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Transcriptional Control of the Bovine Leukemia Virus Genome: Role and Characterization of a Non-Immunoglobulin Plasma Protein from Bovine Leukemia Virus-Infected Cattle

PHALGUNI GUPTA,* S. V. S. KASHMIRI, AND J. F. FERRER

Section of Viral Oncology, Comparative Leukemia Studies Unit, School of Veterinary Medicine, University of Pennsylvania, New Bolton Center, Kennett Square, Pennsylvania 19348

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Using cloned bovine leukemia virus (BLV) DNA as a probe in the dot blot hybridization technique, we demonstrated that the expression of the BLV genome in infected lymphocytes is blocked in vivo at the transcriptional level. This blocking effect is due to a non-immunoglobulin protein present in the plasma but not in the serum of BLV-infected cattle. The plasma BLV-blocking protein also blocks the expression of the BLV genome in fibroblast cells of bovine and nonbovine origin infected with BLV in vitro. The plasma BLV-blocking factor has no inhibitory effect on the expression of Rauscher murine leukemia virus and feline leukemia virus in monolayer culture. The plasma BLV-blocking factor is not an interferon molecule. As determined by gel filtration chromatography, the plasma BLV-blocking factor has an apparent molecular weight of ca. 150,000.

Bovine leukemia virus (BLV), an exogenous C-type leukemogenic virus of cattle, is unique among retroviruses. This is indicated by the result of immunological analysis of the virion proteins, by nucleic acid hybridization studies, by biochemical and immunological comparison of the virion reverse transcriptase, and by the ability of the virus to induce syncytia formation in nontransformed cells (6, 8). In addition, the virus-host interactions in the BLV system differ significantly from those of the other leukemia virus systems. Unlike the murine leukemia virus and the feline leukemia virus (FeLV), BLV seems to infect only the lymphocytes in vivo. Viral particles, viral antigens, and viral RNA have not been detected in BLV-infected lymphocytes before cultivation. However, cultivation of the infected lymphocytes results in rapid derepression of the BLV genome, leading to the synthesis of complete virus particles that can be detected as early as 3 h after in vitro cultivation (1, 8, 16, 20).

We have recently shown that the plasma of BLV-infected cattle frequently contains a factor capable of blocking the synthesis of the major BLV core protein (p25) in short-term bovine lymphocyte cultures (12). This factor, termed the plasma BLV-blocking (PBB) factor, has been detected in a large majority of the cattle infected with BLV, regardless of whether or not they had persistent lymphocytosis, a benign condition caused by BLV (6) (P. Gupta, unpublished observation). The PBB factor has not been found in plasma from BLV-free cattle, including those infected with common bovine viruses. BLV blocking activity was not detected in sera derived from PBB-positive plasmas or in PBB-negative plasma mixed with bovine gamma globulin with high titers of anti-BLV antibodies. Thus, it seems clear that the PBB factor is not an antibody molecule. The blocking effect is reversible. The factor can exert its inhibitory effect when added to cultured lymphocytes that are already expressing the p25 antigen. Conversely, the synthesis of p25 resumes when the PBB factor is removed from the lymphocyte culture (12). Sensitivity tests with various enzymes have shown that the PBB factor is a protein (12).

In this report we show that the repression of the BLV genome in lymphocytes by the PBB factor occurs at the transcriptional level. Results of further biological and biochemical characterization of the PBB factor are also reported.

In previous studies, liquid hybridization with a ^{3}H-labeled BLV cDNA probe failed to demonstrate the presence of BLV RNA in total cytoplasmic RNA isolated from nonneoplastic or neoplastic BLV-infected bovine lymphocytes without cultivation (8, 16). To determine more rigorously whether viral RNA synthesis takes place in vivo, we examined the presence of viral messages in polyadenylate [poly(A)]-containing RNA from noncultured infected lymphocytes by using the dot blot hybridization technique with a ^{32}P-labeled BLV probe prepared by nick translation of the cloned BLV proviral DNA. The results are shown in Fig. 1A. No hybridization was detected with poly(A)-containing RNA from either BLV-infected lymphocytes before cultivation (spot a) or from noninfected lymphocytes (spot c). However, the probe hybridized strongly with RNA isolated from BLV-infected lymphocytes that had been cultured for 48 h (spot b). Thus, these results confirm that in vivo the expression of the BLV genome is repressed at the transcriptional level.

