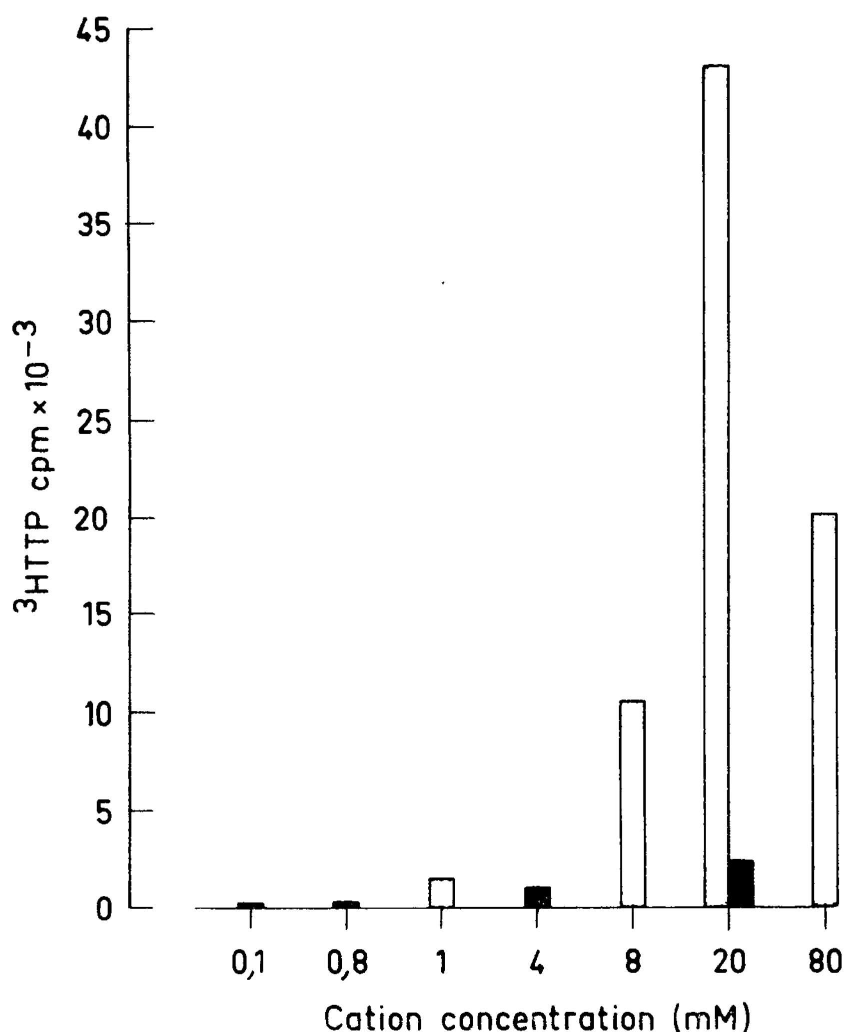


Fig. 2: *Panel A.*

BLV-DNA polymerase activity as a function of detergent concentration. ^{3}H cDNA syntheses were run in the presence of the indicated concentrations of Triton X-100 (□) or NP-40 (■) and analyzed by sucrose gradient sedimentation. Results are expressed as TCA precipitable counts in the 60–70S region of the gradient versus indicated detergent concentration.



Panel B. BLV-DNA polymerase activity as a function of Mg^{++} (□) or Mn^{++} (■) concentration in the reaction mixture. ^{3}H cDNA syntheses were run in the presence of the indicated concentrations of Mg^{++} or Mn^{++} and analyzed by sedimentation in sucrose gradients. Results are expressed as TCA precipitable counts in the 60–70S region of the gradient versus indicated cation concentration.

the protein concentration used (3 mg/ml) maximum synthesis of 60–70S ^{3}H cDNA occurred at 0.01 % of NP40 or 0.03 % of Triton X-100 in the reaction mixture. Higher detergent concentrations practically abolish the reaction (Fig. 2a).

2/ *Divalent cation.*

The preference of viral polymerase for Mg^{++} or Mn^{++} depends on the template-primer that is being used (34, 35). It has been shown also (36) that more similarity exists between the DNA polymerases from viruses of the same type than between the polymerases from viruses of different types but from closely related species. Divalent cation requirement may therefore be informative for the biochemical characterization of new oncornaviruses.

