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# BOVINE LEUKEMIA VIRUS POST-ENVELOPE GENE CODED PROTEIN: EVIDENCE FOR EXPRESSION IN NATURAL INFECTION

Yoshiyuki Yoshinaka and Stephen Oroszlan

Laboratory of Molecular Virology and Carcinogenesis,
LBI-Basic Research Program, NCI-Frederick Cancer Research Facility,
Frederick, Maryland 21701

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Summary. Partial sequence analysis of a 14 kilodalton protein (p14), synthesized by in vitro translation of bovine leukemia virus genomic RNA, showed that it is encoded in the 'X' region of proviral DNA, located between the env gene and the 3' long terminal repeat. The 'X' gene contains a short and a long open reading frame (X-SORF and X-LORF) which overlap. BLV $p14^{X}$ is specified by X-SORF and not X-LORF as seen with the related human T-cell leukemia virus which expresses $p38\text{-}40^{X}$. Antibodies in sera from animals with BLV induced tumors were shown to recognize $p14^{X}$. Expression of this protein in natural infection might be important for virus replication and/or for BLV induced oncogenesis. 

Bovine leukemia virus (BLV) is a B cell lymphotropic retrovirus associated with enzootic bovine leukosis (for review see ref. 1). The genome contains the long terminal repeat (LTR), gag, pol, and env sequences characteristic of all replication competent retroviruses. Immunological and protein sequence studies indicate that BLV and the human T-cell leukemia virus type I (HTLV-I) are related (2,3). Recently, the nucleotide sequences of both BLV and HTLV genome have been determined. BLV, like HTLV, has a region of unknown function of 1800 nucleotides 3' to the env gene (4-6). In BLV there are two possible open reading frames in this 'X' region, a short (X-SORF) and a long (X-LORF) one, corresponding to pX-III and pX-IV (LORF) respectively in HTLV. HTLV X-LORF is expressed in infected cells, resulting in a 38-40 KDa protein (7-10). It has been proposed that this protein mediates transcriptional transactivation of the LTRs as well as transformation by HTLV (11,12). Transcriptional trans-

Abbreviations:

BLV, bovine leukemia virus; HTLV, human T-cell leukemia virus; LTR, long terminal repeat; FLK, fetal lamb kidney; RSV, Rous sarcoma virus.

0006-291X/85 $1.50

activation has also been described for BLV (13,14) but there is no evidence for expression of the BLV 'X' region as yet.

In the course of our studies on the in vitro translation of BLV genomic RNA, we found that in addition to gag related proteins (66K and 44K) a 14K protein was also synthesized. In this report, we describe the purification and partial amino acid sequencing of the 14K protein. We give evidence that it has a sequence corresponding to X-SORF (4,5) and that X-SORF is expressed in natural infection.

## MATERIALS AND METHODS

Virus. BLV was grown in fetal lamb kidney (FLK) cells and purified by sucrose gradient centrifugation (15).

Viral RNA. Viral RNA (70S) was isolated with SDS-phenol extraction (16) followed by fractionation on a 5 to 20% (w/v) sucrose density gradient [in 0.02M Tris-HCl (pH 7.5), 0.1M NaCl, 0.001M EDTA and 0.1% SDS] centrifugation at 30,000 rpm for 150 min in a SW 40.1 rotor at 20°C. An aliquot of 5 µl of each fraction (solubilized in 100 µl of water after precipitated by ethanol) was analyzed for size of RNA species in 1% agarose gel electrophoresis (16) using bovine liver RNA as marker.

Cell free protein synthesis and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The in vitro translation experiments were performed in 25 µl incubation mixture at 37°C for 60 minutes using a New England Nuclear (NEN) in vitro translation kit (rabbit reticulocyte lysate) with 25 µci of L-[$^{35}$S]-methionine (NEN), reduced concentration of potassium acetate (75 mM), magnesium acetate (1.0 mM) and sucrose density gradient purified RNA which was ice-quenced following heating at 90°C for 2 min. The radioactivity incorporated into the proteins was determined by analyzing 1 µl aliquots by 8 to 18% (w/v) gradient SDS-PAGE (17).

