Activation of Bovine Leukemia Virus Transcription in Lymphocytes from Infected Sheep: Rapid Transition through Early to Late Gene Expression

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Bovine leukemia virus (BLV) expression is mostly silent in peripheral blood mononuclear cells (PBMCs) of infected animals. However, when infected cells are cultured, they are stimulated to produce virus. We studied viral transcription in PBMCs taken from BLV-infected sheep because the pattern of transcriptional activation in these cells should closely mimic activation of virus expression within mononuclear cells in vivo. BLV transcription was activated as early as 30 min after PBMCs were cultured. Expression was characterized by early and late stages, each distinguished by a unique pattern of cytoplasmic RNAs. In early expression, cytoplasmic viral RNA was exclusively the doubly spliced *tax/rax* transcript, although all transcripts were present in the nucleus. Early expression gave way rapidly to late expression, in which all viral transcripts accumulated in the cytoplasm. The polyclonal B-cell activator lipopolysaccharide increased the amount of viral RNA by at least twofold but did not alter the pattern of transcription. The transition from early to late expression required new protein synthesis and was blocked by the inhibitor cycloheximide. This requirement reflects the essential role of the viral Rex protein in the transition, but synthesis of cellular factors may be required as well. These results provide the first demonstration of staged viral expression in lymphocytes naturally infected by either BLV or the closely related human T-cell leukemia virus (HTLV) and validate the model of BLV and HTLV gene expression that previously was derived from transfection experiments performed mainly in nonlymphoid cells.

Bovine leukemia virus (BLV) is an oncogenic retrovirus that infects predominantly B cells of cattle and, under experimental conditions, of sheep. BLV is structurally and functionally related to human T-cell leukemia virus types I and II (HTLV-I and -II) and simian T-cell leukemia virus, although this group of viruses shares only limited sequence homology (3, 4). Infection is characterized by long clinical latency during which virus expression appears to be silent in most infected cells. Circulating lymphocytes carry integrated proviral DNA but do not produce detectable virus (23). In contrast, an antiviral immune response persists throughout infection. Thus, viral protein production is probably compartmentalized within immune tissues and may occur intermittently throughout the course of infection. The spread of viral infection to new host cells in these tissues increases the number of cells at risk for tumorigenic conversion. To understand how individuals progress from asymptomatic infection to disease, we need to understand mechanisms that control latency and reactivation of viral expression within the infected cell.

Although BLV transcripts are not readily detected in circulating lymphocytes, rare BLV RNA-positive cells have been found among freshly isolated peripheral blood mononuclear cells (PBMCs), using the very sensitive technique of in situ hybridization (28). Similarly, rare HTLV-transcribing cells have been found among freshly isolated PBMCs from patients with HTLV-associated disease (13). Results obtained by using the polymerase chain reaction show that the presence of rare BLV or HTLV transcripts is independent of

clinical disease (22, 26). Many additional cells are stimulated to express virus when PBMCs from infected individuals are cultured with polyclonal activators of lymphocytes (28). This finding suggests that immune activation of a latently infected cell initiates viral expression within the host.

Expression of BLV and HTLV is proposed to occur in two stages (18, 20, 40). In the early stage, cytoplasmic viral RNA consists of a doubly spliced, 2.0-kb transcript that encodes the viral regulatory proteins, Tax and Rex. Tax interacts with cellular factors to further activate transcription from the viral long terminal repeat (8, 21, 39, 43). Accumulation of Rex initiates a switch to the late stage of expression, when the full-length and singly spliced mRNAs become cytoplasmic; these transcripts are translated into viral structural proteins, which are then assembled into virions. Several mechanisms for the Rex-initiated transition have been proposed, including the regulation of RNA stability, RNA splicing, or RNA transport (18, 20, 40).

While Rex from BLV has been studied directly (9, 11), details of its function have often been drawn from analogies to Rex of HTLV-I or to the functionally related Rev of human immunodeficiency virus (HIV). For the latter viruses, much evidence supporting temporal models of gene expression has been obtained by transfection of subgenomic constructs into nonlymphoid cell lines and examination of cytoplasmic RNAs (10, 14, 15, 20, 30, 34, 40). However, Hidaka et al. (18) transfected the intact HTLV-I provirus into fetal amnion cells to demonstrate that Rex is required for full-length and singly spliced viral mRNA to appear in the cytoplasm. Derse (9) similarly transfected mutated BLV proviruses into transformed canine fibroblasts and showed that the presence of full-length RNA in the cytoplasm requires an intact *rex* gene. Interestingly, in those experi-

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ments, significant amounts of the singly spliced *env* transcript appeared in the cytoplasm of cells transfected with a construct that should have been incapable of Rex synthesis. Recent studies of lymphoid cells led Arrigo and Chen to modify the model of HIV expression; they found that in the absence of Rev, the singly spliced *env* transcript is exported from the nucleus but is not efficiently translated (1). In this light, Derse's results suggested to us that BLV expression in lymphocytes might be more complex than was previously thought.

