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Unique Epitope of Bovine Immunodeficiency Virus Gag Protein Spans the Cleavage Site between p16^{MA} and p2L†

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Bovine immunodeficiency virus (BIV) and Jembrana disease virus (JDV) are closely related bovine lentiviruses that are difficult to distinguish by presently available diagnostic methods. Recently, in our laboratory, a monoclonal antibody (MAb; MAb 10H1) against the BIV Gag protein identified a differential epitope, located at the 6.4-kDa N terminus of a 29-kDa Gag capsid protein, which was absent in JDV. To define the essential amino acids of the epitope, a series of primers within the 163 bp of DNA corresponding to the 6.4-kDa protein were designed. The full-length 163-bp DNA fragment and the smaller DNA fragments with deletions were amplified by PCR and then cloned into pQE32 vectors for protein expression studies. The expressed proteins were analyzed with MAb 10H1 by Western blotting. The differential epitope has been narrowed to a 26-amino-acid region (R121 to R146), which includes 6 residues of p16^{MA} (where MA represents the matrix protein) and 20 residues of p2L. A synthetic peptide corresponding to the putative 26-amino-acid epitope blocked MAb 10H1 binding to the expressed peptide. These experiments revealed that the epitope spans the cleavage site between p16^{MA} and p2L and presumably will be valuable in distinguishing the two viruses.

Bovine immunodeficiency virus (BIV) is an RNA lentivirus of the family *Retroviridae*. BIV causes lymphadenopathy, lymphocytosis, central nervous system lesions, progressive weakness, and associated secondary diseases (8, 18, 21, 24). Although a causal relationship between BIV infections and these conditions has not been clearly established, BIV infection has been recognized to result in a high incidence of common diseases that reduce the economic viability of dairy cattle (17). BIV infection occurs worldwide and has recently been reported in Italy (5) and Australia (3). In Mississippi, the seroprevalence of BIV among cattle herds is higher than 50% (19); however, in western Canada, the prevalence of BIV infection in cattle is very low (9). Jembrana disease virus (JDV) is also a lentivirus and is closely related to BIV in terms of its morphogenesis, protein structure, and antigenic reactivity and by sequence analysis (6, 11). JDV causes an acute and sometimes fatal disease of domesticated banteng or Bali cattle and is endemic in parts of Indonesia (23). Both bovine lentiviruses may be present in Bali cattle populations in some districts (2, 3).

Both BIV and JDV resemble human immunodeficiency virus (HIV) in their biological, antigenic, and structural properties. Among the three major structural proteins (encoded by *gag*, *pol*, and *env*), the Gag protein develops the earliest and strongest antibodies in infected animals. The BIV Gag precursor (Pr53^{gag}) can be processed into three major proteins (the matrix [MA], capsid [CA], and nucleocapsid [NC] proteins) and three smaller proteins (proteins p2L, p3, and p2). The order of these six proteins in a Gag precursor is NH$_2$-p16^{MA}-

p2L-p26^{CA}-p3- p13^{NC}-p2-COOH. The proteins have molecular masses of 14.6, 2.5, 24.6, 2.7, 7.3, and 1.9 kDa, respectively (20). The strong conservation of Gag epitopes of BIV and HIV type 1 (HIV-1) has been demonstrated by the serological cross-reactivity between the two virus capsid proteins. The cross-reactivity of sera from BIV-infected cattle against JDV recombinant capsid protein has been reported (4, 11). The similarity of the *gag* genes between BIV and JDV is approximately 62% at the amino acid level, and the capsid proteins of the two viruses have a higher degree of amino acid identity (75%). Serological tests that detect antibody to the capsid protein of JDV or BIV may not differentiate the two viruses (23).

The BIV *gag* gene has been cloned, and monoclonal antibodies (MAbs) to recombinant Gag protein have been developed (22, 25, 26). One of our MAbs, designated 10H1, reacts specifically with the BIV Gag protein but not with the JDV Gag protein, indicating that BIV has at least one epitope different from JDV epitopes. The epitope has already been located at the 6.4-kDa N terminus of a pATH *gag-3* clone (1, 25) which expresses a 29-kDa Gag protein. Detailed knowledge of the epitope would help to elucidate the structural basis of antigen-antibody recognition to evaluate protein-protein interactions. Identification of antigenic epitopes may also be important to explore future vaccine strategies (12, 16). The present study was aimed at defining the makeup of amino acids required for the unique epitope and elucidating the molecular mechanisms by which MAb 10H1 differentiates the BIV Gag protein from the JDV Gag protein.