Antisera and immunoprecipitation. Antisera against BLV major core protein p24, the DNA binding protein p12 and the glycoprotein gp60 were as described (15,18). Sera from cows naturally infected with BLV and having lymphosarcoma were obtained from Dr. M. Van der Maaten. Protein A-Sepharose (15 mg, Pharmacia Fine Chemicals) in 0.5 ml phosphate buffered saline (Dulbecco's PBS) and 10 µl of serum were incubated for 1 hour at 4°C. After washing with PBS twice, the beads were incubated with translation mixture which was diluted 5 times with 0.02M Tris-HCl (pH 7.5) buffer containing 0.14M NaCl and 1% Nonidet P40 (NP40), for 16 hours at 4°C. Then the beads were washed three times with the buffer and once with water, and analyzed on SDS-PAGE.

Protein purification. After separation by SDS-PAGE, polypeptides were electrophoretically eluted from gel pieces (19) cut from KCl stained gel. As the 14K polypeptide migrated just at the upper edge of the KCl stained globin band, this was used as a guide for excising the gel pieces. The eluate, in 0.05 M $NH_4HCO_3$ and 0.1% SDS, was lyophilized and then solubilized in 1 ml of water, and precipitated with cold (-20°C) acetone to remove SDS. The precipitate was collected by low speed centrifugation, solubilized in 1 ml of water and dialyzed against 5% $CH_3COOH$ at 4°C. The protein in the dialysate was used for $NH_2$-terminal amino acid analysis either directly, or after further purification by RP-HPLC.

Radiosequencing of [$^{3}$H]-proline and [$^{35}$S]-methionine labeled proteins. Edman degradation in the Beckman liquid phase sequenator Model C was done as previously described (20) using myoglobin as carrier. After concentration with $N_2$ gas flow, 0.5 ml of each fraction collected at each cycle was measured for radioactivity.

## RESULTS AND DISCUSSION

Results from a typical *in vitro* translation experiment using increasing amounts of genomic RNA and L-[$^{35}$S]-methionine are shown in Fig. 1. Three major proteins, 66K, 44K, and 14K were detected when the complete incubation mixtures were analyzed by SDS-PAGE. Only a few minor proteins were detected in the control (minus mRNA system). To determine whether the products synthesized are related to viral structural proteins or not, immunoprecipi-