We wanted to examine viral regulatory mechanisms within naturally infected cells, and so we chose to study BLV expression in latently infected PBMCs taken from BLV-infected sheep. This system should closely approximate the activation of viral expression within the animal host. BLV transcription was induced very rapidly upon culture of latently infected cells. Transcription of viral RNA was increased twofold by addition of lipopolysaccharide (LPS). Early expression was transient and was characterized by the exclusive presence of 2.0-kb *tax/rex* mRNA in the cytoplasm. However, all viral transcripts were present in the nucleus, and cultured PBMCs progressed rapidly to late expression when all viral RNAs became cytoplasmic. Viral expression was blocked at the early stage by inhibition of new protein synthesis, including that of Tax and Rex, in keeping with the idea that the switch to late-stage expression depends on production of the Rex protein. These results provide the first demonstration for either BLV or HTLV that viral gene expression proceeds through early and late stages in lymphocytes taken from infected individuals.

MATERIALS AND METHODS

Preparation and culture of cells from infected sheep. Blood was collected by jugular venipuncture from sheep that had been infected 5 years previously with BLV, and buffy coats were prepared. Mononuclear cells were further enriched by centrifugation over Histopaque 1077 (Sigma, St. Louis, Mo.) as previously described (29). PBMCs were suspended in Iscove's medium, counted, and diluted to 10^6 /ml in minimal Eagle's medium (MEM) supplemented with 0.1 mM MEM nonessential amino acids, 2 mM L-glutamine, 100 U of penicillin and 100 μ g of streptomycin per ml, and 5% fetal bovine serum. When incubation times were short, media and culture flasks were prewarmed to 37°C before the addition of PBMCs. LPS (from *Salmonella minnesota*; Sigma) was used at 10 μ g/ml.

Purification of RNA from PBMCs. Samples of approximately 3×10^7 PBMCs were cultured for the indicated times. During the first several hours, lymphocytes often adhered transiently to the flask; therefore, recovery was improved by a brief (1- to 2-min) agitation of the emptied 75-cm² flask with 2 to 3 ml of 1 mM EDTA in Hanks' balanced salt solution. This treatment did not release tightly adherent macrophages. Released cells were pooled with the rest of the sample on ice, and cells were pelleted at $300 \times g$ for 10 min at 4°C, washed once with ice-cold Hanks' balanced salt solution, and pelleted again. The cell pellet was vortexed in 4 ml of denaturing solution (4 M guanidinium thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% [wt/vol] sarcosyl, 0.1 M 2-mercaptoethanol) (6) until cells were completely lysed. At this point, lysates could be frozen in liquid N₂ and stored at -80°C. To prepare total RNA, lysates were extracted with acid phenol and RNA was precipitated with isopropanol (6). The yield was typically 10 μ g of RNA from 10^7 cells but

varied with the individual animal and the amount of time for which cells were cultured.

To prepare poly(A)⁺ RNA, we adapted the method of Aviv and Leder (2) to a batch procedure scaled for small amounts of RNA. Total RNA, 25 to 50 μ g in 200 μ l of H₂O, was heated for 5 min at 65°C and quickly cooled on ice, and 50 μ l of 5 $\times$ binding buffer (1 $\times$ binding buffer is 20 mM Tris-hydrochloride [pH 7.5], 0.5 M NaCl, and 1 mM EDTA) was added. To this mixture was added 100 μ l of a 10% (wt/vol) suspension of oligo(dT)-cellulose (type 7; Pharmacia, Piscataway, N.J.) in 1 $\times$ binding buffer, and samples were incubated at room temperature for 30 to 60 min with mixing, to allow binding. Oligo(dT)-cellulose with bound RNA was then collected by centrifugation (15 s, 12,500 $\times g$) in a microcentrifuge equipped with a horizontal rotor and was washed three times with 400 μ l of 1 $\times$ binding buffer and once with wash buffer (20 mM Tris-hydrochloride [pH 7.5], 0.1 M NaCl, 1 mM EDTA). Poly(A)⁺ RNA was recovered by three successive washes with 150 μ l of elution buffer (10 mM Tris-hydrochloride [pH 7.5], 1 mM EDTA) that had been preheated to 65°C. The eluates were pooled and quantitated by spectroscopy, and RNA was precipitated in the presence of 20 μ g of glycogen carrier (Boehringer Mannheim, Indianapolis, Ind.). The yield after one round of selection was typically 4 to 6% of the input RNA.

Fractionation of nuclear and cytoplasmic RNAs. To prepare nuclear and cytoplasmic RNAs, PBMCs were homogenized in 2 ml of lysis buffer (10 mM Tris-hydrochloride [pH 7.8], 150 mM NaCl, 0.65% Nonidet P-40, 1.5 mM MgCl₂, 1 mM dithiothreitol, 400 U of RNasin [Promega Corp., Madison, Wis.] per ml) and incubated for 10 min on ice, and nuclei were collected by centrifugation at $800 \times g$ for 5 min at 4°C. The cytoplasmic supernatant was mixed with an equal volume of denaturing solution, extracted once with neutral phenol-chloroform, and precipitated. RNA was dissolved in denaturing solution and reprecipitated, and poly(A)⁺ RNA was selected as described above. The nuclear pellet was lysed in denaturing solution and processed as for total cellular RNA. Nuclear RNA was not selected on oligo(dT)-cellulose, as contamination with rRNA was minimal and further enrichment was not necessary to allow loading of samples. Recovery of nuclear RNA was enhanced by addition of 20 μ g of glycogen carrier per ml just before the first isopropanol precipitation.