Endogenous ^{3}H cDNA synthesis was run in the conditions described in Methods except for divalent cation concentration. ^{3}H cDNA counts associated with high molecular weight RNA after sucrose gradient sedimentation were recorded in Fig. 2b as functions of divalent cation concentration used. As can be seen, no

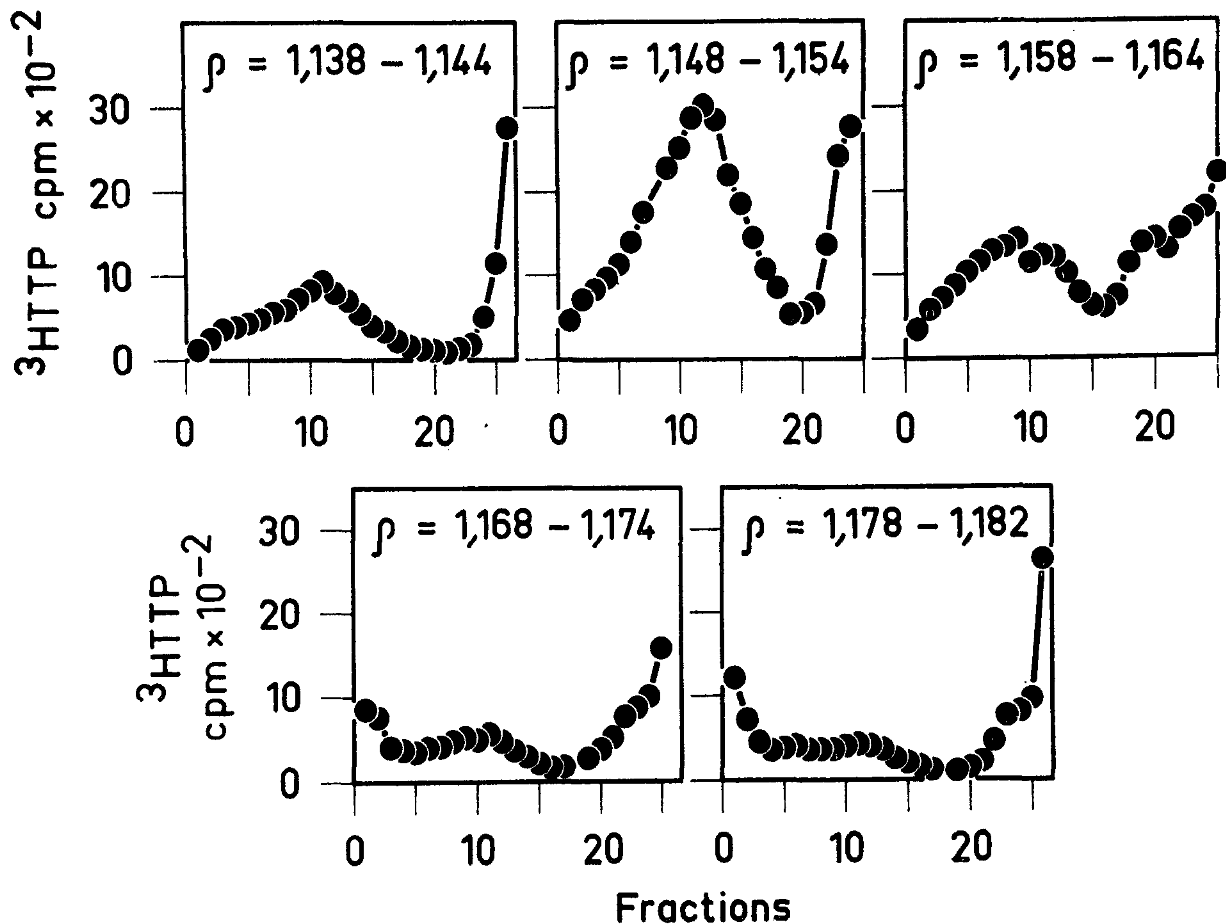


Fig. 3: Determination of BLV equilibrium density in sucrose solutions.
A BLV concentrate, prepared from short term culture supernatant (1200 ml) as described in Methods, was suspended in TNE buffer, layered on a linear gradient 20–50 sucrose in TNE buffer and centrifuged overnight at 25000 rpm in a Spinco SW 27 rotor at 4 °C. Fractions within regions of the indicated densities were pooled and assayed by the simultaneous detection test as described in Methods.

notable incorporation of ^{3}H TMP into DNA occurred at any of the Mn^{++} cation concentrations tested. Mg^{++}, on the other hand, clearly stimulated BLV reverse transcriptase. The optimum concentration for the reverse transcriptase activity in this assay was 20 mM Mg^{++}.

