Defibrinated bovine plasma inhibits retroviral transcription by blocking p52 activation of the NFκB element in the long terminal repeat

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Abstract

Bovine leukemia virus (BLV) induces a persistent but latent infection in cattle. Viral latency is invoked by a protein known as plasma blocking factor (PBF) that is found in both bovine and human plasma. We report here on pathways that mediate latency in the presence of PBF. Reporter-gene constructs driven by the promoters of 6 retroviruses were used to measure the production of chloramphenicol acetyl transferase (CAT) in cell lines cultured with or without defibrinated bovine plasma. Plasma inhibited CAT production only in constructs containing an NFκB-binding element proximal to the initiation site (BLV, human immunodeficiency virus, and human T-cell leukemia virus). The promoters of *Bovine immunodeficiency virus*, *Feline immunodeficiency virus*, or *Feline leukemia virus* were not inhibited in the presence of bovine plasma. Using gel mobility shift assays, we demonstrated that activation of viral transcription upon stimulation with phorbol esters and ionomycin was mediated through the NFκB element and that this was abrogated in the presence of plasma. Furthermore, analysis of individual NFκB proteins in nuclear extracts of mononuclear cells or Jurkat cells showed that all 5 members of the NFκB family were upregulated in response to stimulation, but only p52 was significantly downregulated in the presence of bovine plasma. Thus, we infer that plasma effects are mediated through interference with either p52 translocation to the nucleus or p52 synthesis.

Résumé

Le virus de la leucémie bovine (BLV) induit une infection persistante mais latente chez les bovins. La latence virale est causée par une protéine connue sous l'appellation de facteur plasmatique bloquant (PBF) et retrouvée dans le plasma bovin et humain. La présente étude fait état de voies qui conduisent à la latence en présence de PBF. Des constructions de gènes rapporteurs menées par les promoteurs de 6 rétrovirus ont été utilisés afin de mesurer la production d'acétyl transférase du chloramphénicol (CAT) dans des lignées cellulaires cultivées avec et sans plasma bovin défibriné. Le plasma a inhibé la production de CAT seulement chez les constructions contenant un élément liant le NFκB proximal au site d'initiation (BLV, virus de l'immunodéficience humaine, et virus de la leucémie humaine à cellules T). Les promoteurs du virus de l'immunodéficience bovine, du virus de l'immunodéficience féline, ou du virus de la leucémie féline n'étaient pas inhibés par la présence de plasma bovin. Au moyen d'une électrophorèse en gel avec décalage de la migration, nous avons démontré que l'activation de la transcription virale suite à une stimulation par les esters de phorbol et l'ionomycine était sous médiation de l'élément NFκB et qu'elle était abrogée par la présence de plasma. De plus, l'analyse des protéines individuelles NFκB dans les extraits nucléaires des cellules mononucléaires et des cellules de Jurkat a démontré que les 5 membres de la famille NFκB étaient régulés à la hausse en réponse à une stimulation mais que seulement p52 était régulée à la baisse de manière significative en présence de plasma bovin. Ainsi, nous proposons que les effets du plasma se produisent par interférence soit avec la translocation de p52 vers le noyau ou la synthèse de p52.

(Traduit par Docteur Serge Messier)

Introduction

Bovine leukemia virus (BLV) is a member of the human T-cell leukemia virus (HTLV)/BLV group of retroviruses, which naturally infect cattle. It is the etiologic agent of enzootic bovine leucosis, a B-cell lymphoma that develops in less than 10% of infected cattle, after a lengthy latency (1). Infection is asymptomatic in most cattle, but persistent lymphocytosis (PL), a benign polyclonal expansion of peripheral blood lymphocytes (B cells), develops in approximately one-third (1). The mechanisms that maintain BLV latency in vivo are of interest, as they have potential relevance in other retroviral infections.

Transcription of the BLV genome, like that of HTLV, is regulated by the transactivating viral gene products tax and rex, which are encoded by overlapping reading frames from a doubly spliced transcript (2,3). Tax acts in concert with the host factors cyclic adenosine monophosphate (cAMP) binding protein (CBP) (4) and activating transcription factors I and II (ATF I and ATF II) (5), which bind to 3 *cis*-acting cyclic AMP response elements (CREs) 21 base pairs (bp) long, which are overlapped by E box motifs (6) in the

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3′ untranslated (U3) region of the BLV 5′ long terminal repeat (LTR) to stimulate viral expression (7–9). Additional protein-binding sites in the promoter region include an NFκB element (10) and a glucocorticoid response element (GRE) (11) in the U3 region, which act alternatively as stimulatory or inhibitory regions, depending on the cell line used to demonstrate activation of the LTR (12). The 5′ untranslated region (U5) and the repeated (R) region also have roles in viral replication.

Despite the presence of both cellular factors and viral proteins conducive to rapid replication of viral transcripts, BLV expression is rarely detected in fresh cells or tissues (13,14). However, the use of polymerase chain reaction (PCR) enables detection of the integrated provirus in infected cells (15). The replication of BLV appears to be related to the activation of B cells. Since viral infection is detected in cells delayed in the cell cycle, not expressing viral proteins (16), infected cells are thought to be maintained in a quiescent state in vivo and thus in a state of latency, which is conducive to evasion of immune detection. In vitro, however, factors present in fetal bovine serum (FBS) induce viral transcription within 3 to 6 h and are suspected to affect spontaneous cell proliferation, which peaks at 72 h of culture (17). The expression of BLV is upregulated in vitro independently of T cells or other stimulation but can be further increased by the addition of lectins such as concavalin A or phytohaemagglutinin A (17,18), lipopolysaccharide (LPS), and pokeweed mitogen (PWM) (19), by the addition of dexamethasone and insulin (11), by the addition of phorbol esters, which activate the protein kinase C pathway, by the addition of calcium ionophores, which enhance the influx of calcium ions, or by the engagement of surface receptors, which results in an influx of calcium into the cell (20).

