Isolation and Characterization of a 2.3-Kilobase-Pair cDNA Fragment Encoding the Binding Domain of the Bovine Leukemia Virus Cell Receptor

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An immunoscreening strategy was used to isolate a cDNA clone encoding the binding domain for the external glycoprotein gp51 of the bovine leukemia virus (BLV). Three recombinant phages demonstrating BLV binding activity and containing 2.3-kbp cDNA inserts with identical nucleotide sequences were isolated from a lambda gt11 cDNA library of bovine kidney cells (MDBK). One clone, BLVRcp1, hybridized with a 4.8-kb mRNA from cells of bovine origin and was also found to be conserved as a single-copy gene in murine, bovine, ovine, primate, canine, feline, and porcine DNAs. The same gene is amplified in caprine DNA isolated from a BLV-induced tumor. The longest open reading frame of BLVRcp1 encodes a protein fragment of 729 amino acids with a putative receptor structure. BLVRcp1 cDNA was cloned in the eucaryotic expression vector pXT-1 and transfected into murine NIH 3T3 and human HEp-2 cells. Cells expressing BLVRcp1 mRNA became susceptible to BLV infection. BLVRcp1 has no known physiological function and has no significant homology with sequences registered in the GenBank and EMBL data libraries (31 July 1992). Expression of deleted constructs of BLVRcp1 indicates that the BLV binding region is encoded at the 5' side of the receptor clone.

Retrovirus host range and cell tropism are determined in part by the presence of receptors at the cell surface. The external envelope protein of the virus interacts with the receptor with a given affinity constant, a parameter that governs the phenomenon known as virus interference (32). Several classes of viral receptors have been described. Receptors for human immunodeficiency virus (14, 22, 25) and other human viruses such as poliovirus (27) and rhinovirus (18) belong to the immunoglobulin superfamily. Another group of retroviral receptors contains amino acid and phosphate permeases. For example, Rec 1, the receptor for ecotropic murine leukemia virus, is a basic amino acid transporter (1, 21, 36), and the receptor for gibbon ape leukemia virus, GLVR1, was found to be homologous to a phosphate permease of *Neurospora crassa* (19).

Bovine leukemia virus (BLV), a naturally occurring exogenous B-lymphotropic retrovirus, is the etiologic agent of enzootic bovine leukosis (11). BLV infects a variety of cells in vitro and propagates in various animal species (2, 5, 6, 7, 26, 28). BLV and human T-cell lymphotropic virus types I and II (HTLV-I and HTLV-II) are related retroviruses. They share properties such as genome organization, presence of regulatory proteins Tax and Rex, nucleotide sequence homology, lack of viremia in the infected host, and integration at multiple sites in the host cell DNA. Because BLV and the HTLVs do not infect the same target cells, it is anticipated that they do not share the same receptor (32).

In the present study, we isolated and characterized a cDNA encoding a polypeptide to which the BLV envelope glycoprotein gp51 selectively binds.

MATERIALS AND METHODS

Cells and viruses. Cells were cultivated in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. The bovine kidney cell line (MDBK; ATCC CCL 22) and the human larynx cell line (HEp-2; ATCC CCL 23) were obtained from the American Type Culture Collection.

Immunoscreening was carried out by using either the crude supernatant of BLV-producing cells (35) or a cell lysate obtained from Vero cells (31) infected by a vaccinia virus recombinant that expressed BLV envelope proteins gp51 and gp30. Sensitivity to BLV infection was estimated by counting the number of cells expressing *lacZ* after infection of the culture by a recombinant BLV carrying the *lacZ* gene. Such a recombinant is expressed by the cell line FLK-BLV/BLV$_s$SV*nls*LacZ as described by Milan and Nicolas (28). The culture supernatant was filtered through 0.22-μm-pore filters and used for infection.

Escherichia coli N 4830-1 cells expressing the carboxylic part of gp51 (amino acids [aa] 167 to 268) and the complete gp30 were used as a source of recombinant BLV envelope proteins (8).

Isolation of BLV gp51. The external glycoprotein gp51 was purified by immunoaffinity chromatography from BLV virions produced by the VP-1 subclone of FLK cells (3, 4).

Northern (RNA) and Southern blot hybridization. RNA was isolated from cells by the guanidinium isothiocyanate-cesium chloride centrifugation method (13), and poly(A)$^+$ RNA was selected on oligo(dT)-cellulose columns. mRNA was treated with glyoxal and either 1 μg per sample was dotted onto nitrocellulose membranes (34) or 5 μg per sample was electrophoresed for Northern blot analysis (17). Cellular DNAs were analyzed under Southern blot stringent

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hybridization conditions (in 3× SSC [1× SSC is 0.15 M NaCl plus 0.015 M Na citrate]).

Immunoscreening. After a 4-h incubation at 42°C, the plates containing lambda gt11 plaques were covered with nylon membranes impregnated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) and transfer was allowed to proceed for 4 h at 37°C. The membrane lifts with immobilized plaques were then saturated in 10 mM Tris-HCl (pH 7.4)-150 mM NaCl (TBS) containing 10% skim milk at room temperature. They were subsequently immersed into a solution containing the gp51 antigen (tissue culture fluid from virus-producing cells treated with 0.1% N-octylglucoside for 30 min on ice before use or 1 μg of immunoaffinity-purified gp51 per ml). After a 16-h incubation at 4°C, the membranes were washed in TBS containing 0.05% Tween 20 and incubated with a mixture of mouse monoclonal antibodies (MAbs) directed against BLV gp51 for 1 h at room temperature. The mixture contains MAbs directed against epitopes A to H (10), each of them at a final concentration of 1 μg/ml. After being washed in TBS-0.05% Tween 20, the filters were incubated with an alkaline phosphatase conjugate of anti-mouse immunoglobulin G antibodies (diluted 7,500-fold; Promega) for 1 h at room temperature. After being washed, the plaques were visualized by using a mixture of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt.