Northern (RNA) blot analysis. RNA pellets were dissolved in formaldehyde gel sample mix (32), and RNAs were separated by electrophoresis through a 1% agarose gel containing 2.2 M formaldehyde. RNA was transferred overnight by capillary blotting with 20 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) onto a nylon membrane (Hybond N; Amersham, Arlington Heights, Ill.). The membrane was baked at 80°C for 1 h and then was cross-linked with 12,000 μ J of UV light (Stratalinker; Stratagene, La Jolla, Calif.). Membranes were prehybridized for 2 to 4 h at 42°C in 50% formamide-5 $\times$ SSPE (1 $\times$ SSPE is 0.15 M NaCl, 10 mM NaH₂PO₄ [pH 7.4], and 1 mM EDTA)-2 $\times$ Denhardt's solution-0.1% sodium dodecyl sulfate (SDS)-100 μ g of salmon sperm DNA per ml. Hybridization was for 20 to 24 h at 60°C in fresh prehybridization solution containing 100 μ g of tRNA and 4 $\times 10^6$ cpm of ³²P-labeled RNA probe per ml. The probe, complementary to the 1,122-base *Clal*₇₁₁₃-to-*SacI*₈₂₃₅ fragment of the BLV genome (numbering according to reference 37), hybridizes equally to all BLV mRNAs and enables direct comparison of their amounts. The probe was transcribed by T7 polymerase in the Riboprobe system (Promega), using 100 μ Ci of [³²P]CTP (800 Ci/mmol; Amer-

sham) in a 20- μ l reaction. After hybridization, membranes were washed twice at room temperature in $1\times$ SSC-0.1% SDS for 15 min, twice at 42°C in $1\times$ SSC-0.1% SDS for 30 min, once at 68°C in $0.2\times$ SSC-0.1% SDS for 30 min, and once at 68°C in $0.1\times$ SSC-0.1% SDS for 30 min. Blots were exposed to Kodak X-AR5 film with an intensifying screen at -80°C for the indicated times. Autoradiographic band intensities were quantitated on a Visage 70 densitometer (BioImage, Ann Arbor, Mich.), usually by using exposures shorter than those illustrated in the figures. Two different exposures of each figure were analyzed to ensure that signals were in the linear range.

Immunohistochemical detection of BLV capsid protein. Capsid protein (CA or p24) was detected in PBMCs by immunocytochemistry as described previously (24). Briefly, cultured PBMCs were deposited onto slides, and proteins were fixed with paraformaldehyde and then with acetone. CA was detected by incubation with rabbit anti-CA serum and then with biotinylated goat anti-rabbit immunoglobulin G and biotinylated alkaline phosphatase complexed with avidin. Assays were developed by using the HistoMark Red substrate kit (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). Positive cells were scored under $\times 400$ magnification, using the $40\times$ planachromat objective of a photomicroscope.

Immunoprecipitation. PBMCs that had been purified from the blood of a BLV-infected sheep and from an uninfected control were cultured in duplicate. Each culture contained 4×10^7 PBMCs in 8 ml of methionine- and cysteine-deficient MEM supplemented with 7.5% fetal bovine serum, 1 mCi of [^{35} S]methionine (1,000 Ci/mmol; Amersham), and 1 mCi of [^{35} S]cysteine (1,300 Ci/mmol; Amersham). Cycloheximide (CHX; Sigma) was included at 10 μ g/ml in one of each pair of cultures. After 4 h at 37°C, cells were washed twice in cold phosphate-buffered saline (140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4 , 1.5 mM KH_2PO_4). DNase I (DPFF; 50 U; Worthington, Freehold, N.J.) was added directly to each cell pellet, and then cells were resuspended in 1 ml of cold 50 mM Tris-hydrochloride (pH 7.55)-1 mM CaCl_2 -1 mM MgCl_2 -1.0% Nonidet P-40-1.0% sodium deoxycholate-1% (vol/vol) aprotinin-100 μ g of phenylmethylsulfonyl fluoride. SDS was added to 0.1% before lysates were incubated on ice for 5 min. EDTA was then added to 5 mM, and NaCl was added to 150 mM, making the solution equivalent to RIPA buffer. DNA was sheared by drawing the viscous lysates through 22- and 25-gauge needles and by sonication. Insoluble material was removed by centrifugation of the lysates at $27,000 \times g$ for 20 min at 4°C.