Transcription of BLV is also regulated by host plasma factors (21–23). Zandomeni et al (23) found both stimulatory and inhibitory factors in plasma; bovine plasma enriched for IgG stimulated viral expression, whereas IgG-free plasma protein strongly inhibited viral transcription. The stimulatory plasma fraction from BLV-infected cattle was more potent than that isolated from BLV-free cattle. In this study, we sought to identify the pathway by which the plasma factor exerts its transcription-blocking effect in retroviruses that have or lack common *cis*-acting elements, with the use of plasmid constructs expressing a chloramphenicol acetyl transferase (CAT) gene driven by the LTRs of BLV, *Bovine immunodeficiency virus* (BIV), *Feline immunodeficiency virus* (FIV), *Feline leukemia virus* (FeLV), HTLV, or human immunodeficiency virus (HIV).

Materials and methods

Animals

A BLV-seropositive Holstein cow with PL, housed according to the guidelines of the Canadian Council on Animal Care, was selected. Blood was drawn from the tail vein into evacuated blood collection tubes containing acid citrate dextrose (ACD) for plasma separation and lymphocyte isolation as described previously (24). Additional samples were obtained from BLV-seropositive cows with elevated lymphocyte counts in a commercial dairy herd.

Plasmid constructs

Plasmids pHIV LTR and pHTLV-I LTR (25) were supplied by Dr. Karen Copeland, Department of Medicine, University of Ottawa, Ottawa, Ontario. Plasmids pRSVtax (7) and pBL-1, a BLV 5′LTR inserted into a pSV0 CAT construct (26), were gifts of Dr. David Derse, National Cancer Institute, Frederick, Maryland, USA. Plasmids pBIV LTR CAT and pSVtat (27) were kindly supplied by Dr. Susan Nadin-Davis, Animal Disease Research Institute, Ottawa. The FeLV LTR (subcloned into p16A1), pPPR LTR (the LTR of the FIV, subcloned into the CAT vector p22A2s), and pF34-NF-κB (derived from a mutation of the Petaluma strain of FIV, which consists of a 37-bp region in the LTR encoding the NFκB binding site and subcloned into p22A2s) (28) were provided by Dr. Ellen Sparger, University of California, Davis, California, USA.

The plasmids (10 μg/mL) were electroporated into competent DH5α bacterial cells. Ampicillin-resistant clones derived from individual colonies were purified by means of the Qiagen tip kit (Qiagen Inc., Mississauga, Ontario) according to the manufacturer's instructions. After precipitation with isopropanol and a wash with 70% ethanol, the DNA pellet was air-dried for 10 min and its concentration adjusted to 1 mg/mL in Tris ethylene diamine tetraacetic acid (EDTA) buffer.

Cell culture

Suspension cell lines included the following: a human T-cell leukemia cell line (Jurkat; American Type Culture Collection [ATCC] TIB 152), selected owing to previous success in transfection with retroviral constructs; a human B-cell line (Raji; ATCC CCL 86), selected as a model of B-cell support of retroviral transcription; an undifferentiated human myelogenous leukemia cell line (K-562; ATCC CCL 243); and a bovine leukemia B-cell line (BL-3; ATCC CRL 8037), also selected owing to previous success in transfection with retroviral constructs. These were cultured in Roswell Park Memorial Institute tissue culture medium (RPMI) (Sigma-Aldrich Canada, Oakville, Ontario) supplemented with 10% FBS and L-glutamine for 3 to 5 d before transfection with CAT constructs. Adherent cell lines consisting of bat lung cells and fetal lamb kidney (FLK) cells already infected with BLV were cultured for 7 to 10 d in RPMI with 10% FBS before transfection.

Transfection of mammalian cells

A lipid reagent, DMRIE-C (1,2-dimyristyloxypropyl-3-dimethyl-hydroxyethyl ammonium bromide; Life Technologies, Mississauga, Ontario), was used as a vehicle to transfect cells. Variability in transfection efficiency within each experiment was controlled for by making 1 transfection preparation for each experiment and then testing aliquots of transfected cells with various culture treatments. A transfection medium of 1.5 mL of OPTI-MEM (Life Technologies) and 7.2 μL of DMRIE-C was allowed to equilibrate at room temperature for 15 min before the addition of 10 μg of plasmid DNA with or without 5 μg of the pSVtat or pRSVtax vector, then was incubated for 30 min at room temperature.

Adherent cells were cultured in 6 mL of RPMI with 10% FBS in a 6-well culture plate until 75% confluence was attained. After 3 washes in serum-free medium (OPTI-MEM), adherent cells were

reconstituted in transfection medium containing BLV constructs prepared as described above, then cultured for 6 h. The medium was aspirated, and the transfected cells were trypsinized and resuspended at a concentration of 10^7 cells/mL in OPTI-MEM.

Suspension cell lines were pelleted and resuspended at a concentration of 5×10^5 cells/mL for 3 h of culture in RPMI supplemented with 10% FBS and L-glutamine. Cells were collected and resuspended in OPTI-MEM at a concentration of 10^7 cells/mL, and 240 μL of each cell solution was added to the prepared plasmids, which were incubated at 37°C in 5% CO_2 for 4 h. After transfection, 5×10^4 live cells were placed in each of 6 wells of a 24-well plate.

Bovine plasma from ACD-anticoagulated blood was heated to 65°C for 10 min to precipitate fibrin and clarified by centrifugation at 3000 × *g*. Ten-fold dilutions of the defibrinated (DF) plasma were prepared such that the plasma concentration ranged from 100% to 0.01% in RPMI containing 10% FBS. Two potent stimulators of viral transcription were then added: phorbol-12-myristate 13-acetate (PMA) (25 ng/mL), which activates protein kinase C by binding to its diacylglycerol binding site; and ionomycin (Sigma-Aldrich) (2 μM), which mimics the resulting calcium flux upon binding of inositol triphosphate to intracellular receptors (analogous to antigen receptor signaling in T cells). One milliliter of 1 of the plasma dilutions or of the medium control was added to each well in the culture plate and incubation continued for 48 h. The cells were pelleted and washed with phosphate-buffered saline.