Buoyant density in sucrose gradients.

The buoyant density values of oncornaviruses in sucrose solutions vary according to the virus type (37). The B-type MMTV equilibrates at a density of 1.18 g/ml in sucrose while C type viruses have a density of 1.16 g/ml in sucrose.

In order to determine the density values of BLV, the virus released in the supernatant of 1200 ml of culture was processed as described in the legend to Fig. 3. The simultaneous detection profiles obtained reveal that BLV equilibrates in these conditions between 1.148 and 1.164 g/ml, the density region of C type viruses.

Preparation of BLV ³H cDNA.

Once the optimum conditions of the BLV reverse transcriptase reaction were determined, we prepared BLV ³H cDNA and tested it for its representativity of the BLV genome. A virus concentrate was prepared from five liters of lymphocyte culture supernatant and used to generate an ³H-DNA probe. The high molecular weight RNA-³H cDNA complex was purified by sucrose gradient centrifugation (25), alkali treated to destroy RNA and fractionated on hydroxyapatite to separate single stranded material from the small proportion of double stranded molecules. Single stranded ³H cDNA was further characterized by self annealing, annealing to globin mRNA and to BLV 60–70S RNA. Fig. 4a shows a Cs_2SO_4 equilibrium density profile of the self annealed single stranded material. All the ³H counts band as a sharp peak in the density region where DNA is expected. This profile is not significantly altered if BLV ³H cDNA has been previously hybridized to globin mRNA (Fig. 4b). This eliminates the possibility of BLV ³H cDNA contamination by poly thymidylic acid stretches. After annealing to BLV high molecular weight RNA (Fig. 4c), all the ³H cDNA counts equilibrate in the Cs_2SO_4 gradient in a broad density region from 1.66 g/ml (he RNA density region) up to 1.48 g/ml covering the whole region of RNA-DNA hybrids. Virtually no single stranded DNA molecule remains in the DNA region. This set of experiments shows that purified single stranded DNA molecules of the BLV probe specifically hybridize to BLV RNA sequences.

Relatedness of BLV to other RNA oncogenic viruses.

A constant amount (2000 cpm; SA ≐ 2 x 10^7 cpm/µg) of single stranded molecules was annealed to increasing amounts of BLV RNA. Percentages of hybrid-

