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Expression in *Escherichia coli* and Sequencing of the Coding Region for the Capsid Protein of Dutch Maedi-Visna Virus Strain ZZV 1050: Application of Recombinant Protein in Enzyme-Linked Immunosorbent Assay for the Detection of Caprine and Ovine Lentiviruses

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Maedi-visna in sheep and caprine arthritis-encephalitis in goats are caused by two closely related and widespread lentiviruses. The infections are characterized by life-long virus persistence and slow induction of antiviral antibodies. The diagnosis is based on the detection of antiviral antibodies. We have used the polymerase chain reaction (PCR) to amplify a part of the *gag* gene coding for the entire capsid protein and for parts of the matrix and nucleocapsid proteins. Sequencing of the PCR fragment of the Dutch maedi-visna virus strain ZZV 1050 revealed 85 and 92% homology to the DNA and deduced amino acid sequences, respectively, of the distantly related Icelandic visna virus strain 1514. The respective homologies with caprine arthritis-encephalitis virus strain CO were 76 and 80%. The PCR fragment was cloned into pGEX-2T and expressed as a glutathione *S*-transferase fusion protein. The recombinant protein could be detected on immunoblots by using a monoclonal antibody and polyclonal antisera and was further purified by glutathione-based affinity chromatography. Enzyme-linked immunosorbent assay with purified recombinant fusion protein is shown to be a sensitive and specific diagnostic tool for the detection of lentiviral infection in goats and sheep.

Caprine arthritis-encephalitis is a widespread and economically important lentiviral infection of goats. The main clinical manifestations are arthritis, chronic subclinical mastitis, and interstitial pneumonia. Encephalitis is a rare feature of caprine arthritis-encephalitis virus (CAEV) infection in goat kids (for reviews, see references 7 and 31). The disease is characterized by a slowly progressive course, with sporadic recrudescence of inflammation. In spite of life-long virus persistence, only 30 to 40% of infected animals develop clinical symptoms (6, 10, 21). CAEV is genetically and antigenically closely related to maedi-visna virus (MVV) (12, 34), which induces a similar syndrome with progressive pneumonia, mastitis, and occasionally encephalitis or arthritis in sheep (33). The laboratory diagnosis of both infections is based on the demonstration of antiviral antibodies in agar gel immunodiffusion assays (1, 19) or enzyme-linked immunosorbent assays (ELISAs) (14, 16, 44). MVV antigens are frequently used for the diagnosis of maedi-visna in sheep as well as for the diagnosis of caprine arthritis-encephalitis in goats (9, 16, 47). Recently, recombinant viral proteins have found wide application in second-generation assays for the detection of antiviral antibodies (4, 22, 37). In particular, the use of such proteins is well documented in human and simian lentiviral serology (3, 8, 32, 41).

To circumvent the time-consuming and inefficient production of small-ruminant lentiviral antigens in cell culture, we have cloned and expressed a *gag* (gene for group-specific antigens) fragment encompassing the entire coding region for the major cross-reactive and immunogenic capsid protein (15, 17, 30, 35) and parts of the coding sequence for the matrix and nucleocapsid proteins of MVV strain ZZV 1050 (14). Sequencing of the cloned *gag* fragment revealed homologies with the published sequence of the distantly related Icelandic visna virus strain 1514 (43) of 85% for DNA and 92% for deduced amino acid sequences. Most of the changes in the deduced amino acid sequence are conservative. The homologies with CAEV strain CO (38) are 76% for DNA and 80% for deduced amino acid sequences. Used as an antigen in ELISA, the recombinant Gag protein proved to be superior to antigen prepared from whole virus, indicating that the recombinant Gag protein is a good candidate for broad diagnostic use in small-ruminant lentivirus serology.

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MATERIALS AND METHODS

PCR. The coding region of the capsid protein of MVV strain ZZV 1050 (originally obtained from D. Houwers, Lelystad, The Netherlands) was amplified as an 850-bp *gag* fragment by the polymerase chain reaction (PCR) essentially as described before (45). As primers, we used 20-mers starting at positions 824 (sense; 5′-AAGTATGAAAGAAG GACTAC-3′) and 1673 (antisense; 5′-ATGTCCTGGTTTTC CACAAT-3′) of the published sequence of Icelandic visna virus strain 1514 (43). Briefly, the reaction mixture contained approximately 1 μg of DNA (crude cell extract) as the template, primers and deoxyribonucleotides at 1 μM and 1.5 mM, respectively, and 1 U of *Taq* polymerase (Boehringer Mannheim) in *Taq* buffer [67 mM Tris-HCl (pH 8.8), 6.7 mM $MgCl_2$, 16.6 mM $(NH_4)_2SO_4$, 10 mM β-mercaptoethanol, 6.7 μM EDTA, 170 μg of human serum albumin]. The samples were amplified for 30 cycles, consisting of denaturation at 94°C for 30 s, followed by annealing at 56°C for 60 s and extension at 74°C for 60 s. The final extension step was lengthened by 10 min to allow complete extension of all products.