DNA sequencing. The BLVRcp1 cDNA was subcloned into the EcoRI site of plasmid pBluescript II SK⁺ (Stratagene) and partially deleted by successive ExoIII exonuclease digestions with the Erase-a-Base system (Promega). The nucleotide sequence was determined with a Sequenase 2.0 (U.S. Biochemical) sequencing kit according to the manufacturer's recommendations.

Construction of BLVRcp1 deletion mutants. Insertion of a 2.3-kbp EcoRI fragment of BLVRcp1 cDNA into plasmid pUC8-2 (Pharmacia) places these coding sequences in frame with the initiation ATG codon of the β-galactosidase gene. This recombinant plasmid was named pβ-gal-BLVRcp1. Several mutants were derived from pβ-gal-BLVRcp1 by sequence deletions: (i) pβ-gal-BLVRcp1/del1, which contained sequences from the 5' end of BLVRcp1 defined by the EcoRI-SmaI fragment, encoding the first 148 aa of BLVRcp1 (deletion of the SmaI fragment in pβ-gal-BLVRcp1); (ii) pβ-gal-BLVRcp1/del2, defined by the EcoRI site and the first SacI site, encoding the first 262 aa of BLVRcp1; and (iii) pβ-gal-BLVRcp1/del3, defined by the EcoRI-HindIII fragment, encoding the first 331 aa of BLVRcp1 (deletion of the HindIII fragment in pβ-gal-BLVRcp1). The constructs pβ-gal-BLVRcp1/del4 and pβ-gal-BLVRcp1/del5 were made in the same way by deleting parts defined by the BglII and BamHI fragments and PstI fragments in pβ-gal-BLVRcp1, respectively. The construct pβ-gal-BLVRcp1/del6 was made by deleting the EcoRI-HindIII fragment in pβ-gal-BLVRcp1, encoding the protein segment expanding between aa 332 and the carboxylic end of the protein.

Construction of the pXT-1-BLVRcp1 eucaryotic expression vector and its transfection into NIH 3T3 and HEp-2 cells. The BamHI-XhoI fragment from pBluescript-BLVRcp1 was inserted into BglII- and XhoI-digested pXT-1 (Stratagene), generating the pXT1-BLVRcp1 plasmid containing BLVR cp1 in the correct orientation for expression.

NIH 3T3 murine fibroblasts or HEp-2 human cells were transfected with pXT-1-BLVRcp1 or pXT-1 (10 μg of DNA per 4 × 10⁴ cells) by using the calcium phosphate procedure with 5 μg/ml of Polybrene followed by a dimethyl sulfoxide shock (10% dimethyl sulfoxide in Dulbecco's modified Ea-

gle's medium). When necessary, selection was performed in the presence of G418 at a concentration of 800 μg/ml for 2 weeks. Single G418-resistant colonies were picked and grown separately.

Immunoprecipitation. Cultured cells were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine (48 TBq; 0.5 mCi of each per 60-mm dish; Amersham) for 8 h and then treated as previously described (3). The cell extracts were immunoprecipitated after an overnight incubation at 4°C with rabbit anti-peptide RP1 (aa 25 to 36 of the BLVRcp1 protein, PENALPSDEDDK) serum at a 1:10 dilution followed by protein A-Sepharose affinity precipitation. The radiolabeled proteins recognized by this antibody were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE [10% polyacrylamide]) followed by autoradiography. Peptide RP1 was prepared by continuous-flow solid-phase multiple peptide synthesis (24), and rabbit antipeptide antibodies were prepared as previously described (12) by using glutaraldehyde as the coupling agent. The sequence of the peptide RP1 was derived from the nucleotide sequence data.

Nucleotide sequence accession number. The nucleotide sequence presented in Fig. 1A is available through the GenBank and EMBL data libraries (accession number M98430).

RESULTS

Isolation of a cDNA encoding a polypeptide binding to BLV gp51. In order to isolate a cDNA potentially corresponding to the BLV receptor, a lambda gt11 expression library was screened by using a solution containing BLV envelope glycoprotein gp51 as the probe. This strategy assumes that (i) the recombinant receptor protein is able to bind the virion external glycoprotein gp51 and that (ii) the ligand-receptor complex is detectable by subsequently binding to a mixture of MAbs directed against various epitopes of gp51. Therefore, a cDNA library was constructed with mRNAs from bovine cells susceptible to BLV infection (MDBK cell line). The screening of 5 × 10⁵ recombinant phages from an amplified library yielded three recombinant phages having identical inserts of about 2.3 kbp each (confirmed by DNA sequencing). The cDNA used for further characterization is referred to as BLVRcp1.

The binding of the BLVRcp1 expression product to BLV gp51 has been confirmed by using immunoaffinity-purified gp51, gp51 produced by recombinant vaccinia virus, or recombinant gp51 produced in bacteria in the immunoscreening assay. No reaction occurred in the absence of BLV gp51 or when gp51-unrelated MAbs (anti-BLV p24, bovine growth hormone, or pig phosphohexose isomerase) were used (data not shown).

Nucleotide sequence of BLVRcp1 and its deduced amino acid sequence. The nucleotide sequence of BLVRcp1 and its deduced amino acid sequence were determined as follows. The BLVRcp1 insert was subcloned into pBluescript II SK⁺. The nucleotide sequence of the BLVRcp1 cDNA and the predicted amino acid sequence are shown in Fig. 1A. The longest open reading frame shows a first ATG located at position 70 (position 1 is the first nucleotide after the β-galactosidase sequences). The first TAG stop codon in the same frame is at position 2188 and is followed by a polyadenylation signal (AATAAA) at position 2336. That open reading frame, in frame with β-galactosidase, encodes a protein of 729 aa with a calculated molecular mass of 80.3 kDa in the absence of posttranslational modifications. The