Assay of CAT protein

A commercial enzyme-linked immunosorbent assay (ELISA) kit (Roche Diagnostics, Mississauga, Ontario) was used to quantify the production of CAT protein. Briefly, 150 μL of CAT lysis buffer was added to each pellet and the mixture incubated for 30 min at room temperature, then centrifuged at 12 000 × *g* for 10 min at 4°C. The protein concentrations of a 6.25% concentration of cell lysate mixed in water were determined by a Bradford dye-binding assay (BioRad Laboratories, Mississauga, Ontario). Duplicate 100-μL samples of protein or standards of 0, 12.5, 25, 50, and 100 ng of CAT were applied to individual wells of the ELISA plate, which was precoated with an anti-CAT antibody. The CAT protein was detected by the addition of anti-CAT-digoxigenin (DIG) antibody, anti-DIG-peroxidase (POD) antibody, and then a POD substrate, according to the manufacturer's instructions. The CAT assays were repeated from 2 to 6 times in each cell line for each plasmid construct. The data were recorded as milligrams of CAT produced per milligram of cell lysate, then expressed as a percentage of the value for the medium control within each experiment to account for differences in transcription in individual experiments.

Electrophoretic mobility shift assay (EMSA)

Peripheral blood mononuclear cells (PBMCs) from the BLV-infected cows with PL were isolated as described previously (24). Briefly, blood was collected in evacuated 10-mL tubes containing ACD and centrifuged at 800 × *g*. The buffy coat was removed and layered onto a density gradient (Histopaque 1077; Sigma-Aldrich). Mononuclear cells were collected and washed, and aliquots of 10^7 cells were added to 75-mm culture flasks and cultured for 24 h in RPMI with 10% FBS. Defibrinated plasma (25% v/v) was added to 2 flasks. After 12 h, PMA (25 ng/mL) and ionomycin (2 μM) were added to 1 flask each of a culture in medium and a culture in 25% DF plasma, and incubation was continued for 3 h. Thereafter, nuclear extracts from the 4 cultures were prepared by standard procedures (29).

Complementary double-stranded oligonucleotides of the NFκB (52 bp) and CRE (21 bp) binding sites (Gibco-BRL, Burlington, Ontario) in the BLV LTR were end-labeled with adenosine triphosphate labeled with radioactive phosphorus (^{32}P) (Mandel Scientific Company, Guelph, Ontario) with the use of T4 polynucleotide kinase (Pharmacia Inc., Mississauga, Ontario) according to the manufacturers' instructions but allowing the reaction to continue for 1 h at 37°C. Nucleotide sequences of the top strand were 5′-CTGGTGGCTAGA ATCCCCGTACCTCCCCAACTTCCCCTTTCCCGAAAAATCCA-3′ and 5′-GATCTCCATGACGTCAATTGA-3′. The probes were passed through columns (Quick Spin; Roche) and precipitated with 0.1 volume of 3 M sodium acetate, pH 5.2, and 2 volumes of 95% ethanol. After a 2nd wash in 70% ethanol, the probes were resuspended in 75 μL of water.

In binding reactions, 0.5 ng of the radiolabeled probe and 5 μg of nuclear extract in buffer (1 mM Tris, 0.1 mM EDTA, 4 mM NaCl, 0.1 mM beta-mercaptoethanol, 0.4% glycerol, 1 mM dithiothreitol, 50 μg/mL of poly dI-dC [Sigma-Aldrich], and 50 μg/mL of bovine serum albumin) were incubated for 30 min at room temperature. In competition assays, a 50-fold excess of unlabeled probe was added before the labeled probe. The mixture was resolved on a 6% polyacrylamide gel in a buffer of 22 mM Tris, 22 mM boric acid, and 0.5 mM EDTA. The gels were air-dried and then exposed to radiographic film for 6 h at −80°C.

Assay of NFκB proteins

To support the EMSA results, NFκB proteins were quantified with an ELISA (TransAM NFκB Family Transcription Factor Assay; Active Motif, Carlsbad, California, USA) according to the manufacturer's instructions. Briefly, 30 μL of binding buffer and 20 μg (1 μg/μL) of nuclear lysate from BLV-infected lymphocytes or Jurkat cells cultured in medium alone, in medium with PMA and ionomycin, in medium containing 25% (v/v) autologous DF plasma, or in medium containing 25% autologous DF plasma, PMA, and ionomycin were added to individual wells of a supplied 96-well plate. Control wells received 5 μg of provided Raji nuclear extract (0.25 μg/μL) or 20 μL of buffer only. In competitive-binding assays, 30 μL of binding buffer containing 20 pmol of either NFκB wild-type or NFκB mutated oligonucleotide was added to appropriate wells. The plates were sealed and incubated for 1 h at room temperature rotating at 25 × *g*. Proteins were detected with antibody to 1 of NFκB p50, p52, p65, c-rel, or rel-b, followed by secondary antibody against rabbit IgG conjugated with horseradish peroxidase and developing solution. Absorbance was read within 5 min. The assay was repeated 3 times.

Statistical analysis

With the use of SigmaStat (Systat Software Inc., Richmond, California, USA), 1-way repeated-measures analysis of variance was performed to test for differences between treatments. Pairwise comparisons of treatments were done by means of the Holm-Sidak post-test. Differences were considered significant if the *P*-value was 0.05 or less.