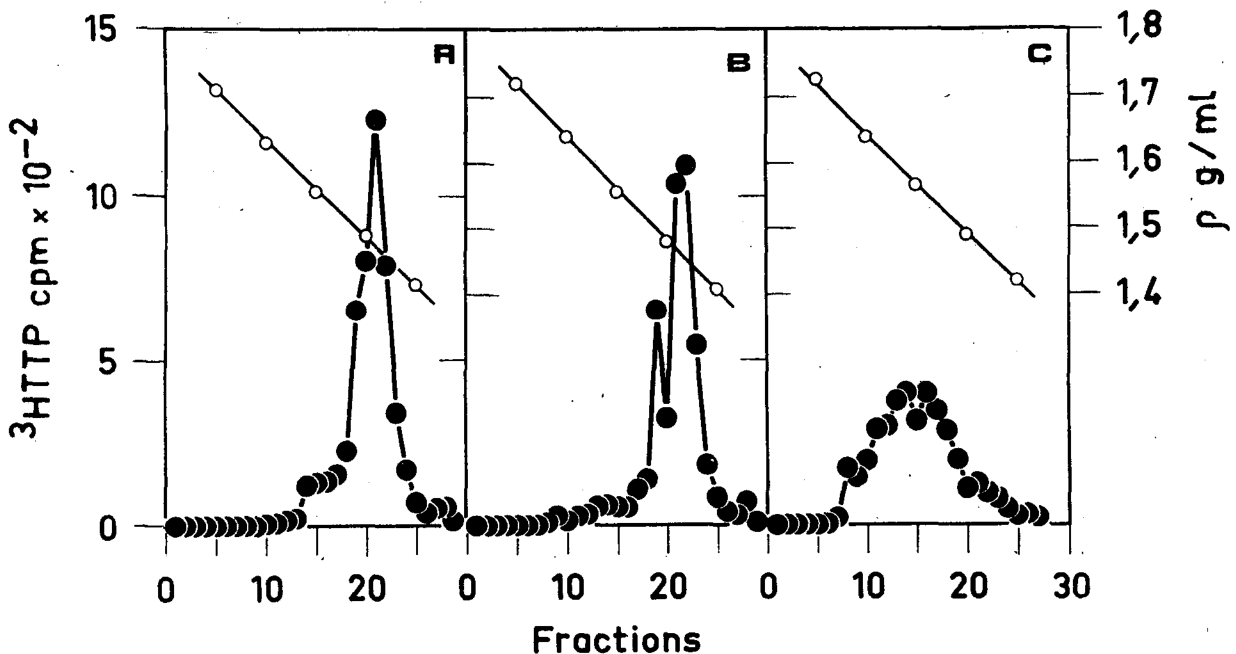


Fig. 4: Characterization of BLV cDNA probe.
Cs_2SO_4 equilibrium density gradient centrifugation of the purified BLV-cDNA alone (a) and after annealing at 68 °C for 3 days to 4.5 µg of globin 9S mRNA (b) and 0.16 µg of BLV 70S RNA (c) ● = cpm; ○ = density.

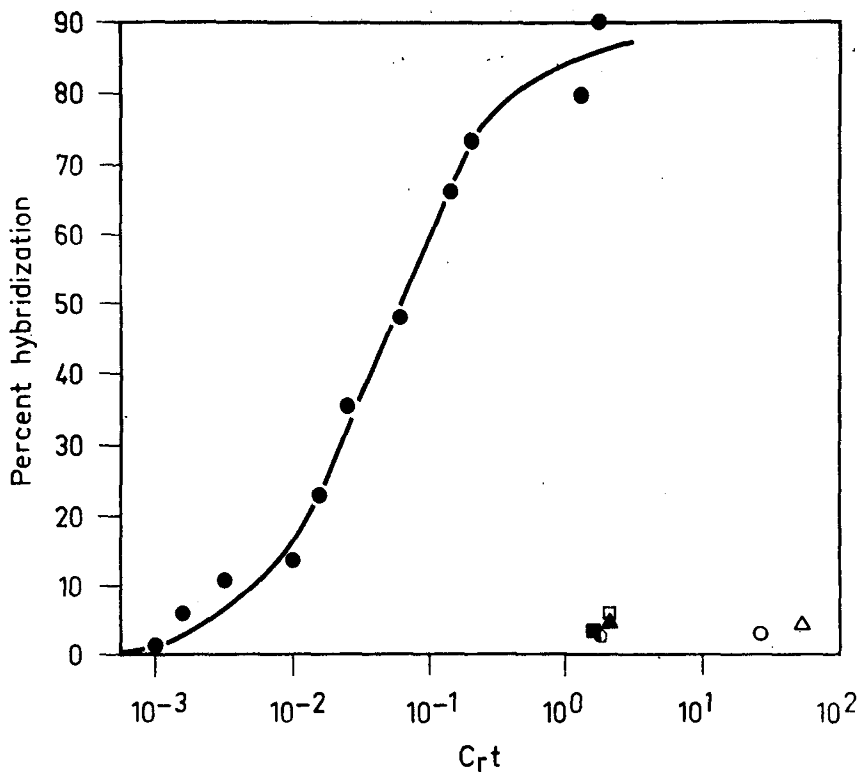


Fig. 5: Kinetics of annealing of BLV-cDNA to 60–70S BLV RNA. Crt is the product of nucleic acid concentration (in OD_{260}) and hybridization time (in hours/2). No corrections were made for salt concentrations. The hybridization mixtures contained from 0.078 ng to 160 ng of BLV 70S and were all incubated at 68 °C for 3 days. The extent of hybridization was determined by S1 nuclease treatment (●—●).