Using the same technique, we sought to determine whether the inhibition of expression of the BLV genome by the PBB factor in cultured lymphocytes also occurs at the transcriptional level. In this study total cytoplasmic RNA was examined because to obtain sufficient quantities of poly(A)-containing RNA it would have been necessary to start the culture with an inordinate number of lymphocytes. First, to determine the sensitivity of the BLV RNA detection procedure, we examined cytoplasmic RNA isolated from lymphocyte mixtures containing varying proportions of BLV-expressing cells. These mixtures were made of noninfected lymphocytes and lymphocytes induced to express BLV p25 antigen by precultivation (48 h). The immunofluorescence technique (7) was used to identify BLV p25-expressing cells. As shown in Fig. 1B, viral messages were detected neither in noninfected lymphocytes (spot a) nor in noncultured BLV-infected lymphocytes (spot b). However,

* Corresponding author.

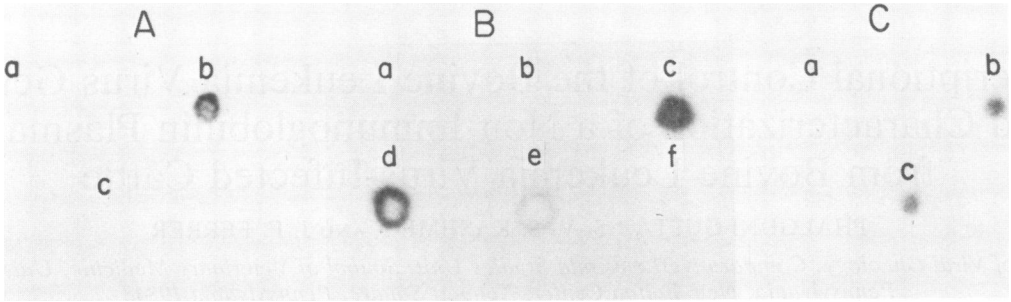

FIG. 1. Dot blot hybridization of viral RNA from lymphocytes of BLV-infected cattle. 2×10^9 buffy-coat lymphocytes from a BLV-infected cow were cultured at 37°C for 48 h at an initial density of 5×10^6 cells per ml of 100% PBB-positive plasma, 100% PBB-negative plasma, or MEM, as previously described (12). After incubation, cells were harvested and washed three times with phosphate-buffered saline. Cytoplasmic RNA was extracted from the cell pellet by the hot phenol procedure of Britten et al. (3). Poly(A)-containing RNA was isolated from total cytoplasmic RNA by affinity chromatography on oligodeoxythymidylate-cellulose (24). ^{32}P-labeled BLV DNA probe was prepared from cloned viral DNA (14) by the method of Maniatis et al. (17). The specific activity of the nick translated probe was 8×10^8 cpm/μg. The dot blot hybridization assay was done according to the procedure described by Thomas (22), except that bovine rRNA was added to the hybridization mixture at a concentration of 100 μg/ml. (A) Analysis of poly(A)-containing RNA isolated from BLV-infected lymphocytes before (spot a) and after (spot b) cultivation in normal growth medium and from noninfected lymphocytes (spot c). (B) Analysis of viral RNA in total RNA from noninfected lymphocytes (spot a) and from BLV-infected lymphocytes before (spot b) and after (spot c) cultivation and from lymphocyte mixtures containing 10% (spot d), 1% (spot e), and 0.5% (spot f) BLV-expressing cells. (C) Expression of viral RNA in BLV-infected lymphocytes cultured in the presence of PBB-positive plasma (spot a), PBB-negative plasma (spot b), or MEM (spot c).