Results

Since viral production relied upon the addition of FBS to the culture medium, presumably to provide nutrients necessary to sustain cell health, we concluded that FBS does not contain PBF activity (24). We did not measure CAT activity but indirectly measured LTR-driven transcription of CAT by assessing the amount of CAT protein in cells. We were able to detect production of CAT driven by the BLV LTR only if the cells were already infected with BLV. Cotransfection of noninfected cells by the tax construct along with the BLV construct yielded no CAT protein. With the addition of DF plasma to the cell culture, CAT production in infected cells was reduced significantly, in a dose-dependent manner, for all 3 cell lines tested (Figure 1A). The blocking effect was strongest in the FLK line but was significant ($P < 0.001$) in all 3 cell lines to a concentration of 0.01% plasma. The addition of DF plasma at any concentration did not significantly affect the production of CAT in the BIV-LTR-CAT construct in any of the suspension cell lines (Figure 1B). In experiments with the FeLV-LTR-CAT construct (Figure 1C), an inverse dose response to the addition of DF plasma was observed, such that the amount of CAT increased with incremental amounts of plasma, reaching significance at a concentration of 10% plasma in the culture medium. Viability in the Jurkat cells declined significantly if the plasma concentration surpassed 50%, thereby reducing the amount of CAT per milligram of cell lysate. The addition of DF plasma to cultures of Raji and Jurkat cell lines transfected with the FIV LTR (Figure 1D) had no effect on CAT production. Unexpectedly, the K-562 cell line did not support FIV LTR-driven CAT production in any experiment. As shown in Figures 1E and 1F, respectively, the production of CAT from the HTLV and HIV LTRs was studied in Jurkat cells only. Both of these constructs demonstrated a dose response to the addition of DF plasma to culture similar to that of the BLV LTR. The effect disappeared at $\leq 0.08\%$ plasma with the HIV construct and at 1% with the HTLV-LTR construct. Figure 1G shows the results of experiments with a construct containing only the NFκB element (from the Petaluma strain of FIV) fused to the CAT gene, tested in Jurkat cells: a significant reduction in CAT was detected at $\geq 1\%$ plasma.

The EMSAs, performed on BLV-infected PBMCs cultured in RPMI or in RPMI with 25% DF plasma with or without added stimulation by PMA and ionomycin, showed binding of the 21-bp probe for the CRE to 4 proteins from nuclear extracts (Figure 2A). The largest oligonucleotide–protein complex was detected only in cultures including 25% DF plasma but was not upregulated in response to stimulation with PMA and ionomycin. The 2nd-largest complex was present in very low amounts in all treatments but responded to PMA and ionomycin only in the presence of plasma. A 3rd complex was found in very low amounts in the medium treatments only. The final band represents cold competition, wherein a 50-fold excess of unlabeled probe competed for binding sites with the radiolabeled probe. The unlabeled probe outcompeted the labeled probe, and radiolabeled nuclear complexes were not seen.

As Figure 2B shows, EMSA demonstrated a low level of binding to the NFκB element in extracts from cells cultured in medium alone. However, binding greatly increased when the cells were stimulated with PMA and ionomycin. There was reduced binding of the NFκB probe in extracts of cells cultured in medium supplemented with 25% DF plasma. Importantly, stimulation with PMA and ionomycin of cells cultured with plasma did not relieve the block imposed by plasma alone.

To confirm that individual NFκB proteins differed in the stimulated nuclear extracts as compared with the unstimulated and to define the specific proteins involved, we performed NFκB ELISA on all 4 nuclear-extract samples described above. A sandwich ELISA was used to capture 1 of 5 NFκB proteins in the extract, and the amount of protein captured was determined by absorbance at 450 nm. Preliminary experiments were done in which NFκB wild-type DNA or a mutated form of NFκB DNA not recognized by NFκB proteins was added to nuclear extracts before assay. The ELISA uses antibodies that recognize the DNA binding site on NFκB proteins; therefore, competition with wild-type DNA revealed significantly reduced absorbance of all treatments ($P < 0.001$) as compared with the mutated DNA, which had no effect on treatments (data not shown). The ELISA detected the amount of each NFκB protein in the nuclear component of Jurkat cells (Figure 3, left-side panels) and in PBMCs from 3 BLV-infected cows (Figure 3, right-side panels).

The individual NFκB proteins varied considerably in amount, but each was present in relatively similar amounts in the Jurkat and the BLV+ lymphocyte nuclei. The most highly detected NFκB proteins were c-rel and p65; the least abundant were p50 and p52. Although abundance of proteins was similar in the 2 types of cell in untreated cultures, there were differences between the cell types in response to plasma components. In Jurkat cells, the levels of 4 members of the NFκB family increased in nuclear extracts from cells stimulated with PMA and ionomycin, whereas p50 displayed no change. In contrast, the nuclei of the PBMCs of 3 cows grown in identical culture treatments showed alterations in only 2 of the 5 NFκB proteins: p52 and rel-b.

In both cell types, the amount of detected p50 in nuclear extracts did not change in response to treatment conditions. In contrast, the amount of p52 was always greater in extracts from the stimulated-medium treatment than in those from the plasma treatment ($P < 0.001$), even if the plasma-treated cells were also stimulated with PMA and ionomycin ($P = 0.001$). The amount of p52 in the nuclei of stimulated cells grown in medium was also greater than that in cells grown in medium alone ($P = 0.002$ for Jurkat cells and $P = 0.031$ for PBMCs). In addition, the amount of p52 in the nuclei of Jurkat cells grown in medium alone was greater than the amount in cells grown with plasma, whether without stimulation ($P = 0.007$) or with stimulation ($P = 0.005$). However, there was no difference in nuclear localization of p52 between the PBMCs cultured in medium and those grown with plasma.

