THE BOVINE LEUKEMIA VIRUS X REGION ENCODES A TRANS-ACTIVATOR OF ITS LONG TERMINAL REPEAT

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We constructed a fusion plasmid, pMX-I, by which the major open reading frame, X-I, of the bovine leukemia virus (BLV) X gene was expressed under control of the mouse metallothionein promoter. pMX-I was cotransfected into CV1 monkey kidney cells together with another construct containing the BLV long terminal repeat (LTR) linked to the chloramphenicol acetyltransferase (CAT) structural gene. The result of assay of CAT synthesis suggests that the X-I product functions as a trans-acting activation factor of the BLV LTR.

Key words: Bovine leukemia virus — X gene — Trans-activator — Chloramphenicol acetyltransferase assay

The bovine leukemia virus (BLV), and the human T-cell leukemia virus types I and II (HTLV-I and II) are distinguished from other retroviruses by many structural, biological and pathological features. One of the structural features is the presence of the X region located 3' to the env-gene but 5' to the long terminal repeat (LTR).1-5

In BLV, the major open reading frame among several found in the X region (here referred to as the X-I frame) was shown to be expressed as a 38 kilodalton (kD) protein in a fetal lamb kidney cell line productively infected with BLV (FLK/BLV).6 Northern blot analysis data suggested that the X-I protein was synthesized by translation of a double-spliced mRNA which had a 5'LTR-pol/env junction-X structure.7 From a structural analysis of the X mRNA, it was predicted that another open reading frame (X-II frame) might be expressed by the same mRNA.7 An in vitro translation experiment revealed that a protein, whose coding sequence was found in the X-II frame, was synthesized from BLV genomic mRNA.8 Moreover, sera obtained from cows infected with BLV reacted with the in vitro-synthesized protein, suggesting that the X-II frame might be expressed in BLV-infected animals.

Another interesting structural feature of the HTLV-BLV family is that the LTR sequences include an unusually long R region, a polyadenylation signal located far from the expected position, and a region which has the potential to form a loop in the sequence between U3 and R.9,10

In studies focusing on gene expression directed by the LTR, it was observed to be common to HTLV-I,11,12 HTLV-II13 and BLV14,15 that the transcriptional activity of the LTR was stimulated in cells infected with the corresponding virus. In HTLV-I, it has been recently determined that the factor responsible for the transcriptional activation of its LTR is a product of the XIV frame16 or X-LOR,17 namely, the major open reading frame in the X region.

Although BLV belongs to the HTLV group, BLV induces B-cell transformation. It would be interesting to know what growth-related gene in B-cells can be trans-activated by BLV infection. Here, we attempted to examine the trans-activating capacity of the products of its X frames.

To test the capability of the X-I product of BLV to activate the BLV LTR, we expressed the X-I gene in mammalian cells using a fusion plasmid, pMX-I, in which the transcription of the X-I gene was directed by the mouse metallothionein I (MT-I) promoter unit. The construction of pMX-I is shown in Fig. 1. The parental plasmid was pMK,18 which contained the structural gene for thymidine kinase (TK) from herpes simplex
virus fused to the promoter/regulatory region of the MT-I gene. The TK structural sequence was deleted from pMK by complete digestion with BglII followed by partial digestion with SmaI, and replaced by the X-I sequence. Based on the BLV sequence published by Sagata et al., the X-I frame starts at nucleotide 7246 and ends at 8172. The ClaI-PvuII fragment containing the X-I sequence from nucleotide 7319 to 8247 was obtained from pBLV12, which had the BLV sequences inclusive of 5' and 3' LTR and the flanking cellular sequences cloned into pBR322 at the EcoRI site. Synthetic nucleotides (86/84 base double strand) were inserted to code for the MT-I sequence from the BglII site to its translation start codon and the X-I sequence of positions 7246–7318. Thus, the intact X-I frame was placed 3' to the MT-I promoter, so that the expression of the X-I gene was subjected to regulation by heavy metals.

Fig. 1. Construction of pMX-I. pMX-I was constructed by ligating three fragments: the BglII-SmaI fragment obtained from pMK, the ClaI-PvuII fragment obtained from pBLV-12, and the synthetic nucleotide of 86/84 base pairs as described in the text. The resulting plasmid contained the following elements: the MT-I promoter/regulatory region, its initiation codon, the intact X-I frame, the polyadenylation signal derived from TK gene, and pBR322 sequences of Amp' cistron and the origin of replication. The structure of the plasmid DNA was confirmed by extensive restriction enzyme analysis. E. coli strain HB101 was used in transformation to isolate the plasmid.

Fig. 2. Immunoblot analysis for the expression of the X-I gene in CV1 cells. Approximately 5 x 10^6 cells were plated on a 100-mm dish 24 hr prior to transfection. Calcium phosphate precipitate (500 µl) containing pMX-I (10 µg) was added to the culture. Cells were fed with medium containing 1 µM CdCl2 24 hr after transfection. Cell extract prepared 48 hr after transfection was analyzed by SDS-PAGE of an 8 to 18% polyacrylamide gradient followed by electroblotting onto diazobenzyl-oxymethyl paper. Immunoautoradiography was carried out using the rabbit antiserum to a synthetic peptide described elsewhere. Lane 1, Tb1Lu/BLV cells; lane 2, CV1 cells transfected with pMX-I; lane 3, control CV1 cells. The band of the X-I protein is shown by the arrow.
To examine whether the X-I product is synthesized, pMX-I was introduced into CV1 monkey kidney cells by the calcium phosphate precipitation technique. Cells were incubated in the medium containing 1 µM CdCl₂ to induce that transcription of the X-I gene for 24 hr before harvesting. The cell lysate made at 48 hr after transfection was analyzed by immunoblotting after sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). We used an antiserum to a synthetic peptide which has the carboxy-terminal amino acid sequences of the X-I product. Figure 2 shows that a 38 kD band of the X-I protein specifically reacted with the antiserum in TblLu bat cells producing BLV (TblLu/BLV) (lane 1). This protein also appeared to be synthesized in CV1 cells transfected with pMX-I (lane 2). Without introducing pMX-I, the protein was not detectable in CV1 cells (lane 3).