Hybridizations with hemoglobin messenger RNA as a control (△); RLV 70S RNA (□) MPMV 70S RNA (■) AMV 70S RNA (○) SSV-1 70S RNA (▲) FeLV 70S RNA (◐) were run up to indicated Crt values.

ization were calculated from the ratios of S_1 resistant counts over total ^3H cDNA counts in the controls. The values obtained were recorded (Fig. 5) as functions of Crt (concentration of viral RNA in moles/liter times time in seconds (38). Crt 1/2 equals 7×10^{-2} moles x sec/liter. In similar experiments with AMV and RLV, values such as 3×10^{-2} (39) and 1.5×10^{-2} (40) were reported. The somewhat higher value obtained here in the BLV system is probably due to some contamination of BLV 60–70S RNA. As BLV is produced by degenerating cells, contamination of the 60–70S region of sucrose gradients by cellular nucleic acid cannot be ruled out. Such a Crt curve also shows that 90 % of the cDNA engaged in the reaction was hybridized at Crt values of 1 and above. Annealing experiments tending to detect an hypothetical relatedness between BLV and other known RNA oncogenic viruses must be carried out up to, at least, Crt values of 1. Such experiments were performed with globin mRNA as control and MPMV, RLV, FeLV, AMV, SSV-1, RNAs. Within the limits of our experiments, we can conclude that 4 of the 5 viruses tested do not share common RNA sequences with BLV. RLV, however, showed a slight but reproducible relatedness to BLV. BLV ^3H cDNA systematically showed some 2 to 3 % of hybridization to RLV RNA. A control experiment was then performed where BLV 60–70S RNA was annealed to ^3H DNA synthesized in the above five viruses tested (Table 1). Again, MPMV,

Table I: % Hybridization of ^{3}H cDNA probes synthesized in various RNA viruses with globin mRNA (as control) and various viral 60–70S RNAs*.

^{3}H cDNA probes	globin mRNA	AMV	SSV-1	RNAs MPMV	FeLV	RLV	BLV
BLV	4.3	3.1	4.9	3.6	–	6.1	85.1
SSV-1	0.0	–	90.0	–	–	–	0.3
AMV	0.0	90.2	–	–	–	–	2.6
RLV	0.0	–	–	–	–	80.1	8.8
FeLV	0.0	–	–	–	43.1	–	0.8

* Hybridizations were run at Crt values $\geqq$ 2.

AMV, SSV-1, FeLV appeared to be unrelated to BLV but RLV ^{3}H cDNA repeatedly hybridized to some extent (3 % to 8 %) to BLV-RNA.

BLV genome sequences in DNA from normal bovine cells and bovine lymphosarcoma cells.

A constant amount (2000 cpm; SA = 2 x 10^7 cpm/µg) of single stranded BLV ^{3}H cDNA was annealed to increasing amounts of normal and leukemic bovine