The amount of p65 detected in nuclei of Jurkat cells grown in medium with PMA and ionomycin was significantly increased compared with the amount after any of the other 3 treatments ($P < 0.001$). However, the amount of p65 in BLV+ PBMC nuclei did not alter in response to either the presence of plasma or stimulation. Paralleling the EMSA results, nuclear localization of rel-b was always greatest with the stimulated-medium treatments, particularly in Jurkat cells treated with either plasma or plasma and PMA plus ionomycin ($P < 0.001$). However, the difference between cells in medium and stimulated medium was less dramatic ($P = 0.041$). Interestingly, the amount of nuclear rel-b detected in the

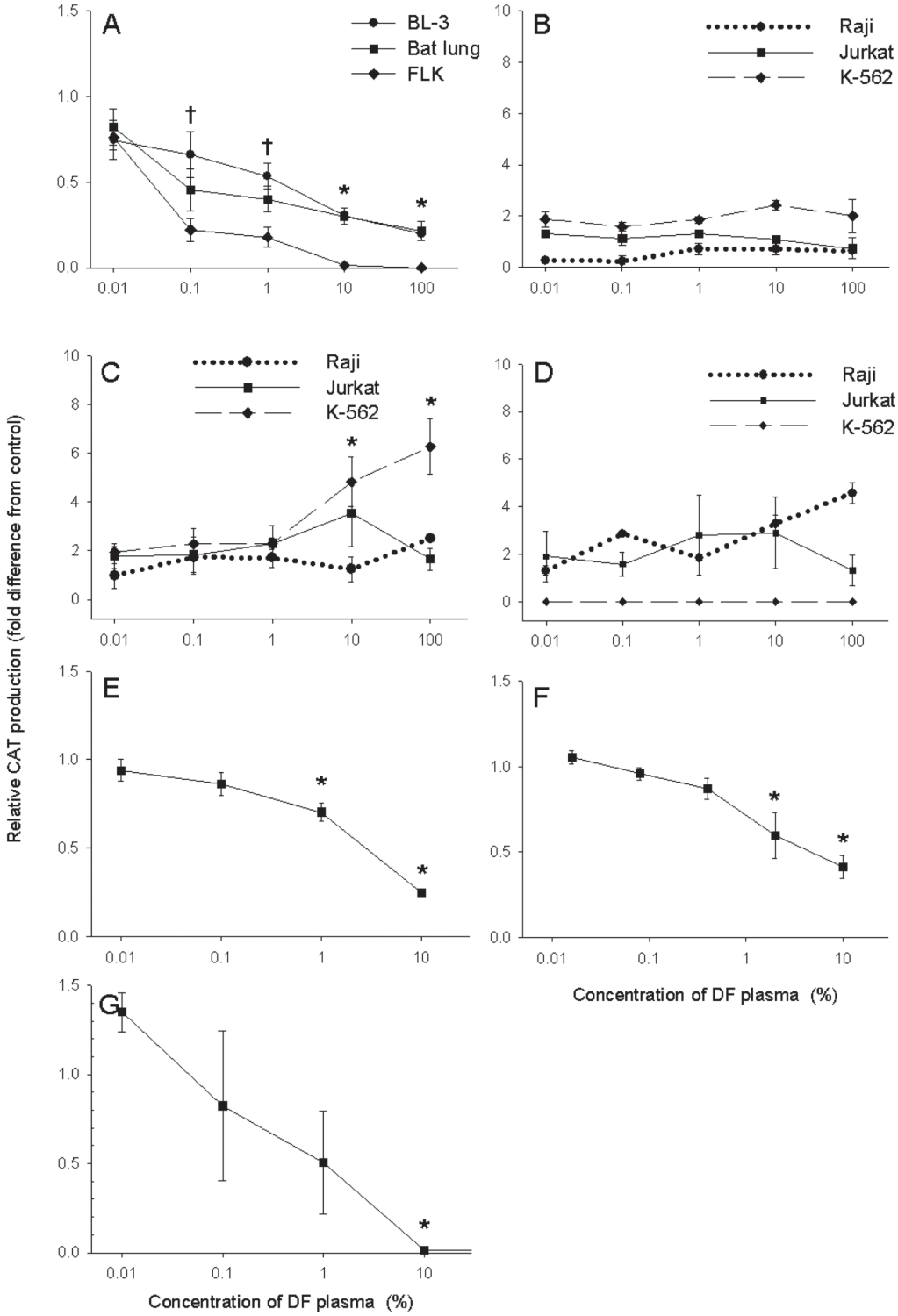

Figure 1. Effect of various concentrations of defibrinated (DF) plasma from cattle infected with *Bovine leukemia virus* (BLV) on the production of chloramphenicol acetyl transferase (CAT) protein in cell cultures transfected with plasmids containing a retroviral long terminal repeat (LTR) fused to the CAT gene. Each data point and bar represent the mean and standard error of 2 to 6 independent experiments. Production of CAT in cells cultured in medium alone (control) was assigned a value of 1. Production of CAT in cell cultures treated with various concentrations of DF plasma was expressed as fold differences from the control. (A) Bat lung and fetal lamb kidney (FLK) cells infected with BLV and BL-3 (bovine B-cell leukemia) cells transfected with pBL-1 (a BLV 5′ LTR inserted into a pSVO CAT construct). The significant-difference symbols pertain to all cell lines in this panel. (B) Raji (human B cell), Jurkat (human T-cell leukemia), and K-562 (undifferentiated human myelogenous leukemia) cell lines cotransfected with pBIV LTR and pSVtat. (C) Raji, Jurkat, and K-562 cells transfected with a *Feline leukemia virus* (FeLV) LTR subcloned into p16A1. The significant-difference symbols apply only to the K-562 cell line in this panel. (D) Raji, Jurkat, and K-562 cells transfected with pPPR LTR (the LTR of *Feline immunodeficiency virus* [FIV], subcloned into CAT vector p22A2s). (E) Jurkat cells cotransfected with pRSVtax and pHTLV-1 LTR. (F) Jurkat cells cotransfected with tat and pHIV LTR. (G) Jurkat cells transfected with pF34 NFκB subcloned into p22A2s. * — significantly different from the control at $P < 0.001$. † — significantly different from the control at $P < 0.01$.