A

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  1 R  S  L  H  T  E  S  D  E  D  I  A  P  A  Q  R  V  D  I  V  T  E  E  M  P  E  N  A  L  P  S  D  E  D  D  K  D  P  N  D
  1 CGCTCGCTGCACACGGAGAGCGACGAGGACATCGCACCTGCCCAGCGCGTGGACATCGTCACCGAGGAGATGCCTGAGAACGCCCTGCCCAGTGACGAGGACGACAAAGATCCCAATGAC

 41 P  Y  S  A  L  D  I  D  L  D  K  P  L  A  D  S  E  K  L  P  V  Q  K  H  R  N  A  E  T  S  K  S  P  E  K  E  D  V  P  L
121 CCTTACAGGGCCCTGGACATCGACCTGGATAAGCCCCTAGCTGATAGTGAGAAGCTGCCTGTCCAGAAACACAGAAACGCTGAGACCTCCAAGTCCCCTGAGAAAGAGGATGTCCCCCTT

 81 V  E  K  K  S  K  K  P  K  K  K  E  K  K  H  K  E  K  E  R  E  K  K  K  K  E  V  E  K  G  E  D  L  D  F  W  L  S  T  T
241 GTGGAGAAGAAGAGCAAGAAACCCAAGAAGAAGGAGAAGAAGCACAAGGAGAAGGAGCGGGAGAAGAAGAAGAAGGAGGTGGAGAAGGGTGAGGACTTGGACTTCTGGTTGTCTACTACG

121 P  P  A  A  T  P  A  L  E  E  L  E  V  N* T* T* V  T  V  L  K  E  G  Q  E  E  P  R  E  R  N  R  M  P  K  R  T  G  S  R
361 CCGCCTGCTGCCACACCAGCCCTGGAAGAGCTGGAGGTGAACACCACAGTCACTGTCCTGAAGGAGGGGCAGGAGGAGCCCCGGGAGAGGAACAGGATGCCGAAGAGGACAGGGAGCAGG

161 T  W  R  R  N  P  P  S  T  R  R R N  T  R  K  T  R  R  S  G  P  R  T  R  G  S  P  R  R  R  C  H  Q  R  M  R  R  L  L
481 ACCTGGAGAAGAAACCCTCCAAGCACAAGAAGAAGAAACACAAGAAAGACAAGGAGGAGCGGCCCAAGGACAAGAGGAAGTCCAAGAAGAAGGTGCCACCAGCGGATGAGGAGGCTGCTG

201 S  P  W  R  T  A  A  L  E  E  E  P  L  P  P  M  S  S  Y  I  L  L  A  E  N  S  Y  I  K  M  T  Y  D  V  Q  G  S  L  Q  K
601 AGCCCGTGGAGAACGGCAGCACTCGAAGAGGAGCCCCTGCCGCCCATGTCCAGCTACATTCTTCTGGCTGAAAATTCTTATATCAAGATGACGTACGATGTACAGGGCAGCCTACAGAAG

241 D  S  Q  V  T  V  S  V  V  L  E  N* Q* S* D  T  F  L  K  S  M  E  L  N  V  L  D  S  L  N  A  R  L  A  R  P  E  G  S  S
721 GACAGCCAGGTCACAGTCTCTGTGGTCCTGGAGAACCAGAGCGACACGTTTCTCAAGAGCATGGAGCTCAATGTGCTGGACTCGCTCAATGCCCGGCTGGCCCGGCCCGAGGGCTCCTCG

281 V  H  D  G  V  P  V  P  F  Q  L  P  P  G  I  S  N  E  A  Q  F  V  F  T  I  Q  S  I  V  M  A  Q  K  L  K  G  T  L  S  P
841 GTCCACGACGGTGTCCCCGTGCCTTTCCAGCTGCCCCCTGGTATTTCCAACGAGGCCCAGTTTGTGTTCACCATTCAGAGCATCGTCATGGCCCAGAAGCTCAAGGGGACACTGTCTTTC

321 I  A  K  N  D  E  G  S  T  H  E  K  L  D  F  K  L  H  F  T  C  T  S  Y  L  V  T  T  P  C  Y  S  D  A  F  A  K  L  L  E
961 ATCGCCAAGAACGACGAAGGCTCCACCCACGAGAAGCTTGACTTCAAACTGCACTTCACCTGCACCTCGTACCTGGTCACCACACCGTGCTACAGCGATGCCTTTGCCAAGTTGCTGGAG

361 S  G  D  L  S  M  S  S  I  K  V  D  G  I  S  M  S  F  H  N  L  L  A  K  I  C  F  H  H  R  F  S  V  V  R  R  V  D  S  C
1081 TCTGGGGACCTGAGCATGAGCTCGATCAAGGTGGATGGCATTAGTATGTCCTTCCATAACCTCCTGGCAAAGATCTGTTTTCATCACCGTTTTTCTGTTGTGGAGCGAGTGGACTCCTGT

401 A  S  M  Y  S  R  S  I  Q  G  H  H  V  C  L  L  V  K  K  G  E  K  S  V  S  V  D  G  K  C  S  D  P  T  L  L  S  N  L  L
1201 GCCTCCATGTACAGCCGCTCCATCCAGGGCCACCACGTCTGCCTCCTGGTGAAGAAGGGCGAAAGTCAGTGTCGGTGGATGGGAAGTGCAGCGACCCAACGCTGCTGAGCAACCTGCTG

441 E  E  M  K  E  T  S  G  H  V  L  S  A  A  S  H  R  R  A  A  V  V  C  T  H  W  V  Q  P  G  L  T  K  S  T  I  V  H  C  V
1321 GAGGAAATGAAGGAGACGTCTGGCCACGTGCTGAGTGCCGCCTCCCACAGGAGGGCGGCCGTGGTCTGCACACACTGGGTCCAGCCAGGCTTGACCAAGTCCACCATCGTCCACTGTGTA

481 D  L  A  V  H  V  A  L  I  Y  L  V  I  F  F  Y  F  A  S  L  Y  S  L  A  S  S  P  C  Q  S  P  S  Q  P  G  L  F  W  L  E
1441 GACCTCGCAGTTCACGTTGCGTTGATTTACCTTGTCATCTTTTTTTACTTTGCTTCTCTCTACTCTCTAGCGAGCAGCCCTTGCCAGTCCCCAAGCCAGCCAGGGCTCTTCTGGCTGGAG