MATERIALS AND METHODS

***gag* gene clones and MAb 10H1.** A pATH *gag-3* clone containing 781 bp of the *gag*-coding region from BIV strain R29 was used to generate BIV MAbs, including MAb 10H1. The production and characterization of MAb 10H1 were described earlier (25). One 751-bp fragment of the *gag* gene insert was subcloned into prokaryotic expression vector pQE32, designated pQE32-*gag* (25, 26), and was used as a template for PCR amplification of *gag* gene fragments in this study.

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Construction of expression plasmids. We designed primers with sequences specific within the 163-bp DNA region corresponding to the 6.4-kDa protein (25). For cloning of BIV *gag* fragments into the pQE32 expression vector (QIA-GEN), the forward primers had *Bam*HI restriction sites at their 5′ ends, and the reverse primers had *Hin*dIII sites at their 3′ ends. For cloning of the deduced epitope into the pQE30Xa expression vector (QIAGEN), the forward primer had an *Stu*I restriction site at the 5′ end, and the reverse primer had an *Hin*dIII site at the 3′ end. PCR amplification with the primers added the restriction sites to the ends of the PCR products. The PCR products were cloned into pGEM-T Easy Vectors by a standard procedure (Promega), and the T vectors were transformed into *Escherichia coli* strain JM109. Subsequently, the PCR products were purified, digested with the corresponding restriction enzymes, and separated by electrophoresis on a 1% agarose gel. The BIV *gag* fragments were cloned into the pQE32 expression vector at the *Bam*HI-*Hin*dIII restriction sites, and the deduced epitope was cloned into a pQE30Xa expression vector at the *Stu*I-*Hin*dIII restriction sites. The sequences and the reading frames were confirmed by the dideoxyribonucleotide chain termination method.

Expression of proteins. *E. coli* strain M15 was transformed with a pQE32-*gag* construct, a pQE30Xa-epitope construct, or a relevant vector alone as a negative control and was grown at 37°C overnight in 1 ml of Luria-Bertani (LB) broth containing ampicillin and kanamycin. The cultures were then inoculated with 4 ml of fresh LB broth containing the same antibiotics and were incubated for 30 min at 37°C. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 2 mM to induce protein expression. After 4 h of induction, the cells were harvested by centrifugation at $3,000 \times g$ for 15 min and lysed by heating for 5 min in 500 ml of a 1:1 mixture of phosphate-buffered saline buffer and 2× sample loading buffer (0.125 M Tris, 20% glycerol, 4% sodium dodecyl sulfate, 1% 2-mercaptoethanol).

Western blot assay. The protein samples were loaded onto a discontinuous Tricine gel (16.5% T, 6% C) (15). Electrophoresis was run at a constant voltage of 30 V for 1 h and then at 100 V for about 4 h. The proteins were then transferred onto a nitrocellulose membrane at a constant voltage of 100 V for 1 h with a transblot apparatus. The membrane was blocked with 2% bovine serum albumin in phosphate-buffered saline containing 0.1% Tween 20 and incubated with MAb 10H1 at 1:2,000 dilutions at room temperature for 2 h or at 4°C overnight. Anti-mouse immunoglobulin G peroxidase conjugate (Promega) was added at 1:3,000, and the mixture was left for 30 min at room temperature. Specific protein bands were detected with the standard enhanced chemiluminescence reagent (Amersham Pharmacia Biotech).

Dot and block blotting. A 6-amino-acid peptide (S-6aa) from the C terminus of the BIV matrix protein, a 20-amino-acid peptide (S-20aa) from the N-terminal portion of the p2L protein, and the combined 26-amino-acid peptide (S-26aa) were synthesized (courtesy of the Biotechnology Core Facility of Kansas State University). For dot blotting, the peptides were dissolved in distilled water at 0.5 μg/ml and the solutions were dotted onto a nitrocellulose membrane. The dotted membrane was air dried and used to complete the Western blotting as described above. For block blotting, two synthetic peptides, S-26aa and S-20aa, along with the proteins expressed from the pQE30Xa-epitope clone were separated by use of the Tricine gel and transferred onto nitrocellulose membranes. The membranes were prepared and blotted in the same way as described above, except that MAb 10H1 was preabsorbed with synthetic peptides at a concentration of 1 mg in 20 ml of the blocking buffer for 1 h at room temperature.