Lysates were each divided into two portions that were precleared by immunoprecipitation with 4 μ l of preimmune sera (a 1:1 mixture from the rabbits used to raise antibodies against Tax and Rex peptides) and with 50 μ l of a 50% (vol/vol) suspension of protein A-Sepharose (Sigma) in RIPA buffer. After 20 h of incubation at 4°C, immunoprecipitates were washed twice in RIPA buffer and once in 50 mM Tris-hydrochloride (pH 8.0) and then were eluted into double-strength Laemmli gel sample buffer containing 100 mM dithiothreitol. Cleared supernatants were then sequentially immunoprecipitated, first with 4 μ l of rabbit anti-Rex serum raised against the C-terminal 12 amino acids of Rex (36) and then with 4 μ l of rabbit anti-Tax serum. The latter serum was raised against 15 amino acids (RFPRDTSEPPLSEFE; residues 215 to 229) of Tax in the manner already described for the Rex antibody (36). The appropriate competing peptide was included at 5 μ g/ml in one of each pair of lysates. The final immunoprecipitation was with rabbit anti-CA serum or

with the corresponding preimmune serum. Products of each immunoprecipitation were resolved on SDS-10% polyacrylamide gels. The gels were impregnated with fluorors and exposed to X-AR5 film.

RESULTS

All BLV RNAs appear rapidly in cultured PBMCs. To determine the timing and pattern with which BLV mRNAs were synthesized following activation from transcriptional latency, we cultured PBMCs newly harvested from a BLV-infected sheep and analyzed the resulting viral RNAs by Northern blots. To maximize the signal, PBMCs were stimulated with LPS. We had previously shown that this B-cell mitogen increased both the number of cells transcribing BLV and the amount of viral RNA per cell (28). Whole cell poly(A)⁺ RNA was prepared from samples of 3.25×10^7 PBMCs harvested over the course of 24 h of culture. Because some viral transcription might take place during the 3 h required to purify this large quantity of mononuclear cells (3.5×10^8 PBMCs from 400 ml of blood), RNA was also prepared from PBMCs harvested from buffy coats within 1 h of bleeding the animal. The results of Northern analyses (Fig. 1A) showed that neither the rapidly harvested sample (lane B) nor the gradient-purified, uncultured sample (lane 0) contained detectable levels of BLV transcripts. Thus, in the freshly isolated population of primary cells, the BLV genome was silent.

BLV transcription was initiated very rapidly when infected PBMCs were cultured with LPS; all three species of viral RNA appeared simultaneously by 1 h. BLV transcripts consist of an 8.4-kb full-length RNA that acts both as genomic RNA in virions and as mRNA encoding the *gag/pol* proteins, a 4.4-kb singly spliced RNA encoding the *env* proteins, and a 2.0-kb doubly spliced RNA encoding the *tax/rex* proteins. Since the ^{32}P -labeled antisense RNA probe corresponds to the *tax/rex* region which is present in all viral messages, signal intensities are in direct proportion to the moles of each mRNA species. Initially, the 8.4-kb transcript predominated, comprising about 50% of the viral RNA. By 2 h, the 2.0-kb *tax/rex* transcript had increased 40-fold, while the levels of 8.4- and 4.4-kb mRNAs rose by 3- and 5-fold, respectively. At this point, a pattern became established in which all mRNAs continued to accumulate, but the 2.0-kb transcript greatly predominated. BLV RNA levels remained high through 3 to 12 h of culture but decreased four- to sixfold by 24 h. This decline in mRNA levels between 12 and 24 h was consistently found in PBMCs from three infected sheep.

To determine when viral RNAs served as templates for translation, the number of PBMCs expressing the BLV capsid protein (p24 or CA) was determined by immunocytochemical staining (Fig. 1B). CA-positive cells were first detected after 3 h in culture, 2 h subsequent to the appearance of viral RNA. CA-positive cells continued to increase in number through 20 h, despite the decline in BLV RNA. This difference probably reflects a slower turnover of CA protein compared with viral RNA, since in subsequent experiments, CA-positive cells decreased by 48 h (data not shown).

BLV is transcribed in the absence of LPS. Early and late stages of viral expression have been observed in cells latently infected with HIV, as judged by the differential accumulation of viral transcripts in whole cell RNA (25, 27, 33, 35). Because there was no temporal separation in the appearance of different BLV transcripts, we thought that

FIG. 1. Activation of BLV expression in LPS-stimulated PBMCs. (A) PBMCs (3.25×10^7 per sample) from BLV-infected sheep 468 were cultured with $10 \mu\text{g}$ of LPS per ml for the number of hours indicated above each lane. Lane B contains RNA from 3.25×10^7 buffy coat cells (85% mononuclear cells) harvested without being cultured. Poly(A)⁺ RNA was prepared from cell lysates, electrophoresed through a 1% agarose-formaldehyde gel, and analyzed by Northern blotting with a ³²P-labeled, *tax/rex* region-specific RNA probe followed by autoradiography. Sizes of viral mRNAs are indicated on the left. The blot was exposed to film for 10 h. (B) Cells expressing the BLV capsid protein were identified by immunohistochemical staining with anti-CA serum at a 1:500 dilution. Positive cells were counted under $\times 400$ magnification.