521 R  T  V  P  V  L  W  G  L  P  E  G  E  R  D  R  Y  R  G  L  R  T  S  G  G  W  M  G  T  T  M  L  L  A  T  S  V  K  S  S
1561 CGGACTGTGCCTGTGCTATGGGGGCTGCCTGAGGGTGAGCGTGACCGCTATCGAGGTCTCAGGACCTCGGGGGGCTGGATGGGGACGACGATGCTGCTCGCCACTTCTGTCAAGTCGTCC

561 E  S  M  S  S  R  L  L  P  L  G  L  S  R  W  R  P  W  P  A  A  W  G  D  I  P  L  H  P  T  P  R  G  T  S  S  P  G  H  T
1681 GAGTCCATGAGTTCCAGATTGCTGCCGCTAGGCCTTAGTCGGTGGAGGCCATGGCCAGCTGCTTGGGGAGACATTCCTCTTCACCCCACGCCTAGGGGCACTTCCTCCCCAGGACACACT

601 M  G  Q  S  L  A  G  C  R  P  S  H  P  V  C  S  T  T  V  S  C  S  A  E  G  S  A  Q  R  G  P  G  P  W  P  P  C  P  A  A
1801 ATGGGGCAGAGTCTTGCAGGCTGCCGTCCATCACATCCTGTTTGTTCTACCACCGTGTCCTGTTCTGCTGAGGGGAGTGCCCAGCGGGGTCCAGGGCCTTGGCCTCCGTGCCCAGCTGCG

641 C  C  G  E  W  W  R  A  T  A  L  A  L  L  S  S  L  D  A  L  Q  V  C  V  C  T  C  G  R  A  W  V  P  G  L  F  P  C  W  E
1921 TGTTGCGGTGAGTGGTGGAGAGCTACAGCTCTGGCTCTCCTGTCATCACTGGATGCTCTGCAGGTCTGTGTCTGCACCTGTGGAAGGGCCTGGGTACCTGGCCTGTTTCCTTGCTGGGAA

681 A  R  G  S  G  G  G  W  A  L  R  C  T  S  G  A  G  G  E  P  T  P  R  E  T  E  L  S  S  N  M  M  V  L  N  D  I  L  T  S
2041 GCACGTGGGTCCGGGGGTGGCTGGGCCCTGAGATGCACCTCAGGGGCAGGGGGGGAACCGACCCCCCGTGAGACTGAACTTTCCTCAAACATGATGGTCCTCAATGACATTTTAACTTCT

721 F  D  E  N  C  H  F  S  M  ***
2161 TTTGATGAAAACTGTCACTTTAGCATGTAGAGTAACCTATTACAGAATCCTGTGCAGTGATTCTAGAATCTCTAAAATTGTATGATGTGTTATTAAGAATTTATTTGATATCGACATTCC

2281 CGTGTAAAGGAGAGACATATCATGCTGCTGTAATGATTTTGTGTCAAGATGATCCAATAAACTTGCGAAACAGGAAAAAAAAAAAAAAAA 2369
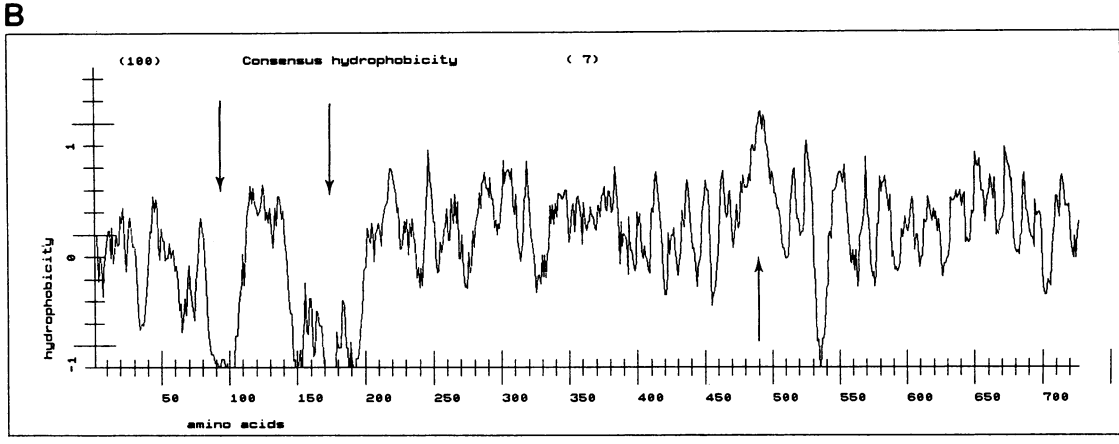```



# B

Consensus hydrophobicity

FIG. 1. (A) Nucleotide and deduced amino acid sequences of BLVRcp1 cDNA. The putative starting ATG codon at position 25 is indicated with boldface letters. Two consensus N-glycosylation sites (N-X-S/T; aa 134 to 136 and 252 to 254) are indicated by asterisks. The consensus site for phosphorylation by cAMP-dependent protein kinase (R-R/K-X-S/T; aa 171 to 174) is underlined. The putative transmembrane domain (aa 486 to 512) is underlined twice. The in-frame terminator TAG codon (nucleotide positions 2188 to 2190) is indicated by asterisks. The poly(A)+ track at the end of the cDNA begins 13 nucleotides after the AATAAA sequence, which is underlined twice. (B) Hydropathy plot of the deduced BLVRcp1 protein according to Eisenberg et al. (16). Numbers indicate the amino acid positions. Arrows indicate the presence of highly hydrophobic or hydrophilic regions.