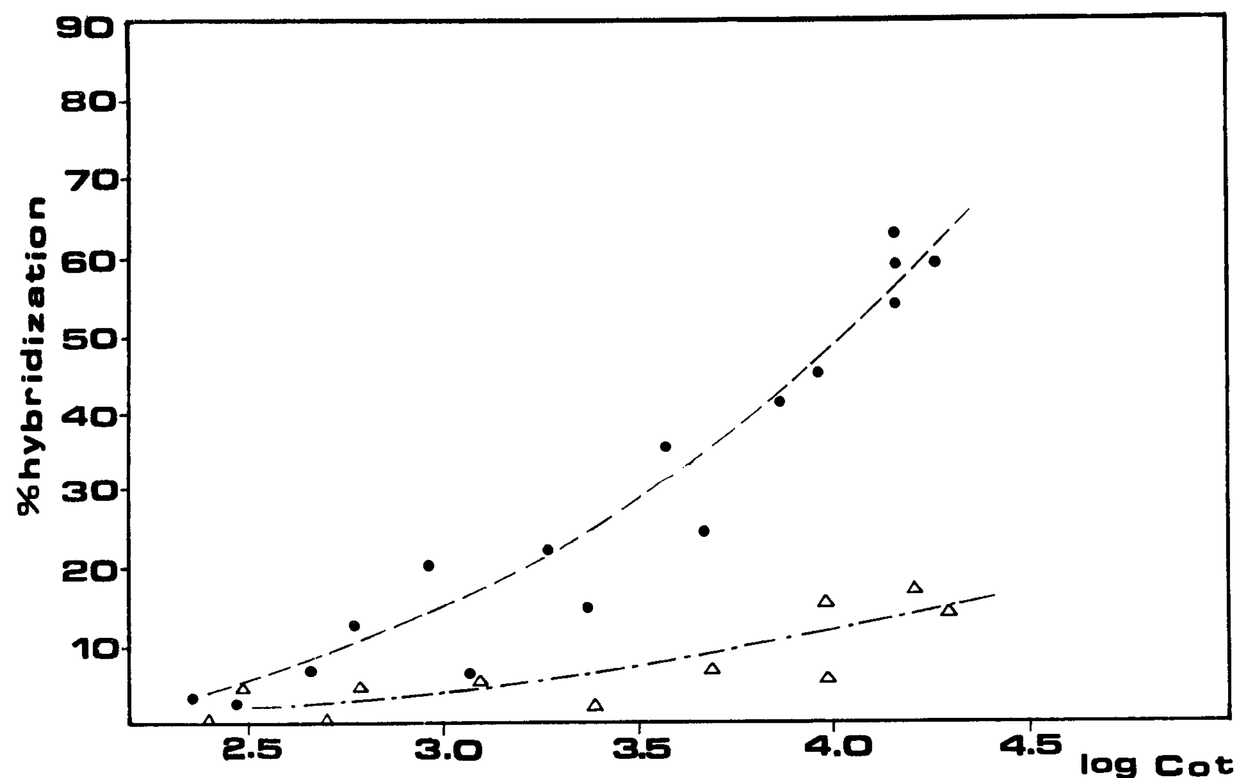


Fig. 6: Hybridization kinetics of BLV ^{3}H cDNA with normal (△—△) and leukemic bovine DNA (●—●). Hybridization reactions were carried out with 2000 input counts/min of BLV ^{3}H cDNA at a specific activity of 2 x 10^7 cpm/µg, at 68 °C in Tris-HCl, 0.01 M, pH = 7.7; NaCl : 0.4 M; EDTA : 0.001 M; SDS : 0.1 %. The dashed lines are not mathematically derived, but simply fit to the data for illustrative purposes.

DNA. After about 20 days, the percentages of hybridization were determined and recorded as functions of Cot. Fig. 6 shows the kinetics of hybridization of BLV x ^{3}H cDNA with normal (△–△) and leukemic bovine DNA (●–●). About 60 % of the radioactive probe enters S_1 nuclease-resistant hybrids after annealing with bovine leukemic DNA. The same probe formed hybrids with normal bovine DNA at a much slower rate, reaching only 15 % at log Cot values of about 4.5.

These observations are consistent with the proposition that BLV contributes genome sequences to the leukemic cell which are not detectable by this technique, in normal bovine DNA. Definite proof that this proposition is indeed correct must await further experimental evidence. Recycling experiments (29, 41, 42) and thermal stability analysis of the hybrids are presently under way.

Discussion

Bovine leukemia is by far the best known natural model system from epidemiological studies. As outlined in the introduction of this report, the disease spreads by horizontal and vertical transmission. In the latter case, however, experiments strongly suggest that transmission of the disease is most probably due to perinatal infection (3). It was therefore of obvious interest to try to identify the putative agent, and characterize it, biologically and biochemically. A major step towards this goal was accomplished when virus production was achieved in short term cultures of leukemic lymphocytes. We report here on our attempt to study biochemical features of the virus. The positive outcome of simultaneous detection tests, the sensitivity of the reaction to ribonuclease treatment, and the strict requirement for the four deoxyribonucleoside triphosphates (Fig. 1) practically identifies BLV as an RNA tumor virus. The definite proof, however, that BLV possesses a high molecular weight RNA-reverse transcriptase complex could only be obtained through back hybridization of the DNA synthesized in vitro with the viral 60–70S RNA. Fig. 4 and 5 show that this is, indeed, the case.