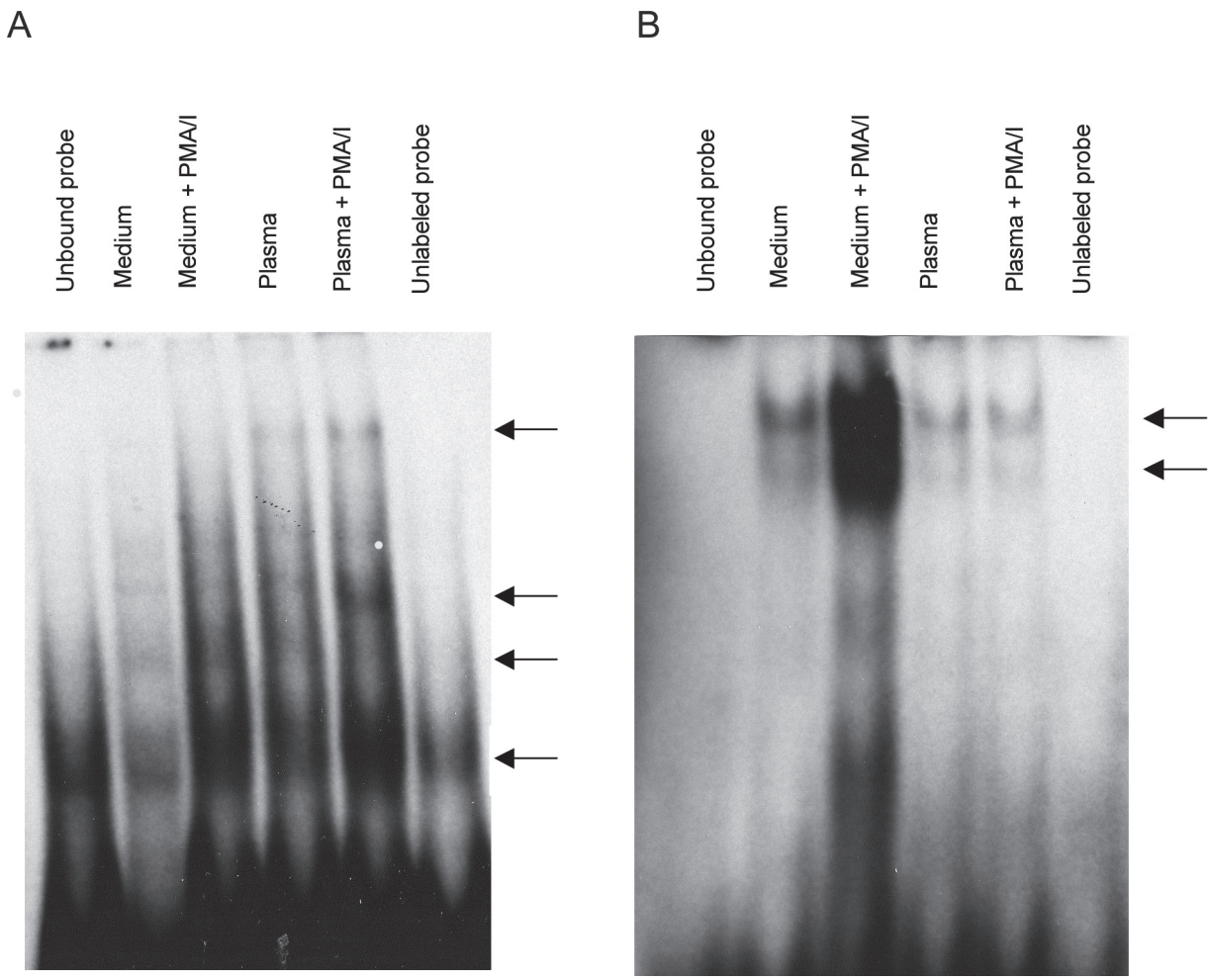

Figure 2. Autoradiographs of polyacrylamide gels revealing complexes formed when probes labeled with radioactive phosphorus (^{32}P) encoding the binding sites of the cyclic adenosine monophosphate response element (A) and NFκB (B) of the BLV LTR were mixed with nuclear extracts from BLV-infected peripheral blood mononuclear cells (PBMCs) cultured in medium alone or medium containing DF plasma from BLV-infected cows, with or without stimulation with phorbol-12-myristate 13-acetate (PMA) or ionomycin (I). Unbound probe — radiolabeled probe with no nuclear extract. Unlabeled probe — nuclear extracts from the medium control with radiolabeled probe and a 50-fold excess of unlabeled probe.

medium-cultured Jurkat cells also differed from the amounts detected in the plasma-treated ($P = 0.008$) and the stimulated plasma-treated Jurkat cells ($P < 0.001$). Similarly, nuclear localization of rel-b in stimulated PBMCs grown in medium differed from that of PBMCs grown in medium ($P = 0.009$) or plasma ($P = 0.004$) without stimulation or in plasma with stimulation ($P = 0.035$), but the unstimulated medium samples did not differ from either of the plasma-treated samples.

Finally, c-rel also gave contrasting results for the 2 cell types: there was no change in nuclear amounts of c-rel in PBMCs, but in Jurkat cells the amount was significantly increased by stimulated medium treatment as compared with medium alone ($P = 0.008$), plasma alone ($P < 0.001$), and plasma with stimulation ($P = 0.013$). However, the c-rel amount in Jurkat cells cultured in medium alone did not differ from that in cells receiving either of the plasma treatments.

In bovine PBMC extracts, there were no significant changes in nuclear translocation of p50, p65, or c-rel in response to treatment of culture medium with plasma, PMA and ionomycin, or all 3. Importantly, levels of p52 and rel-b in cell nuclei increased in response to PMA and ionomycin but only in the absence of plasma. Thus, these 2 forms of NFκB are the most likely mediators in upregulating BLV transcription in PBMCs, as their absence in plasma-cultured cells implies that transcription is inhibited.