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hydropathy plot of the predicted BLVRcp1 amino acid sequence suggests a long extracellular region encompassing two N-glycosylation sites (positions 134 to 136 and 252 to 254) and one consensus site for phosphorylation by the cyclic AMP (cAMP)-dependent protein kinase (R-R/K-X-S/T) at positions 171 to 174, a transmembrane domain (positions 486 to 512), and probably an intracellular, cytoplasmic region (Fig. 1B). The ATG codon mentioned above is not consistent with the sequence AXX ATG G, defined as an efficient translation start site by Kozak (23). Searches (31 July 1992) in the GenBank and EMBL data bases for homology with known nucleotide and amino acid sequences failed to identify any significant homology with previously reported sequences.

**Northern analysis of bovine mRNAs.** To determine the size of the mRNA transcript that encodes the BLV receptor in bovine cells, a Northern blot experiment was performed with poly(A)$^+$ RNAs isolated from MDBK cells and bovine kidney tissue. The hybridization probe was BLVRcp1. After the filters were washed under stringent conditions, the presence of a single band of approximately 4.8 kb was detected in both MDBK cells and bovine kidney tissue RNA (Fig. 2A). These data suggest that the BLVRcp1 cDNA represents only part of the genetic message for the BLV receptor.

**Presence of sequences homologous to BLVRcp1 in cells of different species.** The wide host range of BLV among species other than bovine indicates that the BLV receptor is widespread and is conserved among animal species. To test the conservation of the isolated sequence described here, Southern analysis was performed with DNA obtained from different species. Hybridization with a BLVRcp1 probe under stringent conditions permits the detection of a limited number of hybridizing fragments in EcoRI digests of murine, bovine (DNA from MDBK cells), ovine, primate, caprine, canine, feline, and porcine DNAs (Fig. 2B). These data suggest the presence of a single-copy gene that is highly conserved in DNA from various species. In caprine DNA isolated from a BLV-induced tumor (Fig. 2B, lane 5), BLVRcp1 sequences are probably amplified.

**Expression of BLVRcp1 cDNA in murine and human cells confers sensitivity to BLV infection.** To examine the ability of BLVRcp1 protein to render cells permissive to infection by BLV, the BLVRcp1 cDNA was placed under the thymidine kinase promoter in the pXT-1 expression vector, generating pXT-1-BLVRcp1. pXT-1-BLVRcp1 or pXT-1 alone was transfected into murine NIH 3T3 and human HEp-2 cells and tested for transient expression of BLVRcp1. Forty-eight hours after transfection, cells were tested for their susceptibility to BLV infection by using a recombinant BLV expressing lacZ (BLV$_s$SVnls LacZ) (28). The number of BLV-sensitive cells was estimated by counting the number of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)-positive cells. In untransfected control cells, 17 (for NIH 3T3) and 85 (for HEp-2) positive cells were detected out of 2 × 10$^5$ cells. Cells transfected with the parental plasmid pXT-1 did not show any increase in BLV infectivity. However, a significant increase of BLV sensitivity was observed after transfection with pXT-1-BLVRcp1, because about 150 and 300 BLV-sensitive cells were detected in 2 × 10$^5$ NIH 3T3 and HEp-2 cells, respectively.

In order to ensure that the increased sensitivity to BLV infection observed in the transient expression experiment is due to expression of BLVRcp1, we performed the following experiment. pXT-1-BLVRcp1 or pXT-1 alone was transfected into NIH 3T3 cells, and cells were selected in the
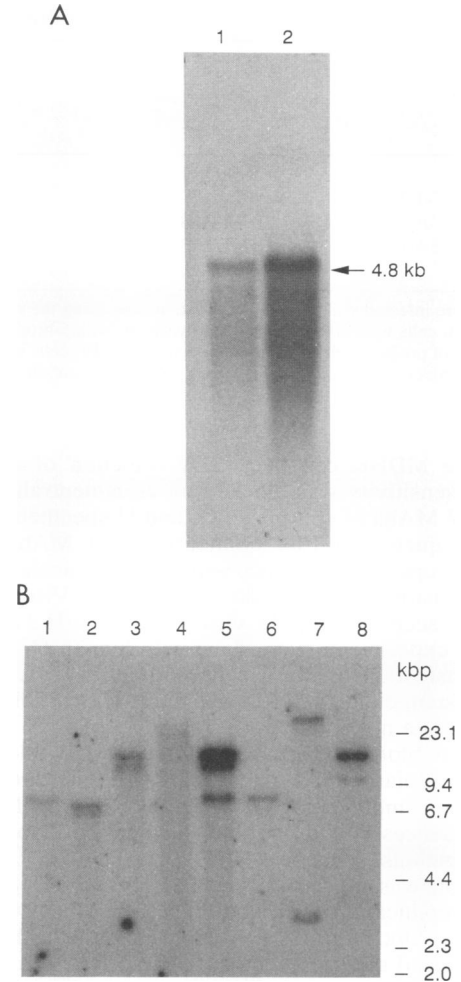


FIG. 2. (A) Expression of BLVRcp1 mRNA in MDBK cells and bovine kidney tissue. Poly(A)$^+$-selected RNAs isolated from MDBK cells (lane 1) and bovine kidney tissue (lane 2) were fractionated on an agarose-formaldehyde gel, blotted, and hybridized with the BLVRcp1 probe. (B) Southern blot hybridization with the BLVRcp1 probe of cellular DNAs from different animal species. Genomic DNAs (10 μg) digested with EcoRI were electrophoresed on a 0.9% agarose gel, blotted and hybridized. DNAs (by lane) are as follows: 1, murine; 2, bovine-MDBK; 3, ovine; 4, primate; 5, caprine (isolated from a BLV-induced tumor); 6, canine; 7, feline; and 8, porcine.