Cloning of 850-bp *gag* fragment. The 850-bp PCR fragment was purified in a 4% polyacrylamide gel, filled in with

Klenow polymerase, and chromatographed on a Sephadex G-25 spin column (Pharmacia). The purified *gag* fragment was cloned into the *Sma*I site of pUC19 (Boehringer Mannheim) and subcloned in the *Sma*I site of pGEX-2T (Pharmacia) after double digestion with *Bam*HI and *Eco*RI and filling in with Klenow polymerase. All recombinant DNA techniques were performed by standard methods (29).

Expression of Gag fusion protein in *Escherichia coli* and purification. The *gag* fragment was expressed in *E. coli* JM107 as a fusion protein of glutathione *S*-transferase, extracted from bacteria and affinity purified as described before (42). The recovery of the fusion protein could be enhanced significantly by using chromatography instead of batch extraction with glutathione-agarose beads (G4510; Sigma). Briefly, bacterial extracts were loaded on a column filled with 10 ml of glutathione-agarose beads (preswollen in phosphate-buffered saline [pH 7.3]) and the fusion protein or glutathione *S*-transferase, respectively, was eluted with 5 mM reduced glutathione (Sigma) in 50 mM Tris-HCl (pH 8.0) after extensive washing with phosphate-buffered saline.

ELISA and immunoblotting. ELISA with recombinant Gag fusion protein and whole-virus preparations, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), silver staining, and immunoblotting were performed as previously described (44). The glutathione *S*-transferase extracted from *E. coli* transformed with wild-type pGEX-2T served as the control antigen in the ELISA with recombinant protein. Briefly, microelisa plates (Immulon M 129 A; Dynatech) were sensitized overnight at 4°C with recombinant- or whole-virus-derived antigens diluted in phosphate-buffered saline (pH 7.2; 100 μl per well). Plates were then stored at −20°C until use. Sera were diluted 1:100 in Tris buffer (20 mM Tris-HCl [pH 8.0], 0.5 M NaCl, 5% skim milk, 0.1% Tween 20, 0.05% NaN_3) and incubated overnight at 4°C (200 μl per well). After washing (0.01 M phosphate buffer [pH 7.2], 1.5 M NaCl, 0.1% Tween 20), peroxidase-conjugated protein G (Bioreba AG, Basel, Switzerland) was added in dilution buffer (0.01 M phosphate buffer [pH 7.2], 0.5 M NaCl, 1% Tween 20) and incubated for 2 h at room temperature (100 μl per well). After washing as above, the substrate-chromogen solution containing 2.5 mM hydrogen peroxide and 0.002 M ABTS (2,2′-azino-di-[3-ethyl-benzthiazoline sulfonate]; Boehringer Mannheim) was added and incubated for 15 min at room temperature (100 μl per well). The OD_{405} of each well was read on an automatic ELISA plate reader. A total of 400 serum samples from 346 goats and 54 sheep were used for comparison of the two ELISAs.

Sera with extinction higher than the weak positive control serum included on each plate were recorded as positive in the ELISA. The criteria for a positive recording in the immunoblot were strong reactivity with one viral protein (e.g., capsid protein) or weak reactions with at least two viral proteins (capsid protein and/or matrix protein and/or surface protein).

Sequencing. The 850-bp *gag* fragment was digested with *Sau*3AI, and each fragment was subcloned into the *Bam*HI site of pUC19. From 1 to 3 μg of plasmid DNA from plasmid minipreparations was subjected to double-strand sequencing. Briefly, plasmid DNA was denatured with NaOH for 10 min at room temperature and then neutralized with sodium acetate (pH 5.5) before ethanol precipitation (46). The subsequent steps of the sequencing procedure were performed according to the Sequenase (United States Biochemicals) protocol. The single sequences were assembled with the PcGene program, and the resulting fragments were aligned with the FASTA program package (27).