Discussion

Using CAT constructs, EMSA, and quantification of NFκB proteins in cell nuclei, we demonstrated the effect of an inhibitory plasma factor on LTR-driven transcription. In CAT ELISA, detection of CAT protein varied widely from cell line to cell line but was highest in Jurkat cells for most constructs, except for the BLV LTR, in which CAT was undetectable. In agreement with previous reports, CAT production as a result of transfection with the pBL1 construct was found only in established BLV-producing cell lines — FLK cells, bat lung cells (26,30), and BL-3 cells that we infected with BLV by culture in supernatants from BLV+ cells. This has been shown to be due to the necessity of transactivation by tax to initiate viral transcription

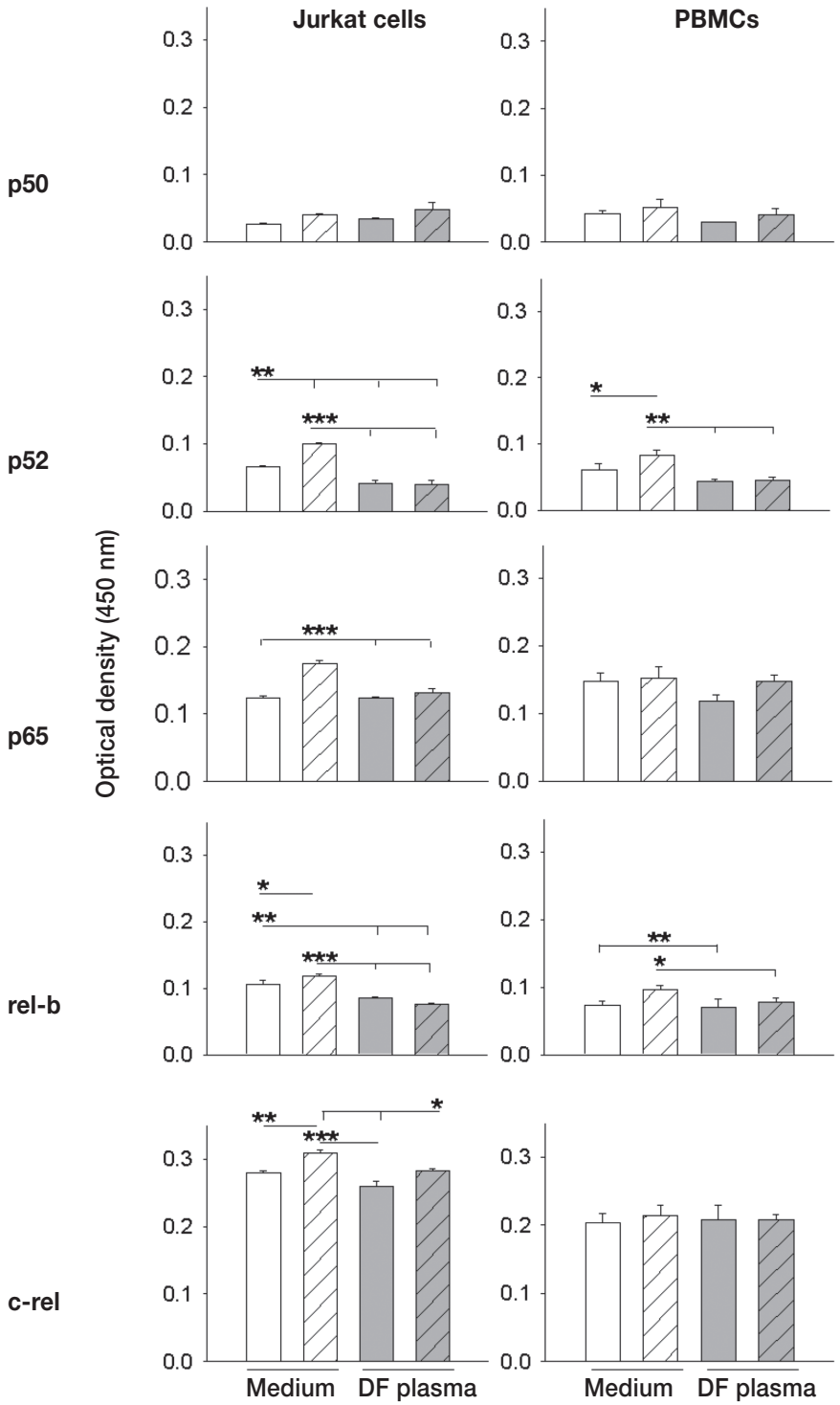

Figure 3. Quantities, expressed as optical density at 450 nm, of NFκB proteins p50, p52, p65, rel-b, and c-rel detected in nuclear extracts from Jurkat cells and PBMCs of 3 BLV-infected cows. The cells were cultured in medium alone (white bars), medium with added PMA and ionomycin (white cross-hatched bars), medium containing 25% (v/v) DF plasma from BLV-infected cows (grey bars), or medium containing DF plasma with added PMA and ionomycin (grey cross-hatched bars). Each bar and whisker represent the mean and standard error of 3 experiments. The significant-difference symbols indicate that the results differ for the treatment underneath, compared with the other treatments, at *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.

(2). In the present study, however, cotransfection with pRSVtax did not permit sufficient CAT expression for detection by the CAT assay. It is possible that the tax vector transfected poorly in comparison with the pBL-1 LTR construct.