LPS might have driven expression very rapidly, making an early stage of expression difficult to discern. Previous work had shown that components of fetal bovine serum activated BLV transcription, although to a lesser extent than did LPS (28). To test whether the weaker stimulus provided by serum might result in slower progress through an early stage of expression, PBMCs from infected sheep were cultured without LPS, and RNA was isolated at early times (Fig. 2). With such short incubations, the time required for temperature equilibration could significantly affect transcription. Consequently, for this and all subsequent experiments, culture media were prewarmed to 37°C before the addition of PBMCs. BLV RNA was detectable by 30 min of culture when the major species was the 8.4-kb full-length RNA, and transcription proceeded rapidly in the absence of LPS. By 1 h of culture, the 2.0 kb *tax/rex* transcript was predominant. All species of RNA increased to a maximum at 4 h, and the 2.0-kb transcript remained the major species throughout. All transcripts decreased in number by 8 h. For comparison with

FIG. 2. Activation of BLV transcription in the absence of LPS. PBMCs from BLV-infected sheep 468 were cultured for the indicated number of hours. Poly(A)⁺ RNAs were prepared from whole cell lysates (3.45×10^7 PBMCs per sample) and analyzed by Northern blotting. In parallel, RNA was prepared from PBMCs cultured for 0.5 and 1 h with $10 \mu\text{g}$ of LPS per ml. Lane U contains RNA from 3.3×10^7 uninfected PBMCs cultured for 1 h with $10 \mu\text{g}$ of LPS per ml. The blot was exposed to film for 2 h.

LPS-stimulated transcription, parallel samples were prepared after incubation with LPS for 30 min or 1 h. At these early times, LPS made no difference in the pattern of transcripts and little difference in the overall level of BLV transcription. Thus, in contrast to HIV, no selective accumulation of 2.0-kb BLV transcripts was evident in early samples of whole cell RNA.

LPS did not appear to alter the pattern of BLV transcription over longer incubations, but its effect on RNA levels could not be determined by comparison of results from experiments performed on separate days. To directly assess the influence of LPS on the level of BLV transcription, we harvested RNA over the course of 24 h from BLV-infected PBMCs cultured in parallel, both with and without LPS. After Northern analysis, levels of the three mRNAs were quantitated by densitometry (Fig. 3). LPS elevated the level of BLV RNA approximately twofold. The effect of LPS was most pronounced between 4 and 8 h of culture, when the 8.4- and 4.4-kb transcripts were elevated fivefold. In addition, all species of mRNA reached their maximum levels earlier in the presence of LPS. Curiously, transient decreases in some mRNAs were frequently seen at 8 h (see also Fig. 1A, lane 8). In the case of the 8.4-kb transcript, this decrease could result from packaging of genomic RNA into virions. Alternatively, nuclease activity may be enhanced at this time, causing some loss of signal.

Early BLV expression is defined by compartmentalization of mRNAs. The BLV-encoded regulatory protein, Rex, and its functional analogs, HTLV Rex and HIV Rev, have been proposed to regulate the cytoplasmic localization or utilization of viral *gag/pol* and *env* mRNAs. Since we had not observed temporal regulation of individual transcripts within whole cell RNA, comparison of cytoplasmic RNA populations over time appeared to be crucial to understanding the activation of BLV expression. The very early prevalence of 8.4-kb RNA in our initial experiments could have reflected newly transcribed nuclear RNA which subsequently served as a precursor for splicing. To determine whether there was

FIG. 3. Quantitation of BLV RNA in cultured PBMCs. PBMCs from BLV-infected sheep 410 were cultured in the presence or absence of $10 \mu\text{g}$ of LPS per ml. Poly(A)⁺ RNA was prepared from samples of 3.5×10^7 cells lysed after the indicated times in culture and was analyzed by Northern blotting. Integrated optical densities (OD) were determined by scanning nonsaturating 3-h exposures of autoradiograms.

early compartmentalization of transcripts, nuclear and cytoplasmic fractions were prepared from PBMCs cultured for 1 h with or without LPS (Fig. 4). Quantities of nuclear RNA were low; accordingly, selection for poly(A)⁺ RNA was not necessary to load all of the nuclear RNA from 3.0×10^7 cells. Cytoplasmic fractions were enriched for poly(A)⁺ RNA as usual. When nuclear and cytoplasmic transcripts were compared, compartmentalization of viral mRNAs was observed. Despite the presence of all three species of BLV mRNA in the nucleus after cells were cultured for 1 h, only the 2.0-kb *tax/rex* RNA was present in the cytoplasm. This transcript appeared to be specifically enriched in the cytoplasm, where its level greatly exceeded that in the nucleus. In fractionated cells, an effect of LPS stimulation became apparent very early; cells cultured for 1 h with LPS showed significant levels of 4.4- and 8.4-kb RNAs in the cytoplasm.