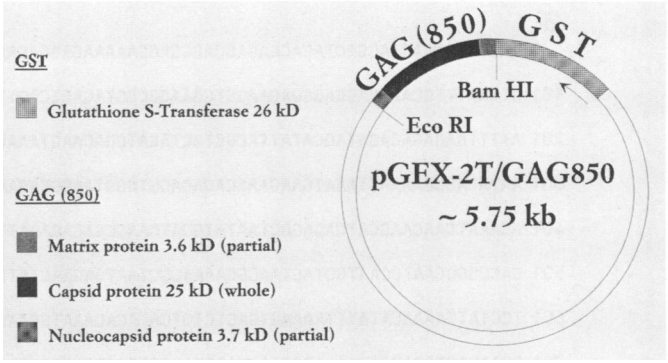

FIG. 1. Expression clone pGEX-2T/GAG850. Approximate sizes of expressed *gag* gene products are given in kilodaltons.

Nucleotide sequence accession number. The sequence discussed herein has been assigned EMBL accession number X54379.

RESULTS

Expression and purification of recombinant Gag fusion protein. The Gag proteins of retroviruses are expressed as a polyprotein precursor, which is then cleaved into the matrix, capsid, and nucleocapsid proteins (23, 28, 43). An 850-bp *gag* fragment of Dutch maedi-visna virus strain ZZV 1050, consisting of 94 bases coding for the carboxy terminus of the presumptive 15-kDa matrix protein (44), the entire coding sequence of the 25-kDa capsid protein, and 96 bases coding for the amino terminus of the 14-kDa nucleocapsid protein (43) was amplified by PCR and cloned into pUC19 and subsequently into pGEX-2T (Fig. 1). The Gag–glutathione *S*-transferase fusion protein expressed in *E. coli* had an apparent molecular mass of 58 kDa in SDS-PAGE, whereas glutathione *S*-transferase migrated with an apparent molecular mass of 26 kDa (Fig. 2). The 58-kDa Gag fusion protein migrated consistently as a double band, which might be due to an early and specific degradation of this protein in *E. coli* extracts. Purification of the fusion protein and unfused

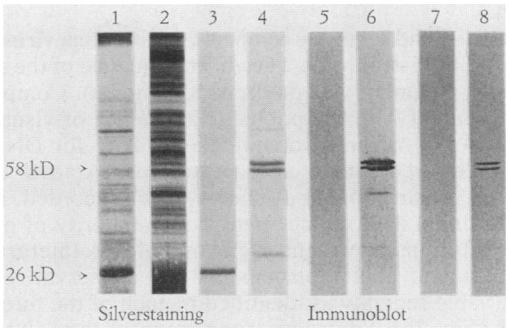

FIG. 2. SDS-PAGE and immunoblotting of extracted recombinant proteins. Even-numbered lanes represent cell lysates of *E. coli* transformed with recombinant pGEX-2T/GAG850; odd-numbered lanes refer to lysates of bacteria transformed with wild-type pGEX-2T. Lanes 1 and 2, crude cell lysates. Lanes 3 to 8, affinity-purified cell lysates. Lanes 1 to 4, silver staining. Lanes 5 and 6, immunoblot with CAEV-positive goat serum. Lanes 7 and 8, immunoblot with monoclonal antibody to capsid protein. The 58 and 26 kD indicate the positions of Gag fusion protein and glutathione *S*-transferase, respectively.