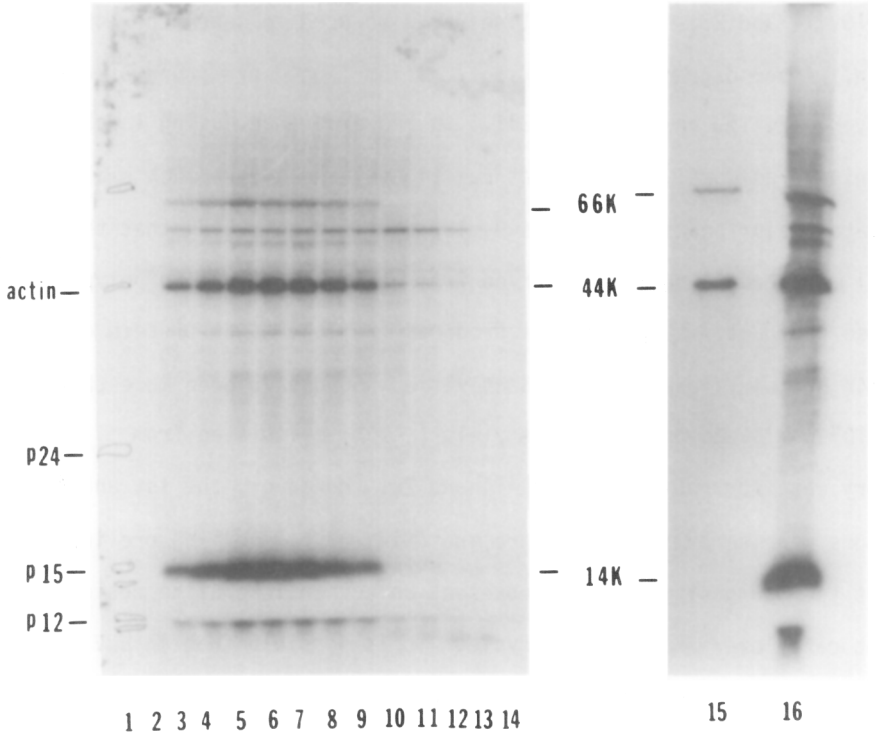


Fig. 1. Cell free synthesis of BLV proteins. After staining the gel with coomassie brilliant blue, fluorography was done using a sodium salicylate solution (27). Lane 1: tracing of stained BLV proteins. Lanes 2-6: 0, 0.125, 0.25, 0.5, and 1 µg 70S RNA, respectively; the RNA in these lanes was obtained from pooled sucrose gradient fractions 1 to 3 (70S RNA). Lanes 7 to 9: RNA (~ 1 µg) from sucrose gradient fractions 1 to 3, respectively. Lanes 10-13: gradient fractions 7-10 respectively and lane 14 pellet. Only the fractions which contained significant amounts of RNA were analyzed. Lane 15: immunoprecipitation with anti-p24. Lane 16: sample before immunoprecipitation.

tation studies were carried out using antisera against the p24, p12 and gp60. Both 66K and 44K proteins were precipitated with anti-p24 (Fig. 1, lane 15) and anti-p12 (not shown) but not with anti-gp60 serum and normal calf serum (Gibco). None of these antisera was reactive with the third major translated product, the 14K protein. Previously the 66K and 44K proteins have been described as gag related polyprotein precursors (21). The 14K protein was purified by a combination of SDS-PAGE, electroelution and reverse-phase high performance liquid chromatography (RP-HPLC) (Fig. 2A). The partial $NH_2$-terminal amino acid sequence was determined by Edman degradation of radiolabeled protein (Fig. 2B and C). As shown in Fig. 2C L-[$^{35}$S]-methionine radioactivity was recovered at cycles 1, 4, 9, 10, 14 and 23. L-[$^{3}$H]-proline was also used to label the 14K protein. After degradation, peaks of tritium label were found at cycles 1, 4-5, 8, 12, 15, and 18 (Fig. 2B). In the experiment using labeled methionine, radioactivity peaks at positions 1 and 4 were much smaller than later major peaks (at cycles 9, 10, and 14) indicating that the initial minor peaks could be derived from other contaminating polypeptide(s); if those radioactivity peaks were produced from the major protein chain (the 14K protein), expected radioactivities would have been more than 3-4 x $10^4$ cpm based on repetitive yield (~ 90%) calculated from recovery data for residues 9, 10, 14 and 23. Moreover, the 1st and 4th positions were determined to be proline (Fig. 2B). The [$^{3}$H]-proline peak at the 5th position might represent an authentic proline residue and not carry over from the 4th cycle. It is possible that in BLV grown in the FLK cells, the 5th position of the translated 14K protein is proline instead of the tryptophan indicated by the sequence of proviral DNA (4,5) cloned from virus free tumor cells. Based on the data presented in Fig. 2B and C we can assign proline in positions 1, 4, 8, 12, 15, and 18 and methionine in positions 9, 10, 14 and 23. These assignments give a perfect fit with the amino acid sequence (see Fig. 2B) deduced from the DNA sequence in the X-SORF (originally designated pX-1) region (4,5).