presence of G418. Twenty colonies were isolated from NIH 3T3 cells transfected with pXT-1-BLVRcp1, and 5 colonies were isolated from NIH 3T3 cells transfected with the control pXT-1. The sensitivity of these colonies to BLV infection was estimated as mentioned above. The data obtained for some of the NIH 3T3 transfectant clones, as well as negative and positive controls infected with the recombinant BLV expressing lacZ, are presented in Table 1. In the untransfected control NIH 3T3 cells, 20 positive cells among 2 × 10$^5$ were detected. The clones transfected with the parental plasmid pXT-1 did not show any increase in staining efficiency, as examplified by clone NIH 3T3 XT-1/1. In one out of 20 clones (clone NIH 3T3 3x6A6) corresponding to cells transfected with pXT-1-BLVRcp1, a significant increase in BLV sensitivity was observed. This clone was found to be as sensitive to BLV infection as the BLV-

TABLE 1. Susceptibility of various cell clones to BLV infectivity

| Cells[a] | DNA transfected | RV[b] | No. of β-galactosidase-positive cells infected with: | | | |
|---|---|---|---|---|---|---|
| | | | RV + MAbs[c] | | | |
| | | | F | G | H | E |
| NIH 3T3 | None | 20 | 10 | 10 | 10 | 20 |
| NIH 3T3 XT-1/1 | pXT-1 | 23 | 8 | 10 | 8 | 20 |
| NIH 3T3 3x3A1 | pXT-1-BLVRcp1 | 15 | 12 | 12 | 11 | 17 |
| NIH 3T3 3x3A6 | pXT-1-BLVRcp1 | 2,350 | 198 | 240 | 185 | 2,275 |
| MDBK | None | 2,366 | 175 | 244 | 165 | 2,370 |

[a] Cells were infected with 1 ml of supernatant containing the retroviral vector (RV) harvested from FLK-BLV/BLV$_s$SVnlsLacZ cells. At approximately 24 h postinfection, cells were fixed and stained overnight with 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
[b] Number of positive cells after transfection of $2 \times 10^5$ cells with the recombinant BLV expressing lacZ.
[c] Prior to infection, the recombinant BLV (RV) was incubated with MAbs of different epitope specificities (F, G, H, and E) for 1 h on ice.

permissive MDBK cell line. BLV infection of cells with acquired sensitivity was blocked by virus-neutralizing anti-gp51 BLV MAbs of epitope F, G, and H specificity but not by the sequential (virus nonneutralizing) MAb directed against epitope E. At variance with that spectacular increase in BLV sensitivity, transfection of pXT-1-BLVRcp1 did not enhance susceptibility to the virus in clone NIH 3T3 3x3A1.

Similar experiments were done with human HEp-2 cells. After transfection of these cells with pXT-1-BLVRcp1, 1 G418-resistant clone out of 18 was found susceptible to BLV infection (data not shown).

Southern blot hybridization of undigested and EcoRI-digested cellular DNAs from the NIH 3T3 3x6A6 cells revealed an amplification of integrated 2.3-kbp BLVRcp1 DNA sequences in addition to a fragment corresponding to the endogenous sequences (Fig. 3A, lane 7). A similar observation was made for the NIH 3T3 3x3A1 clone but without amplification of the additional copy (Fig. 3A, lane 5).

Northern blot hybridization analysis with the BLVRcp1 probe revealed the presence of BLVRcp1 transcripts in the NIH 3T3 3x6A6 clone only (Fig. 3B, lane 4). Specific mRNA expression was not found in NIH 3T3 cells that had integrated only one additional copy of the BLVRcp1 sequences or in untransfected control cells (Fig. 3B, lanes 1, 2, and 3). Similar levels of BLVRcp1 expression were found in BLV-permissive MDBK cells and in the NIH 3T3 3x6A6 cells that had acquired BLV sensitivity (data not shown).

Expression of the BLVRcp1 gene product in NIH 3T3 3x6A6 cells was demonstrated by immunoprecipitation of cell extracts with a rabbit antipeptide RP1 serum reactive against the sequence aa 25 to 36 (PENALPSDEDDK) in the BLVRcp1. A protein with a molecular mass of about 70 kDa was precipitated from the transfected NIH 3T3 cells (clone 3x6A6) (Fig. 4, lane 3) but not from normal murine cells (lane 1). The same antipeptide serum immunoprecipitated a product with an apparent molecular mass of 90 kDa (Fig. 4, lanes 5 and 6) from MDBK cells. This protein probably represents the entire BLV receptor in its native structure.

**Localization of the binding domain of BLV gp51 on the BLVRcp1 product.** The hydropathy profile of the BLVRcp1 product revealed the existence of two hydrophilic regions (aa 80 to 112 and 140 to 200) flanking a hydrophobic region at the amino side of BLVRcp1 (16) (Fig. 1B).

To determine whether these regions are involved in the binding of gp51, several deletion mutants of BLVRcp1 (Fig. 5) were prepared and expressed in pUC8-2. Their lysates were tested by dot blot immune assays for their capacity to bind gp51. The reactions were visualized by using the mixture of antiglycoprotein MAbs and nitroblue tetrazolium
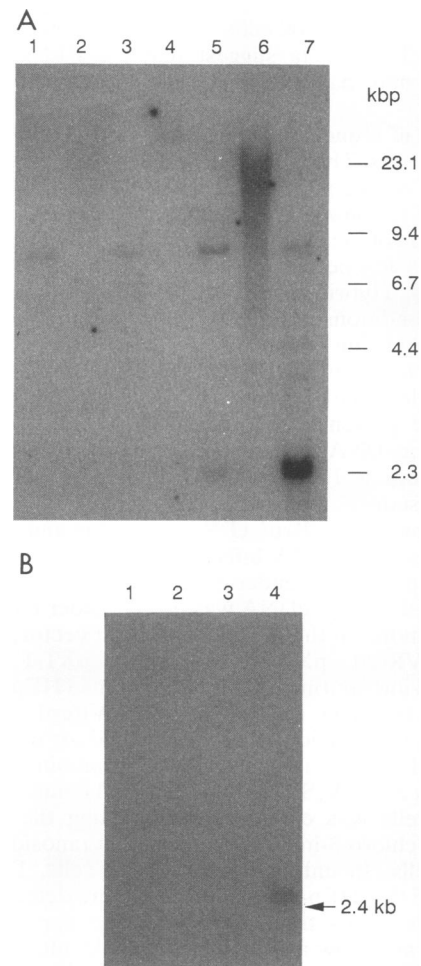