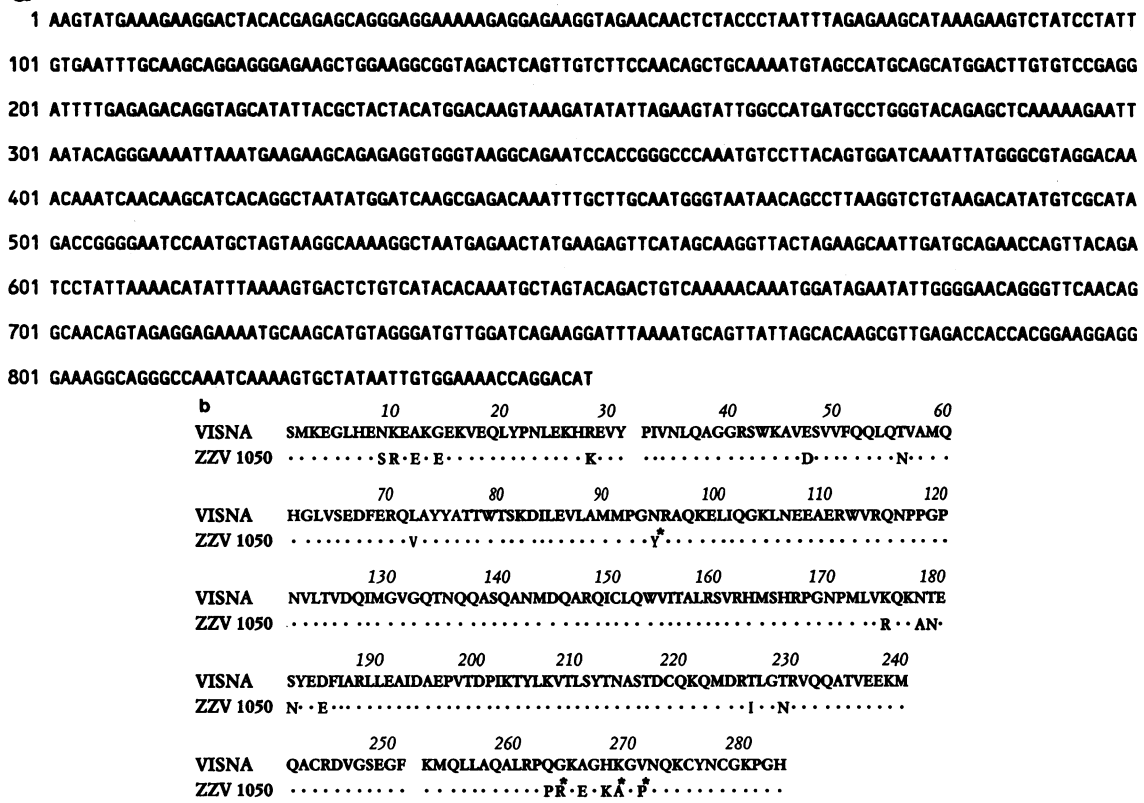

FIG. 3. (a) Nucleotide sequence of 850-bp *gag* fragment. (b) Comparison of deduced amino acid sequence of recombinant Gag protein with the sequence of Icelandic visna virus. Asterisks indicate amino acid changes which are not conservative. Dots show identical amino acids. Spaces in the sequence indicate the locations of presumed cleavage of the Gag precursor protein into the three core proteins. Note hypervariable regions starting at positions 9, 175, and 263.

glutathione *S*-transferase resulted in a dramatic reduction in *E. coli* contaminants (Fig. 2, lanes 1 and 2 versus lanes 3 and 4). Immunoblotting with polyclonal and monoclonal sera (16) confirmed the specificity and antigenicity of the Gag fusion protein (Fig. 2, lanes 5 to 8). The yield of purified recombinant protein was in the range of 10 mg/liter of culture medium.

Sequencing and sequence comparison with visna virus strain 1514 and CAEV strain CO. The entire sequence of the cloned 850-bp *gag* fragment was determined (Fig. 3a). Comparison of this fragment with the published sequence of visna virus strain 1514 (43) revealed homologies of 85% for DNA and 92% for deduced amino acid sequences. A total of 128 nucleotide substitutions (not shown) were recorded, resulting in 22 amino acid replacements. The majority of nucleotide substitutions represent silent mutations. Interestingly, 18 of 22 amino acid replacements are conservative. One hypervariable region was identified in each of the three Gag proteins (Fig. 3b). Sequence comparison of the 850-bp *gag* fragment with the corresponding sequence of CAEV strain CO (38) revealed homologies of 76% for DNA and 80% for deduced amino acid sequences (not shown).

ELISA. Affinity-purified recombinant Gag fusion protein was compared with whole virus as the antigen in an ELISA for the serological detection of lentiviral infection in goats (346 serum samples) and sheep (54 serum samples). Glutathione *S*-transferase and antigen derived from mock-infected cell cultures served as the respective negative control antigens (Table 1). Immunoblotting was used as the reference test for resolving conflicting results. Of 400 sera, the results for 345 (86%) were in accordance in both assays. Of the 55 discrepancies, a higher proportion (29 versus 18) were resolved correctly and fewer samples were recorded as unresolved in the recombinant ELISA (12 versus 31). Whereas the sensitivities of the assays are comparable, the specificity of whole-virus ELISA is superior to that of recombinant ELISA (one versus eight false-positive results). Absorption of false-positive sera with high concentrations of lysed *E. coli* (20 mg/10 μl of serum for 1 h at room temperature) did not reduce their reactivity significantly.

