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METHYLATION AND EXPRESSION OF BOVINE LEUKEMIA PROVIRAL DNA

S.V.S. Kashmiri*, Rafia Mehdi, Phalguni Gupta, and Jorge F. Ferrer

University of Pennsylvania, School of Veterinary Medicine,
New Bolton Center, Kennett Square, PA 19348

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SUMMARY: In vivo, the BLV proviral DNA usually resides in a transcriptionally inert state, and is hypermethylated. Upon short-term in vitro cultivation of the neoplastic or non-neoplastic lymphoid cells, the viral genome becomes transcriptionally active but without detectable change in its methylation state. Proviral DNA was found to be methylated in one but not in the other long-term BLV producer cell line examined. These data indicate that hypermethylation of proviral DNA may not be responsible for the covert nature of BLV infection in vivo.

Bovine leukemia virus (BLV), an exogenous retrovirus related to the human T-cell lymphotropic viruses (HTLVs) (1-3), has been established as the causative agent of enzootic bovine leukemia (lymphosarcoma) (for review see Ref. 4), an invariably fatal neoplasia of cattle. The virus is horizontally transmitted among cattle and more often leads to clinically asymptomatic infection or persistent lymphocytosis (PL), which is characterized by a permanent increase in the number of lymphocytes in the peripheral blood of the infected animals (5). In cattle, BLV has been detected only in B lymphocytes (6). In these cells the provirus usually resides in a transcriptionally inert state (7-10), regardless of whether or not they are neoplastic. However, only a few hours after in vitro cultivation, the infected lymphocytes begin to synthesize viral RNA, viral antigens and virus particles (7,10-13). A non-immunoglobulin protein present in the plasma and not in the serum of the BLV-infected cattle has been implicated in the transcriptional repression of the BLV genome in vivo (10,13). The molecular basis for the transcriptional inactivity of the BLV genome in vivo and the mechanism of genome derepression following in vitro cultivation remains obscure.

*To whom reprint requests should be addressed.

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An inverse correlation between the degree of methylation of cytosine residues at CpG dinucleotide sites in genomic DNA and the transcriptional activity of these genes have been found in several nonviral and viral systems (for review see Ref. 14). It has become apparent from studies in these systems that the absence of DNA methylation is an essential, though not sufficient, prerequisite for gene expression. Cytosine methylation has been implicated in transcriptional control of both murine and avian endogenous retroviruses (15-18). The present study was undertaken to investigate whether cytosine methylation of the BLV proviral DNA is responsible for the covert nature of BLV infection. The results show that in tumor cells and peripheral blood lymphocytes from BLV-infected cattle with lymphosarcoma transcriptionally inactive BLV proviral DNA exists in hypermethylated state. However, the derepression of BLV genome following in vitro cultivation of these cells is not accompanied by any detectable demethylation of the proviral DNA.

MATERIALS AND METHODS

BLV producing bat cells, BLV-bat clone (19), which were established by infecting a bat monolayer cell culture, CCL88, with BLV, were grown in Eagle's minimum essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Cell line NBC-13, which was established from buffy coat cells of a leukemic cow (20), was maintained in RPMI 1640 medium supplemented with 20% FCS. Suspension of single cells from BLV-induced tumors was prepared by perfusion with MEM. Cells were washed twice with MEM supplemented with 5% FCS before extraction of DNA or setting up short-term culture.

The peripheral blood lymphocytes were isolated, without hypotonic shock, from the buffy coat of heparinized peripheral blood by Ficoll Hypaque centrifugation (21).

Conditions for the short-term cultivation have been described (13). Briefly, the lymphocytes were suspended in MEM supplemented with 20% FCS in the presence and absence of purified phytohemagglutinin (PHA) at a final concentration of 1.5 ug/ml. The cells were adjusted at a density of 3×10^6 per ml and were incubated at 37°C for 24 hr. Following incubation, the cells were harvested and washed twice before the high molecular weight DNA was isolated from them.

High molecular weight DNA was prepared from tissues or cultured cells as described by Marmur (22). Digestion of DNA with restriction endonucleases was performed using conditions specified by the suppliers of the enzymes (New England BioLabs, Boston, MA). For in situ hybridization, the DNA was subjected to horizontal gel electrophoresis and it was transferred to nitrocellulose filter by the Southern procedure (23). [^{32}P]-labeled DNA probe was prepared by nick translating the cloned BLV DNA insert as described previous-

ly (24). The conditions of filter hybridization and washing have previously been described (25).