FIG. 3. (A) Southern analysis of NIH 3T3 transfectant clones with the BLVRcp1 cDNA as probe. Cell types (by lane) are as follows: 1, untransfected NIH 3T3; 2 and 3, NIH 3T3 XT-1/1; 4 and 5, NIH 3T3 3x3A1; and 6 and 7, NIH 3T3 3x6A6. Lanes 1, 3, 5, and 7 contained DNAs digested with EcoRI; lanes 2, 4, and 6 contained undigested DNAs. (B) Northern blot analysis of NIH 3T3 transfectant clones with the BLVRcp1 probe. Each lane contains 30 μg of total RNA. Cell types (by lane) are as follows: 1, untransfected NIH 3T3; 2, NIH 3T3 XT-1/1; 3, NIH 3T3 3x3A1; and 4, NIH 3T3 3x6A6.
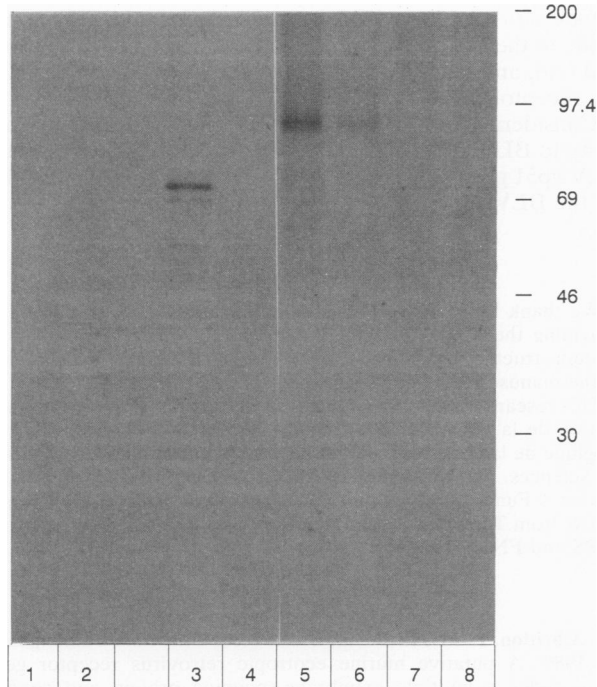
FIG. 4. Detection of the BLV receptor products by immunoprecipitation. NIH 3T3 (lanes 1 and 2), transfected NIH 3T3 (clone 3x6A6) (lanes 3 and 4), and MDBK (lanes 5 to 8) cells were metabolically labeled with [³⁵S]methionine and [³⁵S]cysteine as described in Materials and Methods. Cell extracts were immunoprecipitated with a normal rabbit serum (lanes 2, 4, and 7, dilution of 1:10; lane 8, dilution of 1:15) or with the rabbit anti-peptide RP1 serum (lanes 1, 3, and 5, dilution of 1:10; lane 6, dilution of 1:15) before SDS-PAGE and autoradiography (24-h exposure for lanes 1 to 4 and 5 days for lanes 5 to 8).

chloride–5-bromo-4-chloro-3-indolylphosphate   p-toluidine salt reagents as substrate for an anti-mouse antibody conjugated with alkaline phosphatase. When the first 993 bp from the 5' end of BLVRcp1 DNA (fragments EcoRI to HindIII, the first 331 aa) was deleted, the binding activity was abolished (pβ-gal-BLVRcp1/del6). When the 789-bp fragment defined by sites EcoRI to SacI (construct pβ-gal-BLVRcp1/del2) was present, the binding activity was unaltered. Positive binding activities were also observed for other constructs carrying different portions of the BLVRcp1 gene (constructs pβ-gal-BLVRcp1/del3, -del4 and -del5). Reduced binding activity was observed with the pβ-gal-BLVRcp1/del1 construct that encodes the first 148 aa of the protein, thus suggesting that the protein segment encoded by the SmaI-SacI DNA fragment plays a major role in gp51 binding.

## DISCUSSION

In this article, we report the identification and characterization of a cDNA, BLVRcp1, that encodes a polypeptide behaving as a receptor for BLV. The BLVRcp1 product was first identified in three recombinant lambda gt11 phages that displayed specific binding activity for BLV gp51. Two cell lines (murine NIH 3T3 and human HEp-2), found to be quite resistant to BLV infection compared with other cells (i.e., bovine cells) (28), were used in transfection experiments with the BLVRcp1-expressing plasmid. Expression of BLVRcp1 in these cells conferred sensitivity to BLV infection.