TABLE 1. Comparison of whole-virus and recombinant ELISA results[a]

Recombinant ELISA	Whole-virus ELISA			
	−	+/−	+	Total
−	196	10 (8−, 2+)	5 (1−, 4+)	211
+/−	4 (4+)	6	8 (8+)	18
+	7 (6−, 1+)	21 (2−, 19+)	143	171
Total	207	37	156	400

[a] Immunoblot results are shown in parentheses for discordant results. Fifty-five discrepancies were resolved by immunoblot between whole-virus ELISA (W) and recombinant ELISA (R): correct: W, 18 (33%); R, 29 (53%); unresolved: W, 31 (56%); R, 12 (22%); false-negative: W, 5 (9%); R, 6 (11%); false-positive: W, 1 (2%); R, 8 (15%).

However, preliminary data indicate that the proportion of false-positive reactions can be significantly reduced by further purification of the recombinant protein with ion-exchange chromatography (Mono Q, Pharmacia; data not shown).

DISCUSSION

We describe a simple procedure for the cloning and high-yield expression of an 850-bp *gag* fragment including the entire coding region of the capsid protein of Dutch MVV strain ZZV 1050 and parts of the coding sequences for the matrix and nucleocapsid proteins. The high degree of homology of the DNA and deduced protein sequences (85 and 92%, respectively) of this *gag* fragment derived from Dutch MVV strain ZZV 1050 (14) with the corresponding sequences of the distantly related Icelandic visna virus strain 1514 (40) is in line with observations made for other lentiviruses (25, 36). Taking into account a *Taq* enzyme error rate of 0.1 to 3 per 400 bp after 30 cycles of PCR (11, 13, 18), a maximum of 6 out of 128 nucleotide substitutions can be estimated to be PCR artifacts, indicating that such changes do not translate into major errors in sequence comparisons. Taking also into account that most of the changes in the deduced amino acid sequence are of a conservative nature, it becomes clear that PCR-derived *gag* gene products are good candidates for use as antigens for the detection of antibody to lentiviruses (3). This conclusion is further substantiated by the homologies of DNA and deduced protein sequences (76 and 80%, respectively) between the 850-bp *gag* fragment of Dutch MVV strain ZZV 1050 and the corresponding sequence of CAEV strain CO (38).

The fusion of the recombinant Gag protein with glutathione *S*-transferase allows simple one-step purification with glutathione beads. The purified fusion protein proved to be a very potent antigen in ELISA for the detection of lentiviral infection in goats and sheep. Reactions with wild-type glutathione *S*-transferase antigen were seen in less than 0.5% of all sera (data not shown). Nevertheless, the use of glutathione *S*-transferase as the control antigen is necessary to avoid misinterpretation of positive reactions with the recombinant Gag fusion protein. The use of second-generation ELISA with recombinant Gag protein has several advantages over conventional ELISA with whole-virus antigens. The large-scale production in *E. coli* and one-step purification of recombinant Gag fusion protein are fast, simple, and efficient compared with cell culture-derived viral antigens. The yield of viral antigen is about 200-fold higher in *E. coli* extracts than in equal volumes of supernatants of infected-cell cultures, obviating the need for work-intensive concentration of antigen. The time frame for the production of recombinant protein is overnight instead of weeks for whole-virus production.

This new ELISA displays higher accuracy, concurrent with a higher tendency toward false-positive results. Several reasons can account for positive reactions in recombinant ELISA which cannot be confirmed in whole-virus immunoblot. Some ELISA-positive sera might not react in the immunoblot due to denaturation of the proteins and might be recorded as positive in further supplemental testing (24). Alternatively, reactions with *E. coli* antigens could be responsible for false-positive results in recombinant ELISA (41) despite the use of glutathione *S*-transferase as a negative control. Preliminary data indicate that ion-exchange chromatography of the recombinant protein reduces the proportion of false-positive reactions in ELISA.

It is not clear at present whether proteins derived from *env* (envelope), the least conserved gene (25), should also be incorporated as antigens in this assay to improve overall sensitivity (2, 20) or to compensate for the loss of antibody reactivity with *gag*-derived proteins during the course of infection (15, 17), as seen often in human immunodeficiency virus infection (5, 26, 39). Systematic studies of the antibody reaction in small ruminants during the course of experimental and natural infections are required before these points can be settled.

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