FIG. 4. Nuclear and cytoplasmic BLV RNAs in briefly cultured PBMCs. PBMCs from sheep 468 were cultured for 1 h with or without $10 \mu\text{g}$ of LPS per ml. Nuclear (lanes N) and cytoplasmic (lanes C) fractions were prepared by lysis of 3.0×10^7 cells with nonionic detergent. Total nuclear and poly(A)⁺ cytoplasmic RNAs were isolated and analyzed by Northern blotting. The blot was exposed to film for 11 h.

Late-stage expression is dependent on new protein synthesis.

Transcription in the absence of protein synthesis is characteristic of the α or immediate-early genes of a number of DNA viruses, such as adenovirus and herpes simplex virus, which also undergo temporal regulation of gene expression (19, 38). BLV RNA has been shown to be transcribed during 20 h in the presence of the protein synthesis inhibitor CHX (5); however, it is impossible to know which viral messages were present because individual transcripts were not distinguished in that experiment. When silently infected cells are activated in the presence of protein synthesis inhibitors, production of the viral Tax and Rex proteins should be blocked. To investigate this question, we cultured PBMCs in the presence of $10 \mu\text{g}$ of CHX per ml. This procedure reduced overall incorporation of ^{35}S -amino acids into proteins by 95% (data not shown). However, it was formally possible that synthesis of Tax or Rex could proceed in the presence of this inhibitor. To address this possibility, PBMCs freshly harvested from an infected sheep were cultured for 4 h in the presence or absence of CHX, and proteins were labeled by metabolic incorporation of [^{35}S] methionine and [^{35}S]cysteine. Cell lysates were sequentially immunoprecipitated with antisera specific for BLV proteins. Each lane on the autoradiograms (Fig. 5) represents lysate from 10^7 cells, approximately one-third the number used to generate one lane on Northern blots. Rex was readily apparent as a 19-kDa polypeptide in lysates from normally cultured PBMCs (Fig. 5A), and its precipitation was blocked by the peptide used to raise antibodies against its C terminus. In cells cultured with CHX, only a trace of Rex was immunoprecipitated, amounting to 4% of that precipitated from cells cultured without the inhibitor. Tax was detectable as a 36-kDa polypeptide when protein synthesis was not inhibited (Fig. 5B), and its precipitation was also competed for by the corresponding peptide. No Tax could be detected when protein synthesis was blocked by CHX. *gag* precursor polyproteins were immunoprecipitated by anti-CA serum from lysates of cells cultured without inhibitor, but within the first 4 h of culture, p24^{CA} had not yet been generated by proteolytic processing (data not shown). *gag* precursors were not detectable when cells had been cultured with CHX.

FIG. 5. Synthesis of BLV regulatory proteins inhibited by CHX during the first 4 h of culture. PBMCs from uninfected sheep 471 and infected sheep 468 were cultured with or without the addition of 10 μ g of CHX per ml in medium containing [^{35} S]methionine and [^{35} S]cysteine. Cell lysates were cleared with preimmune serum and then were serially immunoprecipitated with antisera specific for peptides of the BLV Rex or Tax protein. Each lane on the autoradiograms represents radiolabeled proteins immunoprecipitated from 10^7 PBMCs by rabbit serum specific for the C-terminal 12 amino acids of Rex (A) or a 15-amino-acid peptide of Tax (B). Appropriate competing peptides were added as indicated. Some Rex-antibody complex was carried over into the infected cell sample in which immunoprecipitation of Tax was blocked by Tax peptide. Positions of ^{14} C-methylated protein standards are shown in kilodaltons. Autoradiograms were exposed for 4 days.

Lysates of PBMCs from an uninfected sheep showed no BLV-specific proteins. However, they did contain an 18-kDa polypeptide that was precipitated only in the presence of blocking peptide (Fig. 5A); this protein was also precipitated under the same conditions from lysates of PBMCs from the infected sheep.

Thus, new synthesis of Tax and of *gag* polyproteins was completely undetectable in the presence of CHX, and new synthesis of Rex was at trace levels. New synthesis of any host cell proteins that might be required for Tax or Rex function was presumably also at trace levels. It is important to note that this analysis could not indicate whether small amounts of Tax and Rex were already present before PBMCs were cultured.

Without Tax, a reduced level of viral transcription would be expected. Comparison of whole cell poly(A)⁺ RNA from PBMCs cultured for 1 h in the presence of LPS, with or without CHX (Fig. 6A), showed that despite inhibition of protein synthesis, all BLV mRNAs were produced. Surprisingly, the level of BLV RNA was slightly increased in these cells cultured briefly with CHX. A similar phenomenon has been reported for cellular early-response genes (17). The presence of all BLV transcripts in CHX-treated cells indicated that neither early transcription nor splicing of viral RNA requires new protein synthesis.