RESULTS

Cloning and expression of epitope region. Previously, we mapped the MAb 10H1-specific epitope by chemical cleavage analysis and located the differential epitope at the 6.4-kDa N terminus of the 29-kDa capsid proteins expressed by two different BIV constructs, pATH *gag-3* and pQE32-*gag* (24). To confirm the result, a pair of primers, primers BIV*gag*-1F (GG ATCCAGGCCAGAGCTGATAAGGAA) and BIV*gag*-1R (AAGCTTGCTGCTTCATGAGGTCAG), were used to amplify the 163-bp fragment corresponding to nucleotides 655 to 817 in a BIV complete proviral genome. The PCR product was further purified and subcloned into prokaryotic expression vector pQE32. The 54 amino acids, Gag protein residue R114–R167, were translated from the vector part with 13 additional amino acids at the N terminus and 3 amino acids at the C terminus. The N-terminal 6.4-kDa protein and the 29-kDa BIV
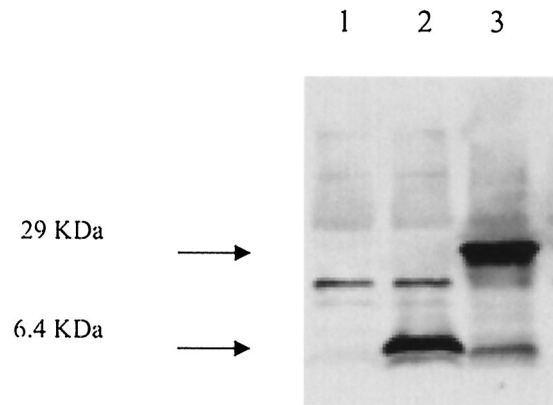

FIG. 1. Expression of the 6.4-kDa N terminus of the BIV Gag capsid protein (pATH clone). The full-length 29-kDa Gag capsid protein expressed by the pQE32-*gag* construct (lane 3) and N-terminal 6.4-kDa protein R114–R167 expressed in vector pQE32 (lane 2) reacted positively with MAb 10H1 by Western blotting. *E. coli* strain M15 transformed with vector pQE32 (lane 1) was used as the negative control.

Gag protein were well expressed and reacted positively with MAb 10H1 in the Western blot, while *E. coli* strain M15 transformed with vector pQE32 was negative (Fig. 1). This result verified the location of the MAb 10H1-specific epitope.

Mapping and expression of epitope. To define the epitope location, we made serial constructs which expressed overlapping peptides. The Gag R114–R146 fragment reacted positively with MAb 10H1 by Western blotting, the Gag R114–R145 fragment gave a weak positive reaction, while the Gag R114–R144 fragment gave a negative response (Fig. 2). These results indicate that the glutamine at R146 is necessary for the C terminus in terms of epitope function. Comparison of Western blotting results for the Gag R121–R167 and Gag R122–R167 fragments demonstrated that the glutamic acid at R121 is crucial for the N terminus (Fig. 2). The deduced epitope sequence covered 26 amino acids from residues R121 to R146. To verify the sequence, we engineered the corresponding 78-bp DNA fragment into a pQE30Xa expression vector. The Gag R121–R146 fragment was expressed at a high level in bacterial strain *E. coli* M15 and reacted well with MAb 10H1 by Western blotting (Fig. 3, lane 4 of the nonblocking plot). The results confirmed the mapped position and, therefore, defined the epitope sequence.