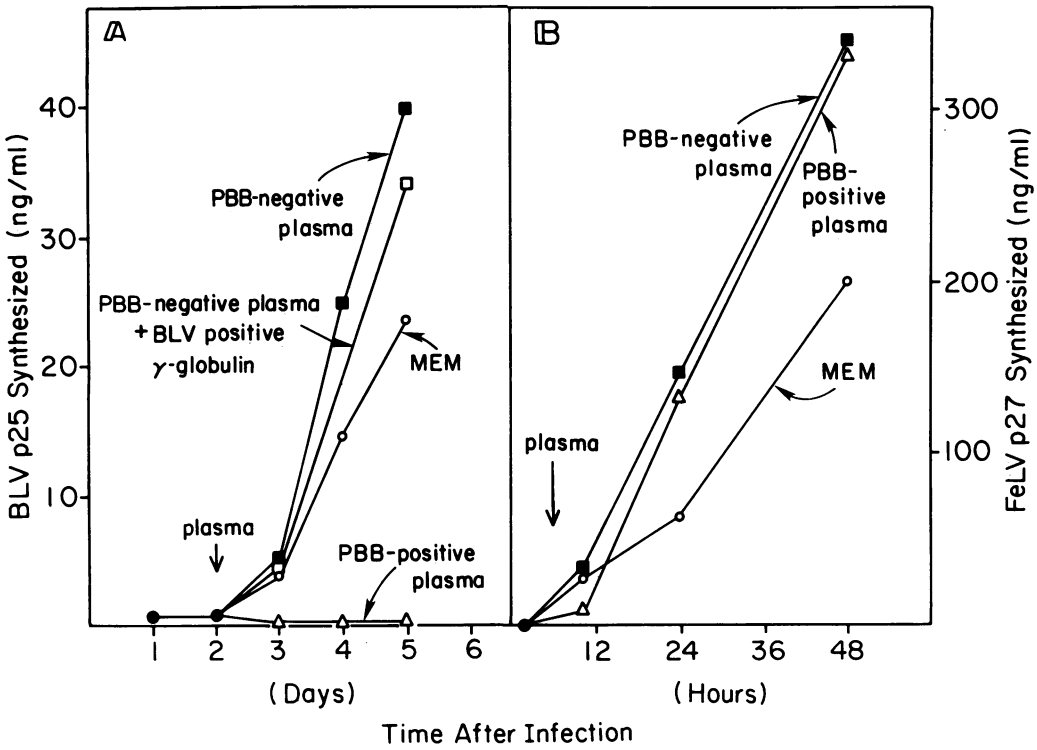

FIG. 2. Effect of the PBB factor on the synthesis of BLV p25 and FeLV p27 antigens. (A) 3×10^5 CEHC cells were seeded in 60-mm petri dishes. Twenty-four hours later the cell monolayers were treated with DEAE-dextran (25 μg/ml) for 30 min and incubated with supernatant fluid from the BLV-bat clone (5, 10) or BLV-infected fetal lamb kidney (23) cell lines. After virus adsorption for 2 h at 37°C, the cell monolayer was washed three times and reincubated. Twenty-two to forty-eight hours after infection, the medium was replaced by 4 ml of PBB-positive or PBB-negative plasma, and the incubation was continued. Cells were harvested by trypsinization at indicated intervals, washed once, and tested for the presence of the BLV p25 protein by competitive radioimmunoassay (11). (B) 3×10^5 feline cells (FLf_3) were seeded in complete medium containing 5 μg of Polybrene per ml. Twenty-four hours later the cells were infected with FeLV. After virus adsorption for 1 h at 37°C, the cell monolayer was washed three times and reincubated. Six hours after infection, the medium was replaced by 4 ml of PBB-positive or PBB-negative plasma. Cells were harvested at the indicated times, and the expression of the FeLV p27 was quantitated by competitive radioimmunoassay (21).

viral RNA was readily detected in lymphocyte mixtures containing 1% or more BLV antigen-positive lymphocytes (spots c, d, and e). No positive signal was detected in RNA from lymphocyte preparations containing 0.5% antigen-positive cells (spot f).

Next, the technique was applied to determine whether viral RNA synthesis takes place in BLV-infected lymphocytes cultured for 48 h in the presence of the PBB factor. As shown in Fig. 1C, whereas the BLV probe hybridized strongly with RNA from lymphocytes cultured either in 100% PBB-negative plasma (spot b) or in growth medium (spot c), it did not hybridize with RNA from infected lymphocytes cultured in 100% PBB-positive plasma (spot a). Thus, we conclude that the PBB factor blocks the transcription of the BLV genome.