Parameters of the endogenous reverse transcriptase reaction were then determined. As a rule, reverse transcriptases from mammalian leukemogenic viruses are extremely sensitive to non-ionic detergent concentrations (32). According to our titration experiments, BLV reverse transcriptase exhibits maximum activity when Triton X-100 reaches a concentration of 0.03 % in the solution where protein concentration averages 1.5 mg/ml. In the same conditions of protein concentration, the optimal NP-40 concentration is 0.01% (Fig. 2).

It could also be informative to investigate the metal requirements of BLV endogenous DNA synthesis. The optimum Mg^{++} concentration averages 20 mM while Mn^{++} is ineffective at any of the concentrations tested (Fig. 3). As pointed out by Waters and Yang (35), divalent metal requirements of a given reverse transcriptase reaction depends primarily on the template-primers used. If, however, the endogenous synthesis of DNA on an RNA template is considered, the following rules seem to obtain:

– Mammalian type C viruses: DNA synthesis proceeds equally well in the presence of Mg^{++} or Mn^{++} provided optimal concentrations are used. The reaction may even be stimulated if both cations are present at a given concentration (36).

– Type B viruses: Mg^{++} is a mandatory requirement for DNA synthesis (43).

– MPMV (36, 43), GPV (43), BLV: they are morphologically different from type B viruses but share their divalent cations requirements.

Further characterization of BLV included an equilibrium density gradient centrifugation in sucrose gradients. The simultaneous detection tests performed on material banding at the indicated densities (Fig. 4) show that BLV equilibrates between 1.148 and 1.164 g/ml, a density region characteristic of type C virus particles.

The potential relatedness of BLV to other known RNA oncogenic viruses was screened by hybridization of the various viral RNAs to BLV ^{3}H cDNA (Fig. 5) and, *mutatis mutandis,* of bovine viral RNA to the various viral ^{3}H cDNAs. The results of this double check are quite clearcut: AMV, MPMV, FeLV and SSV-1 are unrelated to BLV. Surprisingly enough, RLV showed in both tests a slight but reproducible relatedness to BLV (some 3 to 8 %) (Table 1). A comparable situation obtains when RLV ^{3}H DNA probes are used to search viral sequences in human leukemias, lymphomas and Hodgkin diseases (44, 45, 46). One would obviously want to understand the exact significance of this observation.

Epidemiological and experimental evidence strongly suggests bovine leukemia to be an infectious disease. It was therefore of crucial interest to try to classify BLV as an exogenous or an endogenous virus. The biochemical strategy of such experiments has been designed (41) and includes a recycling step. The recycling step is a mandatory prerequisite before classification of a virus, if the normal cell DNA of the species studies contains, at least, one copy equivalent of the viral genome. In this case indeed one could interpret leukemogenesis as an amplification of existing DNA sequences.

That viral transformation implies *de novo* insertion of viral DNA sequences into the genome of the infected host, is easy to demonstrate in cells transformed by a non-indigenous virus (47–49). That the same phenomenon holds true for cells infected by an indigenous virus, was clearly demonstrated by Baxt and Spiegelman (41) in the case of human leukemias. From studies on the leukemic member of identical twins (42), it was further concluded that leukemia specific information must have been inserted subsequent to fertilization. These facts observed in human systems have been extended to avian lymphoma (50) and leukemia (51). The data we present here (Fig. 6) about bovine leukemia, a "field" leukemia, suggest that leukemia specific sequences are present in the DNA of the leukemic cell. Further evidence in support of this proposition are presently being sought, i. e. recycling of BLV ^{3}H cDNA on normal bovine DNA, thermal stability analysis of the hybrids . . .

What is the information carried by the extra-sequences? From studies with a transformation defective mutant of Prague RSV-C, it has been suggested (52) that the sarcoma virus adds transformation specific sequences to the DNA of normal cells. The clearcut identification of the viral sequences responsible for each viral function should help answering the question.

In conclusion, we tend to believe, without having at the present time the definite proof, that bovine leukemia is an infectious disease both on epidemiological as well as on biochemical grounds. It seems also highly probable that it will be the first natural system in which Koch's postulate will be fulfilled.

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