RESULTS

In vivo, the provirus in BLV-induced tumors and BLV-infected lymphocytes is usually in a transcriptionally inactive state (7-10). To investigate a possible correlation between the expression of the proviral DNA and its state of methylation, we studied methylation patterns of the proviral DNA in cells from BLV-induced tumor tissue and circulating lymphocytes of leukemic animals using the isoschizomeric pair of restriction endonucleases, MspI and HpaII. Of these isoschizomers, which recoginze the sequence CCGG, MspI cuts the DNA regardless of methylation state of the internal cytosine, whereas HpaII cuts only when the internal cytosine is not methylated. We took advantage of the cleavage by SstI, which has sites on the BLV LTR in addition to a unique internal site (24), to generate two internal fragments of 7.0 and 1.35 kilobase pair (Kbp) from each of the proviral DNA copies. These two fragments encompass almost the total BLV information. SstI generated fragments were then treated with HpaII or MspI. Results of the Southern analysis of DNAs from tumor tissues and circulating lymphocytes of leukemic animals presented in Fig. 1 show that MspI treatment of SstI fragments of the proviral DNA

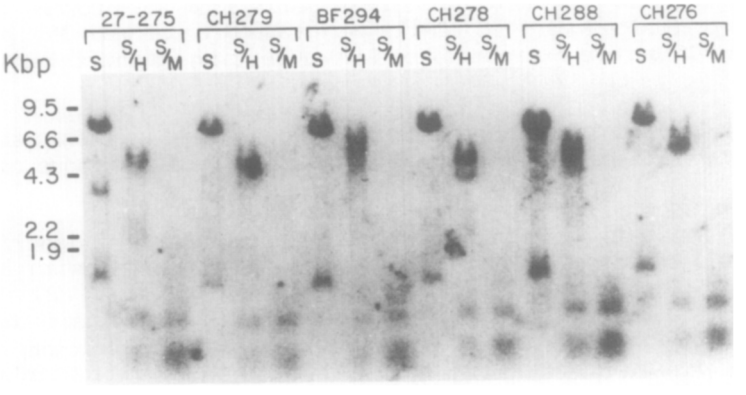

Fig. 1. Hypermethylation of proviral DNA in BLV-induced tumor cells and circulating lymphocytes of leukemic cattle. High molecular weight DNA was prepared from fresh bovine tumors (27-275, CH279, BF294 and CH278) and circulating lymphocytes of leukemic animals (CH288 and CH276). Approximately 20 ug of DNA was digested with either SstI alone (S) or with SstI and subsequently with HpaII (S/H) or MspI (S/M). The DNA was electrophoresed in 0.5% agarose gel, transferred to nitrocellulose filter and hybridized to the BLV [^{32}P] DNA as described in Methods. The numbers on the left represent the lengths of HindIII fragments of wild type λ DNA used as markers.

invariably generated fragments smaller than 1.0 kilobase pair in length. In contrast, the double digestion of the DNA with SstI and methylation sensitive enzyme HpaII, consistently produced DNA fragments ranging in size from approximately 5.0 to 6.5 Kbp. The lack of sensitivity of most of the CCGG sequences of the BLV proviral DNA to HpaII shows that their internal cytosine residues are methylated. Several other methylation sensitive enzymes (AvaI, HhaI, SalI, and XhoI), which have sites on the replicative form of BLV DNA, fail to cleave the BLV proviral DNA in tumor cells (data not shown).

We have also examined the state of methylation of BLV proviral DNA in circulating lymphocytes of clinically normal BLV-infected cattle with PL. We have found that the state of methylation of the proviral DNA in these cells is similar to that of the proviral DNA in tumor tissue (data not shown).

To investigate a possible correlation between methylation and expression of the BLV proviral DNA, we investigated whether derepression of the BLV genome following short-term cultivation of the lymphocytes from the tumor tissue, is accompanied by any change in the degree of cytosine methylation of the viral DNA. Cells from the BLV-induced tumors were cultured with and without PHA. The transcriptional activation of the BLV genome following short-term cultivation (7,10,13) was confirmed by measuring the level of the major core BLV antigen (p25) in the cells by competitive radioimmunoassay (26). A six-fold increase in p25 antigen expression was observed when PHA was added to the lymphocyte culture (data not shown). Such effect of PHA has been shown to be due to stimulation of viral transcription (27). High molecular weight DNA was prepared from the BLV-induced tumor cells before and after their short-term cultivation in the presence and absence of PHA. The DNAs were doubly digested with SstI and the methylation sensitive enzyme, HpaII. Results of a Southern analysis presented in Fig. 2 show that the cleavage patterns of the BLV proviral DNAs isolated from the uncultured tumor cells or the cells cultured with and without PHA were identical. This analysis of the proviral DNA did not include parts of the BLV LTR because the DNA was initially cleaved with SstI, which has sites on

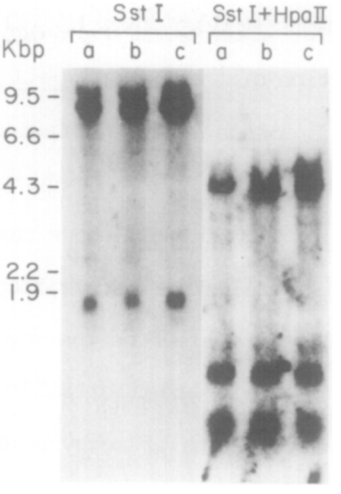

Fig. 2. State of methylation of proviral DNA before and after in vitro cultivation of BLV-infected tumor cells. High molecular weight DNA was prepared either from fresh tumor cells (a) or from tumor cells cultivated for 24 hours in medium without (b) or with (c) 1.5 ug/ml of PHA as described in Methods. 20 ug of DNA was digested with the indicated enzymes and electrophoresed in 0.8% agarose gel. Blotting and hybridization of DNA to the BLV [^{32}P] DNA was performed as described in Fig. 1.