In another experiment we found that the incorporation of [^{3}H]uridine in BLV-infected lymphocytes cultured in the presence of PBB-positive or PBB-negative plasma was not inhibited (data not shown), thus ruling out the possibility that the blocking effect of the PBB factor is due to inhibition of total cellular RNA synthesis.

We sought to determine if the PBB factor blocks the expression of BLV in nonlymphoid cells. Calf embryonic heart cells (CEHC) (5) were used in this study because they are highly susceptible to BLV infection (15) and because the kinetics of the expression of viral antigen in these cells was known from previous studies. The PBB factor was added to the CEHC cells after virus penetration but before the time at which they are known to express viral antigens. Synthesis of p25 in the CEHC cells was detected 3 days after infection and increased progressively thereafter in the cultures grown in the presence of minimal essential medium (MEM), PBB-negative plasma, or PBB-negative plasma supplemented with serum gamma globulin (30 mg/ml) from a BLV-infected animal (Fig. 2A). In contrast, the synthesis of BLV p25 was completely inhibited when the CEHC cells were grown in the presence of PBB-positive plasma (Fig. 2A). The synthesis of BLV gp51 in CEHC cultures was also completely inhibited by PBB-positive plasma but not by PBB-negative plasma, whereas serum derived from the PBB-positive plasma did not have any blocking effect on BLV expression in the CEHC cultures (data not shown).

We studied the effect of the PBB factor on the expression of BLV in nonbovine fibroblasts and found that PBB-positive plasma, but not control plasma, prevented the synthesis of p25 in bat lung cells (CCL88). Thus, it is clear that the PBB factor can manifest its blocking effect in cells of both bovine and nonbovine origin.

To determine the specificity of the PBB factor, we examined its effect on the expression of leukemia viruses of other species. The experiments in Fig. 2B show that PBB-positive plasma did not block the expression of the major internal FeLV protein (p27) in feline cells. Similarly, PBB-positive plasma did not have any blocking effect on the expression of the Rauscher murine leukemia virus p30 antigen in mouse cells (data not shown). Thus, it appears that the PBB factor is specific for BLV. The possibility should be considered, however, that the effect of the factor encompasses a certain group or family of C-type leukemia viruses which, like BLV and unlike the Rauscher murine leukemia virus and FeLV, are not expressed in the host. In this regard, it is pertinent to note that, as in the case of BLV, human T-cell leukemia virus and adult T-cell leukemia virus, two closely related retroviruses associated with human T-cell lymphomas, were isolated only after the infected cells were cultured in vitro (9, 18). Furthermore, it has been reported that the expression of the human T-cell leukemia virus genome is blocked by the plasma but not by the serum of a human T-cell leukemia virus-infected patient (13).

The ability of the PBB factor to inhibit BLV expression in cells of diverse species is in contrast with the species specificity displayed by most of the interferon molecules (19). As determined by the vesicular stomatitis virus plaque reduction assay (4), as well as by the cytopathic assay (19) with bovine indicator cells, no correlation was observed between BLV blocking activity and interferon activity in bovine plasmas (data not shown). Thus, it is clear that the PBB factor is not interferon.

Using the CEHC indicator system, we repeated and extended our earlier studies on the characterization of the