Determination of epitope location. BIV Gag precursor protein Pr53^{gag} has six proteolytic cleavage products in the order p16^{MA}, p2L, p26^{CA}, p3, p7^{MC}, and p2 (Fig. 4A). The 54-amino-acid region (residues R114 to R167) spans three of the six Gag products: 13 residues at the C terminus of p16^{MA}, 22 residues of p2L, and 19 residues at the N terminus of p26^{CA} (Fig. 4B). The defined epitope (represented in boldface in Fig. 4B) consists of 6 residues of p16^{MA} and 20 residues of p2L. The results indicated that the unique epitope excludes the capsid protein. To further pinpoint the epitope location, we synthesized S-6aa from p16^{MA}, S-20aa from p2L, and the combination of the peptides (S-26aa). Dot blotting of the three synthetic peptides demonstrated that only S-26aa gave a positive reaction with MAb 10H1 (data not shown). Block blotting was performed to further test the interactions between the

Residues	Expressed peptide sequence	Western blot
R114-167	ARADKEGEIKSIYPSLTQNTQNKKQTSNQTNTQSLPAITTQDGTPRFDPDLMKQ	+
R114-146	ARADKEGEIKSIYPSLTQNTQNKKQTSNQTNTQ	+
R114-145	ARADKEGEIKSIYPSLTQNTQNKKQTSNQTNT	+/-
R114-144	ARADKEGEIKSIYPSLTQNTQNKKQTSNQTN	-
R121-167	EIKSIYPSLTQNTQNKKQTSNQTNTQSLPAITTQDGTPRFDPDLMKQ	+
R122-167	IKSIYPSLTQNTQNKKQTSNQTNTQSLPAITTQDGTPRFDPDLMKQ	-

Deduced epitope sequence

R121-146　　　　EIKSIYPSLTQNTQNKKQTSNQTNTQ

FIG. 2. PCR-based mapping of MAb 10H1-specific epitope. The peptide sequences of Gag fragments in the N-terminal 6.4-kDa protein (R114–R167) were expressed. The DNA fragments were amplified by PCR, purified from the gel, and cloned into vector pQE32. The vectors were expressed in *E. coli* strain M15. Western blotting was done with MAb 10H1. The Western blotting results are designated as follows: +, positive; +/−, significantly reduced positive; −, negative.

synthetic peptides and MAb 10H1 (Fig. 3). In the nonblock plot, S-26aa (Fig. 3, lane 1) and the protein expressed by the pQE30Xa-epitope clone (Fig. 3, lane 4) reacted with MAb, while S-20aa (Fig. 3, lane 2) and the negative control (Fig. 3, lane 3) did not react. The results for the synthetic peptides were consistent with the dot blotting results. In the 6aa-block and 20aa-block plots in Fig. 3, S-26aa and the expressed epitope also reacted positively with MAb 10H1 as strongly as they did in the nonblock plot. This indicates that neither of the two synthetic peptides could block the activity of MAb 10H1. However, in the 26aa-block plot, both S-26aa and the expressed epitope did not react with MAb 10H1, indicating that the synthetic epitope completely blocked the activity of the MAb. The results demonstrate that neither S-6aa of p16^MA nor S-20aa of p2L has the epitope complementarity to block MAb 10H1. Therefore, the epitope must span the cleavage site between p16^MA and p2L.

Differential epitope of BIV from JDV Gag protein. The 26-amino-acid sequence was aligned against a sequence in the

JDV Gag protein database. The epitope matched a region that spans the matrix protein and the capsid protein of JDV (Fig. 5). The starting proline for BIV protein p2L matched the starting proline for the JDV capsid protein. Five of the six amino acids of the epitope located at the C terminus of the BIV matrix protein are identical to the corresponding amino acids of the JDV matrix protein, with high degrees of identity between the two viruses in the region. Only 3 of the 20 amino acids in the BIV p2L region match the JDV capsid protein sequence in the corresponding region, showing the difference between the two regions. These results indicate that MAb 10H1 does not react with JDV because the p2L region is missing in JDV.