the BLV LTR. Cleavage with EcoRI, which has a unique site on the BLV genome, generates DNA fragments comprising the proviral DNA carrying the BLV LTRs in their entirety and the flanking cellular sequences. No apparent difference in cleavage pattern of the proviral DNA was detected when the DNAs from uncultured tumor cells and cells cultured with or without PHA were doubly digested with EcoRI and HpaII (data not shown). Similar results were obtained when circulating lymphocytes of a BLV-infected cow with lymphosarcoma were used in the experiment (data not shown).

Next, we examined the state of methylation of BLV proviral DNA in two long-term infected cell lines, NBC-13 and BLV-bat. NBC 13, a BLV-infected lymphoid cell line, which expresses BLV antigens when cultured in RPMI 1640 medium supplemented with 20% FCS, was established from the buffy coat cells of a leukemic cow (20). BLV-bat is a fibroblastic chronic producer cell line, which was established by infecting a monolayer culture of BLV bat cells (19). Results presented in Fig. 3A show that the digestion of the EcoRI fragments of the BLV proviral DNA in BLV-bat cells by the isoschizomeric enzymes HpaII or MspI, generated identical cleavage patterns. Thus, the

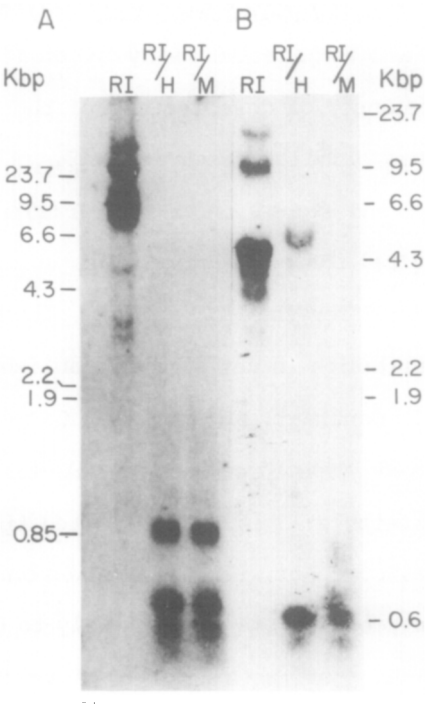

Fig. 3. Southern blot analysis of BLV proviral DNA in producer cells. High molecular weight DNA from the BLV-bat (A) or NBC-13 cells (B) was digested with either EcoRI alone (R1) or with EcoRI and subsequently with HpaII (R1/H) or MspI (R1/M). 10 ug of the enzyme digested DNA was electrophoresed in 0.8% agarose gel. Blotting and hybridization of DNA to the BLV [^{32}P] DNA was performed as described in Fig. 1.

proviral DNA copies present in BLV-bat cells are not methylated. In contrast, a comparison of the cleavage pattern of the EcoRI fragments of BLV proviral DNA in NBC-13 cells by HpaII and MspI (Fig. 3B) shows that BLV DNA harbored by this cell line exist in a hypermethylated state.

DISCUSSION

The data presented here show that the transcriptionally silent BLV proviral DNA in bovine tumor cells and the circulating lymphocytes of cattle with lymphosarcoma and clinically normal cattle with PL is invariably hypermethylated. However, no detectable demethylation of the proviral DNA takes place when the BLV genome in these cells becomes transcriptionally active on short-term cultivation. Neither the PHA stimulated enchanced transcription of BLV genome shows a concomitant change in the state of methylation of the

proviral DNA. However, our analyses may detect only the gross demethylation of the genome and may fail to detect demethylation at a specific site that may be critical for the onset of derepression of the viral genome. While this work was in progress, similar observations have been reported for the proviral DNA methylation and expression of HTLV-I (29). However, our observations on the BLV proviral DNA methylation in established cell lines is at variance with that reported for the HTLV system. Clarke et al (29) did not detect proviral DNA methylation in any of the established HTLV-infected cell lines they examined. In contrast, BLV proviral DNA was found to be hypermethylated in BLV-infected lymphoid cell line, NBC-13, whereas the proviral DNA was found to be not methylated in a BLV-infected fibroblastic cell line. Nevertheless, our data, together with the studies on HTLV system, suggest that hypermethylation of proviral DNA is not likely to be responsible for the quiescent state of BLV/HTLV proviruses in vivo. A non-immunoglobulin protein present in the plasma of the BLV-infected cattle has been implicated in the suppression of BLV expression in vivo (9,10). A similar plasma factor from the HTLV-infected individuals has been thought to be responsible for the inhibition of HTLV expression (29,30).

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