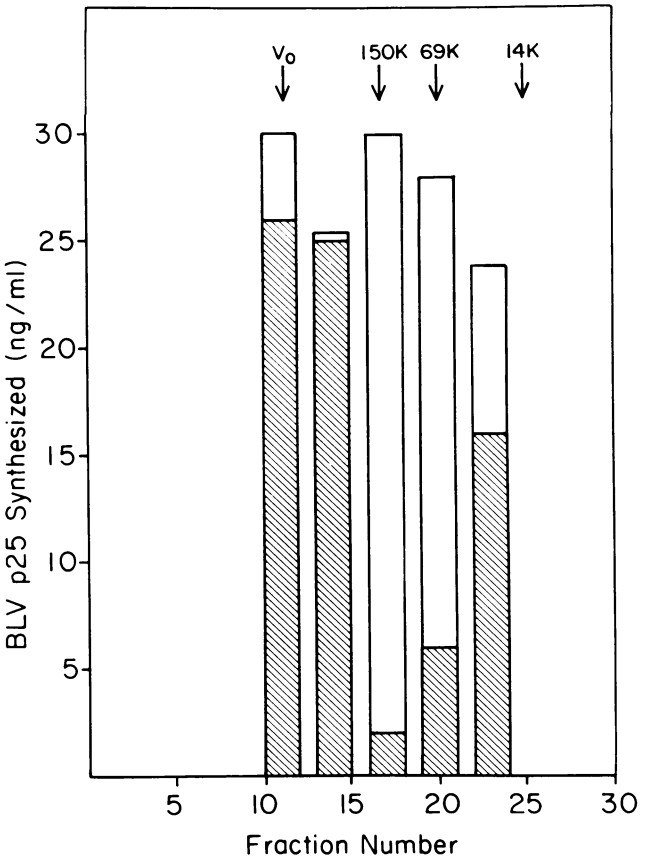

FIG. 3. Gel filtration chromatography of the PBB factor. Four milliliters of plasma was applied on a 100-ml column of Sephadex G-200 equilibrated with MEM. Elution was done under sterile conditions at 4°C with MEM at a flow rate of 4 to 6 ml/h. Two-milliliter fractions were collected, appropriately pooled, concentrated approximately fourfold by ultrafiltration on Amicon filters with a 50,000-molecular-weight cut-off, and tested for BLV blocking activity in a microassay. This microassay was carried out with CEHC indicator cells as described in the legend to Fig. 2, except that: (i) 3 × 10^4 CEHC cells were seeded in each well of a Linbro 26-well microtiter plate, (ii) 1 ml of plasma sample was applied in each assay well, and (iii) indicator cells were harvested for testing in the competitive radioimmunoassay only once, at 4 days after infection. Values in the ordinate represent the amount of p25 synthesized in the presence of the indicated pooled fraction. Blue dextran was used to determine the void volume (Vo) of the column. Bovine immunoglobulin G (molecular weight, 150,000 [150K]), bovine serum albumin (molecular weight, 69,000 [69K]) and lysozyme (molecular weight, 14,000 [14K]) were used as molecular weight markers. Symbols: ▨, PBB-positive plasma; □, PBB-negative plasma.

PBB factor. The results showed that the activity of the PBB-positive plasma was sensitive to protease but not to DNase, RNase, lipase, or amylase (data not shown), thus confirming that the PBB factor is a protein molecule. The factor retained its activity after storage for at least 6 days at 4°C and for at least 3 months after lyophilization. Freezing and thawing and dialysis overnight through a membrane with a molecular weight cut-off of 12,000 did not significantly reduce the PBB activity of positive plasma.

To determine the molecular weight of the PBB factor, PBB-positive plasma was chromatographed on a Sephadex G-200 column. Appropriate column fractions (starting from the void volume) were pooled, concentrated by ultrafiltration, and assayed for blocking activity with indicator CEHC cells. Figure 3 shows the amount of p25 synthesized by CEHC cells in the presence of the indicated pooled column fractions. The elution profile of the column revealed a peak of blocking activity at a position corresponding to the protein with a molecular weight of 150,000. A small decrease in the p25 synthesis in the presence of fractions 15 and 29 obtained from PBB-negative plasma is nonspecific. Thus, the apparent molecular weight of the PBB factor is ca. 150,000. The possibility cannot be ruled out that this value corresponds to an aggregate.

Based on the results presented here, it is reasonable to conclude that the PBB factor is responsible for the repression of the BLV genome in vivo. The present results clearly show that the PBB factor is not an antibody or an interferon molecule. Recently, Baron and McKerlie (2) described a cell-produced inhibitor against a broad range of viruses. This viral inhibitor is clearly different from the PBB factor because it acts extracellularly at the level of virus attachment and penetration.

The observation that, in vivo, the transcription of the BLV genome is blocked by a non-immunoglobulin protein has no precedent in any other leukemia virus. By interfering with the replication of BLV, the PBB factor most likely plays a major role in the control of BLV infection and BLV-induced leukemogenesis. Studies on the mechanism of action of the PBB factor may contribute fundamentally towards our understanding of the control of the expression of other cryptic retroviral genomes.

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