More importantly, the addition of bovine plasma to cell cultures resulted in a dose-dependent decrease in CAT initiated by the LTRs of BLV, HIV, and HTLV-1. Although the optimal concentration of the added plasma differed between constructs, the reductions

induced by the plasma were significant. These results suggest that the pathway used by the inhibitory factor is common to BLV, HIV, and HTLV-1 but does not affect promoters in BIV, FeLV, or FIV. The BLV, HIV, and HTLV LTRs each have an NFκB response element proximal to the transcription initiation site. Neither FeLV nor the FIV strain used in these studies contains this site, and, although BIV does have an NFκB response element, it is comparatively distant from the start site and has not been found to be important in regulating transcription (31). The use of a construct containing only the NFκB element corroborated that CAT expression was reduced by plasma in a dose-dependent manner and was obliterated at a plasma concentration of 1%, an amount comparable to that of cells with constructs containing the LTR of BLV, HTLV, and HIV, which titrated out at 0.01%, 0.8%, and 1%, respectively. This suggested that PBF interferes with NFκB binding to the LTR.

The evaluation of the roles of individual elements in the LTR that regulate retroviral expression is complicated by the fact that not only are some sites synergistic, both negative and positive elements coexisting, but also the function of individual elements varies in different cell lines or tissues. In a series of experiments, Xiao and Buehring (12) introduced a number of point mutations into protein-binding sites within the LTR of BLV-producing Bat2Cl6 (bat lung) cells. They concluded that, in the absence of tax, a completely intact LTR was needed to initiate expression of BLV, because a point mutation in any protein-binding site abrogated viral transcription. In contrast, Brooks et al (32), using viral promoter constructs, observed that NFκB, together with a single CRE site, was able to stimulate low levels of BLV expression independently of tax. In the presence of tax, CRE2 alone maintained viral transcription, but effects of mutation in the NFκB site varied between cell lines.

By means of EMSA, we compared the importance of the NFκB response element and the CRE element in mediating the PBF effect. There was low binding of the nuclear extracts of cells to the CRE probe. However, the same extracts bound strongly to the NFκB probe and responded substantially to stimulation by PMA and ionomycin when grown in medium alone. However, exposure to PBF during culture reduced the basal ability of nuclear extract to bind to the NFκB response element. Importantly, nuclear extracts of plasma-treated cells did not support the strong response to stimulation with PMA and ionomycin. Although differences in binding to the CRE probe were much less dramatic, some slight changes in the binding pattern were observed. Therefore, it cannot be ruled out that the CRE also plays a minor role in mediating transcriptional regulation by PBF.

In resting cells, NFκB is retained in the cytoplasm in an inactive complex with IκBα, such that recognition of the NFκB localization signal (NLS) by nuclear import machinery is blocked. Upon stimulation by cytokines, viral or bacterial products, or mitogens, IκBα is phosphorylated, and its DNA-binding subunits are disrupted (33,34). Thus, NFκB is activated, and its proteins are released for transport to the nucleus, where they can bind to NFκB response elements to regulate gene expression. The NFκB family comprises the DNA-binding proteins c-rel, rel-b, and rel-a (p65), as well as 2 large precursor proteins: p105, which yields the 50-kDa DNA-binding protein p50; and p100, which gives rise to p52. The NFκB proteins, through binding the response element, regulate a number of gene products contributing to inflammation and immune response, cell proliferation, and apoptosis.

We further investigated the role of individual NFκB family members in blocking transcription of BLV by quantifying nuclear localization of these NFκB proteins in Jurkat cells and BLV-infected PBMCs. From our results, it appears that all members of the NFκB family except p50 are more abundant in the nucleus of cells stimulated by PMA but that the presence of DF plasma significantly reduces nuclear translocation of p52 and rel-b in both Jurkat cells and PBMCs, a reduction not abrogated by stimulation with PMA and ionomycin. In addition, Jurkat cells cultured with plasma also show reduced amounts of p65 and c-rel in their nuclei as compared with those cultured in medium alone. Of the 5 members of the NFκB family in the cytoplasm, p65 and p50 are the most abundant. Upon signaling from surface receptors, IκB complexes, which sequester NFκB in the cytoplasm, become phosphorylated and release the proteins, which form homodimers or heterodimers. Homodimers of p50 or p52 inhibit transcription of NFκB-regulated genes, whereas heterodimers upregulate transcription (35). In this study, PBMCs were derived from BLV-infected cattle, but Jurkat cells were not transfected with BLV, so the differences in NFκB-protein translocation to the nucleus support the notion that the effect of PBF is on regulation of sequestration in the cytoplasm rather than on translocation to the nucleus. Thus, it appears that rel-b and p52 are particularly important in BLV transcription. Since rel-b has no transactivating potential, it appears that retention of p52 in the cytoplasm under PBF regulation is the critical factor in initiating transcription of BLV.

The role of extracellular proteins in regulation of transcription is key to maintaining viral latency in vivo. These proteins likely exert their effect via existing cell-signaling pathways. Plasma blocking factor exerts its effect in vitro, even in the presence of added mitogens (22,36). Since the coordinated transcription of genes in B cells in response to stimulation by LPS is mediated by NFκB (37), it appears that PBF acts by blocking activation of NFκB in the cytoplasm. We recently isolated PBF and found it to be a novel form of fibronectin (38). Lymphocyte ability to bind fibronectin via surface receptors is critical to the inflammatory response. Indeed, lymphocytes from patients with chronic inflammation exhibit enhanced affinity for fibronectin, which maintain an inflammatory response (39). This increased affinity could be inhibited by the calcium chelator EDTA. In early experiments, we noted that EDTA-anticoagulated blood did not retain PBF activity (22). We subsequently found that the addition of PBF to cultures of PBMCs had several effects: the expression of BLV and surface immunoglobulin was consistently downregulated, but the net effect was upregulation of protein expression in the cell (40). It remains to been seen if the decrease in expression of viral protein is a result of competition by transcription of cellular genes or, more likely, a result of specific signals that sequester nuclear proteins necessary for BLV transcription. Similar blocking activity has been described in human plasma (23), suggesting that diverse species have conserved the proteins responsible for the activity to minimize the effects of chronic retroviral infection. Characterizing the protein and understanding the pathways through which it functions may reveal novel methods for maintaining the latency of chronic infections by retroviruses such as HIV.

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