With Rex production blocked by CHX, the 2.0-kb transcript should appear in the cytoplasm, but 8.4-kb mRNA should be confined to the nucleus. The location of the 4.4-kb mRNA is more difficult to predict, given conflicting results obtained for HIV. To assess the influence of newly synthesized protein on RNA compartmentalization, nuclear and cytoplasmic fractions were prepared at 1.5 and 4 h from PBMCs cultured both with and without LPS or with CHX in the absence of LPS (Fig. 6B). In this experiment, transcrip-

tional activation proceeded somewhat more slowly than in previous experiments; after 1.5 h, the dominant nuclear message was 8.4 kb, and in the absence of LPS, only the 2.0-kb mRNA was present in the cytoplasm. Both LPS and CHX increased RNA levels as described above and led to

FIG. 6. BLV RNAs in cells cultured with CHX. (A) PBMCs from sheep 468 were cultured for 1 h with 10 μ g of LPS per ml, with and without the addition of 10 μ g of CHX per ml. Whole cell poly(A)⁺ RNA was prepared from lysates of 3.45×10^7 cells and analyzed by Northern blotting. The blot was exposed to film for 2.5 h. (B) PBMCs from sheep 468 were cultured for 1.5 or 4 h in medium alone or in medium supplemented with either 10 μ g of LPS per ml or 10 μ g of CHX per ml. Nuclear (lanes N) and cytoplasmic (lanes C) fractions were prepared from 3.2×10^7 cells. Total nuclear RNA and poly(A)⁺ cytoplasmic RNA were analyzed. Blots were exposed to film for 12 h.

the appearance of very small amounts of 4.4- and 8.4-kb mRNAs in the cytoplasm at 1.5 h. These faint cytoplasmic signals seem unlikely to result from nuclear leakage, since the two transcripts were equally represented in the cytoplasm, while in the nucleus, the level of 8.4-kb mRNA exceeded that of 4.4-kb mRNA by threefold. The appearance of all mRNA species in the cytoplasm despite the marked deficiency of newly synthesized Rex was unexpected. Levels of 2.0-kb mRNA detectable by polymerase chain reaction have been found in PBMCs rapidly harvested from BLV-infected animals (22), indicating that this message may be transcribed at very low levels in some circulating lymphocytes. Our results imply that small amounts of the Tax and Rex regulatory proteins are also present before PBMCs are cultured with CHX.

By 4 h, BLV RNA accumulated to high levels in both nucleus and cytoplasm of cells cultured either with or without LPS, and mRNAs were present in their usual ratios. However, in PBMCs cultured with CHX for 4 h, viral transcription was reduced, probably as a result of a deficiency of newly synthesized Tax protein. However, most striking was the exclusive presence of the 2.0-kb transcript in the cytoplasm. Both 4.4- and 8.4-kb mRNAs were virtually absent from the cytoplasm; faint traces of these mRNAs could be detected only on greatly overexposed autoradiograms. Thus, in the absence of protein synthesis, BLV expression is blocked at the early stage when only the 2.0-kb *tax/rex* transcript appears in the cytoplasm. New synthesis of Rex and perhaps of host proteins is thus required for the population of infected PBMCs to enter the late stage of BLV expression.

DISCUSSION

We used virus-infected, primary lymphocytes to test whether the current model of BLV and HTLV expression holds when transcription is activated in silently infected cells. Enough cells can be obtained from sheep that have been infected with BLV for several years to examine viral RNAs directly, without the use of reporter genes or artificial constructs. Most importantly, activation of BLV in cultured PBMCs should closely mimic transcriptional activation within the infected host. In these infected lymphocytes, BLV expression is characterized by early and late stages, each distinguished by a unique pattern of cytoplasmic RNA. This essential test validates a model based on transfection of nonlymphoid cells and suggests that temporal regulation of BLV and HTLV expression is not dependent on host cell lineage.

During early expression, BLV RNA species were highly compartmentalized. This stage is characterized by the exclusive presence of 2.0-kb *tax/rex* message in the cytoplasm. Although all three classes of mRNA were found in the nucleus, the singly spliced 4.4-kb and the unspliced 8.4-kb RNAs failed to accumulate in the cytoplasm. Viral regulatory proteins are translated during the early stage. Our data demonstrate that protein synthesis is required at this time for the switch to late-stage expression, in which the messages encoding structural proteins are added to the cytoplasmic population. Rex most probably is the protein functioning in this switch, but we cannot exclude the possibility that a cellular factor is required as well. The transition to late-stage expression occurs very rapidly. Viral RNA was detected in cells after only 30 min in culture, and the cytoplasmic RNA profile became that of late-stage expression between 1.5 and 4 h in culture. Cells producing the CA protein first appeared

as early as 3 h, placing the early-to-late-stage transition in some cells at 2 to 3 h after PBMCs are placed in culture.

Certain HIV-infected T-lymphocyte and macrophage cell lines have been proposed to be models of latent HIV infection (33, 35). Virus expression is mostly limited to the early stage in these cells, although some viral particles are evident by electron microscopy, indicating that virus is persistently produced. Progression to late-stage expression is induced by phorbol esters and phytohemagglutinin, which activate the host cells. In contrast, we do not find that polyclonal B-cell activators are essential for the transition to late-stage BLV expression. Unlike the continuously cultured HIV-infected cells, BLV-infected PBMCs are sufficiently stimulated by fetal bovine serum in the culture medium to activate transcription from undetectable levels and to proceed through both early and late expression.