This acquired sensitivity to BLV infection occurred despite the fact that the encoded polypeptide (expressed as a protein with a molecular mass of about 70 kDa in transfected NIH 3T3 cells) (Fig. 4, lane 3) might not be complete. Indeed, (i) the putative ATG codon lacks a classical Kozak environment, (ii) no leader peptide is identifiable at the NH₂ end of BLVRcp1 protein, (iii) the cloned 2.3-kbp cDNA is shorter than the native mRNA (4.8 kb), and (iv) BLVRcp1 expressed from transfected cDNA in NIH 3T3 cells is
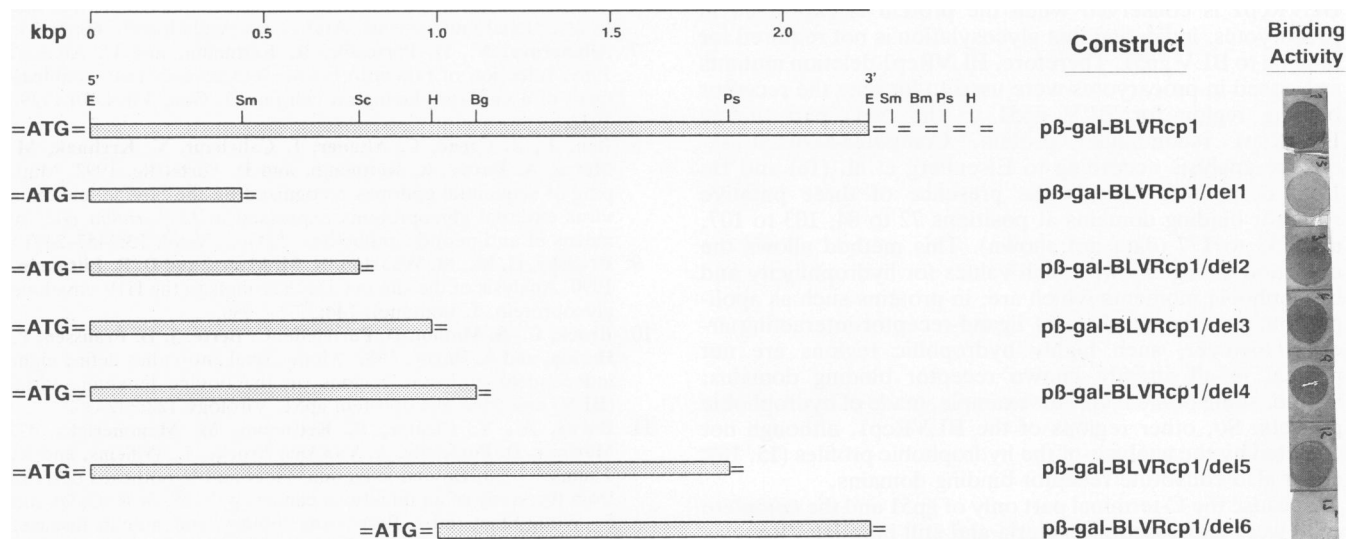


FIG. 5. Schematic representation of the BLVRcp1 deletion constructs and their binding activities to BLV gp51. E, EcoRI; Sm, SmaI; Sc, SacI; H, HindIII; Bg, BglII; Ps, PstI; Bm, BamHI. The pUC8-2 ATG initiation codon is indicated; = represents plasmid sequences; stippled boxes indicate BLVRcp1 sequences. gp51 binding activities of cellular lysates were revealed on dots by immunoreaction, as performed for the immunoscreening (see Materials and Methods).

smaller than the protein precipitated from MDBK cells with the same serum. Obviously, however, the missing protein segment did not prevent correct processing and transport of BLVRcp1 proteins to the cell membrane. The lack of a leader peptide sequence has been observed for the gibbon ape leukemia virus receptor. In this case also, transfection of the cloned receptor sequence and expression in NIH 3T3 cells conferred sensitivity to gibbon ape leukemia virus (29). The gibbon ape leukemia virus receptor, like all of the other members of the permease-like protein family, would not be expected to have a signal peptide.

Analysis of the BLVRcp1 product reveals a protein with a receptor structure. It contains an extracellular domain including a BLV gp51 binding region, a single transmembrane region, and a putative intracellular, cytoplasmic domain. The presence of these domains suggests that the BLVRcp1 protein is an integral membrane protein, as expected for a receptor. No homologous nucleotide or amino acid sequences could be identified in the presently available data bases. This observation again illustrates the notion that retroviral receptors are largely unrelated molecules, despite the fact that a limited number of interference groups have been identified (20, 32, 33). It seems very likely that retroviral receptors have a primary but variable physiological function, such as those of permeases, transporters, and aminopeptidases, and coincidently act as attachment points for retroviral envelope glycoproteins that are responsible for virus entry.

The widespread distribution of BLVRcp1 among mammals and its highly conserved sequence suggest that the molecule recognized by BLV plays a major role in the physiology of many cell types. Elucidation of the reasons and mechanisms by which the virus is confined to a few cell types in vivo must await a much more detailed analysis. However, from the limited data so far available concerning in vitro susceptibility of infection of MDBK cells, NIH 3T3 cells, and one clone of NIH 3T3 cells transfected with pXT-1 BLVRcp1 (Table 1), a positive correlation between the receptor expression levels (Fig. 2A and 3B) and the susceptibility to BLV infection seems to exist.

It is worth mentioning that the gp51 binding ability of BLVRcp1 is conserved when the protein is expressed in procaryotes, indicating that glycosylation is not required for binding to BLV gp51. Therefore, BLVRcp1 deletion mutants expressed in procaryotes were used to localize the receptor binding region for BLV gp51 to the NH₂ part of the BLVRcp1 recombinant protein. Computer-assisted sequence analysis according to Eisenberg et al. (16) and De Loof et al. (15) reveals the presence of three putative receptor binding domains at positions 72 to 84, 103 to 107, and 153 to 177 (data not shown). This method allows the detection of regions with high values for hydrophilicity and hydrophobic moments which are, in proteins such as apolipoproteins, characteristic of ligand-receptor-interacting areas. However, such highly hydrophilic regions are not present in all already known receptor binding domains: indeed, some of them are, for example, made of hydrophobic pockets. So, other regions of the BLVRcp1, although not detected by the analysis of the hydrophobic profiles (15, 16), might also constitute receptor binding domains.

Because the C-terminal part only of gp51 and the complete gp30 were expressed in bacteria and still bind BLVRcp1 as purified gp51 does, we conclude that the COOH region of gp51 (close to epitope B) (12) is the region of binding of gp51 to the receptor (data not shown). Thus, the domains of binding of BLV gp51 to BLVRcp1 and human immunodefi-

ciency virus gp120 to CD4 are similarly located: HIV gp120 binds to the CD4 receptor at a site located on its C-terminal end (30), and binding to CD4 also occurs at the NH₂ end of the receptor protein (9).

Considering the ability of the BLVRcp1 to confer sensitivity to BLV infection and its ability to specifically bind the BLV gp51 glycoprotein, we believe that the protein encoded by the BLVRcp1 cDNA acts as a receptor for BLV.

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