DISCUSSION

The pATH *gag-3* clone contains a 0.8-kb capsid gene from strain R29 of BIV (1) and was used for the expression of the BIV capsid protein. We used the clone to generate MAbs
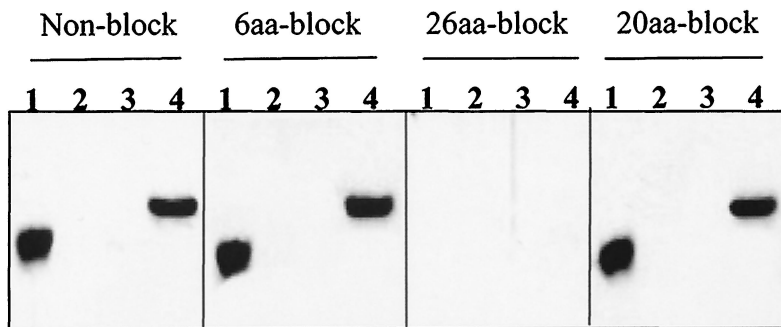

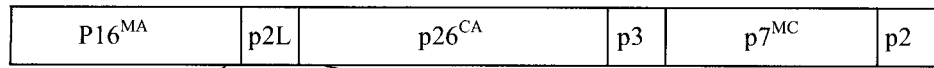FIG. 3. Block blotting of MAb 10H1 by the synthetic epitopes. Lane 1, S-26aa; lane 2, S-20aa; lane 3, *E. coli* strain M15 transformed with the pQE30Xa expression vector as a negative control; lane 4, *E. coli* strain M15 transformed with pQE30Xa engineered with the DNA sequence of the deduced epitope. The four plots were not blocked (Non-block) or were blocked with S-6aa, S-26aa, and S-20aa, as indicted above each panel.
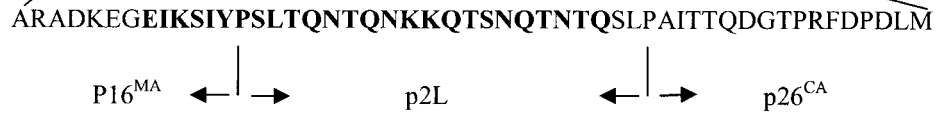
A

B   ARADKEG**EIKSIYPSLTQNTQNKKQTSNQTNTQ**SLPAITTQDGTPRFDPDLM

P16^{MA} ←│→   p2L   ←│→   p26^{CA}

FIG. 4. Diagram of the proteolytic products of BIV Pr53^{gag} and the amino acid sequence (R114–R167) in which the MAb 10H1-specific epitope is located. (A) Proteolytic cleavage products of BIV Gag proteins (Pr53^{gag}). The BIV Gag proteins shown are p16^{MA}, p2L, p26^{CA}, p3, p7^{MC}, and p2. (B) MAb 10H1-specific amino acid sequence that covers 13 residues (R114 to R126) of p16^{MA}, 22 residues (R127 to R148) of p2L, and 19 residues (R149 to R167) of p26^{CA}. The region in boldface type indicates where the epitope mapped.

including MAb 10H1 (25). The protein of the pATH *gag-3* clone expressed in *E. coli* starts with Gag residue R114, while the capsid protein starts with residue R149 (20). The MAb 10H1-specific epitope was previously mapped to the N-terminal 6.4-kDa region, which includes 13 residues (R114 to R126) of p16^{MA}, 22 residues (R127 to R148) of p2L, and 19 residues (R149 to R167) of p26^{CA} (25). We have now excluded the epitope from the capsid protein, narrowed the epitope to a 26-amino-acid region, and defined it to the cleavage site spanning proteins p16^{MA} and p2L.

Numerous epitopes for HIV have been identified in the lentiviruses. HIV-1 *gag*-encoded cytotoxic T-lymphocyte epitopes, for example, are composed of 7 to 10 amino acids. The BIV MAb 10H1-specific epitope has been mapped to a 26-amino-acid region and may not be the minimal size for an epitope. The techniques that we used may not be suitable for the purpose of fine mapping for two reasons. First, the fragments are too small to be engineered into a construct. Second, the flanking amino acids from vector parts may interfere with the reaction between the epitope and the antibody. Our failure to express the 26-amino-acid epitope in vector pQE32 may be due to the second problem (data not shown). This suggests that the epitope could be narrowed to a smaller region around the cleavage site. Alternatively, the epitope may be affected by the structure of the 26-amino-acid sequence, which was predicted to be a helix-turn-helix structure by the Genefold module of Sybyl (Tripos, St. Louis, Mo.). To resolve this problem, residues in the middle of the 26-amino-acid sequence should be mutated to determine their role in antibody specificity. The use of synthesized peptides is a relatively direct and efficient way for final mapping of an epitope, but such peptides are expensive. Techniques based on phage display or gene fragment libraries have been successful for the rapid identification of a tobacco mosaic virus epitope (7, 10). Perhaps these techniques could eliminate some of the problems and may help map the BIV MAb 10H1-specific epitope to a minimal size.