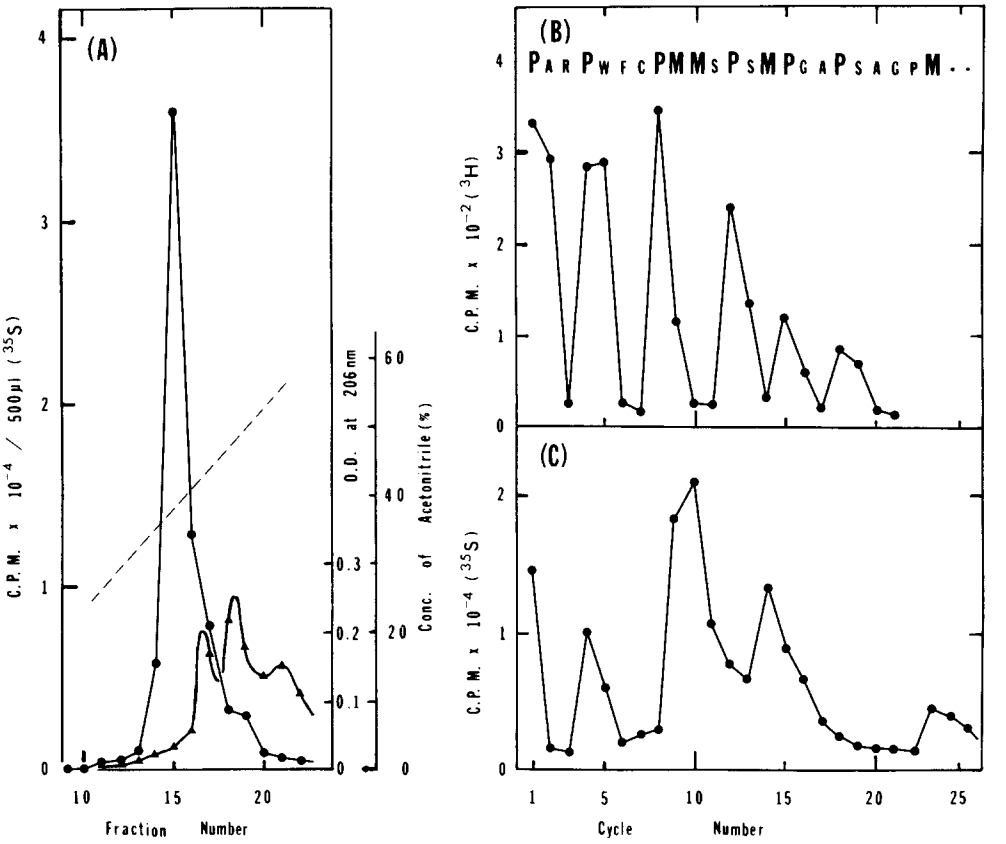


Fig. 2. Purification and $NH_2$-terminal amino acid sequence analysis of 14K protein. (A) For purification by RP-HPLC, proteins were solubilized with 4 M-guanidine HCl adjusting to pH 2.0 with 20% trifluoro-acetic acid and applied to a µBondapak $C_{18}$ column (Waters Associates, 0.39 x 5 cm), and eluted with a linear 0 to 60% acetonitrile gradient. Five ml fractions were collected and 10% was used for measurement of radioactivity. (B) Radiosequencing of L-[2,3,4,5-$^3$H]-proline labeled 14K protein. Labeling with tritiated proline (100 µCi) was carried out as described for methionine. The amino acid sequence deduced from the DNA sequence is shown in the single letter code where the large bold faced letters indicate amino acids confirmed by protein sequencing. (C) Radiosequencing of L-[$^{35}$S]-methionine labeled 14 K protein. The assignment of methionine in positions 9, 10, 14 and 23 is shown in (B).