The rapidity with which transcription becomes activated from latency in PBMCs is striking and raises the interesting question of the exact state of infected cells within the asymptomatic host. BLV latency within cells has been described as active repression of virus expression, mediated by a factor in the plasma of cattle (12). Release from repression is consistent with the rapid onset of transcription that we observe in cultured PBMCs. However, since polyclonal activators induce additional cells to transcribe BLV RNA (28), latency might be more aptly characterized as the lack of activation. In vivo, this would be overcome by immune activation of infected B cells, as during encounters with antigens.

With the recent use of highly sensitive techniques, some circulating PBMCs do appear to have been activated in vivo. PBMCs from infected sheep include a very few cells (less than 1 in 15,000) in which BLV transcripts can be detected by in situ hybridization as soon as the cells are isolated from blood (28). With polymerase chain reaction methods, freshly prepared cells from both sheep and cattle have been shown to contain some BLV *tax/rex* message (22). In patients with HTLV-related disease, *tax/rex* mRNA has been similarly detected in freshly prepared PBMCs (26), as have rare cells that are positive by in situ hybridization (13). However, the stage of viral expression in circulating cells remains unknown. *tax/rex* transcripts may be present, along with 4.4- and 8.4-kb RNAs, in a few circulating cells which have progressed to late-stage expression. Alternatively, *tax/rex* transcripts may be distributed among a larger number of infected cells, each halted in early expression. Until experimental evidence distinguishes between these two circumstances, it is unclear whether infected cell lines blocked in early gene expression provide an accurate model system in which to study activation of latent virus expression in the host.

Rare transcripts would not be detected by Northern analysis, but our data suggest that there may be regulatory proteins present in some freshly isolated cells. Surprisingly, early transcription does not seem to require new synthesis of Tax protein, since cells cultured with CHX had normal or higher levels of viral RNAs. In newly activated primary lymphocytes, the initial rate of transcription may be independent of Tax. Alternatively, a small amount of Tax protein may already be present; if so, it is sufficient to support transcription for a time. However, by 4 h in the absence of new protein synthesis, the level of transcription was noticeably reduced relative to that in cells cultured without CHX. Tax protein which might have functioned initially could be depleted by this time; Tax from HTLV has a half-life of 2 h in lymphoid cell lines (42). We also saw evidence of Rex

function in newly isolated cells. After 1.5 h of culture, some structural gene transcripts were present in cytoplasmic fractions regardless of whether protein synthesis was blocked. By 4 h in the absence of new protein synthesis, these cytoplasmic transcripts were no longer present. These findings are most compatible with the presence of a low level of Rex protein in at least some freshly isolated cells. If this protein were also turned over rapidly in the absence of new synthesis, Rex function would soon be lost, leading to the observed lack of cytoplasmic structural gene transcripts at 4 h.

Rex is a nucleolar protein (41) which recognizes the Rex response element, a highly structured, *cis*-acting RNA sequence found near the 3' end of all BLV and HTLV mRNAs (9, 40). The functionally analogous protein, Rev, of HIV also interacts with a structured viral RNA sequence, the Rev response element (7, 16, 31). Previously, Rex has been studied exclusively in nonlymphoid cell lines. In contrast, Rev function has recently been characterized in a lymphocyte cell line, leading to an alternative model of its function (1). Like Rex, Rev is clearly a prerequisite for structural protein expression. In HeLa and COS cells, Rev appears to facilitate transport of structural protein mRNAs to the cytoplasm (1, 10, 14, 15, 30). In B lymphocytes, however, Rev has little effect on the compartmentalization of the *env* transcript, which is cytoplasmic during all stages of expression (1). Cytoplasmic *env* RNA is maintained in monosomes during early expression, but in the presence of Rev, this mRNA is incorporated into polysomes and translated. Full-length RNA appears to be regulated at the level of transport regardless of cell type.

In contrast to HIV, we find that the location of the BLV *env* transcript parallels that of full-length RNA during both early and late expression. Thus, BLV expression in primary lymphocytes supports the model predicted from fibroblast studies. Our data are most consistent with Rex regulating the transport of viral RNA, rather than its splicing or stability. All BLV RNAs are present in the nucleus during early expression, yet the structural protein messages are specifically absent from the cytoplasm. This compartmentalization is overcome by Rex, probably in concert with host cell proteins, in the late stage of expression. Neither splicing nor stability of RNA in the nucleus is strongly affected by Rex, since nuclear transcripts maintain the same relative proportions (as determined by densitometry) regardless of whether protein synthesis is blocked for a prolonged period. We cannot exclude the possibility that Rex influences the relative stability of transcripts in the cytoplasm, despite its predominantly nucleolar localization.

In summary, the onset of BLV expression in PBMCs is very rapid and does not require polyclonal B-cell activators. Expression is characterized by two stages that are distinguishable in RNA from fractionated cells. Our results suggest that Rex effects the transition between early- and late-stage expression by influencing transport of viral RNA. It will be important to determine whether the rapid activation and progression to late-stage expression is facilitated by the presence of low levels of viral gene products within latently infected cells.

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