We mapped the epitope to a unique region which spans the BIV matrix protein and the short protein p2L. Among the lentiviruses, only the BIV Gag protein contains the intragenic peptide (p2L) between the matrix and capsid proteins. For JDV, the p2L short protein was suggested to be incorporated into the capsid protein (6) or to be absent, as shown in this study. Only 3 of the 20 amino acids between the BIV p2L protein and the JDV capsid protein in the corresponding region are identical. This projects the molecular difference between BIV and JDV Gag proteins and explains why MAb 10H1 does not react with JDV. Field isolates of BIV contain p2L, and despite the p2L sequence heterogeneity, the residues in the cleavage sites are well conserved (20). In general, due to the molecular difference and the conservation of the cleavage sites, the MAb 10H1-specific epitope is unique in distinguishing BIV from JDV. This MAb not only reacts specifically with both the BIV capsid protein and the recombinant fusion protein (25) but also recognizes BIV-infected bovine lung cells (Charles Wood, personal communication; data not shown), suggesting the potential value of MAb 10H1 and the unique epitope.

MA   p2L

←——— ———→

EIKSIYPSLTQNTQNKKQTSNQTNTQ   **BIV**

|| ||||| | ·|

EITSIYPQLRKNFPAVSTSDGSPRYD   **JDV**

←——— ———→
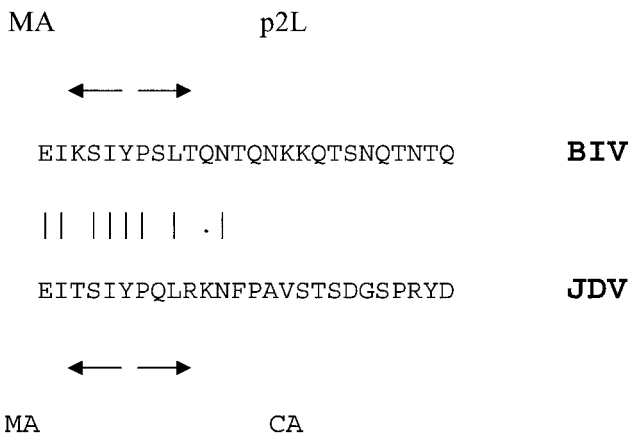
MA   CA

FIG. 5. Alignment of the BIV epitope and the JDV Gag protein. Peptide sequences were aligned by use of the Genetics Computer Group GAP program. The 26-amino-acid peptide of BIV was aligned against 255 amino acids of the JDV Gag protein. The corresponding regions are shown. Both the BIV p2L protein and the JDV capsid protein start with proline. Vertical lines indicate amino acids that are identical in the two sequences. MA, matrix protein; CA, capsid protein.

JDV is a lentivirus closely related to BIV in terms of its morphogenesis, protein structure, and antigenic reactivity and by sequence analysis. Alignment of the *gag* gene products of JDV and BIV showed strong overall amino acid identity (62%), with the matrix protein identity being 60%, the capsid protein identity being 75%, and the nucleocapsid protein identity being 63% (6). The high degree of amino acid conservation explains the difficulty in differentiating the two viruses, either by biological means or by use of molecular technology. BIV is widespread, while JDV is restricted to parts of Indonesia. Distinguishing the two closely related viruses with MAbs will be important in evaluations of the epidemiology of the disease and for quarantine purposes. The matrix protein-p2L cleavage site not only is unique for BIV in relation to JDV but also is highly conserved, so the unique epitope may be useful for future study of BIV.

Similar to the results that we have presented for BIV Gag, MAbs that map to the p17-p24 cleavage site of HIV Gag have been identified by other investigators, with determinants of antibody binding found to be located on both p17 and p24 (14). The MAb was also shown to inhibit cleavage at the p17-p24 site. Another group (13) has shown that MAb to a p17-derived peptide alone inhibits infection of cells by HIV Gag. Taken together, the results suggest that MAbs to Gag cleavage sites in bovine lentiviruses may be further explored for their ability to interact with and/or inhibit different strains of lentiviruses.

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