The probability of having the observed identities (10 out of 23 amino acids) occur by chance alone is $3.2X10^{-14}$. Thus these results provide evidence that the 14K protein is translated from the X-SORF region. As the first amino acid of this $p14^X$ protein is proline, it indicates that most probably the protein is synthesized from an internal initiation site at triplet 7280-7282, the AUG adjacent to CCT (7283-7285) coding for

proline (5). The initiator methinonine is expected to be cotranslationally removed. Results obtained in avian sarcoma virus have shown utilization of internal initiation sites for protein synthesis *in vitro* (22).

The most important question is whether or not the *in vitro* synthesized protein is expressed in infected cells and/or in naturally infected animals. To answer this question, sera obtained from cows naturally infected with BLV were used. Serum 72-7 precipitated the $p14^X$ as well as the 44K and 66K proteins (Fig. 3, lane 2). Three other cow sera (Fig. 3, lanes 1,3 and 4) did not react with the 14K protein although they did precipitate the *gag* proteins. Out of 11 sera (tested at a single dilution of 1:200) from tumor-bearing animals that we examined, two were found positive for $p14^X$. These results strongly suggest that the 'X' region of BLV is expressed in infected animals and the X-SORF product is capable of eliciting antibody in the natural host. Other results from our laboratory indicate expression in BLV-infected cells of a $p14^X$ related larger protein initiated on a spliced message (N. Rice, personal communication). The unique composition and primary structure as deduced from DNA sequence (4,5) of this protein

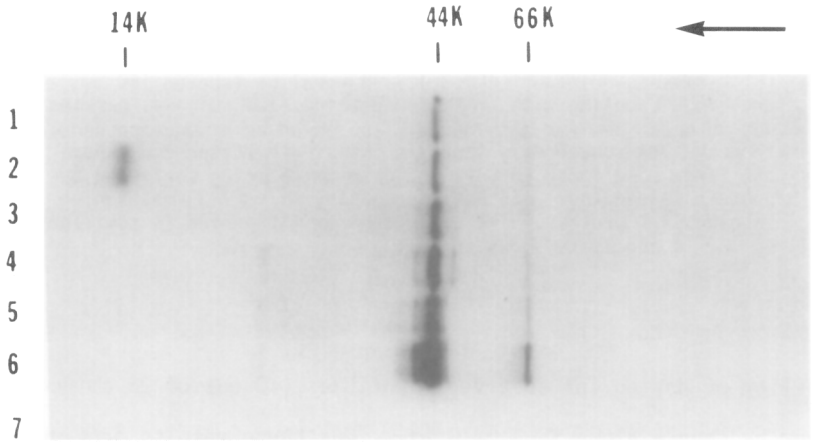


Fig. 3. Immunoprecipitation of *in vitro* translated polypeptide by various BLV positive antiserums from cows with lymphosarcoma and antiserums to BLV p12 and p24. Translation of BLV RNA was done in a 100 µl volume and 10 µl of each was immunoprecipitated as in Fig. 1. Lane 1: cow serum, 70-3; lane 2: cow serum, 72-7; lane 3: cow serum, LS-4; lane 4: cow serum, LS-13; lane 5: anti-p12; lane 6: anti-p24 and lane 7: normal calf serum. Arrow indicates direction of protein migration.

may suggest it having a regulatory function. The high serine and threonine contents indicate that this protein may act as phosphate acceptor. It indeed contains sequences similar to known phosphorylation sites for protein kinases (23,24). Unlike the hydrophobic HTLV-I and II X-LORF proteins (25) BLV $p14^{X}$ is rather hydrophylic in nature, a property shared by the recently described Rous sarcoma virus (RSV) transcriptional activator N-terminal domain (26). In addition three regions of BLV $p14^{X}$ and the putative RSV protein show substantial homologies.

While the function of BLV X-SORF product is unknown, the shared structural properties of this protein with the RSV transcriptional activator may be important for stimulation of expression of LTR and/or other promoters. It remains to be seen whether BLV like HTLV has an X-LORF product expressed, whether HTLVs like BLV also have X-SORF products and whether both products are important for virus replication and/or transformation.

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