To examine the effect of the expressed X-I protein on the BLV LTR, the chloramphenicol acetyltransferase (CAT) gene transient expression assay was used. We constructed another plasmid, pBLCAT, which had the BLV LTR sequences as a promoter 5' to the bacterial CAT gene. The structure of pBLCAT is shown in Fig. 3. pBLCAT was first transfected into the BLV-infected and uninfected cell lines. Forty-eight hours after transfection, the level of the CAT enzyme activity was examined. As shown in Fig. 4A, CAT activity was not detectable in the uninfected TblLu cells (lane 3). In contrast, high levels of activity were detected in TblLu/BLV and FLK/BLV cell lines (lanes 2 and 4). Thus, the BLV LTR was activated in the virus-producing cells, as previously described.

Fig. 3. Structure of pBLCAT. The HaeII fragment containing the entire U3, R, and 5' 61 nucleotides of U5 region (Δ5') was obtained from pBLV12 and inserted into pSV0CAT at the HindIII site. The viral flanking sequences of 133 nucleotides 5' to the LTR were included in the inserted fragment.

pBLCAT was next cotransfected together with pMX-I into CV1 cells. To 5×10⁵ cells, we applied 1 µg of pBLCAT with pMX-I in different amounts: 0, 0.01, 0.1, and 1 µg. Cells were exposed to 1 µM CdCl₂ at 24 hr and harvested at 48 hr after transfection. As shown in Fig. 4B, a low level of CAT enzyme activity was detected in the cells transfected with pBLCAT alone. Upon cotransfection of pBLCAT with pMX-I, the CAT synthesis was substantially increased. On the basis of the percentage of chloramphenicol modified to the monoacetylated form, a 100-fold increase of the CAT activity was observed when 1 µg of pMX-I was applied with 1 µg of pBLCAT. There was a correlation between the increase of CAT activity and the amount of pMX-I transfected. However, if the cells were not incubated with CdCl₂, the CAT activity obtained from the uninduced cell extract was 7-fold lower than that from the induced cells. Corresponding results were obtained using the bat cell line, TblLu, although the CAT activity was 3-fold lower than in CV1 cells.

These results show that enhancement of the BLV LTR transcription activity depends on the expression of the X-I gene. It was therefore suggested that the X-I product, previously characterized as a nuclear protein, is a trans-acting regulatory factor of the LTR. Since the 5' 47 nucleotides of the X-II frame inclusive of the translation start codon are not encoded by pMX-I, there is no possibility of full expression of the X-II. However, pMX-I contains eight ATG codons in the X-II reading frame, which could be initiators for partial expression of the X-II. To test the function of the X-II product(s) we constructed another
plasmid, pMX-II, which encoded the intact X-II frame to be expressed by MT-I promoter but had a mutation of ATG to GTG at the X-I start site. Preliminary data do not show any trans-activating function of the product(s) of pMX-II, confirming that the X-I product is an activator of BLV LTR. Derse and Casey reported the presence of two enhancer elements in the BLV LTR; one of them is located in the U3 region and enhances the transcription directed by heterologous promoter only in BLV-infected cell lines.23) The enhancer element in the U3 may be a target for the action of the X-I protein. Direct or indirect interaction of the X-I protein might bring about the transcription activation. A binding experiment of purified X-I protein to the BLV LTR sequences would be of interest. Very recently Greene et al. have reported the important observation that the X gene product of HTLV-II induced interleukin-2 receptor and interleukin-2 gene expression in a T-lymphoid cell line.24) It is important to investigate the host gene activated by the X-I product to understand the mechanism of the disease induction by BLV infection.

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Fig. 4. Transient expression of the CAT gene. (A) TblLu/BLV cells (lane 2), uninfected TblLu cells (lane 3), and FLK/BLV cells (lane 4) were transfected with pBLCAT as in Fig. 2. Cells were harvested 48 hr after transfection and assayed for CAT activity as described.22) One hundred microliters of the 200 μl extract was incubated with 0.5 μCi of [14C]chloramphenicol and 0.5 mM acetyl coenzyme A for 90 min. [14C]Chloramphenicol (Cm) and the monoacetylated form (AcCm) were detected by ascending thin-layer chromatography followed by autoradiography. The reaction with CAT enzyme (0.1U; P.L. Biochemicals, Inc.) instead of cell extract is shown in lane 1. (B) Cotransfection of pBLCAT (1 μg) into CV1 cells with various amounts (0, 0.01, 0.1 and 1 μg) of pMX-I. Transfection and induction was carried out as in Fig. 2. [14C]Chloramphenicol (0.1 μCi) was used in these enzyme reactions.

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REFERENCES


I. KATOH, ET AL.


